Exploring the wider roles of TetR-family efflux regulators AcrR and EnvR in *E. coli* and *Salmonella*

By

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Abstract

The efflux system AcrAB-TolC extrudes a range of antimicrobials, dyes and detergents in *Salmonella* Typhimurium. Consequently, the overexpression of the *acrAB* genes confers multi-drug resistance. The AcrEF-TolC system is believed to extrude some of the same substrates as AcrAB-TolC, but the conditions which induce the expression of *acrEF* are unknown. Here, induction of *acrA* and *acrE* transcription occurred in response to a variety of conditions and substrates of AcrAB, with both *acrAB* and *acrEF* being induced by the addition of indole or rhodamine 6g.

The expression of the *acrAB* genes is negatively regulated by the TetR-family transcription factors AcrR and EnvR, which are local regulators of the *acrAB* and *acrEF* efflux genes, respectively. However, EnvR also regulates *acrAB,* making *acrAB* a global target of EnvR. Here, AcrR and EnvR protein showed weak binding upstream of multiple, global targets and the overexpression of *acrR* or *envR* altered the expression of these target genes. Therefore, AcrR and EnvR may have roles in addition to regulating *acrAB.* This study also highlights that there is much heterogeneity in the TetR-family of transcription factors found in *Salmonella* and *Escherichia*, even between strains of the same species, with many predicted to have multiple gene targets. Therefore, TetR-family transcription factors, including AcrR and EnvR, may have wider regulatory roles than are currently known.

For Nicole

And My Parents

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Declaration #1

DECLARATION OF AUTHORSHIP

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Signed:

Abigail Colclough

Dr Jessica Blair (corresponding author)

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This is to confirm that Abigail Lucy Colclough was a contributing author to the following publication: Colclough AL, Alav I, Whittle EE, et al. RND efflux pumps in Gram-negative bacteria; regulation, structure and role in antibiotic resistance. *Future Microbiol*. 2020;15:143-157. This was published in *Future Microbiology* in 2020, with Dr Jessica MA Blair as the corresponding author.

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Signed:

Abigail Colclough

Dr Jessica Blair (corresponding author)

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List of abbreviations

Abbreviation	Definition
ml	Millilitres
µg	Micrograms
µg	Micromoles
µl	Microlitres
ABC	ATP-binding cassette
AMR	Antimicrobial Resistance
ANOVA	Analysis of variance
aph	Aminoglycoside phosphotranferase
APS	Ammonium Persulfate
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine serum albumin
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CDC	Centers for Disease Control and Prevention
DBP	Distal binding pocket
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBI	European Bioinformatics Institute
EDTA	Ethylenediaminetetra acetic acid
EMBL	European Molecular Biology Laboratory
EMSA	Electrophoetic Mobility Shift Assay
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FRT	FLP recombinase recognition target
g	Gravity
g	Grams
GFP	Green Fluorescent Protein
HD	Hannah Doherty
HM	Dr Helen McNeil
HTH	Helix-turn-helix
ID	Identification
IPTG	Isopropyl β- d-1-thiogalactopyranoside
IS1	Insertion element 1
IS10	Insertion element 10
IS2	Insertion element 2
JS	Jacob Scadden
KO	Knock out
L	Litre
LB	Luria Bertani
LPS	Lipopolysaccharide

MATE	Multidrug and toxic compound extrusion family
MDR	Multi-drug resistant
MEME	Motif-based sequence analysis tools
MFS	Major facilitator superfamily
MIC	Minimum inhibitory concentration
MJ	Dr Mohammed Jamshad
MOPS	3 -(N -morpholino)propanesulfonic acid
MRSA	Methicillin-resistant Staphylococcus auereus
MUSCLE	Multiple sequence comparison by log-expectation
NCBI	National Center for Biotechnology Information
ng	Nanograms
OD	Optical Density
OMP	Outer membrane protein
PACE	Proteobacterial antimicrobial compound efflux
PAP	Periplasmic adaptor protein
PBP	Proximal binding pocket
PBS	Phosphate Buffered Saline
PCR	Polymerise Chain Reaction
PDB	Protein database
PEF	Protein expression facility (Unviersity of Birmingham)
PVDF	Polyvinylidene difluoride
qRT-PCR/RT-PCR	Quantative Real-Time PCR
RNA	Ribonucleic acid
RND	Resistance Nodulation Division
SDS	Sodium dodecyl sulphate
SDS PAGE	sodium dodecyl sulfate polyacrylamide-based discontinuous gel
SEC	Size exclusion chromatography
SMR	Small multidrug resistance proteins
SNP	Single nucleotide polymorphism
SOC	Super optimal broth with catabolite repression
SPI	Salmonella pathogenicity islands
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TBST	Tris-buffered saline + tween
TBSTB	Tris-buffered saline + tween + bovine serum albumin
TCS	Two-component system
TEMED	N,N,N′,N′-Tetramethyl ethylenediamine
TFTR	TetR-Family Transcription Factor
WHO	World Health Organisation

Chapter One

Introduction

1.0 Introduction

1.1 *Salmonella*

Salmonella are Gram-negative rod-shaped bacteria of the *Enterobacteriaceae* family. The *Salmonella* genus is divided into two species: *S. enterica* and *S. bongori.* These species are then further subdivided based on the presence and identification of O- and H- antigens on the cell surface and flagella (Brenner, Villar, Angulo *et al.*, 2000). There are currently over 2,500 serotypes of *Salmonella* (WHO), but most of these serotypes belong to *S. enterica* subspecies I, with the most common O-antigen serogroups of A, B, C1, C2, D and E responsible for approximately 99% of reported human and animal infections (Popoff, Bockemuhl, & Brenner, 2000). *Salmonella* species are incredibly adaptable and in addition to their survival in hosts, they can survive in both wet and dry environments for up to several months as persistent biofilms (Aviles, Klotz, Eifert *et al.*, 2013). As such, reservoirs of pathogenic *Salmonella* can exist within the environment and can pose a recurrent threat to animal and human health.

1.2 *Salmonella* infections and treatment

Salmonella is a human pathogen responsible for several diseases, including salmonellosis, typhoid fever and bacteraemia. Salmonellosis is caused by ingesting food contaminated with *Salmonella* and is characterised by fever, acute vomiting and diarrhoea (WHO, 2018). Salmonellosis is one of the most common causes of enteric disease and is associated with higher mortality and hospitalisation rates in patients with pre-existing chronic conditions (Cummings, Kuo, Javanbakht *et al.*, 2016). An estimated 150,000 deaths globally are caused by salmonellosis every year, with the majority of deaths occurring outside Western Europe (Majowicz, Musto, Scallan *et al.*,

2010). *Salmonella* infections are usually self-limiting and do not usually require antibiotic treatment. However, individuals who are immunocompromised, (e.g. patients undergoing chemotherapy), are much more at risk from *Salmonella* bacteraemia, especially from non-typhoidal serovars (Gordon, 2008). There are also emerging strains of *Salmonella* which pose an increased risk to human health. For example, an isolate of *S.* Enteritidis was identified as the causative agent of recurrent bacteraemia and this was found to be due to this isolate having a 'super-mutator' phenotype after gaining mutations in DNA mismatch repair gene *mutS* (Klemm, Gkrania-Klotsas, Hadfield *et al.*, 2016) .

1.3.0 Antibiotic resistance

Since the discovery of penicillin in 1928, we have relied heavily on antibiotics to treat bacterial infections. Hailed as the 'Antibiotic Era', over twenty new classes of antibiotics were discovered between 1950 and 1960. Since then, global demand for antibiotics has remained high, with global consumption rising by 40% between 2000 and 2010 (O'Neil, 2014). Despite such demand, the discovery of new antibiotics has slowed dramatically, with only one new novel-class antibiotic, Teixobactin, being discovered in the last 30 years (Ling, Schneider, Peoples *et al.*, 2015). This reliance on existing antibiotics, in combination with widespread over-use of antibiotics in general has led to an increase in antibiotic resistance.

Antibiotic resistance is defined as the ability of bacteria to resist the inhibitory or killing activity of an antibiotic, rendering the treatment ineffective and allowing the bacterial infection to persist. Our over-use and reliance on antibiotics has put selective pressure on bacteria and has promoted the rise of resistant strains. Some bacteria have gained resistance to multiple classes of antibiotics and are referred to as being multi-drug

resistant (MDR). Forecasters predict that up to 10 million deaths a year will be caused by resistant infections by 2050 (O'Neil, 2014). The rise in resistance was predicted by Fleming himself, who stated that: *‘"The thoughtless person playing with penicillin treatment is morally responsible for the death of the man who succumbs to infection with the penicillin-resistant organism"*. Tackling antibiotic over-use and encouraging antimicrobial stewardship are the first steps in preserving any novel compounds discovered in the future.

1.3.1 Mechanisms of resistance

Bacteria have evolved a plethora of mechanisms for developing resistance to antibiotics, some of which are believed to be at least 800 million years old (Baltz, 2006). Bacterial resistance can be pre-determined (*intrinsic*) or can be gained through gaining genetic material or mutations (*acquired*). Intrinsic resistance occurs due to inherent structural or functional features which protect the bacteria from the actions of the antibiotic. The structural differences between Gram-positive and Gram-negative bacterial cell walls, for example, often cause differences in antibiotic susceptibility. The glycopeptide antibiotic vancomycin targets the D-Ala D-Ala residues in the peptidoglycan layer of the cell wall but is unable to penetrate the cell envelope present in Gram-negative bacteria. Consequently, Gram-negative bacteria are intrinsically resistant to glycopeptides.

Acquired resistance, on the other hand, is not pre-determined. Bacteria can acquire genetic material on mobile elements (e.g., plasmids) or can accumulate spontaneous mutations which result in increased resistance to an antibiotic class. Mechanisms of resistance can be grouped into the following categories: (1) those involving the inactivation of the antibiotic, usually by hydrolysis or inactivation (e.g. The action of β-

lactamase enzymes), (2) those that change the antibiotics' target so that it can no longer bind and cause effect (e.g. alterations in penicillin-binding proteins, causing decreased binding efficiency of penicillin) (Zapun, Morlot, & Taha, 2016) and (3) those which prevent intracellular concentrations of antibiotic rising high enough to cause sufficient killing/inhibitory effect, usually by increased efflux and/or reduced influx (e.g. acquiring a plasmid which promotes overexpression of an efflux pump).

In the 1940s, Ernst Chain observed that penicillin could be chemically inactivated and he theorised, (even before the widespread use of antibiotics), that this vulnerability could allow bacteria to become resistant (Abraham & Chain, 1988). We now know that this is an example of acquired resistance and that β-lactamase enzymes are commonly passed between bacteria by horizontal gene transfer, allowing the inactivation of β-lactam antibiotics. Horizontal gene transfer can occur by either conjugation, transformation, transduction or gene transfer agents. Conjugation can allow previously non-mobile elements to become mobilised and therefore transferable between bacteria. Some of these mobile elements are plasmids capable of carrying multiple resistance genes simultaneously. For example, the IncQ family of plasmids are usually non-conjugational (i.e. non-mobile) but are capable of being mobilised for conjugation (Meyer, 2009). A sub-family of IncQ plasmids, IncQ1, were identified in clinical *S*. Typhimurium isolates in Italy, and confer resistance to sulfamethoxazole, streptomycin and tetracycline (Oliva, Monno, D'Addabbo *et al.*, 2017). Worryingly, *Salmonella* containing plasmids with the *mcr-1* colistin resistance gene have also been isolated from clinical samples (Lu, Quan, Zhao *et al.*, 2019). Colistin is often referred to as an antibiotic of 'last resort' as it is the last remaining treatment for some highly resistant infections. A single bacterial strain may therefore gain multiple acquired resistance

mechanisms in addition to any pre-existing intrinsic resistance, producing a multidrug resistant strain.

1.3.2 Efflux-mediated resistance

Efflux pumps are transport proteins that allow the removal of noxious compounds, including antimicrobials, from within the cell back out into the extracellular environment. All efflux pumps are energy-dependent, requiring either a primary (i.e. ATP) or secondary (i.e. coupling to electrochemical gradient) energy source (Webber & Piddock, 2003). These pumps are currently organised into Six superfamilies based on structural similarity and energy source, being classified as either (i) Resistance nodulation division (RND) transporters, (ii) Small multidrug resistance (SMR) transporters, (iii) Major facilitator superfamily (MFS) transporters, (iv) Multidrug and toxic compounds extrusion (MATE) transporters, (v) ATP-binding cassette (ABC) transporters, or (vi) Proteobacterial antimicrobial compound efflux (PACE) (Figure 1.1) (Sun, Deng, & Yan, 2014).

Although some efflux pumps (i.e. Tetracycline pumps) are highly specific, many have multiple binding pockets which can transport structurally dissimilar compounds (Ramos, Martinez-Bueno, Molina-Henares *et al.*, 2005). Consequently, increased expression of these multi-substrate efflux systems enables bacteria to gain multidrug resistance via the overexpression of these systems. For example, increased expression of *acrAB* causes a decreased susceptibility to multiple antibiotic classes including quinolones, tetracycline and chloramphenicol in addition to various other dyes, detergents and antiseptic agents (Baucheron, Tyler, Boyd *et al.*, 2004).

Figure 1.1: The six superfamilies of efflux systems

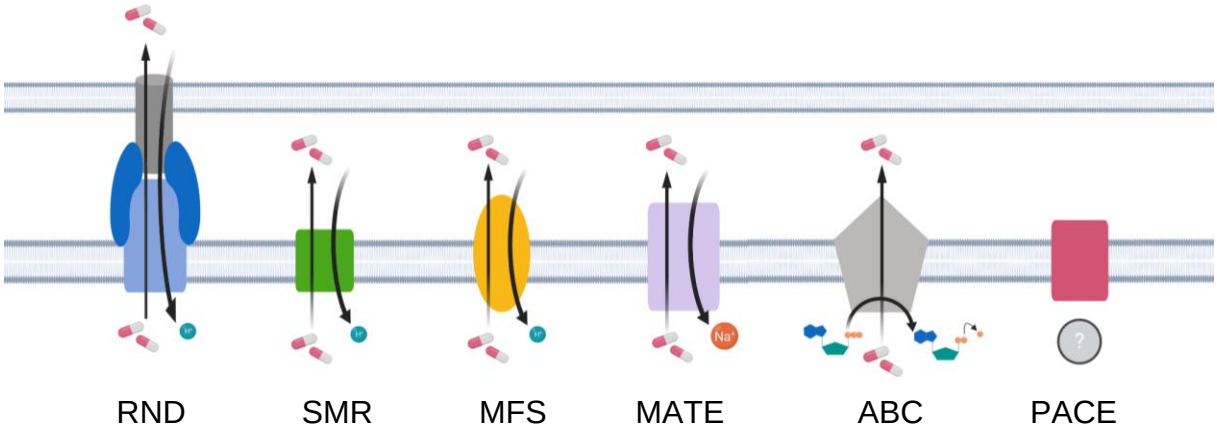


Figure 1.1: The six superfamilies of efflux pumps. Resistance nodulation division (RND) transporters, Small multidrug resistance (SMR) transporters, Major facilitator superfamily (MFS) transporters, Multidrug and toxic compounds extrusion (MATE) transporters, ATP-binding cassette (ABC) transporters and Proteobacterial antimicrobial compound efflux (PACE). The transport mechanism of the PACE family is currently unknown.

Of the nine efflux pumps known to be present in *S.* Typhimurium, AcrAB is known to be constitutively expressed (Nishino, Latifi, & Groisman, 2006) and is also commonly overexpressed in MDR isolates (Giraud, Cloeckaert, Kerboeuf *et al.*, 2000; Piddock, White, Gensberg *et al.*, 2000). Efflux genes are usually chromosomally encoded but some are plasmid-encoded and transmissible between strains, for example the *qacA* (Costa, Ntokou, Martins *et al.*, 2010) and *tetA* genes (Guarddon, Miranda, Rodriguez *et al.*, 2011).

1.4 Physiological roles of efflux systems

Efflux systems are found in all microbiological life and are often chromosomally-encoded, indicating that their function is important and has been conserved. The preservation of efflux mechanisms in bacteria existing in environments lacking antibiotic selective pressure supports the fact that efflux systems have additional roles in bacterial physiology (Martinez, Sanchez, Martinez-Solano *et al.*, 2009). In fact, reduced fitness of *Salmonella* is observed after inhibiting *acrD* expression (Buckner, Blair, La Ragione *et al.*, 2016), and mutants deficient in any of the AcrAB-tolC proteins have altered motility and biofilm-producing phenotypes (Webber, Bailey, Blair *et al.*, 2009; Yamasaki, Wang, Hirata *et al.*, 2015).The deletion of *acrAB* also results in reduced virulence in *Salmonella* (Wang-Kan, Blair, Chirullo *et al.*, 2017), and reduced virulence in *Enterobacter* (Perez, Poza, Fernandez *et al.*, 2012) and *Klebsiella* (Padilla, Llobet, Domenech-Sanchez *et al.*, 2010).

Although efflux pumps are often studied due to their relevance in MDR infections, the broad substrate specificity of pumps such as AcrAB-TolC indicates that efflux of antimicrobial compounds is not the only function of these systems. Efflux pumps can extrude many compounds in addition to antibiotics, including polysaccharides, organic

solvents, host factors (e.g. host lipids) and quorum sensing molecules. Efflux systems are known to be involved with more than just drug resistance, but there is much still to learn about the roles of these systems, and it is difficult to predict what the functions of these systems are *in vivo*. (Piddock, 2006b). AcrAB, in addition to EmrAB and MdtEF, are thought to be responsible for the transport of fatty acid substrates in *E. coli* (Lennen, Politz, Kruziki *et al.*, 2013). Other RNDs are implicated in cell-cell communication, with MexAB-OprM of *Pseudomonas* extruding acylated homoserine lactones, important quorum sensing compounds of *Pseudomonas* species (Minagawa, Inami, Kato *et al.*, 2012). Thus, although efflux systems are mostly studied in relation to their known role and clinical relevance as exporters of antibiotics, this may not represent their original function.

In addition to extruding antibiotics, dyes and detergents, AcrAB provides intrinsic protection against environmental or host factors, such as indole and bile. These are considered natural substrates of AcrAB. Efflux in response to low, sub-lethal concentrations of bile salts has been shown to be essential in the adaptation of *S.* Typhimurium to bile, with prolonged exposure (and therefore prolonged increased *acrAB* expression) leading to the subsequent evolution of resistance genotypes (Urdaneta & Casadesus, 2018).

Loss of function in individual pump components also has consequences for virulence and biofilm formation. A decrease in virulence is seen when cells lack functional AcrA (Blair, La Ragione, Woodward *et al.*, 2009), AcrB (Wang-Kan *et al.*, 2017) and TolC (Baucheron, Mouline, Praud *et al.*, 2005). The interruption of *acrB* is also linked to altered anaerobic growth and reduced expression of virulence determinants (e.g. SPI-1 and SPI-2) (Webber *et al.*, 2009).

1.5 The RND family of efflux systems

The Resistance Nodulation Division (RND) family of secondary transporters are present in all Gram-negative bacteria studied to date, including *S. enterica* (Poole, 2007). Many RNDs have broad substrate specificities (e.g. AcrAB and AcrEF). There are exceptions to this however, for example AcrD is thought to be an aminoglycoside-specific transporter (Rosenberg, Ma, & Nikaido, 2000).

The RNDs have a conserved tripartite structure composed of an outer membrane protein (OMP) and the RND pump itself in the inner membrane, which are in turn joined by a periplasmic adaptor protein (PAP) (Anes, McCusker, Fanning *et al.*, 2015). Some of the OMP proteins of RND transporters are promiscuous and able to complex with components from other efflux families and can therefore 'rescue' the activity of other pumps lacking an OMP. TolC, for example, can also complex with MacA and MacB in both *Salmonella* and *E. coli* (Turlin, Heuck, Simoes Brandao *et al.*, 2014).

Similarly, the PAPs have also been shown to be promiscuous; AcrA is required for the function of AcrD in *S.* Typhimurium (Yamasaki, Nagasawa, Hayashi-Nishino *et al.*, 2011) and in the absence of functional AcrA, AcrE is able to function with AcrB (McNeil, Alav, Torres *et al.*, 2019; Smith & Blair, 2014). This promiscuity may enable the maintenance of efflux function and therefore resistance, even if one or more efflux system is inhibited (Anes *et al.*, 2015) and begins to explain how such redundancy in RND genes can be useful. Moreover, in the absence of functional AcrAB, *acrEF* is upregulated in response to quinolone stress in the absence of functional AcrB (Zhang, Chang, Yang *et al.*, 2018). The conservation of *acrEF* genes may therefore provide a 'back-up' broad-specificity RND in the absence of *acrAB* or in response to currently unknown *acrEF*-inducing signals.

1.6.0 The AcrAB-TolC efflux system

1.6.1 Structure and mechanism of transport

The AcrAB-TolC efflux system is a tripartite system consists of the same components as seen in other RND systems. A trimer of the RND transporter protein AcrB (Murakami, Nakashima, Yamashita *et al.*, 2002) binds a hexamer of the periplasmic adaptor protein AcrA (Mikolosko, Bobyk, Zgurskaya *et al.*, 2006), which then links to OMP protein TolC (Andersen, Hughes, & Koronakis, 2001) to form the final membrane-spanning complex (Figure 1.2). To extrude a substrate, the tripartite structure of an RND pump goes through several conformational changes (rotations denoted as access, binding and extrusion) to move substrates from the binding pockets, through to the funnel TolC and then out to the extracellular environment (Eicher, Cha, Seeger *et al.*, 2012). The assembly of AcrAB-TolC is sequential and contains an intermediate state where AcrAB complexes are not bound to TolC. AcrA has direct contact with the peptidoglycan and can therefore ‘anchor’ the complex in place whilst docking and full assembly with TolC is achieved (Shi, Chen, Yu *et al.*, 2019).

1.6.2 Substrate binding, range and specificity

AcrAB-TolC has a large range of known substrates, including a range of antibiotics (e.g. tetracycline, ciprofloxacin, chloramphenicol, nalidixic acid and minocycline), dyes and detergents (Nikaido & Pages, 2012). The ability to extrude a variety of chemically dissimilar compounds is due in part to the presence of two substrate binding pockets, the proximal binding pocket (PBP) and the distal binding pocket (DBP), which allow the recognition and transport of compounds differing in size and hydrophobicity (Eicher *et al.*, 2012).

Figure 1.2: Cryo-EM structure of AcrAB-TolC

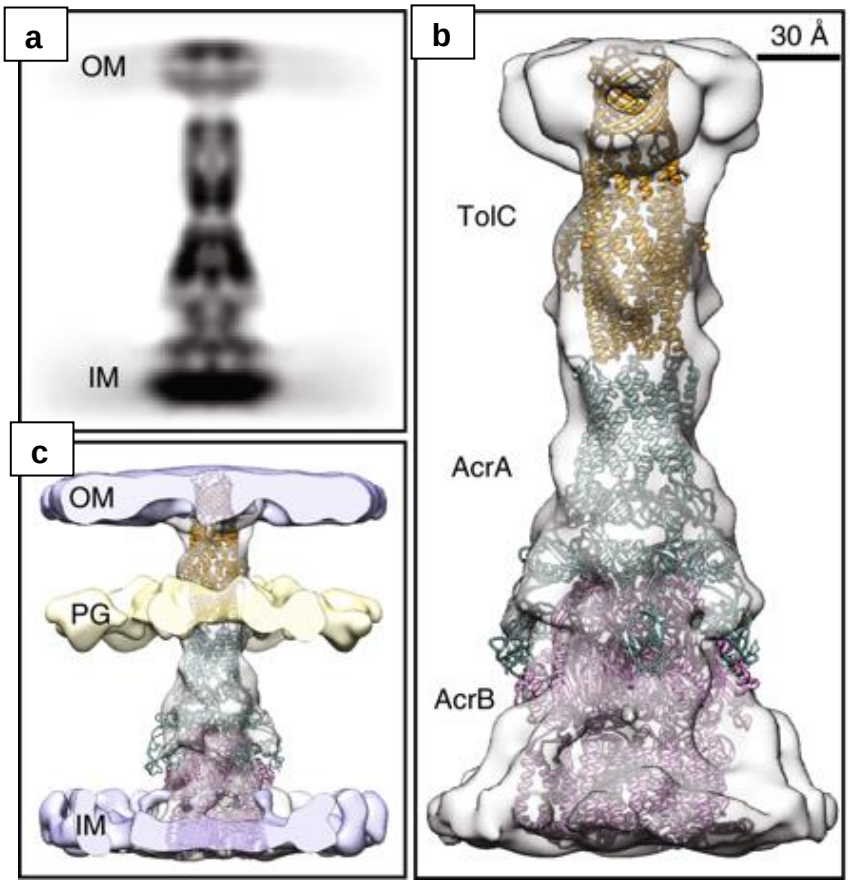


Figure 1.2: Structure of AcrAB-TolC adapted from (Shi *et al.*, 2019). Showing (a) Side-view of AcrAB-TolC complex, (b) Rendering of PDB:5V5S with cryo-EM model and (c) Isosurface rendering superimposed with density map of the cell envelope. OM = outer membrane, IM = inner membrane, PG = peptidoglycan.

Low molecular mass compounds bind directly to the DBP before being extruded, whereas high molecular mass compounds must initially bind the PBP before being translocated to the DBP and extruded (Nakashima, Sakurai, Yamasaki *et al.*, 2011). Therefore, the transport of a given substrate is dependent on the placement of specific residues which enable drug recognition, binding and translocation. Polymorphisms in AcrB can therefore either reduce or increase the susceptibility to a substrate. For example, the G288D mutation decreases susceptibility to ciprofloxacin by altering the distal binding pocket (Blair, Bavro, Ricci *et al.*, 2015). In contrast, the combination of I38F and I671T mutations in AcrB can provide unchanged or decreased susceptibility to larger substrates but increased susceptibility to larger, distal pocket-binding substrates (Schuster, Vavra, & Kern, 2016). Polymorphisms in the periplasmic and transmembrane domains are likely to have vastly different effects on AcrB function, with the former influencing drug binding affinity and the latter influencing the dynamics of drug binding (Soparkar, Kinana, Weeks *et al.*, 2015). It is also possible that substrates also compete for binding (Bohnert, Karamian, & Nikaido, 2010; Takatsuka, Chen, & Nikaido, 2010). In addition to the presence of multiple binding pockets, AcrB also has multiple translocation channels (i.e. pathways through which drugs can migrate). This allows partial structural remodelling of AcrB in response to different substrates, enabling optimal substrate translocation (Zwama, Yamasaki, Nakashima *et al.*, 2018). The true substrate specificity and range of AcrB is still underestimated due to our current limited understanding of the natural substrates of AcrB.

1.6.3 Relevance in AMR

The increased expression of *acrAB* was first identified in 1990's as a mechanism of resistance (McMurry, Oethinger, & Levy, 1998; Okusu, Ma, & Nikaido, 1996) and has since been reported in both clinical (Piddock, 2006a) and veterinary (Sato, Yokota, Ichihashi *et al.*, 2014) isolates. Recently, AcrAB-mediated resistance to tigecycline was reported in *E. cloacae* (Liu, Jia, Zou *et al.*, 2019) and *K. pneumoniae* (Xu, Zhou, Zhai *et al.*, 2016). There is also preliminary evidence that *acrB* expression is increased in response to carbapenems (Chetri, Bhowmik, Paul *et al.*, 2019). However, aside from one molecular docking study (Atzori, Malviya, Malloci *et al.*, 2019) there is no other evidence to suggest that carbapenems are substrates of AcrB. It is possible that other drugs may interact with AcrAB-TolC or it's regulators to influence *acrAB* expression. Due to the broad substrate range of AcrB, increased *acrB* expression confers multidrug resistance and the resulting infections by these *acrB*-overexpressing organisms are more difficult to treat.

Although *acrB* overexpression itself leads to a multi-drug resistant phenotype, the resulting increased fitness of an isolate in response to a given antimicrobial can also promote the acquisition of further resistance-encoding mutations (Frimodt-Moller & Lobner-Olesen, 2019). Chromosomal mutations in resistance determinants such as *acrAB* are also known to predispose to the acquisition and maintenance of further resistance plasmids (Bottery, Wood, & Brockhurst, 2019).

1.7.0 The AcrEF-TolC efflux system

1.7.1 Structure and substrates

The AcrEF-TolC efflux system consists of the periplasmic adaptor protein AcrE, membrane transporter AcrF and the outer membrane channel protein TolC. AcrE and AcrF share homology with AcrA and AcrB, respectively. The crystal structure of AcrEF-TolC has not yet been solved, but due to the homology between AcrB and AcrF, it is hypothesised that AcrEF-TolC will be structurally similar and they have been shown to transport many of the same substrates. Overexpression of *acrEF* decreases susceptibility to known substrates of AcrB (Nishino & Yamaguchi, 2004). There is evidence that AcrF can function with either AcrA or AcrE and requires TolC for function (Kobayashi, Tsukagoshi, & Aono, 2001). It is not clear as to whether shared substrates of AcrB and AcrF have a 'preferred' transporter. It was proposed that indole was preferentially an AcrEF substrate (Kawamura-Sato, Shibayama, Horii *et al.*, 1999). However, the loss of indole efflux in the Δ*acrEF* strain may also be due to indirect, unknown effects of interrupting *acrEF.* The substrates which specifically induce *acrEF* expression are currently unknown.

1.7.2 Relevance in AMR

AcrEF is not thought to play as large a role in antimicrobial resistance compared to AcrAB. Overexpression of *acrEF* does decrease susceptibility to a panel of known AcrB substrates, however this decrease in susceptibility is greater when *acrB* itself is overexpressed (Olliver, Valle, Chaslus-Dancla *et al.*, 2005).

In the absence of AcrAB, the expression of *acrEF* was shown to increase in response to quinolone stress (Zhang *et al.*, 2018). The expression of *acrE* was reported to increase under levofloxacin and ofloxacin stress, in a dose-dependent manner (Chetri,

Dolley, Bhowmik *et al.*, 2018). However, the same authors do not report the same high fold-change increases for *acrF* in response to levofloxacin or ofloxacin. It is known that AcrA and AcrE are promiscuous, with both PAPs able to form tripartite complexes with AcrB (McNeil *et al.*, 2019; Smith & Blair, 2014). It is also known that the stability and degradation of AcrB protein depends on the availability of AcrA (Chai, Webb, Wang *et al.*, 2016). Therefore, it could be possible that the stability of AcrF relies not only on the availability of AcrE protein but also, the relative affinity of AcrF for AcrE compared to AcrB for AcrE. Expression of *acrEF* is therefore thought to occur (1) when there is a lack of functional AcrAB, (2) in conditions which remove H-NS or (3) due to the presence of a currently unknown, AcrEF-specific substrate or induction signal.

1.8.0 Regulation of RND systems in Gram-negative bacteria

The regulation of RND efflux systems is mediated by both local and global regulators acting to fine-tune gene expression in response to a range of signals. Some of these signals, such as increased intracellular antibiotic concentration, lead to temporary increases in RND gene expression. Mutations permitting increased RND gene expression can confer increased tolerance or resistance to antimicrobials. Due to the poly-substrate specificity of some RND systems (e.g. AcrAB), an increase in RND expression can confer multi-drug resistance. Increased *acrAB* expression is achieved via either (1) upregulation of the expression of *acrAB* via the action of an inducer, (2) upregulation of *acrAB* expression via the action of positive regulators (e.g. RamA) or (3) inactivation of a local negative regulator (e.g. AcrR).

The expression of *acrEF* is low in both *Salmonella* and *Escherichia* under laboratory conditions (Eaves, Ricci, & Piddock, 2004; Hirakawa, Takumi-Kobayashi, Theisen *et al.*, 2008). The GC% content of the *acrEF* genes and the associated negative

regulatory gene *envR* is lower than that for the rest of the *Salmonella* genome. As low GC% genes in *Salmonella* are targeted for silencing by H-NS, the *acrEF* genes are thought to be under the negative regulation of H-NS. Hypothetically, substrates of AcrEF could act to upregulate *acrEF* expression, but this has not been tested. It is possible that AcrEF functions only as a “back-up” to AcrAB. However, it is more likely that there are conditions, (in addition to those which allow the removal of H-NS), which can differentially induce *acrEF* expression.

Interruption of the *hns* gene decreases drug susceptibility in *Salmonella* (Nishino, Hayashi-Nishino, & Yamaguchi, 2009). It is hypothesised that the interruption of *hns* results in increased *acrEF* expression and therefore, decreased susceptibility to AcrB/AcrF substrates (Nishino *et al.*, 2009). H-NS is known to be relieved by SPI-2 inducing conditions (Choi, Shin, Yoon *et al.*, 2010) and the *acrEF* operon itself is known to be directly upregulated by LeuO, an antagonist of H-NS (Shimada, Bridier, Briandet *et al.*, 2011). Therefore, although the expression of *acrEF* is silenced under many conditions, it is possible that AcrEF plays a role in the extrusion of AcrB substrates (or other, unknown AcrEF-specific substrates) under SPI-2 inducing conditions.

1.8.1 Positive regulation

The induction of RND efflux expression is mediated primarily by AraC/XylS family regulators in *Enterobacteriacae*. For example, in *E. coli*, MarA, SoxS and Rob are responsible for the induction of *acrAB* in response to the presence of noxious substances or stressors. These global regulators bind a degenerate sequence, denoted the mar/sox/rob box, located upstream of *acrAB* and in other locations across the *E. coli* genome (Martin & Rosner, 2002). MarA is autoregulated by local regulator MarR and mutations in MarR confer AMR through increased *marA* expression and

therefore, increased *acrAB* expression (Ma, Alberti, Lynch *et al.*, 1996a). MarA, SoxS and Rob all activate the *marRAB* promoter in *E. coli* in response to their inducer molecules salicylate, paraquat and decanoate, respectively (Chubiz, Glekas, & Rao, 2012). Both MarA and SoxS are also able to repress the rob promoter (Michan, Manchado, & Pueyo, 2002; Schneiders & Levy, 2006). This cross talk between regulators fine-tunes the induction of *acrAB* in response to different environmental cues. The relative importance of these regulators also varies between *Enterobacteriacae* species, with RamA being the predominant inducer of *acrAB* expression in *S.* Typhimurium and *S.* Cholerasuis (Usui, Nagai, Hiki *et al.*, 2013). *Klebsiella* also has RamA (George, Hall, & Stokes, 1995) along with two further AraC/XylS proteins which regulate *acrAB* expression: RomA (Rosenblum, Khan, Gonzalez *et al.*, 2011) and RarA (Veleba, Higgins, Gonzalez *et al.*, 2012). The levels of the AraC/XylS proteins are further regulated by targeted degradation by Lon protease (Griffith, Shah, & Wolf, 2004).

There is much heterogeneity and variation in how different RNDs are induced in Gram-negative species. Individual or multiple regulators may be responsible for the response to a signal. RamA activates both *acrAB* and *acrEF* expression in *S.* Typhimurium (Zheng, Cui, & Meng, 2009). The expression of *ramA* is in turn negatively regulated by TFTR RamR. Negative regulation by RamR is relived through ligand binding and RamR has been co-crystalised with bile salts and is also thought to interact with other substrates of AcrAB such as ethidium bromide and rhodamine 6g (Yamasaki, Nakashima, Sakurai *et al.*, 2019). However, not every antibiotic substrate of AcrAB increases *ramA* expression, indicating that the induction of AcrAB is not RamA-

dependant for all substrates (Lawler, Ricci, Busby *et al.*, 2013). RamA is also known to have wider roles in *K. pneumoniae*, including RamA-mediated alterations in lipid A of the LPS, permitting better macrophage attachment and uptake (De Majumdar, Yu, Fookes *et al.*, 2016). The inducers of RND systems therefore interact not only with local negative regulators of the RND genes, but also the negative regulators of activators and sometimes also directly with the activator proteins.

SoxR mutants in *A. baumannii* show decreased expression levels of RND genes *adeJ* and *adeG*, but not *adeA* (Li, 2017). Therefore, at least in *A. baumannii,* SoxRS influences the expression of multiple RNDs. Further regulatory roles of these proteins are being discovered, MarA is now known to regulate lipid trafficking and DNA repair in addition to its roles in regulating *acrAB* (Sharma, Haycocks, Middlemiss *et al.*, 2017). The same authors show that MarR, the regulator of MarA is a single-target regulator, which only regulates the expression of *marA.* Recently, it is being acknowledged that regulators are not commonly single-target and that single-target, targeted regulation may be the exception, not the rule (Shimada, Ogasawara, & Ishihama, 2018). The activation of RNDs is therefore complex and the mechanism of activation in response to a signal in one species cannot be assumed to apply to other species.

1.8.2 Negative regulation

Most, but not all, RND systems are encoded alongside, and transcribed divergently from a negative regulator. These regulators are commonly TetR-family regulators (TFTRs), such as AcrR, which negatively regulates expression of *acrAB* (Ma *et al.*, 1996a). Induction of *acrAB* expression therefore first requires the removal of this negative regulation. This is achieved through ligand binding to AcrR, altering the conformation of AcrR and preventing it from binding to and repressing the *acrAB*

promoter. The ligands able to bind these negative regulators often correspond to known substrates of the RND system. These regulators therefore act as one-component signalling proteins. Mutations conferring a loss-of-function of AcrR are reported in *S.* Typhimurium (Olliver, Valle, Chaslus-Dancla *et al.*, 2004), *E. coli* (Webber, Talukder, & Piddock, 2005b) and *K. pneumoniae* (Schneiders, Amyes, & Levy, 2003). Each of these reported mutations were reported to increase the MIC of the *acrR* mutant to ciprofloxacin.

The negative regulation of RNDs is now known to be much more heterogeneous and the classical simple negative regulation acting on the *acrAB* operon does not necessarily apply to all RNDs. For example, non-TFTRs can regulate RNDs, or multiple regulators can work as a network to regulate the same RND. Moreover, not all TFTRs transcribed divergently from RND genes are necessarily negative regulators. For example, VexAB of *V. cholera* is encoded divergently from TFTR VexR, but the *vexRAB* promoter requires VexR for activation, not repression, in a dose-dependent manner (Taylor D.L., 2015). MexR, a MarR-family protein is transcribed divergently of and negatively regulates *mexAB* of *P. aeruginosa* (Evans, Adewoye, & Poole, 2001). However, MexAB is also negatively regulated by NalD and NalC, the genes for which are located elsewhere on the *P. aeruginosa* genome (Cao, Srikumar, & Poole, 2004). Such complexity is achieved through the presence of a tandem promoter in *P. aeruginosa,* with MexR and NalD targeting the distal and proximal promoter, respectively, and NalC regulating *mexAB* through indirect interactions with additional regulators (Tian, Yi, Cho *et al.*, 2016).

In *E. coli*, the TFTR EnvR which is encoded alongside AcrEF is able to negatively regulate *acrAB* with higher binding affinity than the locally-encoded TFTR AcrR

(Hirakawa, Takumi-Kobayashi*, et al.*, 2008). Thus, even negative regulators which seem to have defined, simple roles as the negative regulators of RND systems may have other targets, or may be able to activate and/or repress different targets. A known example of this is MtrR of *Neisseria,* a TFTR which can repress *mtrCDE* but also activates metabolic gene *glnE* as an 'off-target' role (P. J. Johnson & W. M. Shafer, 2015).

It is known that in *Mycobacteria*, the expression of efflux genes promotes remodelling in many metabolic pathways (Black, Warren, Louw *et al.*, 2014). The expression of RND efflux systems in *Vibrio* also influences virulence factor production (Bina, Howard, Taylor-Mulneix *et al.*, 2018). Therefore, for some organisms, the activation (or repression) of efflux genes may have wider consequences.

1.8.3 Two-component systems involved in RND regulation

Many RND systems in *Enterobacteriaceae* are also regulated by two-component regulatory systems. For example, AdeABC of *A. baumanii* is negatively regulated by AdeRS (Lari, Ardebili, & Hashemi, 2018). Two-component systems (TCS) rely on a signalling (e.g. AdeS) and response regulator (AdeR). The signal for inducing the sensor kinase AdeS is unknown, but after phosphorylation and subsequent transfer to AdeR, phosphorylated AdeR then binds and represses the promoter of *adeABC* (Chang, Huang, Sun *et al.*, 2016). AdeABC and AdeIJK, also RND systems of *Acinetobacter*, are also positively regulated by BaeSR (Lin, Lin, Yeh *et al.*, 2014). TCS may provide a secondary response to a signal (e.g. presence of antimicrobial substrate) or be induced in a concentration-dependant manner. It is hypothesised that TCS AdeRS is responsible for the initial increase in *adeABC* expression but that higher levels of antimicrobials then cause BaeRS involvement and lead to further increases

in *adeABC* expression (Lin, Lin, & Lan, 2015). BaeRS is also implicated in the regulation of *acrD* along with several other genes including RND genes *mdtABC and mdtC* (Ye, Rensing, Su *et al.*, 2017). Another TCS, CzcRS, induces expression of CzcCBA by directly binding the *czcCBA* promoter in *P. putida* in response to heavy metals (Liu, Chen, Huang *et al.*, 2015) and to imipenem in clinical isolates of *P. aeruginosa* (Fournier, Richardot, Muller *et al.*, 2013).

1.8.4 The role of H-NS

The nucleoid-associated protein H-NS also plays a role in negatively regulating RND systems. AcrEF of *E. coli* (Nishino *et al.*, 2009) and EefABC of *Enterobacter* (Masi, Pages et al. 2005) are H-NS silenced in addition to having their own locally-encoded negative regulators. EefABC is also known to present in some, but not all, strains of *Escherichia spp.* (Colclough, Scadden & Blair, 2019). H-NS is known to target and repress low GC% content genes in *S.* Typhimurium (Ali, Soo, Rao *et al.*, 2014) and the interruption of the *hns* gene increases expression of *acrEF* (Hirakawa, Takumi-Kobayashi*, et al.*, 2008). Similarly, a study of clinical isolates of *A. baumannii* found a colistin-resistant isolate with non-functional *hns* which could be complemented by re-introduction of wild-type *hns* on a plasmid (Deveson Lucas, Crane, Wright *et al.*, 2018). However, *Enterobacter* isolates that overexpress *EefABC* did not have mutations in *hns* (Masi, Pages, & Pradel, 2006). Therefore, the global regulation of one RND system does not necessarily predict the regulation of a different RND.

1.8.5 Regulatory mutations that confer AMR

Given the vast regulatory network involved in co-ordinating the expression of RND systems, the mutation of these regulators is a key mechanism of efflux-mediated resistance. Mutations which produce non-functional RamR protein lead to an increase

in *ramA* and therefore RamA-mediated activation of AcrAB. Conversely, mutations in the local negative regulator *acrR* promote increased *acrAB* expression. Mutations in AcrR are common but often co-occur with mutations in other genes (Adler, Anjum, Andersson *et al.*, 2016a). There is also evidence of plasmid-chromosome co-evolution of MDR genes, with *E. coli* carrying the tetracycline-resistance plasmid RK2 able to replicate an order of mutations, which reliably selected for *ompF,* followed by *acrR* mutations (Bottery *et al.*, 2019). The step-wise acquisition of resistance may therefore require the mutation of regulatory genes such as AcrR to permit MDR phenotype whilst compensating for the fitness cost of plasmid carriage.

Mutations in AcrR or the promoter of AcrR confer resistance to fluoroquinolones, tetracycline, amoxicillin and kanamycin (Hoeksema, Jonker, Brul *et al.*, 2019), despite the fact that Kanamycin is not thought to be an AcrAB substrate in *E. coli.* The same authors report mutations in MutL, which is recruited by MutS to enact DNA repair. Recent work has demonstrated that there is single-cell level heterogeneity in *acrAB* expression and that cells with higher *acrAB* expression have lower expression of DNA mismatch repair gene *mutS* (El Meouche & Dunlop, 2018). It is hypothesised that the decrease in DNA repair and subsequent increase in mutation frequency enables the rapid evolution of high-level resistance via the accumulation of point mutations. Whether AcrAB or regulators of this system directly or indirectly regulates *mutS* is unclear, but this highlights the plasticity of these regulatory networks and the importance- and consequences of- variable RND gene expression.

1.9.0 Regulation of *acrAB* and *acrEF* expression in *Salmonella* and *E. coli*

As described above, the regulation of RND systems in Gram-negative bacteria is complex and sometimes, organism-specific. Regulators with one known function in one

species may, for example, regulate multiple RNDs in another species. The negative and positive regulators of RND systems are often further classed into ‘global’ or ‘local’ regulator groups dependant on the location of the regulator gene to the regulated gene(s). The regulation of AcrAB and AcrEF by global and local regulators in *Salmonella* and *E. coli* are discussed below.

1.9.1 Global regulation

Global regulators can have activator or repressor roles and respond directly to changes in the environment. Responses to environmental stimuli therefore involve complex interactions between global regulators. The predominant global regulators in *S. enterica* are the XylS/AraC transcriptional regulators MarA, RamA, Rob and SoxS, which act as activators. The XylS/AraC family contain a conserved DNA polymerase binding domain and variable DNA binding domain which allow binding to target DNA. The XylS/AraC family share a conserved DNA-binding region at the C-terminus (Gallegos, Schleif, Bairoch *et al.*, 1997), which enables binding to the *acrAB* promoter and influence over *acrAB* expression (Martin, Gillette, Rhee *et al.*, 1999). The global regulators of *acrAB* bind to the same degenerate sequence denoted as the marsox box, which is located downstream of the binding site of local repressor protein AcrR and upstream of the *acrA* gene (Duval & Lister, 2013).

Of these global regulators, *ramA* upregulation causes the highest increase in *acrB* expression experimentally. RamA influences gene expression through dual pathways, responding both directly (i.e. to increased indole) and indirectly (i.e. through interactions with bile by pre-existing RamR) to environmental signals (Nikaido, Yamaguchi, & Nishino, 2008). Of the global regulators in *Salmonella*, RamA is also the only one not found in *E. coli*. Mutations in the regulator of RamA, RamR cause

overexpression of both *acrAB* and *acrEF* (Abouzeed, Baucheron, & Cloeckaert, 2008). RamR is usually bound to the promoter regions of the genes that it regulates, but mutations which cause a non-functional RamR protein can release this repression and allow higher expression of *ramA*. This overexpression of *ramA* causes ciprofloxacin resistance due to increased efflux in both in clinical isolates (Rosenblum *et al.*, 2011) and *in vitro S. enterica* (Sun, Dai, Hao *et al.*, 2011). Increased *ramA* expression also increases the expression of both *acrAB* and *acrEF* (Bailey, Ivens, Kingsley *et al.*, 2010). Mutants with a decreased susceptibility to antimicrobials also more commonly overexpress *ramA* compared to any of the other global regulators, indicating the importance of *ramA* mutations in causing resistance (Webber *et al.*, 2009). The induction of *acrAB* or *acrEF* expression in response to indole is believed to be RamA-mediated and does not involve MarA, SoxS or Rob (Nikaido *et al.*, 2008).

The remaining regulators SoxS, MarA and Rob are found in both *Salmonella* and *E. coli*. SoxR is activated in conditions of oxidative stress by either oxidation or nitrosylation (Ding & Demple, 2000), resulting in the increased expression of *soxS*. SoxS promotes upregulation *acrAB* but also upregulates *micF,* an antisense RNA involved in the repression of membrane permeability via regulation of *ompF* (Koutsolioutsou, Pena-Llopis, & Demple, 2005). SoxRS-mediated resistance to quinolones has been observed in clinical isolates of *S. enterica* (Koutsolioutsou, Martins, White *et al.*, 2001) and in combination with other mutations in veterinary isolates of *E. coli* (Webber *et al.*, 2005b).

MarA is able to upregulate *acrAB* and *micF* expression in addition to genes involved in superoxide resistance and DNA repair (Ruiz & Levy, 2010). The overexpression of *marA* is seen in MDR clinical isolates, which frequently have mutations in *marR*. A lack

of functional MarR (and therefore a lack of repression on the *marA* promoter) results in a decreased susceptibility to a range of antimicrobials (Maneewannakul & Levy, 1996), leading to the MDR phenotype.

Rob only shares around 50% structural homology with MarA and SoxS (Jair, Yu, Skarstad *et al.*, 1996). Rob is activated by the presence of bile salts (Rosenberg, Bertenthal, Nilles *et al.*, 2003) and increases expression of the *marRAB* operon, in turn upregulating expression of *acrAB*. Rob plays a role in polymyxin B resistance via upregulating *micF* (Oh, Cajal, Skowronska *et al.*, 2000).

Although the examples listed here show specific interactions between regulators and the *acrAB* operon, the regulation *in vivo* is much more complex. For example, many regulators such as the repressor H-NS target multiple efflux systems, with H-NS acting to balance the ratio of AcrB:AcrF activity *in vivo* (Nishino *et al.*, 2009). The regulation of these systems therefore relies on complex interactions between global and local regulators, plus the presence/absence of inducer compounds. A summary of the positive and negative regulation of *acrAB* and *acrEF* in *Salmonella* and *Escherichia* is shown on Figure 1.3.

1.9.2 Local regulation

The local repressor of the *acrAB* operon in both *E. coli* and *Salmonella* is the TetR-family repressor AcrR. A closely related homolog of AcrR, EnvR, is also able to repress *acrAB.* AcrR is encoded divergently from *acrAB* and *envR* is encoded alongside and divergently of *acrEF* (Hirakawa, Takumi-Kobayashi*, et al.*, 2008).

Figure 1.3: Local and global regulation of *acrAB* and *acrEF*

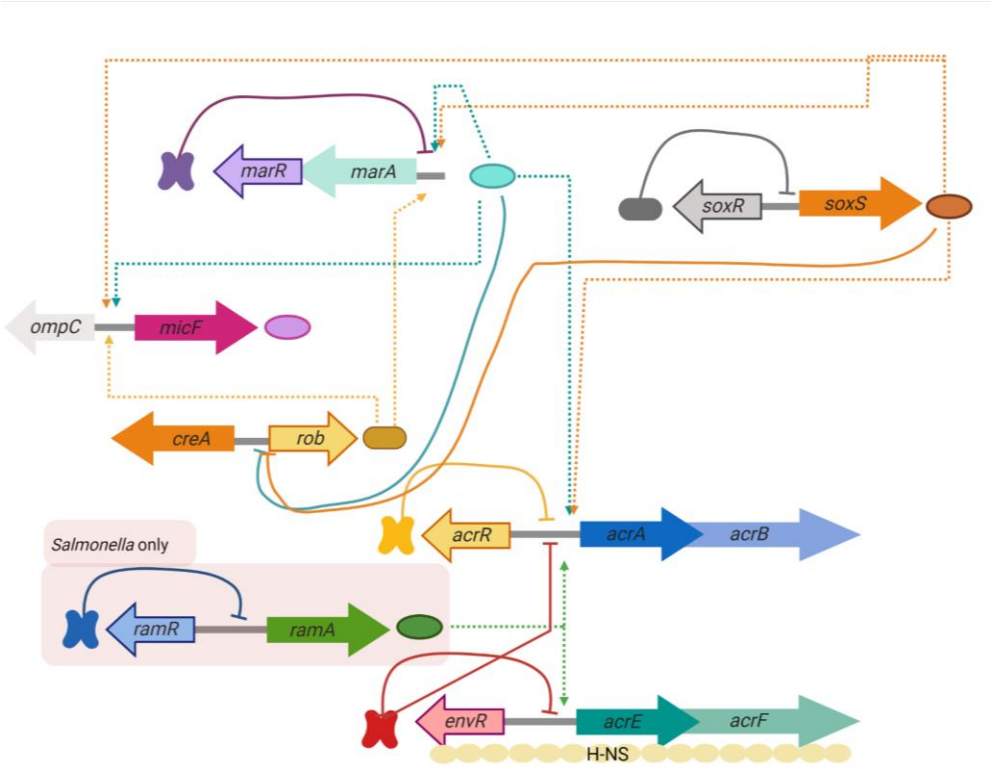


Figure 1.3: The complex network of local and global regulators of *acrAB* and *acrEF* in *Salmonella* and *E. coli.* Positive regulation (dashed arrow lines) and negative regulation (solid lines) are shown in the corresponding colour of the regulator protein. The *ramRA* genes are present in *Salmonella*, not *E. coli*, and this is highlighted by the pink box. A network of global (MarA, SoxS, Rob, RamA, EnvR and H-NS) and local (MarR, SoxR, AcrR and RamR) orchestrate the activation or repression of *acrAB* and *acrEF,* either directly (e.g., EnvR repressing *acrAB*) or indirectly (e.g., Rob upregulates *marA* which in turn upregulates *acrAB*).

1.10.0 The TetR-family of transcriptional regulators

1.10.1 Structure and DNA binding

The TetR-family of transcriptional regulators (TFTRs) are found throughout bacteria, forming single-component regulatory systems enabling fast responses to stimuli (Ramos *et al.*, 2005). The TetR family are named after the *tetR* gene, for which mutants with non-functional TetR protein are resistant to tetracyclines (Beck, Mutzel, Barbe *et al.*, 1982). Members of this family have two variable binding domains, a HTH N-terminal DNA binding domain and a C-terminal ligand binding domain. Although the DNA-binding regions show significant sequence homology between TetR regulators, the C-terminal ligand-binding regions are unique, allowing binding and therefore a regulatory response to different inducing compound(s) (Deng, Li, & Xie, 2013).

TFTRs are often able to bind and respond to the ligands which are also substrates of the locally-regulated efflux gene. For example, AcrR can bind dequilanium, which is able to bind AcrB and is extruded by the AcrAB-TolC pump (Yu, McDermott, Zgurskaya *et al.*, 2003). Currently, all the experimental evidence for AcrR ligand binding is in vitro and therefore, the binding affinities of these potential ligands to AcrR are not likely to be physiologically realistic.

Upon ligand binding to the C-terminal domain, it is proposed that a conformational change allows the separation the DNA-binding domain from target DNA, therefore exposing the RNA polymerase binding sites and allowing the expression of the efflux operon (Gu, Li, Su *et al.*, 2008). Consequently, local regulatory proteins can directly respond to increases in the concentration of toxic compounds to induce the expression of efflux pumps. Thus, in the absence of inducer signals or induction via a positive regulator, the TFTR remains bound to the efflux gene promoter, preventing the

unnecessary expression of efflux genes. In combination with the ability of global regulators to detect and respond to environmental cues, a co-ordinated response between global and local regulation help to further fine-tune bacterial efflux in constantly changing environments.

1.10.2 Roles as regulators of efflux

Up to 25% of identified TFTRs are thought to regulate the expression of efflux genes (Ahn, Cuthbertson, & Nodwell, 2012). Many efflux operons are under the control of TetR regulatory proteins, which compete directly with DNA polymerase for binding at the promoter region of efflux operons, thus preventing their expression (Manjasetty, Halavaty, Luan *et al.*, 2016a). Some substrates of efflux systems are known to also bind the TFTR regulator (i.e. as a ligand) to relieve TFTR-repression and allow the expression of efflux genes. For example, AcrR is known to bind many of the substrates of AcrB including ethidium bromide, rhodamine 6g and proflavine (Su, Rutherford, & Yu, 2007). As single-component systems, this allows the coupling of the detection of the efflux substrate (i.e. presence of antibiotic) with the subsequent activation of the efflux genes required to remove the substrate.

1.10.3 Wider regulatory roles

There are numerous examples of TFTRs regulating local genes, such as AcrR regulating the adjacent *acrAB* efflux genes. However, TFTRs are implicated in the regulation of many processes, including efflux regulation, cell division and the stress response (Cuthbertson & Nodwell, 2013; Ramos *et al.*, 2005). Some TFTRs are global regulators able to alter transcription of multiple targets throughout the genome, including those able to activate and repress multiple targets (e.g. MtrR of *Neisseria gonorrhoea* (P. J. T. Johnson & W. M. Shafer, 2015)). Another example is EnvR, which

regulates the divergently encoded local efflux operon *acrEF*, but also binds upstream and regulates expression of the efflux operon *acrAB*, which is encoded separately on the genome (Hirakawa, Takumi-Kobayashi*, et al.*, 2008).

1.11.0 AcrR and EnvR

1.11.1 Structure and homology

The crystal structure of *E. coli* AcrR has been solved (Li, Gu, Su *et al.*, 2007) and forms a homodimer (Figure 1.4). The sequences of *Salmonella* SL1344 and *E. coli* K-12 are 87% identical (Altschul, Gish, Miller *et al.*, 1990), so the structure of AcrR is expected to be similar in *Salmonella* as has been shown in *E. coli*. AcrR has a particularly large binding pocket and crystal structures of bound ligands show that AcrR may be able to bind multiple compounds simultaneously (Su *et al.*, 2007). The repressor QacR, which regulates the QacA efflux system in *S. aureus* (Grkovic, Brown, Roberts *et al.*, 1998) is the only TetR repressor confirmed to have two distinct binding pockets. The binding of AcrR/EnvR to their target sequences is thought to be similar to that of QacrR to target DNA, with repression released upon ligand binding to the repressor protein. The crystal structure of EnvR is currently unsolved, but is expected to be very similar to AcrR, given their high sequence homology and shared ability to bind upstream and regulate *acrAB* expression.

Figure 1.4: Crystal structure of AcrR

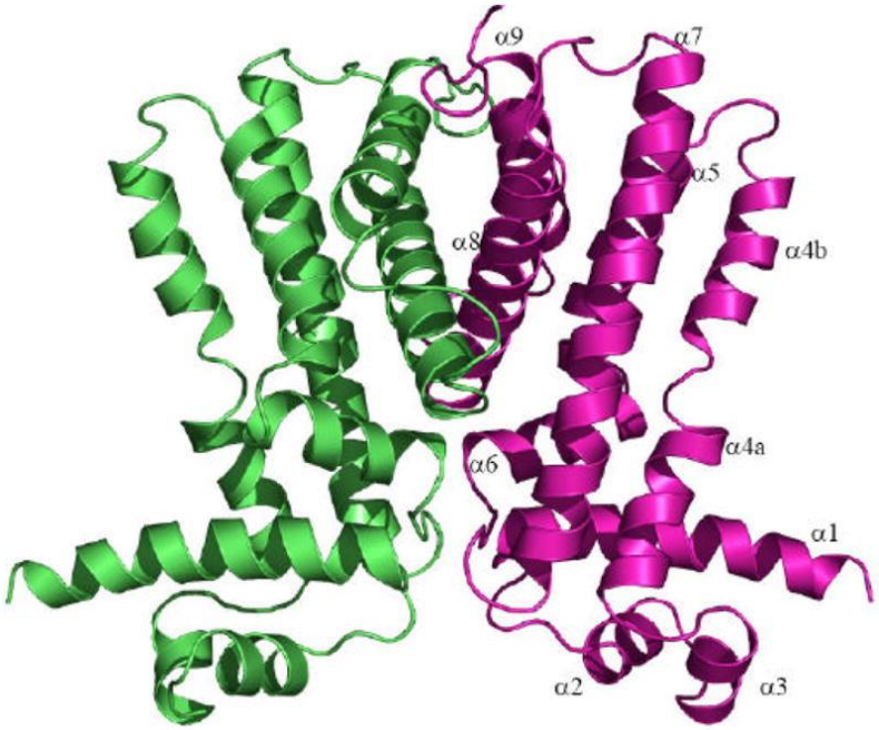


Figure 1.4: Proposed crystal structure of *E. coli* AcrR homodimer adapted from (Li *et al.*, 2007). The DNA-binding N-terminus is formed from helices α1-α3 and the ligand binding C-terminus is formed from helices α4-α9.

1.11.2 Roles as regulators of *acrAB* and *acrEF* expression

Upon its discovery in *E. coli,* the consensus was that the role of AcrR was to prevent excess transcription of *acrAB* in order to fine-tune the efflux response of AcrAB-TolC (Ma *et al.*, 1996a). However, mutations in *acrR* are sufficient to cause ciprofloxacin resistance from increased AcrAB efflux in *Salmonella* and *Klebsiella* (Olliver *et al.*, 2004), illustrating the importance of functional AcrR repression in preventing *acrAB* overexpression. EnvR was initially assumed to repress only the *acrEF* operon. However, interrupting the *envR* gene does not cause an increase in *acrEF* expression, suggesting that either EnvR is not a significant mechanism of *acrEF* repression, or is not the main method of *acrEF* repression (Olliver *et al.*, 2005). It is now known that H-NS is the predominant repressor of the *acrEF* operon, acting to bind and silence the *envR-acrEF* genes. Interestingly, EnvR has a higher binding affinity for *acrAB* than AcrR in *E. coli* and can act to repress the *acrAB* operon (Hirakawa, Takumi-Kobayashi*, et al.*, 2008). Consequently, the inducing conditions of *acrEF* expression also act to cause EnvR-mediated repression of *acrAB.* In this way, EnvR acts as a switch between the AcrAB and AcrEF efflux systems to presumably prevent the expression of both systems simultaneously. There is currently no evidence that AcrR can bind or regulate *acrEF.* The current understanding of AcrAB/AcrEF regulation in *E. coli* is outlined in Figure 1.5.

Figure 1.5: Regulation of *acrAB* and *acrEF* by AcrR and EnvR in *E. coli*

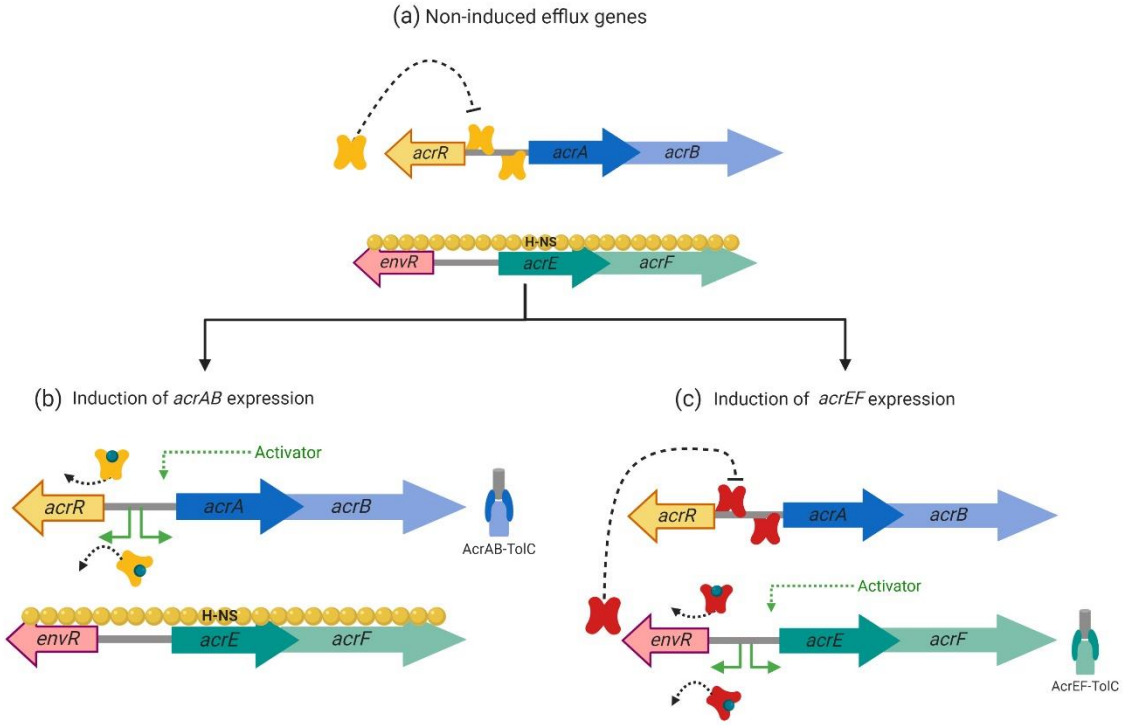


Figure 1.5: Induction of *acrAB* or *acrEF* expression in *E. coli*. (a) Under most conditions, it is believed that AcrEF is H-NS silenced. AcrAB is repressed by local regulator AcrR until induction by either an activator protein or via the binding of a ligand to AcrR. (b) Upon AcrAB induction, a ligand binds to sequester AcrR and remove AcrR-mediated repression of the *acrAB* operon. An activating protein (i.e. positive regulators MarA, SoxS, and Rob) may also bind to promote *acrAB* expression. AcrEF remains H-NS silenced in the absence of an induction signal. (c) Upon AcrEF induction, H-NS has been removed and the *acrEF* operon is now able to be activated by activator proteins. EnvR, much like AcrR, can respond to bound ligands to enable *acrEF* expression. The expression of *acrEF* and *envR* also causes EnvR to bind and repress *acrAB* expression. Therefore, it is proposed that the induction of AcrEF also promotes reduced *acrAB* expression via EnvR-mediated switching.

1.11.3 Evidence for wider roles of AcrR and EnvR

In *E. coli,* AcrR has been shown to bind upstream “off-target” genes. For example, AcrR can bind upstream of a number of regulatory genes, including *marA*, *soxS* and *micF* (Lee, Cho, & Kim, 2014; Rodionov, Gelfand, Mironov *et al.*, 2001). Interrupting the *acrR* gene induces phenotypic changes in cell motility (Kim *et al*., 2016). More recently, AcrR has also implicated in biofilm formation and virulence in *Acinetobacter* (Subhadra, Kim, Kim *et al.*, 2018). Overexpression of *acrR* also increases organic solvent tolerance (Lee *et al.*, 2014; Watanabe & Doukyu, 2012). It is unknown as to whether these functions are conserved across species or if they are species-specific, or whether AcrR and EnvR could share these roles. In addition to it’s roles in regulating *acrAB* and *acrEF,* there is very limited evidence that EnvR is a positive regulator of the RND efflux pump AcrD (Emami, 2014). The similarity of EnvR to AcrR and the fact that they seem to bind to the same region of DNA upstream of *acrAB* suggests that, like AcrR, EnvR could also have further regulatory roles in the cell.

Better understanding the regulatory roles of AcrR and EnvR would also enable a better understanding of the AcrAB and AcrEF efflux systems and could highlight currently unknown roles or substrates of these systems. Therefore, this study investigates the roles of AcrR and EnvR, but also attempts to improve the current understanding of the AcrAB and AcrEF efflux systems in *Salmonella.*

1.12 Overall Aims

1. To investigate the prevalence, conservation and function of TFTRs in *Salmonella* and *E. coli.*
2. To investigate the role of AcrR and EnvR in regulation of *acrAB* in *Salmonella*.
3. To determine whether AcrR and EnvR in *Salmonella* have “off-target” roles.
4. To investigate the conditions which induce the expression of *acrAB* and *acrEF.*

Chapter Two

Materials and Methods

2.0 Materials and Methods

2.1 Identification of TFTR genes in *Escherichia* and *Salmonella*

The purpose of the following bioinformatic analyses was to determine the number and type of TetR-family regulators present across *Escherichia* and *Salmonella* species. These methods have been published and are also available online (Colclough, Scadden, & Blair, 2019).TFTRs contain a highly conserved helix-turn-helix (HTH) domain at the N-terminus which is denoted as IPR001647 on EMBL-EBI Interpro (Mitchell, Attwood, Babbitt *et al.*, 2019). Available deposited proteomes of *Salmonella enterica* serovar Typhimurium and *Escherichia coli* (5 strains of *S.* Typhimurium and 10 strains of *E. coli*) were searched for this conserved domain and these protein sequences downloaded. This approach rapidly provided a proxy for how many TFTRs are present due to the high conservation of the HTH domain. Where possible, sequences were annotated with protein name. All proteins had their annotation manually curated using pBLAST (Altschul *et al.*, 1990)**,** producing a database of TFTR protein sequences with confirmed annotations. Orthologues were aligned using Clustal OMEGA (Goujon, McWilliam, Li *et al.*, 2010; Larkin, Blackshields, Brown *et al.*, 2007) to produce neighbour-joining trees of all TFTRs of *S.* Typhimurium and *E. coli*. For example, the sequence of *bm3R1* shared 100% identity with *ramR* and clustered with other *ramR* sequences, but without this alignment these sequences may have been incorrectly assigned an individual identity. This approach also helped to ensure that proteins with multiple names in use (e.g.. NemR/YdhM and ComR/YcfQ) were identified as one group and not duplicated.

To investigate the variation in TFTR number, type and sequence identity, a total of 15 further proteomes of the wider genera (*Salmonella enterica* subspecies (n = 9), *S. bongori* (n = 1) and *Escherichia* species *E. fergusonii (n = 2) and E. albertii (n = 3)*) were searched in the same way as described above. Any unannotated proteins were searched on pBLAST and all putative TFTRs were aligned with the confirmed ID TFTRs of either *S.* Typhimurium SL1344 or *E. coli* K- 12. TFTRs present in all strains of *Salmonella* or *Escherichia* were denoted as '*core*' for the given genera. TFTR differentially present in our analysed dataset were denoted as '*accessory*'.

2.2 TFTRs in other Gram-negative species

For the selected species on the WHO priority pathogen list the total number of IPR001647 containing-sequences were recorded alongside data on median genome length provided on NCBI. Proteomes, and not genomes, were selected for analyses in this study to enable searching for the specific HTH of TFTRs to prevent false positives.

2.3 Sequence variation of TFTRs and their regulated genes

In order to investigate the variability of TFTRs, all sequences of TFTRs in *Escherichia* and *Salmonella* ($n = 384$) were aligned using Clustal Omega and percentage sequence variation was calculated as the sum of the variable amino acid positions across all sequences of a particular gene in a genera divided by average length of the TFTR gene. Sequence length was therefore accounted for when considering percentage amino acid variation and TFTRs were grouped based on assigned function.

Known and suspected targets of all the TFTRs identified in *Salmonella* and *Escherichia spp.* were curated by searching available published literature. The amino acid sequences of each TFTR were compiled and aligned using Clustal Omega and the number of variable amino acid positions counted. This total was then divided by the mean sequence length for a given TFTR to account for variations in TFTR gene length and multiplied by 100 to give the percentage sequence variance. Here, a variable amino acid position was defined as a position with no consensus amino acid, denoted as either blank, * or ** on Clustal Omega, depending on the possible amino acid substitutions. This process was repeated for the known targets genes of the core TFTRs, excluding targets without conclusive binding studies (i.e. Electrophoretic mobility shift assay) or other proof of binding or regulation (i.e. transcriptomics, ChIP/RNA-seq).

2.4 Phylogenetic analyses

A multiple sequence alignment of the amino acid sequence of AcrB was constructed using MUSCLE (Edgar, 2004) for all strains of *Salmonella* and *Escherichia* in this study. The sequence of AcrB varies between strains of *Escherichia* and *Salmonella* and was therefore an ideal candidate for clustering our strains to our desired level of depth. This alignment was then used to construct maximum-likelihood trees with a 100 bootstrap cut-off using MEGA7 (Kumar, Stecher, & Tamura, 2016). The primary aim of these trees was to separate a small number of very closely-related strains to map whether specific TFTRs are present/absent in species of each genera, not precisely map the evolutionary distance between these strains in depth. Phandango was used to combine the presence/absence metadata with the phylogenetic analysis from MEGA7 (Hadfield, Croucher, Goater *et al.*, 2018).

Phandango then presents a simplified illustration of the phylogeny and overlays this data with the presence/absence data presented in Figures 3.2 – 3.3.

2.5.0 Construction and maintenance of bacterial strains

2.5.1 Bacterial strains

Most strains used in this work are derived from *Salmonella enterica* serovar Typhimurium SL1344 (Wray & Sojka, 1978). This is a pathogenic strain isolated from an infected calf which is used in multiple laboratories globally. Throughout this study, this strain is referred to as SL1344 or Wild type (WT). A selection of pMW82 reporter constructs (gene of interest fused to *gfp* gene) were kindly donated by Dr Mark Webber. These plasmids were transformed into various backgrounds to produce strain for GFP reporter assays.

2.5.2 Storage and growth conditions

Strains were routinely grown on lysogeny broth (LB) agar (Sigma-Aldrich Ltd., UK) or in LB liquid media (Sigma-Aldrich Ltd., UK). Overnight cultures were grown at 37°C overnight with aeration. If required, media was supplemented with antibiotics (Table 2.1). Working and long-term stocks were produced in duplicate using beads (Technical Service Consultants Ltd., UK). *Salmonella* strains were stored at -20°C and *E. coli* at -80°C.

Table 2.1: Strains used in this study

Strain code	Genotype (+plasmid)	Resistance	Reference
	Chapter 4 and 5		
SE01 / WT	*S. enterica* serovar Typhimurium SL1344	-	(Wray & Sojka, 1978)
SE02	SL1344 Δ*acrB*	-	(Eaves *et al.*, 2004)
EC10	BW1125 + pKD4	Amp^R Kan^R	(Datsenko & Wanner, 2000)
SE160	SL1344 + pCP20	Amp	[a]
SE67	SL1344 + pSIM18	Hyg	[a]
SE37	SL1344 *acrR*::*aph*	Kan^R	This study
SE24	SL1344 Δ*acrR*	-	This study
EC16	TOP10 + pTrc *acrR* (+6x his tag)	Amp^R	This study
SE29	SL1344 Δ*acrR* + pTrc *acrR* (+6xhis tag)	Amp^R	This study
SE30	SL1344 Δ*acrR* + pTrc *acrR* (-6xhis tag)	Amp^R	This study
SE35	SL1344 Δ*acrR* + pET20b *acrR*	Amp^R	This study
EC143	BL21 (DE3) + pLysS + pTrc *acrR* (+6xhis tag)	Amp^R Cam^R	This study
SE36	SL1344 *envR*::*aph*	Kan^R	This study

SE23	SL1344 Δ*envR*	-	[a]
EC06	TOP10 + pTrc *envR* (+6x his tag)	Amp^R	This study
SE27	SL1344 Δ*envR* + pTrc *envR* (+6xhis tag)	Amp^R	This study
SE28	SL1344 Δ*envR* + pTrc *envR* (-6xhis tag)	Amp^R	This study
SE34	SL1344 Δ*envR* + pET20b *envR*	Amp^R	This study
EC144	BL21 (DE3) pLysS + pTrc *envR* (+6xhis tag)	Amp^R Cam^R	This study
SE57	SL1344 Δ*acrR* Δ*envR*	-	This study
SE110	SL1344 Δ*acrR* + pSIM18	Hyg^R	This study
SE139	SL1344 Δ*acrR envR*::*aph*	Kan^R	This study
SE25	SL1344 + pET20b (empty vector)	Amp^R	[b]
SE147	SL1344 Δ*acrR* Δ*envR* + pET20b *acrR*	Amp^R	This study
SE148	SL1344 Δ*acrR* Δ*envR* + pET20b *envR*	Amp^R	This study
	Chapter 6		
SE205	SL1344 + pMW82 *acrA*	Amp^R	[b]
SE206	SL1344 + pMW82 *acrE*	Amp^R	[b]
SE59	SL1344 + pMW82 *ramA*	Amp^R	[b]
SE439	SL1344 Δ*acrR* + pMW82 *acrA*	Amp^R	This study
SE440	SL1344 Δ*acrR* + pMW82 *acrE*	Amp^R	This study
SE442	SL1344 Δ*envR* + pMW82 *acrA*	Amp^R	This study
SE443	SL1344 Δ*envR* + pMW82 *acrE*	Amp^R	This study
SE436	SL1344 Δ*acrR* Δ*envR* + pMW82 *acrA*	Amp^R	This study
SE437	SL1344 Δ*acrR* Δ*envR* + pMW82 *acrE*	Amp^R	This study
SE435	SL1344 Δ*acrR* Δ*envR* + pMW82 *ramA*	Amp^R	This study

SE333	4/74 HNS-1::*aph*	Kan^R	(Hinton, Santos, Seirafi *et al.*, 1992)
SE334	SL1344 HNS-1::*aph*	Kan^R	This study
SE336	SL1344 Δ*acrB* HNS-1::*aph*	Kan^R	This study
SE371	SL1344 HNS-1::*aph* + pMW82 *acrA*	Amp^R Kan^R	This study
SE335	SL1344 HNS-1::*aph* + pMW82 acrE	Amp^R Kan^R	This study
SE370	SL1344 HNS-1::*aph* + pMW82 *ramA*	Amp^R Kan^R	This study
SE337	SL1344 Δ*acrB* HNS-1::*aph* + pMW82 *acrE*	Amp^R Kan^R	This study

Table 2.1: A list of strains used in this study. Listed are the strain codes (used at the University of Birmingham), genotypes, resistance phenotypes and the strain origin or reference. [a]Strain created in our laboratory prior to this study, [b]plasmids constructed by Eleftheria Trampari and Mark Webber at the Quadram Institute, UK.

2.6.0 Disruption of *acrR* and *envR* genes in SL1344

The double knockout Δ*acrR* Δ*envR* was constructed by the chromosomal gene inactivation method described by Datsenko and Wanner (Datsenko & Wanner, 2000). This protocol utilises the λ red recombination system employed by bacteriophages. The λ red genes and their promoters are present on the pSIM18 plasmid, which also contains a hygromycin resistance cassette for detection. The first step of gene inactivation is to transform pSIM18 into the strains with genes to be disrupted and then to produce electrocompetent cells of this strain. The second step is to amplify the knockout construct from the pKD4 plasmid by polymerase chain reaction (PCR). The knockout construct is formed of the *aph* gene with flanking regions which have homology to 40 base pairs (bp) up and downstream of the target gene. The third step is to transform this knockout construct into the electrocompetent pSIM18 strain and incubate to allow homologous λ red recombination to occur. The final step is to remove the unwanted *aph* gene cassette using the pCP20 plasmid, producing the double knockout Δ*acrR* Δ*envR*.

2.6.1 Isolation of pDK4, pSIM18 and pCP20 plasmids

The plasmids pKD4, pSIM18 and pCP20 were isolated from strains EC10, SE67 and SE160, respectively (Table 2.2). Cultures (5 ml) of these strains were incubated overnight at 37 °C with aeration in LB media supplemented by the appropriate antibiotic to prevent loss of the plasmid. The QIAprep® Miniprep (QIAgen, UK) was used according to manufacturer's instructions to purify plasmid. Plasmid was quantified using a NanoDrop™ spectrophotometer.

Table 2.2: Plasmids used in this study

Plasmid	Description	Resistance markers	Reference
pKD4	Contains the *aph* gene cassette. Used as a PCR template for inserting the *aph* cassette into a target gene.	Amp^R and Kan^R	(Datsenko & Wanner, 2000)
pSIM18	Encodes λ red recombination system to facilitate integration of *aph* cassette with target gene	Amp^R	(Chan, Costantino, Li *et al.*, 2007)
pCP20	Temperature-sensitive plasmid which encodes Flp recombinase, which remove FRT- sequences such as those that flank the *aph* gene. Used to remove *aph* gene.	Amp^R	(Cherepanov & Wackernagel, 1995)
pTrc His2 TOPO	High copy-number plasmid for protein expression. Gene of interest is inserted adjacent	Amp^R	Invitrogen, UK Cat. K441001

	to the C-terminal polyhistidine (6xHis) tag for protein purification.		
pET20b	High-copy number plasmid with a T7 promoter.	Amp^R	Novagen, UK
pMW82	Suite of pMW82 plasmids donated by Dr M Webber. The promoter of the gene of interested is fused to the *gfp* gene. The promoter of the *gfp* gene is not present, so GFP fluorescence relates to activity on the gene of interest promoter.	Amp^R	(Bumann & Valdivia, 2007)

Table 2.2: Information on the plasmids used in this study. Including description, resistance markers and reference of the plasmid origin.

Table 2.3: Primers used in this study

Code	**Purpose**	**Sequence**
P3	Forward primer for cloning *envR* into pET20b with an NdeI restriction site	GGGGGGGGCATATGGCGAAGAAAACGAAGGCGGAT
P4	Reverse primer for cloning *envR* into pET20b with a HindIII restriction site	GGGGAAGCTTTCAGGCTTCTTCCGCCTGTTGTTCATTTGG
P5	Forward primer for cloning *acrR* into pET20b with an NdeI restriction site	GGGGGGGGCATATGGCACGAAAAACCAAACAACAA
P6	Reverse primer for cloning *acrR* into pET20b with a HindIII restriction site	GGGGAAGCTTTCAGGGGGAGCCGTTGACCGTCGA
P7	Forward primer for cloning *envR* into pTrc using pTrcHis2 TOPO kit	GCGAAGAAAACGAAGGCGGATGC
P8	Reverse primer for cloning *envR* into pTrc using pTrcHis2 TOPO kit without a C terminal tag	TCAGGCTTCTTCCGCCTGTTGTTCA

P9	Forward primer for cloning *acrR* into pTrc using pTrcHis2 TOPO kit	GCACGAAAAACCAAACAACAAGC
P10	Reverse primer for cloning *acrR* into pTrc with a terminal C his tag using pTrcHis2 TOPO kit	GGGGGAGCCGTTGACCGTCGA
P11	Reverse primer for cloning *acrR* into pTrc without the terminal C his tag using pTrcHis2 TOPO kit	TCAGGGGGAGCCGTTGACCG
P189	Forward primer for amplifying upstream *acrA*	TCCCAGATCTCACTGAATA
P190	Reverse primer for amplifying upstream *acrA*	TCAATGGTCAAAGGTCCT
P139	Forward primer for amplifying region upstream of *marR*	AGTTATCACAGCACAATACC
P140	Reverse primer for amplifying region upstream of *marR*	CCAGCGGAATGATTTCATTGA
P141	Forward primer for amplifying region upstream of *soxR*	TTCATCAATCCATTCGATAAGGG

P142	Reverse primer for amplifying region upstream of *soxR*	CGCTACGTTTCGCAACTTC
P143	Forward primer for amplifying region upstream of *micF*	CGTGATAGTTTTAGGCAG
P144	Reverse primer for amplifying region upstream of *micF*	TTCATTCGCAACTAAAATAGT
P147	Forward primer for amplifying region upstream of *acrD*	AGGAAGAGAGTCAGT
P148	Reverse primer for amplifying region upstream of *acrD*	TCGTGTTTCTACATATCG
P149	Forward primer for amplifying region upstream of *acrE*	ATTATGAAACGACAGGGA
P150	Reverse primer for amplifying region upstream of *acrE*	GTCATTACTGTTCCTTAA

P151	Forward primer for amplifying region upstream *ramR*	AGACAGCTTATCGCATGGC
P152	Reverse primer for amplifying region upstream *ramR*	CACTACACGTTACCCTTATGTCTGGA
P153	Forward primer for amplifying region upstream *rrsA*	AAGCGGGAAAGCGTA
P154	Reverse primer for amplifying region upstream *rrsA*	AAGTTTGATGCTCAATG
P155	Forward primer for amplifying region upstream *flhD*	ATGTAAACGTGTAAGGCGA
P156	Reverse primer for amplifying region upstream *flhD*	CCATCCAGAATAACCAACT
P157	Forward primer for amplifying region upstream *flhC*	TGCCGCAGATGGTC

P158	Reverse primer for amplifying region upstream *flhC*	CACTCATTATCATGCCCTT
P159	Forward primer for amplifying region upstream *mdtA*	TTCACCGATGAGAGTTG
P160	Reverse primer for amplifying region upstream *mdtA*	TCATCGGTATAAGTTTCTCA
P161	Forward primer for amplifying region upstream *mdsA*	TGTAACGCCTAGCCTT
P162	Reverse primer for amplifying region upstream *mdsA*	CCGGCTATCAACATAAT
P163	Forward primer for amplifying region upstream *rob*	TGGTTATAGATCACAGGATTAGA
P164	Reverse primer for amplifying region upstream *rob*	ATGCCAGCCTGATCCATA

P165	Forward primer for amplifying region upstream *rpoH*	GCGTCATCTTTATGTCACA
P166	Reverse primer for amplifying region upstream *rpoH*	TGGTCATTCAAATCCTCTCAATC
P171	Forward RealTime primer for measuring *marR* transcription	AAGACTGCCGAATCCTAAT
P172	Reverse RealTime primer for measuring *marR* transcription	TGATGACATTGCTCACAAAT
P173	Forward RealTime primer for measuring *soxR* transcription	CCGTTTAAAAGCCTTACTGA
P174	Reverse RealTime primer for measuring *soxR* transcription	TTAGCCCTTTGCTTTCATAG
P175	Forward RealTime primer for measuring *micF* transcription	GAACGGTCGAGCAGG

P176	Reverse RealTime primer for measuring micF transcription	TGAATGTCTGTTTACCCCTA
P177	Forward RealTime primer for measuring *flhD* transcription	ATATTTACTCCTTGCACAGC
P178	Reverse RealTime primer for measuring *flhD* transcription	CCGGAAATGACAAACTAACT
P179	Forward RealTime primer for measuring *flhC* transcription	TGGAGTTGATTAATCTTGGC
P180	Reverse RealTime primer for measuring *flhC* transcription	ATTCTTTGTACAGCCTGATG
P181	Forward RealTime primer for measuring *rpoH* transcription	CCGTACACTGGATTAAAGC
P182	Reverse RealTime primer for measuring *rpoH* transcription	GTTGCAACTTTAACGATACG

P183	Forward RealTime primer for measuring *mdtA* transcription	CAACTGGCAAAAACCAATC
P184	Reverse RealTime primer for measuring *mdtA* transcription	CATTAGCTTCATCCGCTTTA
P185	Forward Real Time primer for measuring *rob* transcription	TCTGGATATTGCTCTTCAGT
P186	Reverse RealTime primer for measuring *rob* transcription	GGAGCGGCGATACAG
P187	Forward RealTime primer for measuring *mdsA* transcription	GACTATCGTGTCTCACAATC
P188	Reverse RealTime primer for measuring *mdsA* transcription	GGTGGATTTTTGTCGGAG
P326	Forward RealTime primer for measuring *marA* transcription	CAACACTGACGCTATTACTA

P327	Reverse RealTime primer for measuring *marA* transcription	CAGGTGCCATTTGGAA
P328	Forward RealTime primer for measuring *soxS* transcription	CGCATCAGCAGATAATTCAGAC
P329	Reverse RealTime primer for measuring *soxS* transcription	ACTTGGAGTAGCCCGATTT
P330	Forward RealTime primer for measuring *ramA* transcription	TGAATCAGCCGTTACG
P331	Reverse RealTime primer for measuring *ramA* transcription	AGACTCTCCCCTTTGTA
P250	Forward primer for amplifying *hns*	TTGTGCGGTGCCTCAA
P251	Reverse primer for amplifying *hns*	TGGCTTGAAGAAGAAATGG

2.6.2 Generation of knockout constructs

Primers were designed to amplify the *aph* gene from the pKD4 plasmid by PCR and were also engineered to produce a construct with 40 bp homology up and down stream of the gene to be disrupted (Table 2.3). Primers were supplied by Invitrogen and were reconstituted in UltraPure water to a concentration of 100 µM (Invitrogen Ltd., UK). A master mix for PCR reactions was produced by combining 12.5 µl MyTaq™ Red Mix (Bioline, UK), 9.5 µl UltraPure water (Invitrogen Ltd., UK), 1 µl of diluted each primer and 1 µl of isolated pKD4 plasmid DNA was used as a template for the reaction. The PCR cycle started with a 5 minutes denaturation step at 95°C followed by a 30 second denaturation step at 95°C, then 30 seconds of annealing at 52°C and an elongation step at 72°C for 2 minutes. This reaction underwent 30 cycles and was followed by a final elongation step at 72°C for 10 minutes (Veriti PCR machine, Thermo Fisher). The PCR product was then purified using the QIAquick PCR Purification Kit (QIAGEN, 28106) and was eluted in 30 µl elution buffer. Agarose gels (1%) were prepared by adding 1 g of agarose powder (Sigma-Aldrich, UK) to 100 ml 1x Tris-acetate-EDTA (TAE). The solution was boiled in a microwave until the agarose powder was fully dissolved. Once cooled, 1% v/v Midori green (Geneflow, UK) was added. Molten agar was poured into a gel tray to set and a comb placed in the gel to form wells. Gels are then placed in a gel tank filled with 1% TAE. Samples were loaded into the wells along with a 1 kb DNA ladder (Bioline, UK). DNA was separated by size using electrophoresis at 100 V for one hour and then imaged using UV (Amersham, UK). A sample of purified PCR product was quantified using a NanoDrop™.

2.6.3 Transformation of pSIM18 into Δ*acrR*

A single colony of SL1344 Δ*acrR* was incubated overnight in LB broth at 37°C with aeration. The next day, 2 ml of this overnight culture was used to inoculate 50 ml of LB broth and was incubated for around 1.5 hours until the optical density (OD) reached 0.6 at a wavelength of 600nm measured by a spectrophotometer (Jenway 6300, Cole-Parmer Ltd., UK). The culture was poured into two 50 ml falcon tubes and pelleted by centrifugation (Thermo Scientific Ltd., UK) at 4°C for 10 minutes at 4,000 x*g*. Supernatant was discarded and the pellet was washed three times in ice-cold 15% glycerol to produce electrocompetent cells, pelleting by centrifugation between washes. After the final wash, cells were resuspended in 250 µl ice-cold 15% glycerol. An aliquot (50 µl) of electrocompetent Δ*acrR* cells were pipetted into a chilled 1mm electroporation cuvette (Cell Projects Ltd., UK) and 5 µl of pSIM18 was added. The cuvette was incubated on ice for 10 minutes before being electroporated at 1.8V 2.5kV, 200Ω, 25mf (Bio-Rad Ltd., USA). Transformations were immediately added to 1 ml pre-warmed LB broth and were recovered at 30°C for 2 hours with aeration. After incubation, cells were spread on pre-warmed hygromycin (150 µg/ml) agar plates and incubated overnight at 30°C. Colonies from these plates were sub-cultured on to fresh hygromycin plates and incubated at 30°C overnight. An individual colony from these streak plates were used to inoculate an overnight culture, which were incubated at 30°C with aeration. Plasmid was purified from this culture using a QIAprep Spin Miniprep Kit (Qiagen Ltd., 27104), and the presence of pSIM18 was confirmed through both nanodrop quantification and visualisation on a 1% agarose gel following electrophoresis.

2.6.4 Homologous recombination with the pKD4 knockout insert

The knockout construct containing the *aph* gene was then transformed into the Δ*acrR* + pSIM18 strain. An overnight culture of Δ*acrR* + pSIM18 was grown with 150 µg/ml Hygromycin and 2 ml of this overnight culture was used to inoculate 50 ml of LB broth which was incubated at 37°C for around 1.5 hours until optical density (OD) reached 0.6 at OD_{600} measure by a spectrophotometer (Jenway 6300, Cole-Parmer Ltd., UK). The culture was then poured into two 50 ml falcon tubes and heat shocked at 42°C in a water bath for 15 minutes. Cells were then pelleted by centrifugation (Thermo Scientific Ltd., UK) at 4,000 x*g* and 4°C for 10 minutes. Supernatant was discarded and the pellet was washed three times in 25 ml ice-cold 15% glycerol. After the final wash, cells were re-suspended in 250 µl ice-cold 15% glycerol and divided into 45 µl Eppendorf aliquots. All aliquots which were not to be used immediately were stored at -80°C. One 45 µl aliquot was then added to a chilled electroporation cuvette (Cell Projects Ltd., UK) with 5 µl knockout construct and electroporated at 1.8V 2.5kV, 200Ω, 25mf (Bio-Rad Ltd., USA). Transformations were immediately added to 1 ml pre-warmed LB broth and were recovered at 37°C for 2 hours with aeration. After incubation, cells were spread on pre-warmed kanamycin (50 µg/ml) agar plates and incubated overnight at 37°C. Colonies from these plates were sub-cultured on to fresh kanamycin agar plates and incubated at 37°C overnight.

2.6.5 Removal of the *aph* gene using pCP20

An overnight culture of the Δ*acrR envR::aph* strain was grown with 50 µg/ml kanamycin and incubated at 37°C for around 1.5 hours until optical density (OD) reached 0.6 at OD_{600} measure by a spectrophotometer (Jenway 6300, Cole-Parmer Ltd., UK). Competent cells were produced as described in section 2.4.4 and 45 µl of these cells

were electroporated at 1.8V 2.5kV, 200Ω, 25mf with 5 µl of pCP20 plasmid. Cells were recovered in 1ml of pre-warmed LB media and incubated for 1 hour at 30°C with aeration. After recovery, cells were spread on pre-warmed 50 µg/ml ampicillin plates and grown overnight at 30°C. A small number of colonies from these plates were picked using sterile loops and inoculated into LB broth and incubated at 43°C overnight to allow the plasmid to be cured. After overnight growth, a loop of culture was streaked on LB, LB supplemented with 50 µg/ml ampicillin and LB supplemented with 50 µg/ml kanamycin. Colonies grown on LB, but unable to grow on ampicillin or kanamycin (therefore containing neither pCP20 or the *aph* cassette) were checked using PCR.

2.7.0 Construction of strains that over express *acrR* or *envR*

Two over-expression vector plasmids were used here to produce strains which can express at either high (pTrcHis2-TOPO) and lower (pET20b) levels. Preparation of both sets of strains involved producing constructs by PCR using primers with identity to both vector and gene to be expressed (Table 2.3). Constructs were amplified from SL1344 using PCR and were visualised on 1% agarose by electrophoresis to verify that constructs were correct before cloning.

2.7.1 TA TOPO cloning of *acrR* and *envR* into pTrc vector

Constructs were prepared by PCR amplification (Primers P7 – P11) using instructions in the pTrcHis2 TOPO® TA expression kit (Thermo Fisher Scientific Ltd., UK). The PCR to amplify the constructs comprises of an initial denaturation step for 1 minute at 95°C, followed by 30x cycles of 15 seconds at 95°C, 15 seconds at 63°C and 30 seconds at 72°C, followed by a final 20 minutes at 72°C. The pTrc vector contains an ampicillin resistance cassette for detection. For strains requiring a C-terminal his tag, the native stop codon at the end of the gene to be inserted was removed to allow

transcription of the downstream sequence encoding the his-tag. PCR products were purified using the QIAGEN® PCR Purification Kit (QIAGEN, 28106). Then, 2 µl of this PCR product was mixed with 2 µl UltraPure water (Invitrogen) and 1 µl linear vector at room temperature for 5 minutes. Ligation reactions were stored at -20°C.

2.7.2 Cloning of *acrR* and *envR* into pET20b vector

Constructs were prepared by PCR amplification to insert the *acrR* and *envR* genes into the pET20b vector. The binding sites NdeI and HindIII were selected as restriction enzymes and PCR primers (P3 - P6) were designed to include to these restriction sites. Constructs were amplified by PCR comprising of 1 minute 95°C denaturation step, followed by 35x cycles of 15 seconds at 95°C, 15 seconds at 65°C, 45 seconds at 75°C, before a final extension step for 10 minutes at 72°C. The amplified PCR products were purified using QIAGEN® PCR Purification Kit (QIAGEN, 28106) and quantified using a nanodrop. The pET20b empty vector was isolated from strain SE25 using the geneJET maxiprep kit (Fisher Scientific Ltd., UK K0491) using fast spin protocol B. Digestion reactions containing vector, restriction enzymes (HindIII and NdeI), buffer and UltraPure water were prepared as per the instructions provided by the pET20b cloning kit. Digestion reactions were incubated at 37°C for 1 hour in a PCR machine. The reaction was then heat inactivated at 80°C for 10 minutes. The digests were then visualised on a 1% agarose gel by electrophoresis. Bands containing empty plasmid vector digested with HindIII and NdeI were excised and purified using QIAquick Gel Extraction Kit (Qiagen Ltd., 28706). Ligation reactions were prepared to a total volume of 20 µl and contained 3 µl of digested vector, 5 µl of digested PCR product, 1 µl of DNA ligase (Thermo Fisher, UK), 2 µl of 10x buffer and 9 µl of UltraPure water (Invitrogen Ltd., UK). Ligation reactions were incubated at room temperature overnight.

2.7.3 Transforming vectors into *E. coli*

The pET20b and pTrc ligation reactions were transformed into chemically competent One-Shot® TOP10 E. coli cells (Invitrogen Ltd., UK). A range of volumes (2-5 µl) of ligation reaction were incubated with a vial of TOP10 cells on ice for 30 minutes. The cells were then heat shocked at 42°C in a water bath for 30 seconds and immediately transferred to ice for a further 2-minute incubation. Cells were recovered in 250 µl SOC medium (Invitrogen Ltd., UK) and incubated at 37°C for 1 hour. The whole volume of recovered cells was spread onto LB agar plates containing 50 µg/ml ampicillin, as both vectors encode ampicillin resistance. Plasmid was isolated from a successful colony and transferred via electroporation into the parent *Salmonella* strain SL1344. The pTrc *acrR/envR* plasmids were also transformed into *E. coli* BL21 pLysS. The *E. coli* BL21 pLysS strain is preferable for supporting protein expression.

2.7.4 Producing electrocompetent cells for plasmid transformation

The pTrc, pET20b and pMW82 plasmids used in this study were transformed into multiple background strains (Table 2.1). The method to transform these plasmids was the same, irrespective of the plasmid. To transfer a plasmid from one strain to another, the plasmid must be isolated from the donor strain and the recipient strain must be made electrocompetent so that it can receive the plasmid.

To make electrocompetent cells, a 5 ml culture of the recipient strain in LB broth is incubated overnight at 37°C with aeration. The next day, 3 ml of the overnight culture was used to inoculate 50 ml of LB broth and was incubated at 37°C with aeration until an OD_{600} of 0.6 was reached. The cells were then pelleted by centrifugation at 4°C, 4,000 x*g* for 10 minutes. The supernatant was discarded and the pellet resuspended in ice cold 15% glycerol. This was repeated three times. After the third wash with 15%

glycerol, the pellet was resuspended in a final volume of 500 µl of 15% glycerol. 45 µl of these cells were added to a pre-cooled electroporation cuvette (Cell Projects, UK) and 5 ul of the purified plasmid was added. The cells + plasmid were incubated on ice in the electroporation cuvette for 20 minutes. Then, a voltage was applied to the cuvette using an electroporator (Bio-Rad Ltd., USA) set to a 2.5 kV, 25 µF, 200 Ω. Immediately after, 1 ml of pre-warmed LB media was added to the cuvette. Then, the recovered cells were inoculated onto LB agar containing the antibiotic required for selection, depending on the antibiotic resistance marker of the plasmid (Table 2.2). Colonies which grew on selective media were re-inoculated onto fresh media before being made into bead stocks.

2.8 P22 phage transduction of *hns* interruption from *Salmonella* 4/74 to SL1344

P22 phage transduction was used to transduce the *hns*::*aph* interruption from the strain SE333, a *Salmonella* 4/74 strain donated by Prof. Jay Hinton to the background SL1344 strain. The SE333 strain produces a truncated non-functional H-NS protein.

To produce the P22 phage, 5 ml overnight culture of SE333 was grown at 37°C with aeration. This culture was diluted 1:100 in fresh LB broth supplemented with 10 mM MgSO4 and 5 mM CaCl2 and was incubated with aeration for 30 min at 37°C. Then, 5 µl of P22 phage stock was added and the culture was incubated overnight with aeration at 37°C. After overnight incubation, 1 ml of chloroform was added and the mixture was vortexed for 10 seconds. The mixture was then pelleted by centrifugation at 4,000 x*g*, 4°C for 10 minutes. The supernatant containing the P22 phage was transferred to a glass tube and 200 µl chloroform was added.

The strain to receive the P22 phage (SL1344), was inoculated overnight in LB broth at 37°C. Cultured cells were pelleted by centrifugation at 4,000 x*g* at room temperature. Pelleted cells were resuspended in 1 ml of LB broth supplemented with 10 mM $MgSO_4$ + 5mM CaCl2. Transduction reactions were set up with either 0, 5, 10, 50, 100 or 500 µl of phage lysate with 100 µl cells. All reactions were incubated for 15 minutes at 37°C before 1 ml of LB broth supplemented with 100 µl 1M sodium citrate (1M) was added. Transduction cultures were incubated for a further 45 minutes at 37°C with aeration. Finally, 100 µl of each transduction reaction was inoculated onto kanamycin (50 µg/ml) LB agar plates and incubated for 48 hours at 37°C. Individual colonies (n =8) were inoculated on to fresh Kanamycin (50 µg/ml) LB agar plates. Plates were incubated overnight at 37°C. The resulting colonies were checked for the *hns::aph* phenotype using PCR (Primers P250 – P251). The check PCR consisted of 1 x 60 second denaturing step at 95°C, 30 x cycles of 95°C x 25 seconds, 56°C x 25 seconds and 72°C x 60 seconds, followed by a final 10 minutes at 72°C.

2.9 Testing antimicrobial susceptibility

Antimicrobial susceptibility was tested following the standardised agar doubling method (BSAC and J Andrews, 2006). Fresh antibiotic stocks were produced on the day of testing and were used to supplement ISO-sensitest agar (Sigma-Aldich Ltd., UK). The range of antibiotics used was decided based upon EUCAST susceptibility ranges for the selected bacterium (EUCAST, 2020). Bacterial cultures were adjusted to 10^7 -10^8 cfu/ml by diluting in sterile LB broth, with each test spot to contain ~10^4 cfu/ml bacterial cells.

2.10 Assessing curli and cellulose production

Curli and cellulose are components of the extracellular matrix which can be stained using congo red (Sigma-Aldrich, UK). Overnight cultures of test strains were grown at 37 °C. LB agar without salt was prepared from constituents (5 g tryptone, 2.5 g yeast extract and 7.5 g agar per 500 ml Sigma-Aldrich, UK). Congo red was dissolved in the cooling agar to a final concentration of 40 µg/ml before pouring. Overnight cultures were diluted 1:10,000 in sterile LB broth without salt and spotted (5 µl per spot) on to the dry congo red agar plates. Agar plates were incubated at 30 °C for 48 hours. After incubation, colonies were visualised using a light microscope. Smooth red colonies produce cellulose but not curli, whereas pale smooth colonies lack both curli and cellulose.

2.11 Swimming and swarming motility assays

Swimming and swarming motility was measured using semi-sold agar assays. Swimming motility assays required 0.3% agar semi-solid media, whereas swarming required 0.6% semi-solid media, supplemented with glucose. Media is prepared by combining agar (Difco Bacto® agar), LB broth powder and water according to manufacturer's instructions. Glucose (Sigma Aldrich) (5g/L) was added to agar for swarming plates. Plates are left to dry and 5 ml of overnight culture of test strains is adjusted to OD_{600} of 0.5. For swimming plates, a thin metal stick was used to inoculate a small volume of culture below the surface of the semi-solid agar, in triplicate. For swarming, 5 µl of the same adjusted culture was dispensed onto the surface, on n = 3 plates. Plates were read at 7 (swimming) and 20 (swarming) hours post-inoculation. The average diameter of the swimming plates was recorded and averaged for the n =

3 technical replicates. Both the swimming and swarming experiments were repeated on 3 separate occasions. Images of the plates were taken using the Amersham imager.

2.12 Measuring biofilm formation

Overnight cultures were grown in LB broth without salt at 37°C. These cultures were then diluted in 5 ml of salt-free LB broth to an optical density at 600 nm of 0.1. Then, 200 µl of these dilutions were placed in wells of a microtiter tray in triplicate. These were transferred to a shaking platform in a static incubator and gently agitated for 48 hours at 30°C. After this incubation, biofilms were stained and measured. First, the biofilms were washed gently with tap water to remove any unattached cells. Then, 200 µl of 0.1% crystal violet was added to each well and incubated at room temperature for 15 minutes. Crystal violet stain was then removed, and the cells washed again with water. Finally, 200 µl of 70% ethanol was added to each well and absorbance measured at 600 nm (FLUOstar, BMG Labtech). Three independent experiments were performed, and the averages of these optical densities calculated.

2.13.0 Expression of AcrR and EnvR protein

Using a sterile inoculation loop, a single colony of BL21 (DE3) pLysS pTrc *acrR* +6xhis or BL21 (DE3) pLysS pTrc *envR* +6xhis was inoculated into 10 ml of LB broth containing 50 µg/ml ampicillin and 50 µg/ml chloramphenicol. These cultures were incubated at 37°C with aeration overnight. The next day, 10 ml of overnight culture was used to inoculate 250 ml of pre-warmed LB broth supplemented with 50 µg/ml ampicillin. These expression cultures were grown in large sterile conical flasks, sealed with a sterile bung and capped with foil. The flasks were incubated at 37°C with aeration until an optical density at 600 nm of 0.6 was reached. Once this OD was

reached, a 0.5 ml sample was taken. This was the non-induced control. This sample was pelleted by centrifugation and resuspended in 25 µl 5x protein loading buffer (National Diagnostics, UK) and stored at -20°C.

To induce the expression of AcrR and EnvR protein, isopropylthio-β-galactoside (IPTG) (Thermo Fisher Ltd UK) was added to a final concentration of 1 mM. The cultures were then incubated for a further 5 hours at 37°C with aeration. After this second incubation, a second 0.5 ml sample was taken and pelleted by centrifugation. This sample was resuspended in 50 µl of 5x protein loading buffer (National Diagnostics, UK) and frozen at -20°C. The remaining volume of induced cells were then pelleted by centrifugation at 4,000 x*g* for 20 minutes. These pellets were either frozen overnight at -20°C or immediately processed for purification.

2.13.1 Purification of 6xHis tagged AcrR and EnvR

AcrR and EnvR proteins were purified using the Ni-NTA fast start kit (QIAGEN) according to the manufacturer's instructions for purification under native conditions. Protein was snap frozen in 50 µl aliquots and fractions from each stage of the purification were assessed by SDS-PAGE electrophoresis to determine the success of the protein expression and purification. Size exclusion chromatography was carried out at the University of Birmingham's Protein Expression Facility (PEF). Protein concentration was determined with a Qubit 3.0 (Life Technologies, UK) and a protein assay kit (Life Technologies, UK). If necessary, proteins were concentrated using Amicon Ultra 4 mL centrifugal filters (Merck, UK) with a molecular-weight cut-off of 10 kDa according to the manufacturer's instructions.

2.13.2 SDS-PAGE gel electrophoresis

A 4X concentrated stock of SDS-PAGE resolving buffer (Table 2.4) was prepared with 1.5M Tris-HCl and 0.4% (w/v) SDS, the pH was adjusted to 8.8 with hydrochloric acid. A 4X concentrated stock of stacking buffer was purchased from Biorad and SDS was added to a final concentration of 0.4% (w/v). Polyacrylamide gels were cast using a Mini-PROTEAN® Tetra system (Bio-Rad). Resolving gels contained 1X resolving gel buffer with 12% acrylamide (diluted from 30% acrylamide/bis-acrylamide solution; National diagnostics). Stacking gels contained 1X stacking gel buffer with 3.9% acrylamide (diluted from 30% acrylamide/bis-acrylamide solution; National diagnostics). All gels contained a final concentration of 0.1% (w/v) SDS. Polymerisation was catalysed by addition of ammonium persulfate (APS; 0.1% w/v) and Tetramethylethylenediamine (TEMED; 0.1% v/v). Protein samples were diluted in 5X protein loading buffer, which contains 0.5 M DTT (National diagnostics), and then heated at 95°C for 10 minutes prior to loading. Protein samples were loaded onto the SDS-PAGE gel along with a protein size marker (PageRuler, ThermoFisher, UK). SDS-PAGE gels were electrophoresed at 150 V for 1 hour in 1X SDS-PAGE running buffer (National Diagnostics, UK).

Table 2.4: Resolving and stacking gel composition

Reagent	Quantity	
	Resolving gel (12%)	**Stacking gel (3.9%)**
30% Acrylamide	6 ml	0.78 ml
Buffer	3.75 ml	1.5 ml
10% APS	150 µl	60 µl
TEMED	15 µl	6 µl
Deionised water	5.1 ml	3.6 ml

2.13.3 Coomassie staining for protein

After proteins have been separated by size using SDS-PAGE, the gels can be stained for protein using Coomassie blue (Simply Blue™ Safestain, ThermoFisher, UK). The SDS-PAGE gels were rinsed three times in 100 ml deionised water. Then, enough stain was added to submerge the gel (~20 ml). The gel was incubated in the staining solution for an hour with gentle agitation at room temperature. The gel was then briefly rinsed in deionised water before being destained in deionised water for an hour with gentle agitation, at room temperature. Gels were then imaged (Amersham, UK).

2.14 Western Blotting

Protein resolved using SDS-PAGE was transferred onto polyvinylidene difluoride (PVDF) membrane with a pore size of 0.2 µm (Roche, UK) using the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad). A 10X Tris-glycine protein transfer buffer (Sigma, UK) was diluted to 1X with methanol and water to give a final concentration of 25 mM Tris, 192 mM glycine and 20% methanol. The PVDF membrane was submerged in methanol and then the polyacrylamide gel and PDVF membrane were

equilibrated in 1X transfer buffer for 15 minutes at room temperature. The transfer cassette was assembled according to the manufacturer's instructions and placed in the tank with 950 ml of protein transfer buffer. The transfer was carried out at 320 mA (constant voltage) for 25 minutes.

A 10X stock of Tris buffered saline (TBS) was purchased from Sigma and diluted to 1X with deionised water. The transferred membrane was rinsed with deionised water and then TBS with 0.1% Tween® 20 Detergent (TBST). The membrane was blocked with TBST with 3% BSA (w/v) (TBSTB) for one hour at room temperature. The membrane was subsequently probed with an anti-His horseradish peroxidase (HRP)-conjugated monoclonal antibody (Life Technologies, UK, cat. R93125), diluted 1:5000 in TBSTB, overnight at 4°C.After overnight incubation, the PVDF membrane was rinsed 3 times with TBST for 5 minutes each. Bound antibodies were visualised using Clarity Western chemiluminescent substrate (Bio-Rad, UK) with an Amersham 600RGB imaging system. All incubation and washing steps were performed on a rocking platform.

2.15.0 Electrophoretic mobility shift assays

An electrophoretic mobility shift assays (EMSA) is a form of electrophoresis which can be used to study DNA:protein binding interactions. The assay requires purified protein (in this case, AcrR and EnvR) and purified DNA. During an EMSA, purified protein and DNA are combined and separated by native, non-dentaturing electrophoresis. Free DNA migrates must faster than DNA:protein complexes. Once stained for DNA, bands higher in the gel represent possible DNA:protein complexes. Non-denaturing polyacrylamide gels were made (Table 2.5 for constituents) and left to polymerise for 30 minutes before use.

2.15.1 Amplification of promoter regions for EMSA

The upstream regions of multiple genes were amplified by PCR (Primers listed in Table 2.3). The primers were designed so that all the products would be ~200 bp. PCR products were electrophoresed and purified (QIAquick PCR purification kit, QIAGEN, UK).All PCR reactions consisted of 1x 60 second 95°C denaturation step, followed by 30x cycles of 30 seconds at 95°C, 30 seconds at 57°C, 60 seconds at 72°C and a final extension step of 10 minutes at 72°C.

Table 2.5: Constituents to make 2 x 6% native polyacrylamide gels

Constituent	Volume
5 X TBE	2.5 ml
30% acrylamide solution	7.1 ml
Deionised water	16.3 ml
10% Ammonium persulfate (APS)	250 ml
TEMED	50 µl

2.15.2 Preparation of reactions and electrophoresis

A kit was used to prepare reaction mixtures (E33075, Life Technologies UK). Reaction mixtures containing 2 µl DNA, 2 µl purified protein (AcrR or EnvR), 3 µl of sterile distilled water and 2 µl binding buffer (supplied in E33075) were incubated at 37°C for 30 minutes. The concentration of protein varied depending on protein sample used and experiment, see Table 4.1. Wells of the native polyacrylamide gels were rinsed with 0.5x TBE and were then pre-run for 30 minutes at 200V in 0.5X TBE without sample. After incubation of the reaction mixtures, gel-loading dye (3 µl) was added and the

samples were dispensed in the wells of the gel. Samples were then separated by non-denaturing electrophoresis for 1 hour at 200V.

After electrophoresis, gels were rinsed in 0.5x TBE before being stained using SYBR green. A working stock of SYBR green was made by adding 5 µl of SYBR green to 50 ml 0.5 x TBE. Gels were placed in a Tupperware and submerged in this solution. The gel was incubated in darkness on an orbital shaker (50 rpm) for 20 minutes. After incubation, the gel was washed with deionised water before visualisation (Amersham, UK).

2.15.3 Effects of ligands on AcrR and EnvR binding to target DNA

TFTRs have both DNA and ligand-binding regions. If a ligand binds when a TFTR is bound to DNA, it causes a conformational change which causes the TFTR to be released from target DNA. The following experiments with AcrR were undertaken by AC and the experiments with EnvR by Master's student Jacob Scadden.

The EMSA protocol described in the previous section was performed with AcrR and EnvR and the upstream regions of *acrA* and *acrE*. However, the reaction mixtures were supplemented with either proflavine (Sigma-Aldrich, UK), rhodamine 6g (Sigma-Aldrich, UK), novobiocin (Alfa Aesar, UK), tetracycline (Fisher Scientific, UK) or erythromycin (Acros Organics, UK). The final concentrations of these ligands in the EMSA reaction mixtures are given in Table 2.6.

Table 2.6: Final concentration of ligands after addition to EMSA reaction mixtures

Stock concentration (µg/ml)	1	10	50	100	500	1000	10,000
Final concentration (EMSA reaction mixture, µg /ml)	0.077	0.77	3.8	7.7	38	77	770

2.16.0 GFP reporter assays to measure induction

The purpose of these assays was to measure the induction of *acrA, acrE* or *ramA* in response to multiple conditions or inducer compounds. The minimal media MOPS, 96-well plate assay and 96-well plate assay data analysis described below were undertaken by Master's student Hannah Doherty (HD), supervised by AC. The pMW82 plasmid encodes an unstable GFP variant, which has a triple substitution (S65A, V68L and S72A) resulting in increased fluorescence compared to WT GFP (Cormack, Valdivia, & Falkow, 1996). The GFP has a half-life of approximately 85 minutes and in the absence of induction, florescence is reduced (Rollenhagen & Bumann, 2006). The *gfp* gene is encoded downstream of the promoter of interest, meaning that GFP fluorescence is coupled to transcription of the target gene. This rapid-detection, unstable GFP is therefore an ideal candidate for studying gene expression and for example, was recently used to measure efflux gene induction in response to putative efflux inhibitors .

2.16.1 Minimal media MOPs

Minimal media was also used as a growth medium for induction experiments (Chapter 6). Minimal media was made from constituents, but was also purchased (Teknova), to act as a control. As the constituents of minimal media needed to be varied, a minimal

MOPS 10x mixture was made based on the recipe by Neidhardt *et al.,* (Neidhardt, Bloch, & Smith, 1974). To produce minimal MOPS, a micronutrient stock and a 10X MOPS buffer stock first need to be made. Then, the 1 L of 10X stock was aliquoted into 4 x 250 ml bottles. Other additives were then added (sodium chloride, micronutrient stock and magnesium chloride), (Table 2.9). Adding the magnesium chloride at this stage allowed the production of a variety of 10X stocks containing different concentrations of magnesium chloride. Finally, from the 10X minimal MOPS and the micronutrient stock, 1X minimal MOPS supplemented with glucose and L-histidine was made (Table 2.10). By using this method, constituents such as magnesium, potassium, glucose and pH could be varied. Other additives such as indole and other suspected inducers could be added to the minimal MOPs mixtures for testing the induction of *acrEF* and *acrAB*.

Table 2.7: Micronutrient stock constituents

Constituent	Amount
Ammonium molybdate (para) tetrahydrate (Alfa Aesar, UK)	0.036 g
Boric acid (Sigma-Aldrich, UK)	0.248 g
Cobalt Chloride (MP Biomedical, UK)	0.072 g
Cupric sulfate (MP Biomedical, UK)	0.024 g
Manganese chloride (MP Biomedical, UK)	0.16 g
Zinc sulfate (MP Biomedical, UK)	0.028 g
Distilled water	250 ml

Table 2.7: Constituents to make 250 ml of micronutrient stock for making minimal MOPS. Micronutrient stocks were filter sterilised and stored at room temperature.

Table 2.8: Minimal MOPS 10X buffer constituents

Constituent	Amount
3-(N-Morpholino) propanesulfonic acid (MOPS) (Sigma-Aldrich, UK)	83.72 g
Tricine (Sigma-Aldrich, UK)	7.17 g
Iron (II) sulfate heptahydrate (10 mM) (Alfa Aesar, UK)	10 ml
Ammonium chloride (1.9 M) (Alfa Aesar, UK)	50 ml
Potassium sulfate (0.276 M) (MP Biomedical, UK)	10 ml
Calcium chloride dihydrate (20 mM) (Fisher Scientific, UK)	250 µl
Distilled water	440 ml

Table 2.8: Constituents to make 1L minimal MOPS 10X buffer. MOPS 10X buffer was adjusted to pH 7.4 using 10M KOH. The buffer was aliquoted evenly between 4 x 250 ml duran bottles.

Table 2.9: Additives required for each 250 ml 10X aliquot

Constituent	Amount
Sodium Chloride (5 M) (Sigma-Aldrich, UK)	25 ml
Micronutrient stock	5 µl
Magnesium chloride (25 mM)	To final concentrations of either 5.25 mM, 2.63 mM, 10 µM and 5 µM.

Table 2.10: After the 10X minimal MOPS buffer was split into 4 x 250 ml aliquots, additives (sodium chloride, micronutrient stock and magnesium chloride) are added. The amounts of these additives are described in the table above. Adding the

magnesium chloride at this point allows a 1X minimal MOPs with different magnesium chloride concentrations to be made.

Table 2.10: Standard Minimal MOPS 1X buffer

Constituent	Amount
10X MOPS buffer	25 ml
Glucose (20% w/v)	2.5 ml
L-histidine	0.1 g
Distilled water	220 ml

Table 2.10: Minimal MOPS 1X buffer constituents. Buffer is filter sterilised and stored at 4°C.

2.16.2 Overnight induction of *acrA, acrE* and *ramA*

These initial experiments were designed to investigate whether a 96-well plate method could be used to measure induction of pMW82 reporter constructs. Various MOPS buffers were prepared (Table 2.11). The purpose of varying the media was to try and mimic conditions thought to induce *acrA* or *acrE* expression (via H-NS relief).

Here, cultures of strains containing the pMW82 reporter construct with *gfp* fused to either the promoter of *ramA, acrA* or *acrE* were grown overnight in minimal MOPS supplemented with 50 µg/ml ampicillin at 37°C with aeration. 10 µl of the overnight culture was inoculated into 10 ml MOPS minimal media (containing 20% glucose and L-histidine). Then, 100 µl of each diluted overnight culture was inoculated into 900 µl of the MOPS media of each condition. Aliquots containing strains with the pMW82 plasmid were supplemented with 50 µg/ml ampicillin. All aliquots of strain + MOPS were then dispensed (200 µl per well) in a black 96-well plate in triplicate (Clear flat

bottomed, black, Corning Incorporated, USA). The 96-well plate was incubated in a plate reader (FLUOstar, BMG LABTECH, Germany). GFP fluorescence (excitation 485 nm, emission 520 nm) and growth (absorbance at OD_{600}) were measured every 20 minutes for 49 cycles (16 hours).

Table 2.11: Minimal MOPS 1X buffers with varied indole/glucose/$MgCl_2$

MOPS buffer + variant	Description
MOPS + Indole 0.5 mM 1 mM	Minimal MOPS 1X supplemented with indole to the final concentrations of 0.5 or 1 mM.
MOPS + Glucose 0.2% 0.1% 0.075% 0.05%	Minimal MOPS 1X supplemented with glucose w/v to produce a minimal MOPS 1X with 0.2, 0.1, 0.075 or 0.05% glucose.
MOPS + $MgCl_2$ 5.25 mM 2.63 mM 10 µM 5 µM	Minimal MOPS 1X supplemented with $MgCl_2$ to final concentrations of 5.25 mM, 2.63 mM, 10 µM and 5 µM.

Table 2.11: A list of the minimal MOPS 1X variants produced to attempt to induce *acrA* and *acrE* expression.

2.16.3 Analysis of 96-well plate GFP fluorescence data

The raw data was collected and the average of three technical replicate samples was used to calculate average fluorescence/OD_{600} for each time point. From this average, the average of x3 blanks was subtracted for each time point. This gives blank-corrected fluorescence and absorbance data. Then, the average blank-corrected fluorescence for each time point was divided by the average blank-corrected OD600 for each time

point, giving the fluorescence/OD_{600} (Fl/OD). These experiments were repeated three times (biological replicates n=3) and then the average Fl/OD values of the three replicates was used to generate graphs. The peak fluorescence was identified as the peak Fl/OD over the time course and this was plotted for different background strains in response to different conditions (Chapter 6 Figures 6.1, 6.5, 6.8, 6.11 and 6.14). All statistical analyses were done using GraphPad 8 Prism (GraphPad Software Inc, USA) using an ordinary one-way ANOVA.

2.16.4 Single timepoint induction assay to measure induction

A single timepoint induction assay to measure induction of *acrA, acrE* and *ramA* in response to different levels of inducer drugs was also developed. Potential inducers tested were rhodamine 6g, proflavine, tetracycline, novobicin, kanamycin, indole and erythromycin.

Cultures of strains containing the pMW82 reporter construct with *gfp* fused to either the promoter of *ramA* (SE59)*, acrA* (SE205) or *acrE* (SE206) were grown overnight in LB media supplemented with 50 µg/ml ampicillin at 37°C with aeration. Then, 5 x aliquots of 10 ml LB broth in a 50 ml falcon tube were inoculated with 0.5 ml of the overnight culture. These were then incubated at 37°C with aeration until an OD_{600} of 0.6 was reached. The OD_{600} of only one tube in a strain set of 5 tubes was measured to maintain the 10 ml volume in these tubes.

Stocks (10,000 µg/ml) of inducers were prepared and diluted using sterile water to produce two further stocks of 1,000 µg/ml and 100 µg/ml. Then, 100 µl of each stock was added to one 10 ml falcon tube aliquot per strain, giving 4 x tubes per strain, each containing the inducer at 100, 10 and 1 µg/ml. No inducer was added to the final 10 ml

falcon tube. This will therefore act as a non-induced control. These cultures were then incubated at 37°C with aeration for an hour. After incubation with the inducer, cells were pelleted by centrifugation at 4,000 x*g* at room temperature for 15 minutes. Supernatant (containing inducer and LB broth) was discarded. Pellets were resuspended in 10 ml sterile PBS. To remove any residual inducer, cells were again pelleted by centrifugation (4,000 x*g* for 15 minutes at room temperature) and resuspended in 10 ml sterile PBS. Three technical replicates of 200 µl of each strain and condition were then loaded into a black-bottom 96 well plate (Corning Incorporated, USA). The GFP fluorescence (excitation 485 nm, emission 520 nm) was measured using a FLUOstar (BMG LABTECH, Germany). The testing of each inducer was repeated on three separate occasions.

2.16.5 Analysis of the single timepoint induction assay data

The raw data was collected and the average of three technical replicate samples was used to calculate average fluorescence/OD_{600} for each time point. From this average, the average of x3 blanks was subtracted for each time point. As these experiments used inducer compounds (some of which were fluorescent), a set of blank wells of minimal MOPS + inducer compound (at all concentrations) were used for the blank corrections. This ensured that any residual inducer compound fluorescence or absorbance was accounted for in these analyses. Then, the average blank-corrected fluorescence for each time point was divided by the average blank-corrected OD_{600} for each time point, giving the fluorescence/OD_{600} (Fl/OD). These experiments were repeated three times (biological replicates n=3) and then the average Fl/OD values of the three replicates was used to generate graphs. A one-way ANOVA was used to

compare the average Fl/OD of each strain either with or without each inducer compound at all concentrations.

2.17 Measuring the growth of the *hns* interrupted strain

The growth of *hns*-interrupted strain SE334 was compared to wild type SL1344 SE01 using the FLUOstar (BMG LABTECH, Germany). Cultures of SE01 and SE334 were grown overnight in LB broth at 37°C with aeration. Then, these cultures were diluted to approximately 10^7 cfu/ml. Then 90 µl of LB broth was dispensed in clear 96-well plate (Corning Incorporated, USA). 10 µl of diluted culture was then added to LB broth in the 96-well plate, leaving some wells with only LB broth as blank negative controls. The 96-well plate was incubated in a plate reader (FLUOstar, BMG LABTECH, Germany) at 37°C. Growth (absorbance at OD_{600}) was measured every 20 minutes for 49 cycles (16 hours), with shaking after each cycle. The raw data was collected and the average of three technical replicate samples was used to calculate average OD_{600} for each time point. From this average, the average of x3 blanks was subtracted for each time point.

2.18 Measuring ethidium bromide efflux

Ethidium bromide is a known substrate of AcrAB-TolC. When cells are de-energised, ethidium bromide accumulates within cells. Once the cells are re-energised, the ethidium bromide can be extruded from the cell via efflux systems such as AcrAB-TolC. As ethidium bromide is fluorescent, this can be measured (Blair & Piddock, 2016). This experiment was undertaken by HD as part of her Master's project, supervised by AC.

Cultures (5 ml) are prepared in LB media and are incubated overnight at 37°C with aeration. 400 µl of this culture was then used to inoculate 10 ml LB media and was

incubated at 37°C with aeration until OD_{600} = 0.4. After this OD was reached, the cells were pelleted by centrifugation (4, 000 x*g*) for 10 minutes at room temperature. The supernatant was discarded and the pellet was resuspended in 20 mM PPB buffer containing 1mM $MgCl_2$ and the OD_{600} was adjusted to 0.2. To adjust the OD, the following formula was used:

$$Volume\ to\ add = \left[\frac{Measured\ OD}{OD\ required} - 1\right] \times Volume\ of\ culture$$

Then, CCCP was added to a final concentration of 100 µM. The CCCP inhibits bacterial efflux as it de-energises cells. Ethidium bromide was then added to a final concentration of 50 µg/ml and the mixture incubated at 23°C for 1 hour with aeration. After incubation, the mixture was pelleted by centrifugation (4,000 x*g* at 23°C for 10 minutes) and the pellet resuspended in 1 ml 20 mM PPB supplemented with 1 mM $MgCl_2$.

Finally, 9 ml of 20 mM PPB supplemented with 1 mM MgCl2 and 5% glucose was added to re-energise the cells. 200 µl of this was then dispensed in triplicate in a black 96-well plate. The fluorescence (excitation 544, emission 590) was recorded for 200 cycles using the FLUOstar (BMG LABTECH, Germany). Raw data was collected and the average fluorescence for each time point was calculated. This was then blank corrected by subtracting the average fluorescence of wells containing media only. The time taken for fluorescence to drop by 10, 25 and 50% was then calculated. The highest point on the generated graphs (not including the initial ~5 second fluctuation after fluorescence measurements start) is given as 100% fluorescence. The equation of a straight line ($y = mx + c$, where m is the gradient of the line and c is the y-axis

intercept), is then used to determine the time when fluorescence has decreased by 10, 25 or 50%.

2.19 qRT-PCR to measure the transcription of putative AcrR and EnvR targets

The quantitative real-time PCR (qRT-PCR) experiments described in this section were performed by Dr. Helen McNeil using primers designed by AC and HM, Table 2.3. These experiments were performed as previously described (Blair, Smith, Ricci *et al.*, 2015). All primers designed for RT-PCR were designed to amplify products no bigger than 200 bp, with an annealing temperature differing by no more than +/- 0.5 °C. The PCR efficiency was calculated for the housekeeping gene 16S and each test gene (*acrA, acrE, ramA, ramR, rob, soxS, soxR, marA, marR, mdsA, mdtA, micF, rpoH, flhC* and *flhD*). The data were analysed using CFX Manager (Bio-rad, UK) with expression ratios calculated using the ΔΔCt method (Pfaffl, 2001) and normalised to the expression of 16S. Four biological replicates of each strain (WT, *ΔacrR*, *ΔenvR*, *ΔacrR* + pET20b *acrR* and *ΔenvR* + pET20b *envR*) were grown to an OD_{600} of 0.6 in MOPS minimal medium, supplemented with 2.6 mM histidine, at 37°C, 50 µg/ml ampicillin was added to SE34 and SE35 for plasmid maintenance. A Total RNA Purification Plus Kit with an on-column DNase treatment (Norgen) was used to purify RNA according to the manufacturer's instructions. The quantity of RNA and contaminating DNA was measured using a Qubit 3.0 fluorometer (Invitrogen). A second DNase treatment was carried out using a Turbo DNA-free kit (Fisher) and samples were concentrated using an RNA Clean and Concentrator-5 kit (Zymo Research) according to instructions. FastGene 55-Scriptase (Nippon genetics) was used to convert 1 µg of RNA into cDNA, using random hexamers (Invitrogen) at a final concentration of 5 ng/µl and dNTP's (Invitrogen) at a final concentration of 0.5 mM as described by (Wang-Kan *et al.*, 2017).

Beacon Designer 4.0 (Premier Biosoft, USA) was used to design primers with an annealing temperature of 57.3°C and primers were synthesised by Sigma. IQ SYBR green Supermix (Bio-Rad, UK) was used with a final concentration of 0.5 µM of each primer and 1 µl of cDNA (cDNA for the housekeeping gene was diluted 1:1000 with nuclease-free water) to perform qRT-PCR using a CFX96 RT machine (Bio-Rad, UK) with the following cycling conditions: 95°C for 5 min followed by 40 cycles of 95°C for 30 s, 57.3°C for 30 s and 72°C for 30 s. The efficiency of each reaction was calculated using five cDNA standards, which were prepared using a 1:10 dilution series. Differences in gene expression were calculated in Microsoft excel with the threshold cycle (ΔΔCT) method (Pfaffl, 2001) using 16S rRNA as the housekeeping gene.

Chapter Three

Genomic analyses of the TetR-family of transcriptional regulators

3.0 Genomic analyses of the TetR-family of transcriptional regulators

Note: This work is published. Please see the disclaimer at the start of this thesis for more information.

3.1 Background

TetR-family transcriptional regulators (TFTRs) are DNA binding transcription factors that regulate gene expression in bacteria. TFTRs have classically been viewed as simple, single-target regulators. However, recently it has emerged that some TFTRs have multiple targets in *E. coli* (Shimada *et al.*, 2018). Therefore, the classical view of assuming that TFTRs are single-target by default needs to be reconsidered. As some TFTRs regulate essential processes (e.g. metabolism) or processes which are important determinants of resistance and virulence (e.g. biofilm formation and efflux gene expression) and as TFTRs are present throughout pathogenic bacteria, they may be good drug discovery targets for tackling antimicrobial resistant infections.

Many TFTRs act as repressors by binding palindromic DNA sequences which overlap with promoters, preventing the recruitment and binding of RNA polymerase and preventing transcription. Upon ligand binding, a conformational change in the TFTR occurs which releases the regulator from the target DNA, enabling transcription of target genes (Ramos *et al.*, 2005). Some authors have chosen to classify TFTRs based on their location in relation to their target gene (Figure 3.1) and it is believed that the majority of TFTRs regulate genes within 200 base pairs (bp) of the TFTR-encoding gene (Ahn *et al.*, 2012; Balhana, Singla, Sikder *et al.*, 2015). A TFTR classification system proposed by Ahn *et al.,* describes three types of TFTR which bind

Figure 3.1: TFTR regulation classification proposed by Ahn *et al*

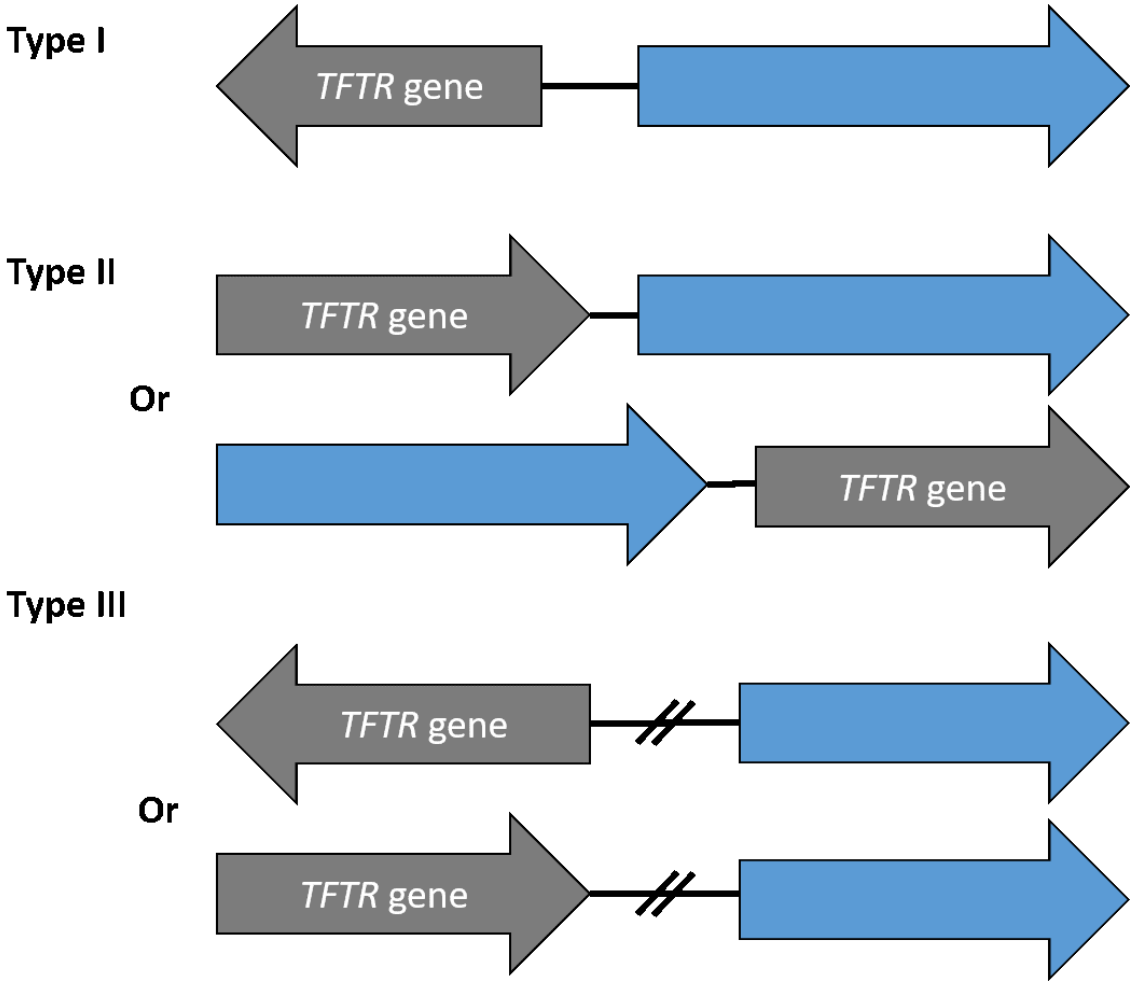


Figure 3.1: Current classification system of TFTRs as proposed by Ahn *et al (Ahn et al., 2012)*. Type I classification involves the TFTR gene regulating a divergently expressed target gene (i.e. AcrR). Type II TFTRs regulate genes directly up/downstream in the same orientation (i.e. ComR). Type III described TFTRs that regulate genes either up/downstream of the *TFTR* gene in any orientation and any location on the genome.

targets which are either divergently encoded (Type I) encoded alongside (Type II) or (Type III), neither I or II (Ahn *et al.*, 2012). Type I TFTRs are more common (e.g. AcrR regulating *acrAB*) than type II TFTRs (e.g. ComR regulating *comAB*). Both Type I and II TFTRs are thought to act on local genes, whereas Type III TFTRs act globally and in any orientation (i.e. RutR). There are numerous examples of TFTRs regulating local genes, such as AcrR regulating the adjacent *acrAB* efflux genes. However, some TFTRs are global regulators able to alter transcription of targets throughout the genome, such as MtrR of *Neisseria gonorrhoea* (P. J. T. Johnson & W. M. Shafer, 2015).

In *Mycobacteria* the number of TFTRs has been shown to increase with genome size and while the number of TFTRs can vary between species, the majority of TFTRs in *Mycobacteria* are believed to regulate targets within 300 bp of the *tftr* gene (Balhana *et al.*, 2015). However, it is now known that some TFTRs act to regulate more than one targets and can therefore act locally and globally and meaning they would fit into multiple categories of the classification system in Figure 3.1. For example, the TFTR EnvR regulates the divergently encoded local efflux operon *acrEF*, but also binds upstream and regulates expression of the efflux operon *acrAB*, which is encoded separately on the genome. Some TFTRs with multiple targets may therefore not fit an individual classification of TFTR. Other TFTRs are activators (Hu & Lidstrom, 2012) and some can act as both activators and repressors (Chattoraj, Mohapatra, Rao *et al.*, 2011). TFTRs have been identified which can bind multiple targets (Hirakawa, Takumi-Kobyashi, Theisen *et al.*, 2008; Liu, Yang, & He, 2016) and intergenic regions (Shimada, Ishihama, Busby *et al.*, 2008). Thus, although some TFTRs are known to

be local repressors, the current classification system is, in some cases, oversimplifying these proteins.

3.2 Aims

- Quantify the number of TFTR genes in *Salmonella* and *Escherichia*.
- Classify identified TFTRs of *Escherichia* and *Salmonella* by function and determine conservation throughout these species.
- Identify the “core” (i.e. present in all strains and species) and “accessory” (i.e. present in some strains/species) TFTRs of *Salmonella* and *Escherichia*.
- Study the variation in the sequences of these regulators between strains, species and genera.

3.3.0 Identifying TFTR genes in *E. coli* and *Salmonella*

While multiple TFTRs have been described in both *Salmonella* and *E. coli*, the total number encoded by each species is not known. To address this, available proteomes (n = 15) of *Escherichia* and *Salmonella* were screened for TFTR genes based on the presence of a highly conserved HTH-motif. The function of the TFTRs detected was determined from the literature and all are shown in Table 3.1. Maximum-likelihood trees of the phylogeny of the *Salmonella* or *Escherichia* strains were constructed using the sequence of *acrB.* It was important to not choose a highly-sequence conserved gene (such as 16S RNA) to produce phylogenetic trees because this would not be different enough between strains to produce a correct phylogenetic tree. These phylogenetic analyses were then used to interpret the following observations by overlaying a simplified tree with the |data on the presence/absence of TFTRs in the *Escherichia* and *Salmonella* genera using Phandango (Hadfield, Croucher, Goater *et al.*, 2017), Figures 3.2 – 3.3

Table 3.1: Proposed biological roles of TFTRs of *Salmonella* and *Escherichia*

TFTR	Core/Accessory (%)	Pathway	Gene(s) or process regulated (organism)	Ligands	References
AcrR	Core**	Multidrug efflux (RND) Multidrug efflux (ABC) Multidrug efflux (MFS) Motility	*acrAB* (Enterobacteriales) *flhDC*	Rhodamine 6g Proflavin Ethidium bromide Ciprofloxacin	(Ma, Alberti, Lynch *et al.*, 1996b) (Su *et al.*, 2007) (Li, Gu, Su *et al.*, 2008)
EnvR	Core**	Multidrug efflux (RND) Multidrug efflux (RND)	*acrAB* (Enterobacteriales) *acrEF* (Enterobacteriales)	No data available	(Hirakawa, Takumi-Kobyashi, *et al.*, 2008)
NemR	Core**	Bleach survival	*nemAB*	Choline	(Gray, Wholey, & Jakob, 2013)
SlmA	Core*	Cell division Chitin catabolism	FtsZ ring formation (Enterobacteriales) *chb operon* (*V. cholera*)	Target DNA sequences FtsZ protein	(Schumacher & Zeng, 2016) (Du & Lutkenhaus, 2014) (Klancher, Hayes, & Dalia, 2017)
YbiH	Core*	Multidrug efflux (ABC) Membrane permeability	*ybhGFSR* (*E. coli*) *rhlE*(*E. coli*)	Chloramphenicol Cephalosporin	(Yamanaka, Shimada, Yamamoto *et al.*, 2016)

BetI	Accessory (67%)	Glycine betaine synthesis	*betT* (Enterobacteriales) *betIBA* (Enterobacteriales)	Choline	(Rkenes, Lamark, & Strøm, 1996)
EefR	Accessory (47%)	Multidrug efflux (RND)	*eefABC* (*Enterobacter spp.*, *K. pneumoniae*)	No data available	(Masi, Pages, Villard *et al.*, 2005) (Masi *et al.*, 2006)
FabR	Core *Accessory (93%)*	Fatty acid biosynthesis	*fabAB* (Enterobacteriales)	Unsaturated thioester	(Zhang, Marrakchi, & Rock, 2002)
RamR	*Core*	Efflux regulation	*ramA* (Enterobacteriales)	Bile Berberine Ethidium bromide Dequalinium Crystal violet Rhodamine 6g	(Yamasaki *et al.*, 2019) (Yamasaki, Nikaido, Nakashima *et al.*, 2013) (Ricci, Busby, & Piddock, 2012)
RutR	Core *Accessory (93%)*	Pyrimidine utilisation Purine degradation Glutamine supply PH homeostasis	*rutABCDEFG* (*E. coli*) *carAB* (*E. coli*) *gadAXW* (*E. coli*) *gadIBC* (*E. coli*) *gly-hyi-glxR-ybbVW-allB-ybbY-glxK* (*E. coli*)	Uracil Thymine	(Shimada, Hirao, & Kori, 2007) (Shimada *et al.*, 2008) (Nguyen Le Minh, de Cima, Bervoets *et al.*, 2015)
TetR	Accessory (40%) *Accessory (20%)*	Multidrug efflux (ABC)	*tetAB* (Enterobacteriales)	Tetracycline	(Aleksandrov, Schuldt, Hinrichs *et al.*, 2009)
UidR	Accessory (67%)	Catalysis of beta-glucuronidase	*uidA* (*E. coli*)	No data available	(Blanco, Ritzenthaler, &

					Mata-Gilsinger, 1986)
U1	*Core*	No data available	No data available	No data available	
YbjK/ RcdA	Accessory (93%) *Accessory (80%)*	Biofilm formation Stress response	*csgD* (*E. coli*) *appY*, *sxy*, *ycgF*, *fimB* (*E. coli*)	No data available	(Shimada, Katayama, Kawakita *et al.*, 2012)
YcfQ/ comR	Accessory (80%) *Core*	Copper transport	*comC* (*E. coli*)	Copper	(Mermod, Magnani, Solioz *et al.*, 2012)
YftA	Accessory (80%)	No data available	No data available	No data available	
YjdC	Accessory (67%) *Core*	Copper tolerance	*cadABC* (*E. coli)*	No data available	(Hwang, Mattei, VanArendonk *et al.*, 2010)
YjgJ/ bdcR	Accessory (60%) *Accessory (93%)*	Biofilm dispersal	*bdcA* (*E. coli*)	No data available	(Ma, Zhang, & Wood, 2011)

Table 3.1: Proposed biological roles of TFTRs from *Salmonella* and *Escherichia*. TFTRs present in all Gram-negatives (*K. pneumoniae, P. aeruginosa, E. coli, E. albertii, E. fergusonii, S. enterica, S. bongori*) tested are denoted as **core****, while those not present in all species but present in all *Escherichia* and *Salmonella* are denoted as **core***. The carriage of the remaining TFTRs found in *Salmonella* and *Escherichia* are listed (%, underlined for *Escherichia,* and italicised for *Salmonella*). This data is combined with biological role as documented in literature. Known targets and ligands are included and targets known to be activated, not repressed, by the TFTR are in bold. A biological role was assigned from the literature if experimental evidence was provided (e.g. binding assays to show TFTR binding to promoter).

3.3.1 TFTRs of *E. coli* and *Escherichia* species

A median number of 14.5 TFTRs were identified in *E. coli*. The *E. coli* strains used were predominantly laboratory strains due to the availability of annotated proteome data. Sequences of *nemR*, *slmA, ybiH*, *envR*, *acrR*, *uidR*, *rutR*, *fabR*, *betI* and *yjdC* were present in all strains of *E. coli*. A further six (*ytfA*, *tetR*, *eefR*, *ycfQ*, *ybjK* and *yjgJ*) were present in some, but not all strains of *E. coli* (Figure 3.2). Strain SMS-3-5 contained the highest number of TFTRs (n = 16) and strain UTI89 the fewest (n = 12). A further two species within the *Escherichia* genera (three strains of *E. albertii* and two strains of *E. fergusonii*, (Table 3.1) were searched for TFTR genes. These strains contained significantly fewer TFTRs than the *E. coli* strains (Student's t test $P < 0.001$), with *E. coli* strains having an average TFTR number of 14 versus 10 for the *E. albertii* and *E. fergusonii* strains.

Six TFTRs (*nemR*, *slmA*, *ybiH*, *envR*, *acrR* and *fabR*) were present in all tested strains of the *Escherichia* genus (Figure 3.2). Using this method, SlmA was identified as a TFTR. However, SlmA directly activates the transcription of the *chb* operon in *V. cholerae* (Klancher *et al.*, 2017), but is not believed to have any direct regulatory roles in *E. coli* (Tonthat, Arold, Pickering *et al.*, 2011). In *E. coli*, SlmA acts as a nucleoid occlusion protein, interacting with target DNA and protein (FtsZ). Thus, although SlmA is included here, this is based on the presence of the HTH motif and not the assumption of direct regulatory roles in either *Salmonella* or *Escherichia.*

Of these regulators, the majority are involved in the removal of toxic compounds through either regulating efflux (AcrR, EnvR and YbiH) or, in the case of NemR, activating enzymatic pathways. The TFTRs *uidR, betI* and *yjdC* were present in all *E.*

coli strains but were not present in all *Escherichia* strains searched. In contrast, these same three TFTRs were absent in all strains of *E. fergusonii* and *E. albertii.* In addition to these, all *E. fergusonii* strains also lacked *eefR, ycfQ* and *yjgJ* and *E. albertii* strains lacked *tetR*. All strains of *E. fergusonii* and *E. albertii* have the *ytfA* gene in all strains. The strains of *E. albertii* also have *ybjK* and *eefR* and all strains of *E. fergusonii* have *tetR.* Both nodes containing *E. fergusonii* and *E. albertii* also contained fewer TFTRs per strain compared to *E. coli*.

3.3.2 TFTRs of *S.* Typhimurium and *Salmonella* species and serovars

All strains of *S.* Typhimurium had 13 TFTRs and all but one strain, DT104, had the same TFTRs present (Figure 3.3). The *tetR* gene was present in DT104 but *ybjK* was absent. A further 9 strains of *S. enterica* of 7 different serotypes (Arizonae, Dublin, Enteritidis, Choleraesuis, Infantis, Newport, Paratyphi) and one strain of species *Salmonella bongori* were searched for TFTRs. As with *S.* Typhimurium, the range of TFTRs in the *Salmonella* genus did not vary considerably (n = 12-14), with *S.* Choleraesuis strain SSC-B67 having the most TFTRs (n = 14). Nine TFTR genes *acrR*, *envR*, *nemR*, *slmA*, *ramR*, *rutR*, *ycfQ*, *yjdC* and *U1* were present in all strains of the *Salmonella* genus (Figure 3.3). As in *Escherichia*, the most frequent biological role of these core TFTRs is regulation of genes encoding efflux pumps, with 3 core TFTRs of *Salmonella* (AcrR, EnvR and RamR) being involved in the regulation of multidrug efflux systems. Two TFTR genes were identified (*ramR* and *U1*) which were not present in any *Escherichia spp.* strain in this study. All nodes of the *Salmonella* tree contained the same TFTRs apart from *S.* Arizonae which lacked *yjgJ*. This lack of diversity is unsurprising as most *Salmonella* strains included here are serovars within the *S. enterica* species and do not show large variation in either the number or type of TFTR

Figure 3.2: Patterns of TFTR presence/absence across *Escherichia* strains

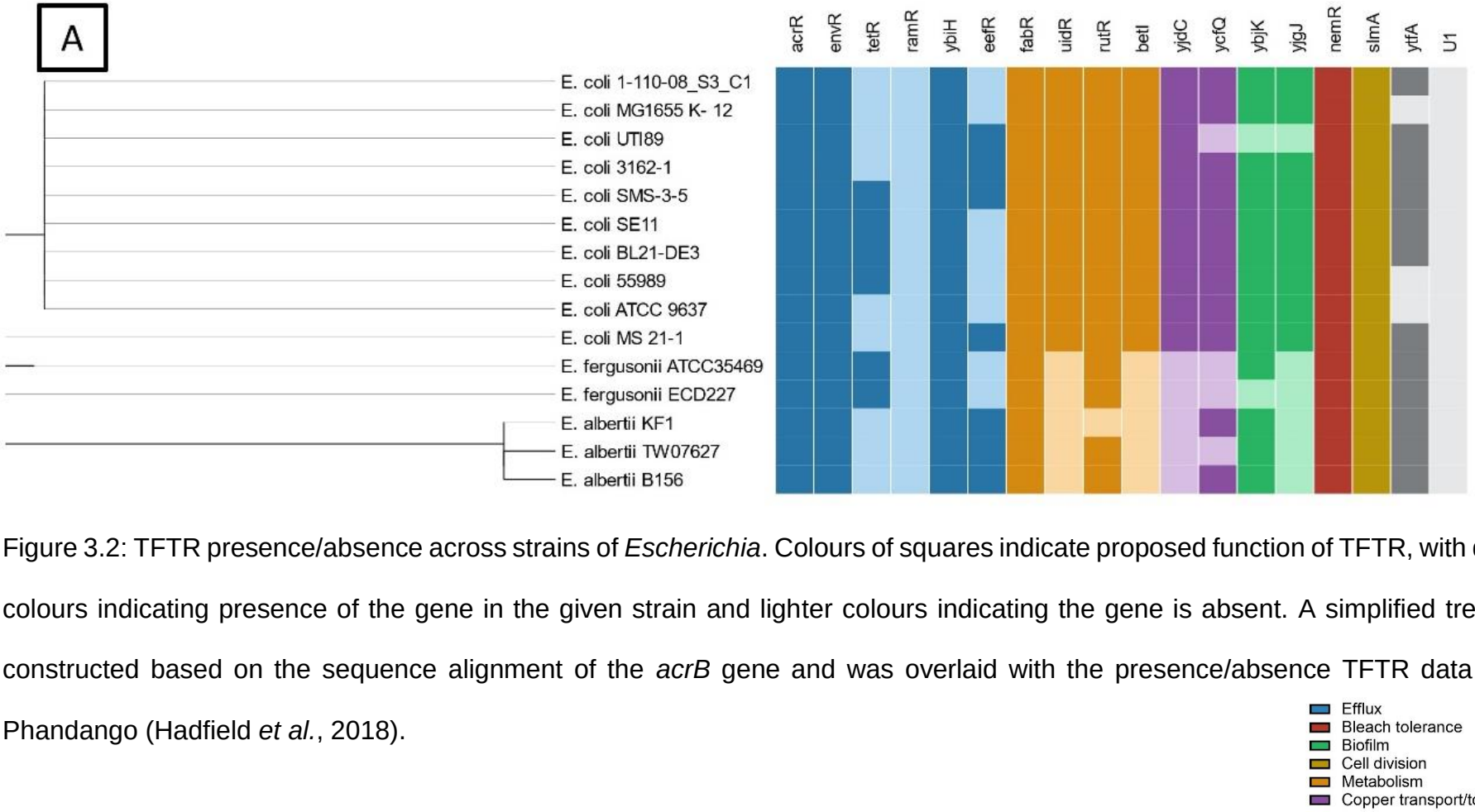


Figure 3.2: TFTR presence/absence across strains of *Escherichia*. Colours of squares indicate proposed function of TFTR, with darker colours indicating presence of the gene in the given strain and lighter colours indicating the gene is absent. A simplified tree was constructed based on the sequence alignment of the *acrB* gene and was overlaid with the presence/absence TFTR data using Phandango (Hadfield *et al.*, 2018).

Figure 3.3: Patterns of TFTR presence/absence across *Salmonella* species

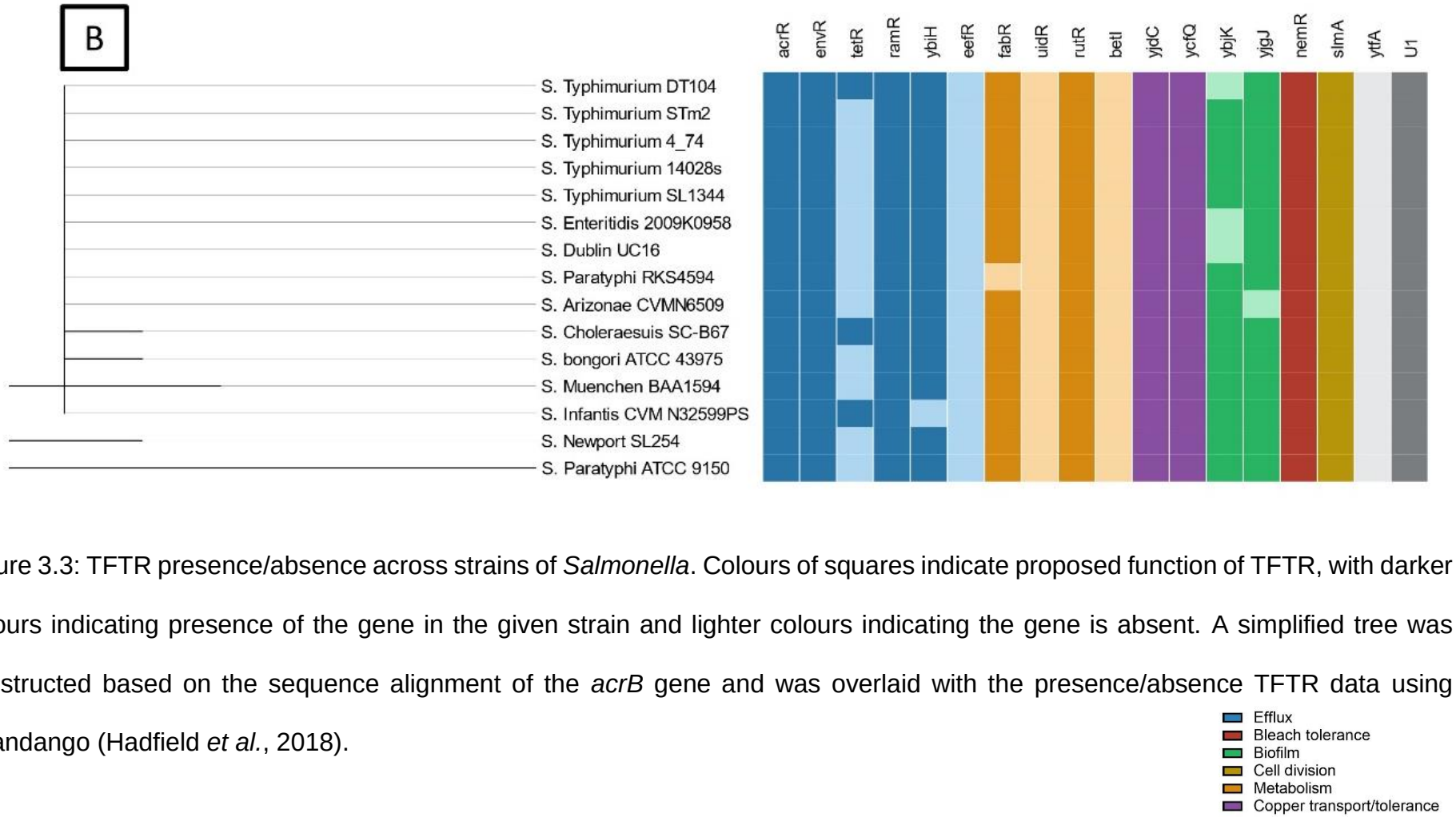


Figure 3.3: TFTR presence/absence across strains of *Salmonella*. Colours of squares indicate proposed function of TFTR, with darker colours indicating presence of the gene in the given strain and lighter colours indicating the gene is absent. A simplified tree was constructed based on the sequence alignment of the *acrB* gene and was overlaid with the presence/absence TFTR data using Phandango (Hadfield *et al.*, 2018).

3.4 Biological roles and conservation of TFTRs of *Salmonella* and *Escherichia*

The biological roles of many TFTRs in this study are known in *E. coli*, but it is not known if the targets, ligands or functions of TFTRs are genera, species or even strain-specific. There were five TFTR genes found in all *Salmonella* and *Escherichia* searched here: (1) *nemR*, (2) *acrR*, (3) *envR* (4) *ybiH* and (5) *slmA*. To classify the TFTRs by role, existing literature was searched for evidence of the regulatory targets and ligands of all TFTRs identified in *Escherichia* and *Salmonella* (Table 3.1). Efflux regulation was the most frequent TFTR function (n = 6) and the majority of TFTRs which are core in both *Salmonella* and *Escherichia* are efflux regulators. *Escherichia spp.* had two extra TFTRs which regulate metabolism, but there were no other differences in the distribution of TFTR role between these genera (Figure 3.4). In addition to the five genes conserved in all Gram-negatives tested here (*acrR*, *envR*, *nemR*, *slmA* and *ybiH*), two were core to *Escherichia* (*fabR* and *rutR*) and a further four (*ramR*, *U1*, *ycfQ* and *yjdC*) were core for *Salmonella*. TFTRs conserved throughout a genera are denoted as ‘core’ and all other TFTRs are therefore ‘accessory’ for this same genera. Therefore *Salmonella* and *Escherichia* have their own set of core and accessory TFTRs. The percentage carriage of each accessory TFTR was calculated for strains of both genera (Table 3.1). Nine TFTRs are, based on current available literature, single-target regulators (Table 3.1). A further seven TFTRs have been shown to either bind upstream of, or affect the transcription of, multiple genes. RutR and YbjK are known activators of at least one of their target genes (Nguyen Le Minh *et al.*, 2015; Shimada *et al.*, 2008). Nucleoid occlusion factor SlmA has no known transcriptional regulatory activity in *E. coli* but is a known activator in *V. chloerae* (Klancher *et al.*, 2017).

Figure 3.4: Biological roles of TFTRs in *Escherichia* and *Salmonella*

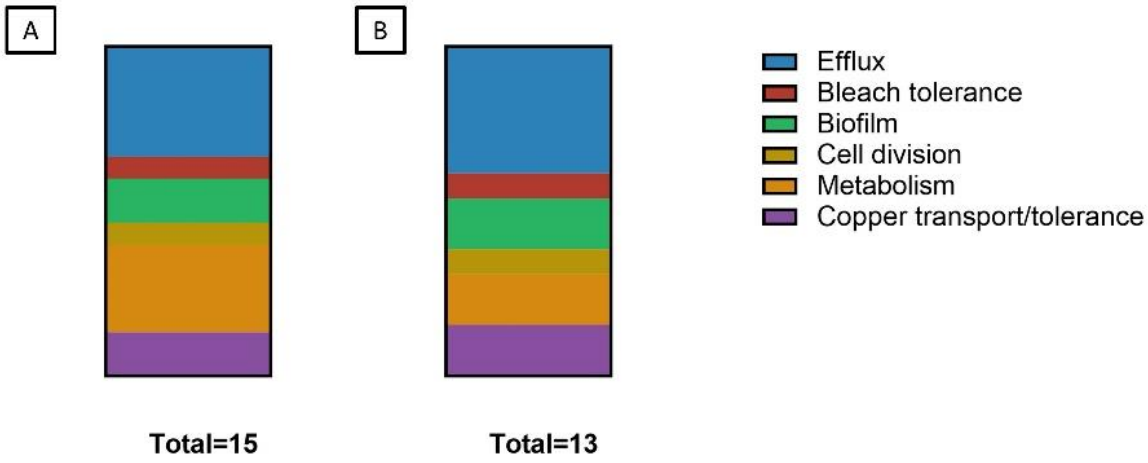


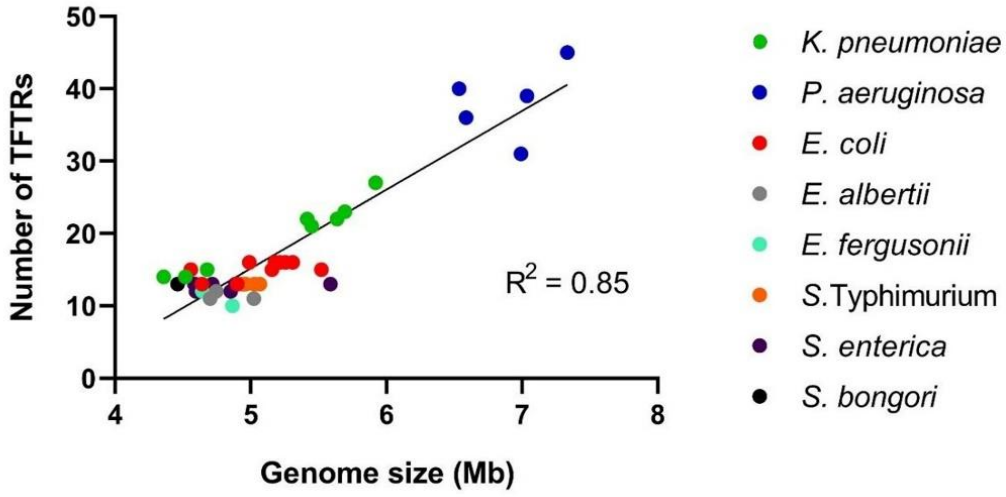
Figure 3.4: Proportion of TFTRs predicted to regulate various processes in (A) *Escherichia spp.* and (B) *Salmonella spp*. Based on the function assigned from literature search (Table 3.1). *Escherichia spp.* have two additional TFTRs involved in regulating metabolism. No other differences between TFTR function in *Escherichia* and *Salmonella* were seen.

3.5 TFTR number increases with genome size

The number of bacterial regulators is known to increase with genome size (Cuthbertson & Nodwell, 2013) and TFTR number is known to be positively correlated with genome size in *Mycobacteria* (Balhana *et al.*, 2015). The number of TFTRs present in Pseudomonas and Klebsiella species was determined. In this study, the number of TFTRs number was significantly positively correlated with genome size for a range of bacterial species ($R^2 = 0.85$, $P < 0.01$) (Figure 3.5a). The median genome sizes and TFTR numbers in this study were also comparable to the large number of genomes deposited on the NCBI database (Figure 3.5b), validating the methodology used here. *P. aeruginosa* has both the largest median genome size and greatest predicted TFTR number (median = 39, range 36-45). All *S.* Typhimurium strains had 13 TFTRs whereas the *Salmonella* genera had a small range of 12 - 14 TFTRs. *E. coli* strains had a slightly larger range of 12 - 16 TFTRs than *Salmonella* and the *Escherichia* genus as a whole had a range of 9 - 16 TFTRs. There was a significant difference in the number of TFTRs found in *E. albertii* and *E. fergusonii* versus *E. coli* and *Pseudomonas spp.* versus *P. aeruginosa,* with the *E. coli* and *P. aeruginosa* strains having a higher number of TFTRs. It is not known whether the number of targets of TFTRs also increases in larger genomes. As many TFTRs have multiple targets this is difficult to ascertain, and it is also possible that targets for individual TFTRs vary between bacterial species.

Figure 3.5: Genome size is positively correlated with the number of TFTRs

A



B

Organism	NCBI median genome size (Mb)	NCBI No. Genome assemblies	Median genome size (This study, Mb)	No. Genomes (This study)	Per Mb	Median predicted TFTR no.
P. aeruginosa	6.6	3,018	6.7	10	5.8	39
K. pneumoniae	5.6	6,101	5.4	10	3.8	21
Escherichia spp.	-	-	5.0	5	2.2	11
E. coli	5.1	13,288	5.2	10	2.9	15.5
Salmonella spp.	4.8	9,436	4.9	10	2.6	13
S. Typhimurium	-	-	5.0	5	2.6	13
A. baumanii	4.0	3,254	4.1	10	-	-

Figure 3.5: TFTR number increases with genome size. **(A)** TFTR number varied between strains, species and genera of bacteria but was significantly positively correlated with genome size (Mb). The largest range of TFTR number was seen in *Pseudomonas spp.* and the smallest in *S.* Typhimurium. **(B)** Table with median genome sizes and n= TFTRs in this study versus NCBI database. The median genome sizes were compared to genomes in this study to check that the genomes selected had a median genome size which is representative of the wider population of isolates. The number of predicted TFTRs was calculated by searching Interpro for IPR001647-containing sequences as previously described. A full list of strains used to produce this Figure are available in Appendix 1A.

3.6 Sequence variation is related to predicted biological function

The variance of these protein sequences was calculated by:

$$\left(\frac{Number\ of\ amino\ acid\ positions\ with\ n => 1\ variants}{Total\ sequence\ length}\right) \times 100$$

This method corrected for sequence length, meaning that variance was not skewed by sequence length. TFTRs which regulate efflux, bleach survival and biofilm formation and dispersal had significantly higher percentage variance (P = 0.01) than those involved in regulating cell division, metabolism or copper transport (Figure 3.6). There was no significant difference in level of TFTR variation between *Escherichia* and *Salmonella*. The lowest variance was seen in nucleoid occlusion factor SlmA. The TFTR genes and their predicted/known targets were also searched for stop codons, to begin to understand whether the resulting protein is likely to be functional.

3.7 Sequence variation is gene and organism- dependant

As the sequence variation of TFTRs was shown to vary due to function (Figure 3.6), the percentage variation in the TFTR target genes was also investigated and compared to variability of the regulator, in order to ascertain if this could be a function or regulator-specific effect. The percentage variation in TFTRs is shown below (Figure 3.7). There was no clear pattern in how level of variation in the regulator sequence relates to variation in target gene sequence. Sequences of *acrR* were more varied than the operon it regulates, *acrAB*, whereas *fabR* was less variable than *fabAB.* The amount of variation seen in a regulator and its target(s) also varied between genera. For example, there was higher variation in the *nemA* sequences in *Salmonella* than *Escherichia.* However, for most other regulator/target pairs, such as *fabR-fabAB,* there were no differences between the genera. Some gene homologs may therefore be

Figure 3.6: Mean percentage variation in TFTRs grouped by biological function

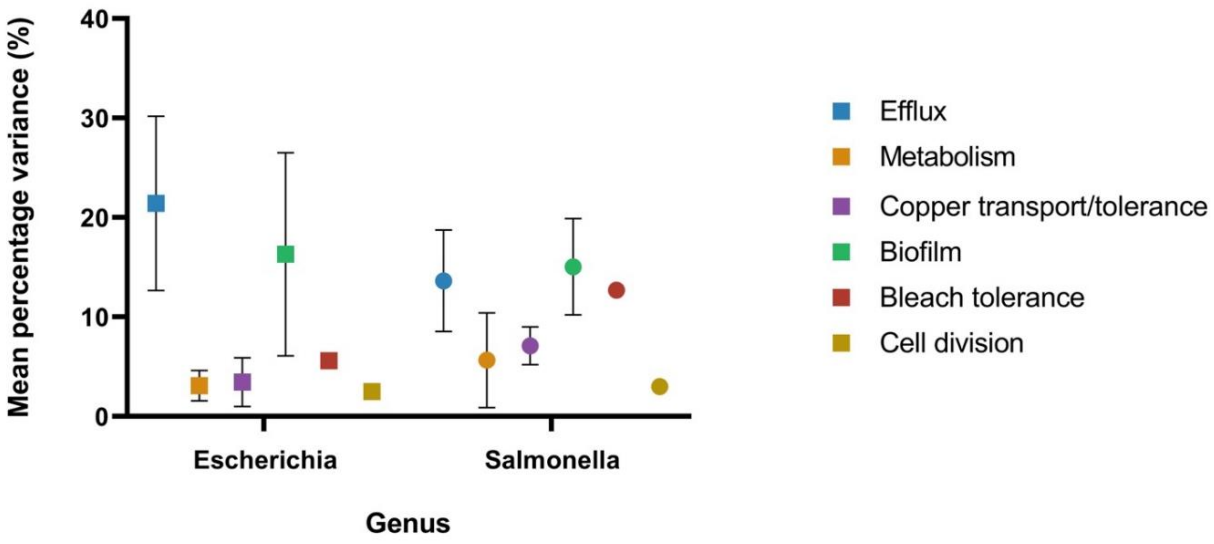


Figure 3.6: Percentage sequence variation of TFTRs grouped by function +/-standard error of the mean. TFTRs regulating efflux regulation, bleach survival or biofilm formation/dispersal have significantly higher percentage variance (one-way ANOVA *P* = 0.01) than those involved in cell division, metabolism or copper transport/tolerance. This was not a genera-dependant effect, with no significant difference between percentage variance of TFTRs between *Escherichia* and *Salmonella* genera.

Figure 3.7: Percentage sequence variation in TFTRs and their targets

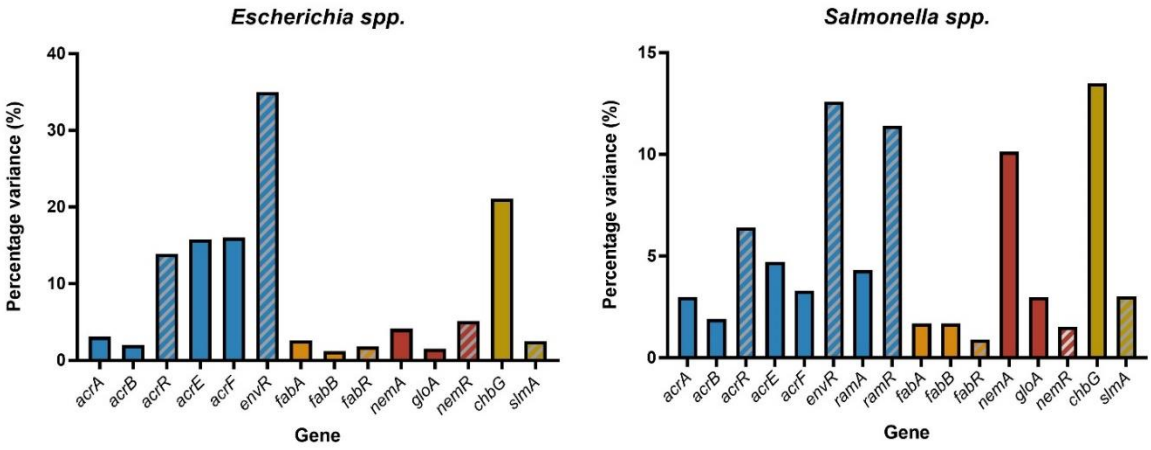


Figure 3.7: Percentage variation in amino acid sequence in TFTRs and their target genes in *Salmonella* and *Escherichia* generated from the sum of polymorphisms after alignment of sequences of the listed genes for each genus.

under similar levels of selective pressure resulting in comparable levels of variance in different genera. Certain TFTRs were genera-specific, i.e. the *eefR* gene was not present in any *Salmonella* strains and *ramR* is absent in *Escherichia* strains. Strains lacking the *eefR* gene were also found to lack *eefA* and *eefB,* components of the EefABC efflux system in *Enterobacter* (Appendix 1B).

3.8 Discovery of EefABCD efflux system and EefR regulator in *E. coli*

During this work, the regulator EefR was found to be present in some isolates of *E. coli.* Studying the genomic context of this regulator reveals it is transcribed alongside putative efflux genes *eefABCD* (Figure 3.8). A literature search revealed that the EefABCD efflux system was first described in *Enterobacter* (Masi, Pages et al. 2005, Masi, Pages et al. 2006), but has not been previously reported in these strains of *E. coli*. Interestingly, the findings here demonstrate that efflux genes can be 'accessory' and not present in all strains of a given species. Further work since this discovery has demonstrated that the *eefABCD* genes are highly conserved and present in the most prominent disease-causing clades of *E. coli* (Pugh *et al.,* in progress). SWISS-MODEL (Waterhouse *et al.,* 2018) was used to generate a structural model of EefR (Figure 3.9). The template selected was a putative TFTR from *Rhodococcus sp*. (PDB number 2nx4.2).

Figure 3.8: Genomic context of the *eefR* gene from EC4115 (O157:H7)

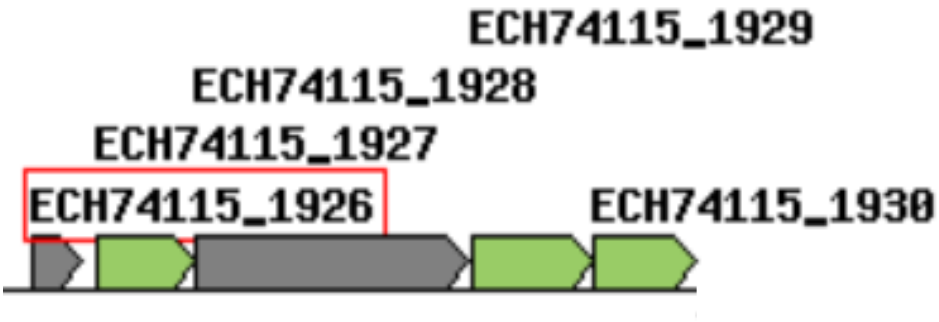


Figure 3.8: The genomic context of the *eefR* gene (ECH74115_1926, red box) generated using KEGG (Kanehisa & Goto, 2000). The *eefR* gene is located upstream of efflux pump component genes *eefA* (ECH74115_1927), *eefB* (ECH74115_1928), *eefC* (ECH74115_1929) and *eefD* (ECH4115_1930).

Figure 3.9: Model of EefR structure based on best-fit template 2nx4.2

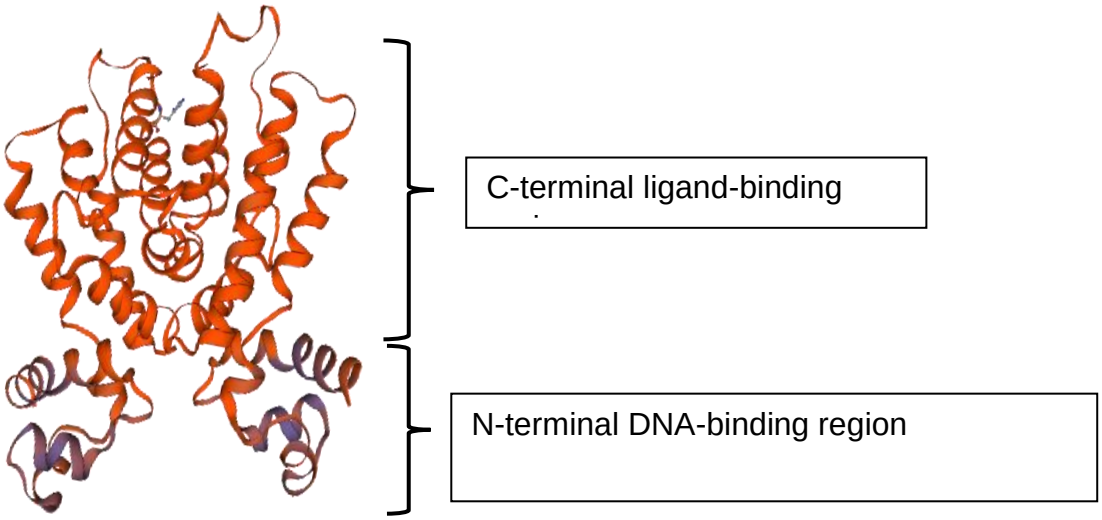


Figure 3.9: The predicted structure of EefR modelled using SWISS-PROT (UniProt, 2019). The structure is representative of TFTR regulators and is has a C-terminal ligand-binding region and an N-terminal DNA-binding region. The EefR protein comprises 2 x homodimers of EefR.

3.9 Discussion

TFTRs are frequently thought of as simple, single-target negative regulators, however, some have been shown to have multiple targets (e.g. EnvR, (Hirakawa, Takumi-Kobyashi*, et al.*, 2008). Some TFTRs can be both activators and/or repressors (e.g. MtrR and MerR) or can repress or activate multiple targets (Brown, Stoyanov, Kidd *et al.*, 2003; de Souza Pinto Lemgruber, Valgepea, Gonzalez Garcia *et al.*, 2019; P. J. Johnson & W. M. Shafer, 2015). Work by Shimada *et al.,* demonstrates that, for multiple classes of transcription factors, single-target function may be the exception, not the rule (Shimada *et al.*, 2018). As it has already been shown that some TFTRs have multiple targets, it is possible that other TFTRs currently classed as single-target regulators may have wider roles.

Even if a given transcription factor has more roles than previously thought, it is not necessarily true that this regulator will be found in every strain of a species, or that the same transcription factor will have the same roles between species or genera. For example, the *yhaJ* gene of *Eshcerichia spp*. is found throughout the genera but binds different locations in different strain genomes, with only 15% of binding sites being shared in all strains tested (Connolly, O'Boyle, & Roe, 2020).

The TFTRs which are most widely characterised, such as AcrR, have been characterised in *E. coli* and *S.* Typhimurium (Gu *et al.*, 2008; Li *et al.*, 2007; Ma *et al.*, 1996a; Manjasetty *et al.*, 2016a; Olliver *et al.*, 2004; Su *et al.*, 2007). Therefore, this work first sought to identify all the TFTRs present in a set of *Escherichia* and *Salmonella* available proteomes, to see if the TFTRs found were present on a strain, species, or genera- level. Proteomes, and not genomes, were searched here because

the specific HTH-motif IPR001647 on Interpro (Mitchell *et al.*, 2019) was used to search for TFTRs. Searching via this motif and not the DNA sequence enabled less false positives to occur. One reason for this is that there are more 'building blocks' of protein (21 amino acids) than DNA (4 bases). So, with fewer proteome sequences, a more accurate conserved sequence motif can be generated. The use of databases such as Interpro to identify proteins by motif are well-documented and reviewed (Mulder & Apweiler, 2002). While this meant that this study was limited to searching proteomes, it also meant that the information gathered via the databases of Interpro was more likely to be a protein containing the HTH-motif of interest. Moreover, the sequences that were available were from a wide variety of sources (i.e. clinical, environmental and laboratory), which provide a representative example of these genera.

The *E. coli* strains had significantly more TFTRs than the other species in the genus, *E. fergusonii* and *E. albertii* (Figure 3.2). All three of these *Escherichia* species have broad host ranges as they are able to colonise and cause infections in both humans and animals (Glover, Wentzel, Jenkins *et al.*, 2017; Yamamoto, Hernandes, Liberatore *et al.*, 2017). Both *E. fergusonii* and *E. albertii* are emerging enteropathogens (Inglis, Merritt, Bzdyl *et al.*, 2015) (Savini, Catavitello, Talia *et al.*, 2008). It is possible that the differences in regulatory genes reflect the different lifestyles and virulence of these species. *Salmonella* species tested here were (aside from one strain) serovars of the species *S. enterica* and therefore it is expected that these strains did not show significantly different TFTR numbers (Figure 3.3).

This work also sought to identify the number and functions of TFTRs across a number of Gram-negative species (*K. pneumoniae, P. aeruginosa, E. coli, E. albertii, E.*

fergusonii, S. enterica, S. bongori). These species were selected because they are featured on the WHO priority pathogen list (WHO, 2017). As many TFTRs are efflux regulators, characterising the TFTR genes of these priority pathogens may provide insight on the antimicrobial resistance mechanisms and virulence of these pathogens.

Of the three TFTRs found to be core across the Gram-negative species studied (AcrR, EnvR and NemR), two are known regulators of efflux (AcrR and EnvR) and the other promotes bleach tolerance (NemR). It has been reported that up to 25% of known TFTRs act as regulators of efflux systems (Ahn *et al.*, 2012). Consistent with this, 33% of TFTRs were predicted to regulate efflux systems in this study. However, when only considering TFTRs found in all strains the majority were efflux regulators. Thus, the most widespread TFTRs in Gram-negative bacteria are those involved in efflux regulation. Efflux is a key mechanism of antimicrobial resistance and the ability to overexpress efflux systems can confer multi-drug resistance, thus understanding the TFTR regulators of these efflux pumps is essential to better understanding efflux-mediated resistance.

It is possible that the prevalence of TFTRs conserved in this dataset (including the high proportion of efflux regulators), was skewed due to the selection of strains from the WHO priority pathogens list of multidrug resistance species, or perhaps the processes regulated by core TFTRs (i.e. efflux) are more widespread than previously thought. However, the strains used in this study originate from multiple sources (including patient samples, environmental and laboratory strains). Therefore, even though the species studied are limited, the strains within this study are diverse and representative of the species.

This work also demonstrated that a higher percentage variation was seen in TFTRs which regulate processes which contribute towards antimicrobial resistance or virulence (i.e. biofilm dispersal and efflux) compared to other TFTRs. This may be because variation in these TFTRs can confer favourable phenotypes, which promotes dissemintation and eventually, fixation, of these genotypes. For example, when challenged with antimicrobials, mutations which cause loss-of-function of the TFTR regulator are selected. Polymorphisms in efflux regulators AcrR (Adler, Anjum, Andersson *et al.*, 2016b; Webber, Talukder, & Piddock, 2005a), EnvR (Olliver *et al.*, 2005), RamR (Chiu, Huang, Chen *et al.*, 2017) and TetR (Henssler, Bertram, Wisshak *et al.*, 2005), have been reported previously. In this study, premature stop codons were observed in the sequences of *envR, acrR*, *acrE* and *acrF* (Appendix 1C).

These patterns in sequence variation were not replicated in the target genes of the TFTRs, i.e. the efflux genes tested did not have significantly higher percentage variation than other target genes (Figure 3.6). This indicates that it is the regulators themselves which are under either positive or negative selective pressure based on the target(s) they regulate and not simply an artefact of the selective pressure which may be applied to the whole local region in general, for example due to proximity to the origin of replication or pathogenicity islands.

The variation between TFTRs and their targets was sometimes similar between *Eshcerichia* and *Salmonella* (Figure 3.7). For example, the variation in sequences of *acrR* and *acrAB* showed a similar pattern in both *Escherichia* and *Salmonella* strains, with higher variation in the sequence of the regulator. Similarly, variation in *fabR* and *fabAB* remained low in both genera. Variation was higher in general in *Escherichia* species, although strains within this group were more genetically distant than those

tested in the *Salmonella* genera. Some patterns of TFTR and target variation did vary between the genera, notably there was much higher variation in sequences of *envR* and *acrEF* in *Escherichia.* The AcrEF efflux pump shares many substrates with AcrAB and the *acrEF* operon is thought to be H-NS silenced under most conditions (Hirakawa, Takumi-Kobyashi*, et al.*, 2008). The operon may not be required in many situations, meaning that the whole region encounters spurious polymorphisms and genetic drift.

Given that variation in the both the presence/absence and sequence of TFTRs was seen here, it is possible that these regulators have species or even strain-specific functions. A transcriptional regulator may evolve differentially in different species, acquiring or losing targets in response to specific requirements of the species (Rogers & Bulyk, 2018). This means that it is not only the number, but the function, targets and sequence similarity of TFTRs and other regulators is likely to vary between bacterial species. The notion that transcriptional regulators can gain targets is not new, for example the CRP regulon of *E. coli* K-12 can be observed to evolve under laboratory conditions in just over 20,000 generations (Cooper, Rozen, & Lenski, 2003).

In addition to a regulator gaining targets, the regulatory gene(s) may undergo a duplication which allows further regulators to evolve. Due to this, multiple transcription factors can sometimes influence the expression of a single target under different conditions, leading to highly diverse networks. This means that if two related species (or strains) with the same set of regulators do not necessarily have the same regulatory network (O'Boyle, Turner, Roe *et al.*, 2020).

During a duplication events, regulatory genes are duplicated and undergo subsequent specialisation in function (Voordeckers, Pougach, & Verstrepen, 2015). This could also

explain some redundancies in regulatory targets and binding sites of TFTRs (for example, AcrR and EnvR both bind to the same site upstream of *acrAB* (Hirakawa, Takumi-Kobayashi*, et al.*, 2008)*)*. The cross-talk of these efflux systems is not well understood but understanding the conservation of these genes gives insight as to their importance in bacterial species. Moreover, understanding the multiple regulators involved in regulating RND efflux could provide opportunity for drug discovery targets to be identified.

The conservation and heterogeneity of TFTRs discussed here highlights the varied and sometimes, underestimated, roles of TFTRs. TFTRs which regulate processes promoting pathogenicity, virulence or multidrug resistance are likely to be more ubiquitous, but contain more sequence variation, throughout Gram-negative bacteria. Our current understanding of TFTRs is largely based on a few well characterised examples for which we have crystal structures, but often leads to the misunderstanding that all TFTRs act in these more simplistic ways. We propose that the current classification system of TFTRs underestimates the roles of TFTRs and that these proteins often regulate many targets, sometimes using multiple different mechanisms.

The number of genes encoding transcription factors varies between bacterial species and this variation depends on both genome size and bacterial lifestyle, with small-genome, niche-restricted species having fewer transcriptional regulators (Merhej, Royer-Carenzi, Pontarotti *et al.*, 2009; Miravet-Verde, Llorens-Rico, & Serrano, 2017). Conversely, bacteria with large genomes and varied lifestyles such as *Pseudomonas* species contain the largest number of regulatory genes of bacterial genomes studied to date (Moradali, Ghods, & Rehm, 2017). Data here supports the observation by others that TFTR number positively correlates with genome size and that this trend

exists throughout *Escherichia* and *Salmonella* in addition to other Gram-negative species (Figure 3.5). The inclusion of pathogenic, environmental and laboratory strains, makes the results reported here more representative of these genera as a whole. Interestingly, strains and species of *Salmonella* and *Escherichia* showed variation in the number of TFTRs present (Figures 3.2 - 3.3), thus even the most recent of evolutionary events are selecting for or against the conservation of certain TFTR genes.

By searching for the HTH motif, it was also possible to identify the presence of TFTRs in strains for the first time. The gene coding for the regulator of the efflux system, *eefR*, was found in four *E. coli* strains and all three *E. albertii* strains (Table 3.1). The TFTR EefR regulates the EefABCD RND efflux system in *Enterobacter* species, which is also under regulation by H-NS (Masi *et al.*, 2006; Masi *et al.*, 2005). This study also identified the EefABCD efflux system in some *Escherichia* species (Appendix 1C). This efflux system has not, to our knowledge, been reported in these *Escherichia* species.

This was, to the knowledge of the authors, the first wide-scale study on TFTRs across Gram-negative pathogens. With rising levels of antimicrobial resistance and limited novel treatment options, we should seek to better understand regulators such as TFTRs which are frequently implicated in multidrug resistant phenotypes.

3.10 Future work

- Increase the number of proteomes searched for the HTH helix to provide a larger dataset.
- Further characterisation of the EefABCD efflux system and the regulator EefR. This should include phenotypic studies, determination of substrates and crystallography. The conservation of this system throughout Gram-negative

bacteria is now being studied at Birmingham University (Pugh *et al.,* in progress).

- An extension to this work would be to understand whether this pattern of conservation is TFTR-specific or also true of other regulators. A small-scale study looking at the conservation (sequence and presence) of another family of regulators could be beneficial to better interpreting this data.
- Characterisation and modelling of the polymorphisms seen in these regulators may be informative, especially as it is known that mutations in a regulator can have consequences for AMR and other cellular processes.
- Further study to classify the regulatory mechanisms of these TFTRs (e.g. DNA bending/looping) and determine whether the mechanism is conserved between species/strains.

3.11 Key findings

- The TFTR family regulators have diverse functions and may commonly act as global “off-target” regulators.
- The most common function of the TFTR regulator in *Salmonella* and *Escherichia* is efflux regulation.
- The TFTRs present can vary, even between strains of the same species. The sequence of these regulators can also vary, with some being more conserved than others. Searching and studying regulators in this way can uncover novel TFTRs or TFTRs present in species not previously documented as having the TFTR. For example, here the regulator EefR was identified in some strains, leading to the discovery of the EefABCD efflux system in *Escherichia* strains.

Chapter Four

Regulation of *acrAB* by AcrR and EnvR in *Salmonella*

4.0 Regulation of *acrAB* by AcrR and EnvR in *Salmonella*

4.1 Background

In both *Salmonella* and *E. coli,* AcrR is the local negative regulator of *acrAB* and is encoded divergently from *acrAB* (Ma *et al.*, 1996a). In *E. coli* EnvR, the local regulator of *acrEF*, is also known to regulate *acrAB* by binding the same 24-bp palindrome upstream of *acrAB* as AcrR (Hirakawa, Takumi-Kobayashi*, et al.*, 2008), Figure 4.1. The palindromic binding site upstream of *acrAB* in *E. coli* that was shown by Hirakawa *et al.,* is present upstream of *acrAB* in *Salmonella*. However, it is not known whether EnvR in *Salmonella* can regulate *acrAB* and whether the binding site is that same as that in *E. coli*.

4.2 Aims

The overall aim of this chapter was to confirm whether EnvR is able to regulate *acrAB* in *Salmonella*. This was split into the following smaller aims:

- Construct strains which can be used to express and purify AcrR and EnvR protein and problem solve any issues with protein expression or purification.
- Test whether purified AcrR/EnvR protein can bind upstream of *Salmonella acrAB* genes.
- Confirm the binding site of AcrR/EnvR by seeing if mutation of the palindromic binding site prevented binding upstream *acrAB*.
- Construct strains which can be used to test the effect of interrupting or overexpressing *acrR* or *envR* genes by using the pET20b vector.
- Investigate the phenotypic effects of interrupting and overexpressing *acrR* and *envR* on growth, antimicrobial susceptibility, cell wall composition and motility.

Figure 4.1: Regulation of *acrAB* and *acrEF* expression by EnvR in *E. coli.*

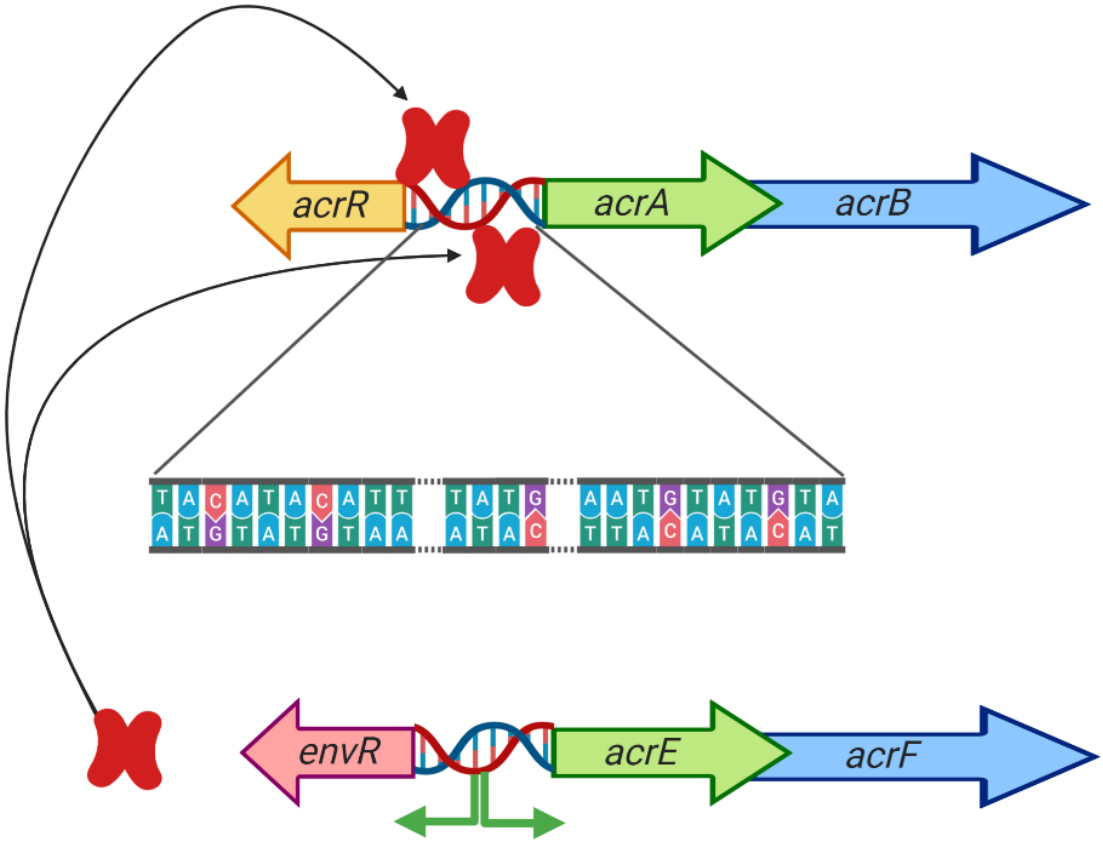


Figure 4.1: Schematic of the regulation of *acrAB* by EnvR in *E. coli*. The EnvR homodimer (red) binds a 24-base pair palindrome upstream *acrAB*. Once bound, EnvR prevents transcription of the *acrAB* and *acrR* genes, presumably via steric inhibition of RNA polymerase binding. Bound EnvR is removed by an unknown mechanism. It is likely that ligands bind EnvR causing a conformational change which reverses binding. This is known to be the case for other TFTRs, but the roles and mechanisms of these proteins is complex (Chapter 3).

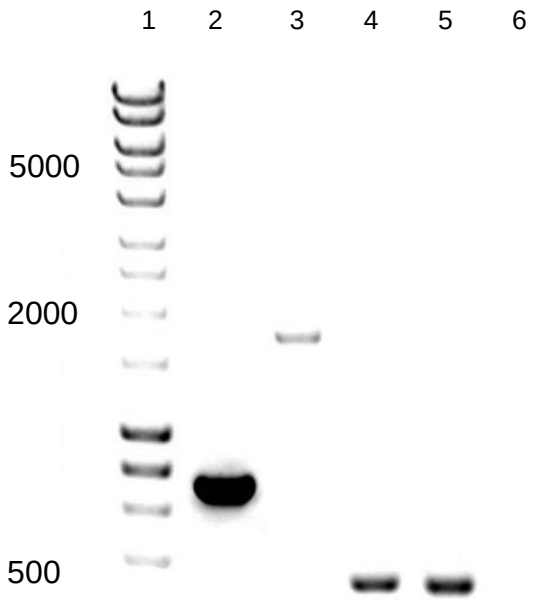
4.3.0 Purification of *Salmonella* AcrR and EnvR protein

To determine whether EnvR and AcrR can bind to the DNA sequence upstream of *acrAB* it was first necessary to express and purify these proteins. This section describes the steps taken to express and purify *Salmonella* AcrR and EnvR protein using an *E. coli* expression system and the problem solving required to obtain functional protein. The *E. coli* strain BL21 pLysS is engineered for protein production and against lysis by lon protease. The pTrc vector also produces high-level and inducible expression of target genes from a T7 promoter. Therefore, the preferred option for purifying the *Salmonella* proteins was to use the BL21 strain in combination with the pTrc vector.

4.3.1 Construction of pTrc expression vector for protein purification

The overexpression vector selected was the pTrc plasmid (pTrc His2 TOPO, Invitrogen), which uses a high-expression *lacO*-controlled *trc* promoter*,* allowing high-level, controlled induction of gene expression. The pTrc plasmid selected encodes a C-terminal 6x his tag, which enables the purification of the protein using ion-affinity chromatography. In this case, the commonly used Ni-NTA matrix was used for purification of the his-tagged protein (Ni-NTA Fast-Start, Cat. 30600, QIAGEN). First, the *acrR* and *envR* genes in *Salmonella* SL1344 were interrupted using a single-step inactivation protocol (Datsenko & Wanner, 2000). The inactivation of *acrR* and *envR* genes was confirmed by PCR (Figures 4.2 – 4.3). The *acrR* and *envR* genes were then cloned into the pTrc vector. Successful constructs were transformed into the *E. coli* strain BL21 pLysS, a strain specifically engineered for protein expression.

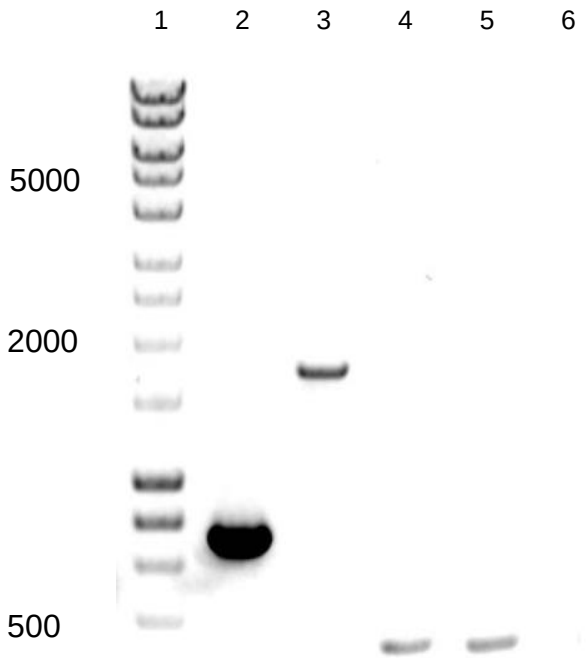
Figure 4.2: PCR to check for successful interruption of the *acrR* gene



Lane	Strain genotype	Fragment size (bp)	Cycling conditions
acrR check PCR			
1	1 kb ladder (Bioline)	-	95°C – 1 min (95°C – 15 sec 52°C – 15 sec 72°C – 2 min) x35 72°C – 10 min
2	SL1344	737	
3	SL1344 *acrR*::*aph*	1729	
4	SL1344 Δ*acrR*	336	
5	SL1344 Δ*acrR* Δ*envR*	336	
6	Negative control	-	

Figure 4.2: The PCR result for checking the length of the *acrR* gene in WT (lane #2), *acrR*::*aph* (lane #3), Δ*acrR* (lane #4) and the double knockout Δ*acrR*Δ*envR* (lane #5). The fragment size (bp) and cycling conditions are also shown.

Figure 4.3: PCR to check for successful interruption of the *envR* gene



Lane	Strain genotype	Fragment size (bp)	Cycling conditions
envR check PCR			
1	1 kb ladder (Bioline)	-	95°C – 1 min (95°C – 15 sec 50°C – 15 sec 72°C – 2 min) x35 72°C – 10 min
2	SL1344	781	
3	SL1344 *envR::aph*	1923	
4	SL1344 Δ*envR*	548	
5	SL1344 Δ*acrR* Δ*envR*	548	
6	Negative control	-	

Figure 4.3: The PCR result for checking the length of the *envR* gene in WT (lane 2), *envR*::*aph* (lane 3), Δ*envR* (lane 4) and the double knockout Δ*acrR Δ*envR* (lane 5). The fragment size (bp) and cycling conditions are also shown.

4.3.2 Purification of AcrR and EnvR protein

The AcrR and EnvR proteins were expressed and purified using the Ni-NTA fast start kit (Ni-NTA Fast-Start, Cat. 30600, QIAGEN). Samples taken prior to IPTG induction were compared to the induced fraction (post-addition of 1 mM IPTG). Comparing the pre- and post- induction fractions serves as a positive control to show that induction of expression was successful. Fractions were separated by SDS-page and stained using Coomassie blue. Over the course of the project, AcrR and EnvR were purified multiple times and an example Coomassie stain is shown in Figure 4.4.

Protein yields for AcrR were consistently higher than for EnvR. This difference in yield can be seen in both the Coomassie gels (Figure 4.4) and a western blot (using Anti-6x His+ antibody, Abcam ab497646), (Figure 4.5). Upon sequencing to check the pTrc constructs, it was found that the native start codon had not been removed in the EnvR pTrc construct. It is recommended that the native start codons are removed prior to cloning as the presence of 2x start codons reduces protein yield. This therefore accounts for the reduced yield of EnvR protein. Information about different protein preparations (name denoted as #no, yields, etc.) are listed in Table 4.1.

Figure 4.4: Coomassie blue protein stain of AcrR and EnvR fractions

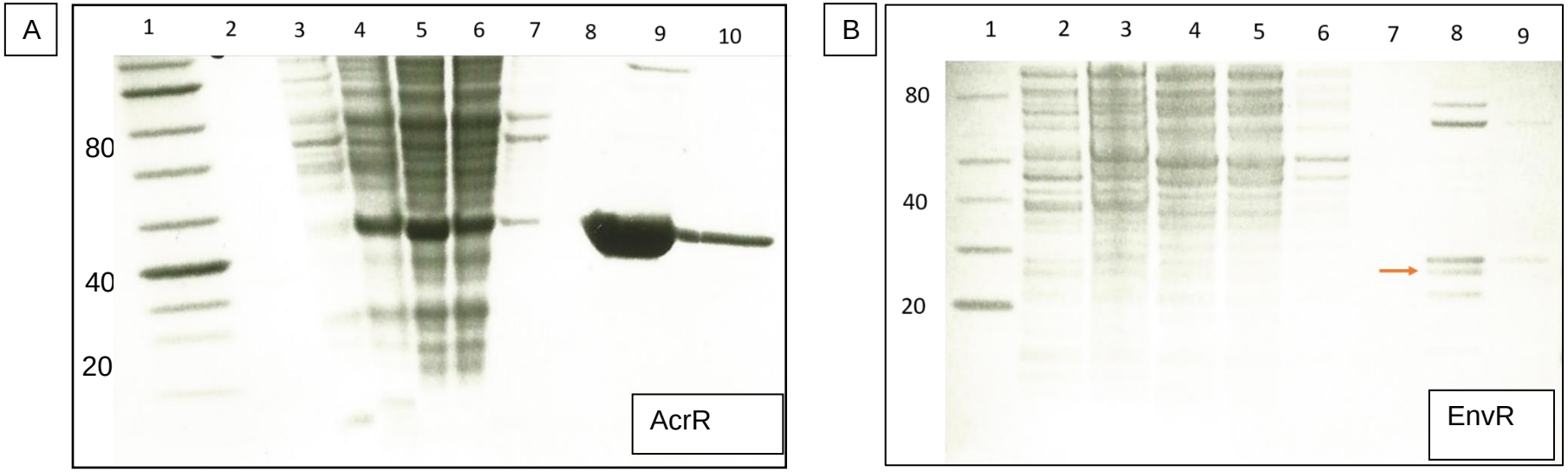


Figure 4.4: Coomassie stain of AcrR and EnvR fractions. The EnvR and AcrR proteins are both ~25 kDa. Fractions of AcrR #1 (Panel A) and EnvR #1 (Panel B) collected during purification were electrophoresed on SDS-PAGE gels and then stained with coomassie blue. **Panel A –** (1) Pageruler marker (Thermo Fisher, UK), (2) Colour mark (NEB, UK) (not visible), (3) Non-induced sample (i.e. before 1 mM IPTG induction), (4) Induced sample (i.e. after 1 mM IPTG induction), (5) Supernatant, (6) Flow-through, (7) Wash #1, (8) Wash #2, (9) Elution #1, (10) Elution #2. **Panel B –** (1) Pageruler marker, (2) Non-induced sample (i.e. before 1 mM IPTG induction), (3) Induced sample (i.e. after 1 mM IPTG induction), (4) Supernatant. (5) Flow-through, (6) Wash #1, (7) Wash #2, (8) Elution #1, (9) Elution #2. EnvR elution #1 presented as doublet (indicated by red arrow). These gels are representative of the many gels undertaken for different protein preps.

Figure 4.5: Western blotting using anti-his antibody for AcrR and EnvR protein

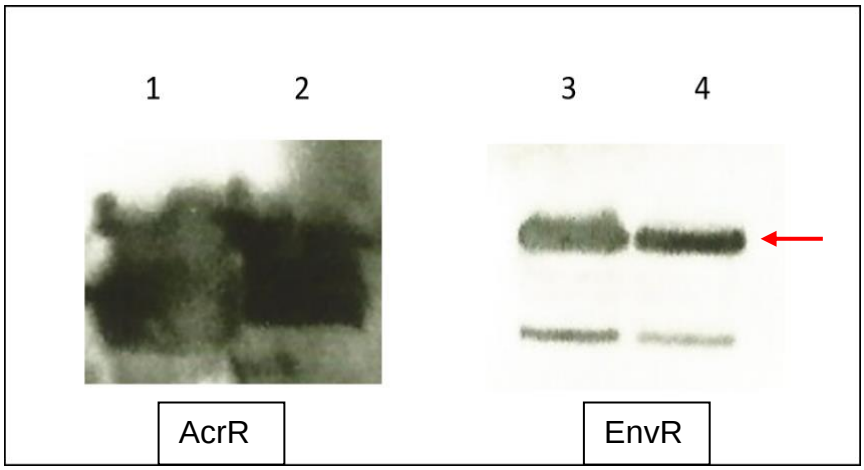


Figure 4.5: Western blot using the anti-6x His+ antibody, (Abcam ab497646) to confirm presence of his-tagged proteins. Colour mark (NEB, UK) marker was also run and the bands shown above are between 20-30 kDa. The AcrR and EnvR are both ~25 kDa. The first and second elution fractions of AcrR #1 (lanes 1 and 2) and EnvR #1 (lanes 3 and 4) are shown. A second band was present in both EnvR elutions. The band believed to be the his-tagged EnvR protein is shown by the red arrow. It is possible that a second band is also in the AcrR elutions, but it is not possible to see this as the concentration of protein was so much higher.

4.3.3 Problem-solving protein functionality and EMSA assays

Purified EnvR appeared as a doublet on a Coomassie gel (Figure 4.4). This could indicate that the protein has cleaved during column purification, producing a second fragment of EnvR protein. It is also possible that there are co-factors or contaminants which are bound to some of the EnvR, influencing the migration of the protein through the gel.

Here, “functional” protein is defined as purified protein able to bind upstream *acrAB,* the known target of AcrR and the predicted target of EnvR. The first batch of purified EnvR was functional (i.e., was shown to bind the promoter of *acrAB*). However, AcrR protein which had been purified at the same time as the functional EnvR was not able to bind upstream *acrAB* in a test EMSA assay. As AcrR is known to bind this region, the protein was deemed non-functional.

It is important to remember that an EMSA reaction comprises many components and therefore many reasons for failure which may be independent of the protein. The EMSAs do not contain all the other factors which would be present *in vivo*, such as other regulators or potential co-factors. Therefore, although such protein batches are referred to as “non-functional”, there are multiple other reasons for a lack of visible binding. These assays should be improved by the addition of further controls, such as running a range of protein concentrations to determine whether binding is concentration-dependent. Furthermore, switching to using radiolabelled DNA probes would enable the addition of further controls such as unlabelled DNA which would improve the reliability of these assays.

The EnvR protein which was able to bind upstream *acrAB* was used to optimise EMSA running conditions and as a positive control in future tests of protein batches. A summary of the different protein preparations is provided in Table 4.1.

The #2 batch of AcrR/EnvR, when combined with DNA in the EMSA reaction, did not allow the free DNA to migrate freely through the acrylamide gel, resulting in no bands present. Others seemed to migrate through the acrylamide very slowly, deforming the band of free DNA during electrophoresis. Examples of these issues are shown in Figure 4.6. These protein samples were later found to contain large amounts of DNA contamination (Figure 4.7). During this work, multiple batches of protein were purified to try and fix these issues. To solve this, the expression and purification of both proteins was repeated under numerous conditions. Unfortunately, while it was often possible to purify functional EnvR protein, purifying AcrR was more difficult. Eventually, a low volume of low concentration AcrR and EnvR was purified at the Protein Expression Facility (PEF). Proteins purified by the PEF were active, albeit at very low concentrations. The PEF pooled and concentrated the proteins. A summary of some of the conditions tested while trying to purify AcrR and EnvR are listed in Table 4.2.

The low concentrations of AcrR and EnvR impacted the results generated here, with EMSAs only able to demonstrate weak binding. After DNA concentration optimisation (Figure 4.9), the DNA concentration used in all further experiments was 15 ng/µl. The molarity of protein:DNA should be kept consistent in future, but this varied depending on the protein preparations used. If the issues with protein function and purification can be solved, future work should ensure that the ratio of DNA:Protein is consistent.

Table 4.1: Summary table of the protein preparations used and referenced in this work.

Prep name	Date purified	Concentration µg/ml	Expression/Purification method	Functional	Notes
AcrR #1	05/04/2017	2,250	Ni-NTA Fast-start protocol	No	-
EnvR #1	05/04/2017	*1020	Ni-NTA Fast-start protocol	Yes	This protein was used: • For optimisation of EMSA assays • **For experiments in this chapter**. • For all work done by Master's student J. Scadden **(Chapter 5).**
AcrR #2	10/08/2017	*886	Ni-NTA Fast start expression protocol, followed by –	No	Further testing revealed this prep was still contaminated with DNA.

			(1) Breaking cells using French press (2) Purification using nickel ion column (with Dr. Jamshad) (3) Homemade wash/elution buffers (constituents the same as in Ni-NTA kit) Salt washes and buffer exchanges to remove DNA		Protein sample was not used for experiments.
EnvR #2	10/08/2017	*744	Ni-NTA Fast start expression protocol, followed by – (1) Breaking cells using French press	No	Further testing revealed this prep was still contaminated with DNA. Protein sample was not used for experiments.

			(2) Purification using nickel ion column (with Dr. Jamshad) (3) Homemade wash/elution buffers (constituents the same as in Ni-NTA kit) (4) Salt washes and buffer exchanges to remove DNA		
AcrR #3	16/10/2017	*912	Same as for #1	Yes	Majority was used problem-solving gel running issues. **Used for this chapter.**
EnvR #3	16/10/2017	*744	Same as for #1	Yes	Majority was used problem-solving gel running issues.

AcrR #4	25/07/2019	*41.16	Ni-NTA Fast start expression protocol, followed by purification of pellets by the PEF facility using Size-exclusion chromatography. Salt washes to remove DNA and buffer exchange performed. Pooled fractions were concentrated.	Yes	Very poor yield- large loss of protein concentration suspected after salt wash. Pressure issues also occurred during SEC resulting in backflow and protein loss. Unable to concentrate this further. Used for assays in Chapter 5.
EnvR #4	25/07/2019	*286.44	Ni-NTA Fast start expression protocol, followed by purification of pellets by the PEF facility using Size-exclusion chromatography. Salt washes to remove DNA and buffer	Yes	Low yield – loss of protein concentration suspected after salt wash. Used for assays in **Chapter 5.**

			exchange performed. Pooled fractions were concentrated.		

Table 4.1: A summary of several protein preps which are discussed throughout this work. Includes information about which protein preparations have been used in different chapters of this thesis. * = Concentrated using a spin column method

Figure 4.6: Example of issues experienced with purified AcrR and EnvR

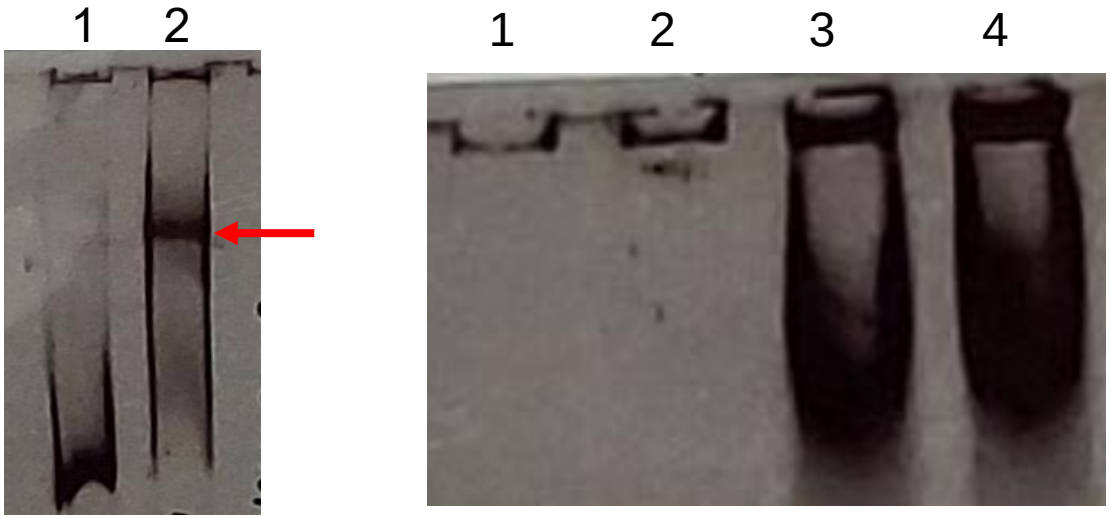


Figure 4.6: EMSA images showing a selection of some of the issues present with select batches of purified protein. Both the images are cropped from the same gel image. EMSA reactions were performed and separated on a native TBE gel by electrophoresis. The gel was then stained for DNA using SYBR green. Bands which are higher in the gel are likely 'shift' bands- which represent protein-bound DNA. Panel A - (1) Free DNA 15 ng/µl (upstream region of *acrAB*) only, (2) 15 ng/µl DNA (upstream region of *acrAB*) + purified EnvR #1. This EnvR protein is functional and has bound the DNA, causing a 'shift' indicated by red arrow and is therefore functional. Panel B - (1) EnvR #2 only, (2) EnvR #2 + 15 ng/µl DNA (upstream region of *acrAB*). No visible DNA bands indicate issues with the formation of protein:DNA complexes. (3) AcrR #2 protein only, (4) AcrR #2 + 15 ng/µl DNA (upstream region of *acrAB*). DNA fluorescence was present in the AcrR #2-only well, indicating DNA contamination. A volume of 3 µl protein was used each time and no dilutions were performed. The concentration of protein used here therefore relates to the information in Table 4.1.

Figure 4.7: DNA contamination in purified AcrR and EnvR protein samples

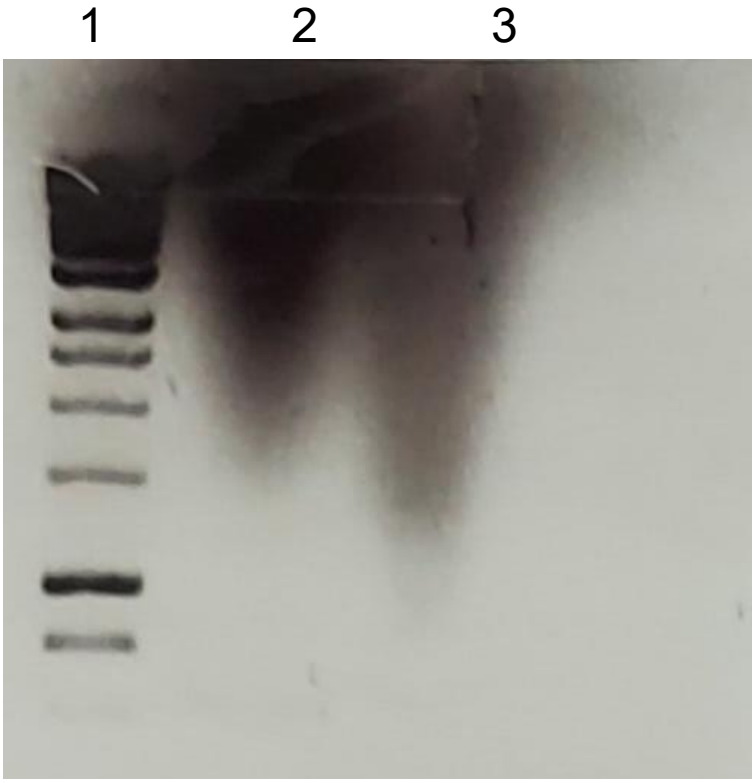


Figure 4.7: DNA contamination visible in AcrR and EnvR protein samples. Samples of protein was boiled for 10 minutes in loading dye and then separated by electrophoresis on an agarose gel containing midori green (Geneflow) for DNA visualisation. (1) 1 kb DNA ladder to show correct running of the gel, (2) 3 µl of AcrR #2 in loading dye, (3) 3 µl of EnvR #2 in loading dye. As no DNA was added to (2) or (3), DNA shown is contamination in the protein samples.

Table 4.2: Summary of conditions tested during problem solving purification

Condition tested	Reason	Outcome
A) Expressing and purifying protein from BL21 pLysS	The Bl21- pLysS strain of *E. coli* has been specifically engineered to support high-volume protein expression. Proteins produced are protected from protease degradation.	Small improvement in protein activity.
B) Undertaking whole procedure at 4C, snap freezing of protein aliquots	Some proteins are temperature sensitive and will denature or precipitate easily.	No improvement in protein activity.
C) Optimisation of expression conditions	Poor growth can cause poor protein yields or a decrease in protein quality. Large conical flasks were used. A concentration gradient of IPTG was tested with aliquots removed for purification to test protein activity.	No improvement in protein activity. Large conical flasks improved aeration and growth of strains.
D) Making buffers from constituents and attempting the expression/purification on a larger scale	Attempted purification using homemade buffers. Help from numerous individuals in the department, specifically Dr. Jamshad. It was hoped that scaling up and using different purification method would improve protein functionality.	Trial of these other methods produced high protein yields, but protein was completely inactive. Upon testing, high levels of DNA contamination were found. This was despite the addition of DNAse to the denaturation buffer and salt washes.

		Hypothesis – protein is co-purifying with DNA so a higher yield of protein also means a higher yield of DNA.
E) Expressing protein and purifying on the same day	The overnight storage of the pellet prior to purification may damage protein	Small increase in protein activity but this was not consistent.
F) Increased wash steps and wash volume of purified protein	The QIAGEN kit used for protein purification (cat. 30600) provides a wash buffer. The volume of wash buffer used and the number of washes was increased.	Small increase in protein activity but a decrease in yield of purified protein.
G) Addition of extra DNAse to buffers during purification	Addition of extra enzymes to degrade contaminating DNA were added to lysis buffers.	DNA contamination still present in these samples.
H) Buffer exchange of purified protein (spin column method)	Imidazole and small amounts of salt in the elution buffer may interfere with protein function. Used a spin column method to buffer exchange.	Protein concentration decreased during this procedure. Hypothesis is that protein precipitated, compromised the membrane of the column.
I) Buffer exchange (membrane dialysis method)	Overnight membrane buffer exchange by Dr. Jamshad.	Buffer exchange successful but no increase in protein activity.
J) Outsourcing the purification to the PEF facility	Decision was made to use the expertise at the protein expression facility (PEF) within the University of Birmingham.	Functional AcrR and EnvR was obtained. However, the PEF also experienced problems with DNA contamination. The resulting concentrations of protein were very low.

4.4.0 Optimisation of EMSA assays to investigate binding of AcrR and EnvR to target genes.

This section explains how the EMSA assays were optimised to test the binding of AcrR and EnvR to the region upstream of *acrAB*.

4.4.1 Optimisation of the running conditions

An electrophoretic mobility shift assay (EMSA) was performed to check that the purified proteins were functional. During an EMSA, purified protein is mixed with purified DNA and fluorescent binding buffers. If the protein can bind the DNA, the band representing the protein:DNA complex will 'shift' up the gel. The protein:DNA complexes migrate more slowly through the gel matrix, causing the shift. During an EMSA, the protein:DNA complexes must migrate through a gel matrix during electrophoresis.

The resolution of these bands after electrophoresis therefore depends on multiple factors (voltage, temperature, strength of protein:DNA binding, size of gel mesh). The size of both the protein and the DNA must also be considered. Therefore, optimisation was required to produce clear, well-resolved gels. To test each condition, functional EnvR #1 protein which had previously been shown to bind upstream *acrAB* was used as a positive control.

A summary of the reasons behind the final running conditions and gel composition are listed in Table 4.3:

Table 4.3: Optimal conditions for EMSA assay

Condition chosen	Reasons
Short (1 hour, 150 V) electrophoresis	Increased voltage can increase the likelihood of protein:DNA dissociation. Heating of buffers during electrophoresis causes smearing of gel lanes.
6 % acrylamide native gel	A 6% acrylamide gel provided a higher resolution of individual bands than a 9% or 12% gel. Lower % acrylamide gels are better at resolving gels with smaller proteins. This is therefore optimal for AcrR and EnvR which are ~25 kDa.
Using 0.5x TBE as a running buffer and in the native gels	TBE was found to produce clearer gels than TAE. TBE is a better conductor of electricity than TAE.
Pre-cooled (4°C) buffers	Keeping the running buffer (0.5 xTBE) in the fridge prior to running the gels increased the resolution of the gel.
Pre-running of the gel at 200 V for 20 minutes	Running the gel without sample for 20 min prior to adding sample increased the resolution of the gel. This, along with careful cleaning of the wells, helps to ensure even distribution of charge across the gel.

Table 4.3: Summary of the reasons behind EMSA running conditions selected. Optimising EMSA assay for the specific proteins used in this assay was important for ensuring that gels were clear and readable.

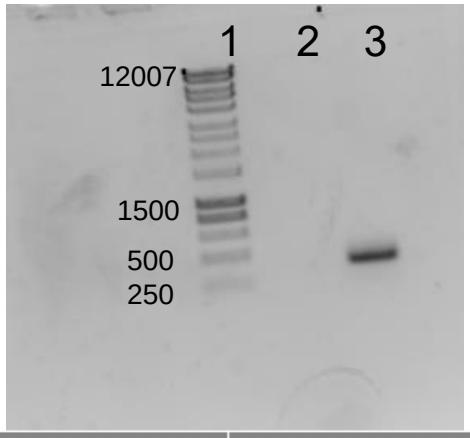
4.4.2 Optimisation of DNA concentration for EMSA assays

The promoter of *acrAB* from SL1344 was amplified by PCR (Figure 4.8). This PCR product was then purified (QIAquick, QIAGEN, UK) ready for use in EMSA assays. The DNA concentration used in the EMSA was also then optimised (Figure 4.9). To avoid overloading the lanes, the optimal concentration of DNA was determined to be 15 ng/µl.

4.4.3 AcrR and EnvR bind upstream *acrAB* of *Salmonella*

After the purification of functional AcrR (prep #3) and EnvR (prep #1), the binding of AcrR and EnvR upstream *acrAB* could be tested. The addition of purified AcrR or EnvR produced weak shifted bands in the EMSA (Figure 4.10). This confirmed that AcrR was able to bind and showed for the first time that EnvR can bind upstream *acrAB* in *S.* Typhimurium. At the same protein concentration, the addition EnvR produced a shifted band which is visibly darker than the band for AcrR. However, the interactions shown here are weak, and only a small proportion of DNA was shifted in the presence of the proteins (Figure 4.10). Therefore, these EMSAs require repeating and optimisation before this result can be confidently reported. See Chapter 7 for further discussion on future work.

Figure 4.8: Amplification of the promoter of *acrAB* from SL1344



Lane	Strain	Predicted fragment size (bp)	Comments
1	1 Kb ladder	-	-
2	-	-	Negative control
3	WT (SL1344)	306	Upstream *acrAB* region successfully amplified from SL1344.

Figure 4.8: Amplification of the region upstream *acrAB* from SL1344. The band of amplified DNA (3) was excised and purified by gel extraction. A concentration of 150 ng/µl after purification was determined using a nanodrop.

Figure 4.9: Optimisation of the DNA concentration used in EMSA assays

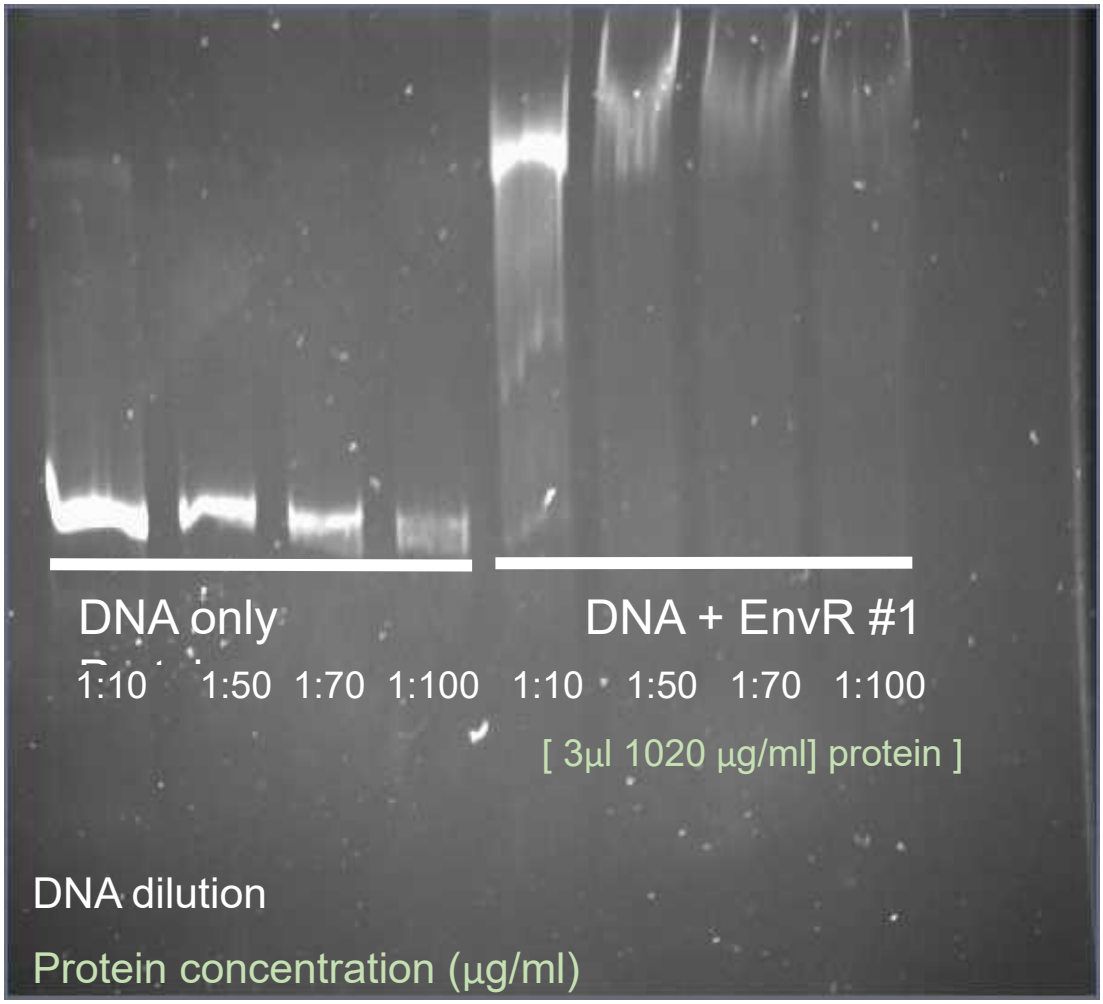


Figure 4.9: Optimisation of the DNA concentration used in EMSA assays. An EMSA assay was performed using 3 µl of EnvR #1 protein (no dilution) with various dilutions of the purified region upstream *acrAB* 1:10 (15 ng/µl) – 1:100 (1.5 ng/µl). The clearest band was visualised when using 15 ng/µl DNA.

Figure 4.10: AcrR and EnvR bind upstream *acrAB*

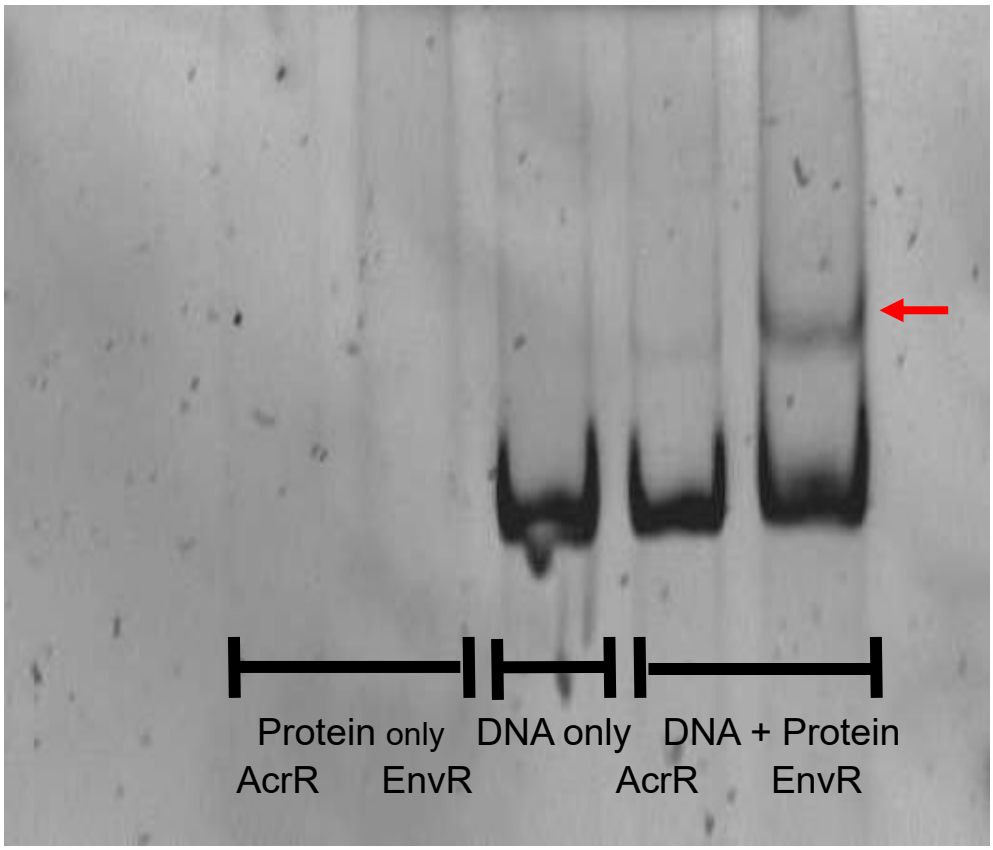
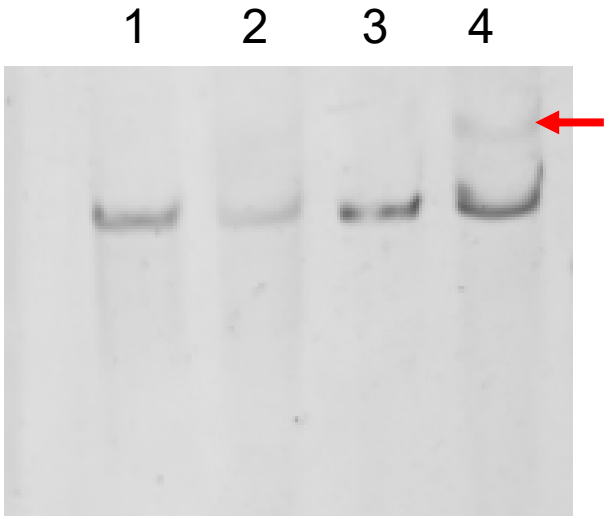


Figure 4.10: AcrR and EnvR bind upstream *acrAB* in SL1344. Active protein preps AcrR #3 and EnvR #3 were used for this assay. They were both diluted to 500 µg/ml and then a final volume of 3 µl of these dilutions were used in an EMSA reaction with 3 µl purified region upstream *acrAB* DNA (15 ng/µl). Both AcrR and EnvR show very weak binding to the *acrAB* promoter, with only a small proportion of the DNA shifted by the addition of EnvR.

4.4.4 EnvR does not bind the mutated promoter of *acrAB*

The binding site of EnvR and AcrR upstream *acrAB* in *E. coli* has been shown to be a 24-base pair palindrome **5'-TACATACATTTGTGAATGTATGTA-3'** (Hirakawa, Takumi-Kobayashi*, et al.*, 2008; Ma *et al.*, 1996a). As AcrR and EnvR are predicted to bind the same site in *E. coli*, it was predicted that EnvR would bind this same site in *Salmonella*. To confirm whether this is the case and to investigate which residues are important for binding, synthesised DNA was purchased (GeneArt, ThermoFisher), containing single nucleotide polymorphisms (SNPs). These SNPs were positioned throughout the palindrome of the known binding site of AcrR in *E. coli*. The purified EnvR protein was able to weakly bind the WT but not the mutated promoter (Figure 4.11) showing that the WT residues (T222, C224, T226, C228, T230, A237, G239, A241, G243 and A245) are required for EnvR to bind upstream of *acrAB*.

Figure 4.11: Binding of EnvR to mutated and WT regions upstream *acrAB*




```
Mut prom  194  ATGTCGGTGAATTTACAGGCGTTAGATTGATAGAAACTTATGGGTTTCTATCCCATAGCA  253
               ||||||||||||||||||||||||||||| | | | | |||||| | | | | ||||||||
WT prom   800  ATGTCGGTGAATTTACAGGCGTTAGATTTACATACATTTATGGATGTATGTACCATAGCA  741

Mut prom  254  CGACGATAATATAAACGCAGCAATGGGTTTAAGGACCTTTGACCATTGACCAA  306
               ||||||||||||||||||||||||||||||||||||||||||||||||||||
WT prom   740  CGACGATAATATAAACGCAGCAATGGGTTTAAGGACCTTTGACCATTGACCAA  688
```

Figure 4.11: EMSA assay showing EnvR is unable to bind upstream *acrAB* when this region is mutated, but can weakly bind the WT promoter. Panel (A) EMSA assay, (1) Mutated DNA (upstream of *acrAB*, 15 ng/µl) only, (2) Mutated DNA (upstream of *acrAB*, 15 ng/µl) + 500 µg/ml EnvR #3, (3) WT DNA (upstream of *acrAB*, 15 ng/µl) only and (4) WT DNA (upstream of *acrAB*, 15 ng/µl) + 500 µg/ml EnvR #3. A shift band is seen for the WT, but not the mutated, DNA.

4.4.5 Overexpression of *acrR* or *envR* reduced *acrB* transcription

Both AcrR and EnvR are known to repress *acrAB* transcription in *E. coli* (Hirakawa, Takumi-Kobayashi*, et al.*, 2008; Ma *et al.*, 1996a). Here, AcrR and EnvR have been shown to bind upstream of *acrAB* in *Salmonella* so it was important to confirm that this binding led to repression of acrAB expression. Expression of acrR is generally low and expression of envR is repressed under laboratory conditions. Therefore, to measure the effect of AcrR and EnvR on expression of *acrAB*, *acrR* and *envR* were cloned into the pET20b plasmid which was then transformed into *Salmonella* SL1344. Expression from this plasmid was not induced due to potential toxic effects of overexpressing *acrR* and *envR* at high levels. It is important to note that this method therefore relied on "leaky" expression from the pET20b vector and the expression of *acrR* and *envR* should be quantified in future work. Expression of *acrB* was measured by RT-PCR. The overexpression of *acrR* or *envR* significantly reduced the transcription of *acrB* (Figure 4.12). The data presented thus far, therefore shows that AcrR and EnvR both bind upstream of *acrAB* in *Salmonella* and repress transcription of the operon.

Figure 4.12: Overexpression of *acrR* or *envR* reduces *acrB* transcription

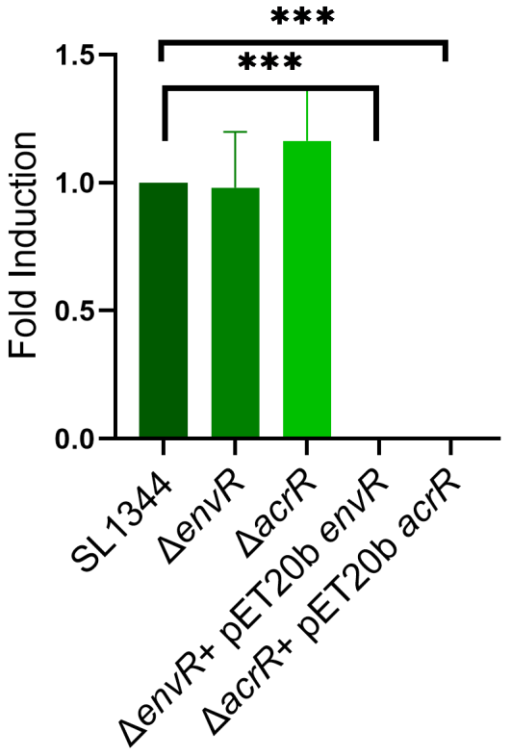


Figure 4.12: Fold changes in transcription of *acrB* in backgrounds which lack or overexpress *acrR* or *envR* were determined by RT-PCR. Efficiency was calculated using a 16S housekeeping gene and normalised to wild-type (SL1344) levels to calculate fold induction (y axis). The error bars represent the standard error of the mean of n = 4 biological replicates. A one-way ANOVA was used to determine whether the differences in mean fold induction of the tested genes were statistically significant, with **** = $P \leq 0.0001$, *** = $P \leq 0.001$, ** = $P \leq 0.005$ and * = $P \leq 0.05$. HM performed the RT-PCR.

4.5.0 Phenotypic effects of overexpressing the *acrR* and *envR* genes

This section describes the effects of overexpressing acrR and envR using the pET20b vector to show that varying the expression of *acrR* and *envR* causes phenotypes associated with increased or decreased *acrB* expression.

4.5.1 Interruption of *acrR* and *envR genes*

First, the *acrR* and *envR* genes were disrupted using the protocol outlined by Datsenko and Wanner (Datsenko & Wanner, 2000). This method allows the single-step inactivation of genes using PCR primers with homology to the target gene and homologous recombination with PCR products containing the *aph* gene, which encodes kanamycin resistance. The *aph* cassette was then removed to produce *ΔacrR*, *ΔenvR* and the double knockout *ΔacrR ΔenvR* strain.

4.5.2 Generation time of strains containing pET20b *acrR* or pET20b *envR*

First, to make sure that the pET20b *acrR* or pET20b *envR* plasmids were not causing a growth defect, the growth of these strains was measured and the generation time calculated. The growth of these strains was not affected by the carriage of the plasmid, with generation times of 20.4 (WT), 18.8 (WT + pET20b *acrR*) and 22.3 minutes (WT + pET20b *envR*) (Figure 4.13).

Figure 4.13: Generation time of pET20b strains

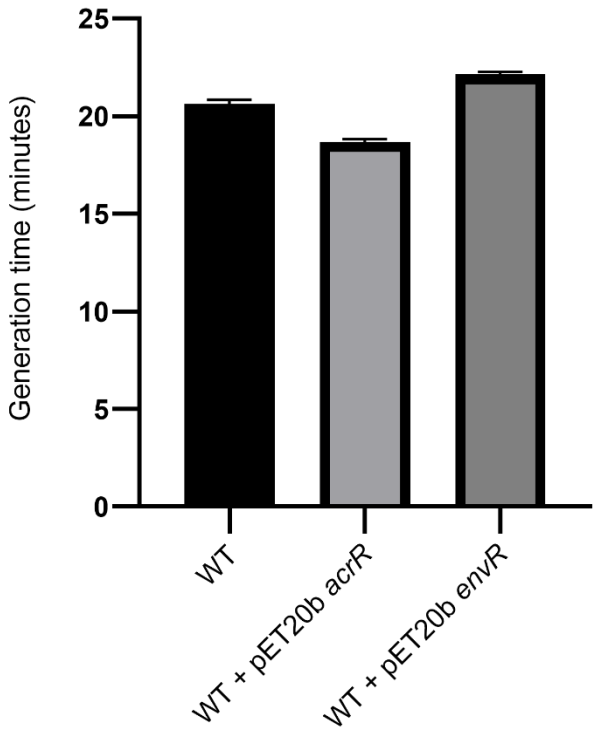


Figure 4.13: Generation time (minutes) of Wild Type SL1344 (WT), and WT + pET20b *acrR* and WT + pET20b *envR*. Data shown is the generation time (minutes) calculated from 3 independent biological replicates which were each performed in duplicate. The average OD_{600} of the strains over time was calculated and blank corrected. The generation time of each strain was therefore calculated from the average blank corrected OD_{600}. Error bars represent ± standard deviation of the mean.

4.5.3 The effect of AcrR and EnvR on antimicrobial susceptibility

As AcrR and EnvR negatively regulate *acrAB*, it was hypothesised that interrupting or overexpressing *acrR* and *envR* would change the susceptibility of SL1344 to substrates of AcrAB. To test this, the antimicrobial susceptibility of the strains to a range of AcrAB substrates was determined using the agar doubling dilution method (Table 4.4). A 2x fold increase or decrease in the minimum inhibitory concentration (MIC) of a compound is considered significant. There were no significant changes in susceptibility of the Δ*acrR*, Δ*envR*, or Δ*acrR* Δe*nvR* strains. However, overexpressing *acrR* or *envR* from the pET20b plasmid caused a decrease in susceptibility to ethidium bromide, ciprofloxacin, crystal violet, nalidixic acid, methyl blue, erythromycin and tetracycline. This is probably due to the increased expression of *acrR* and *envR* repressing *acrAB* gene expression, meaning there are less AcrAB-TolC complexes in the membrane to extrude these compounds.

Table 4.4: Minimum inhibitory concentration of antimicrobial agents to strains lacking or overexpressing *acrR* or *envR*

Compound	SL1344 (WT)	*ΔacrB*	*ΔacrR*	*ΔenvR*	*ΔacrR ΔenvR*	*ΔacrR* pET20b *acrR*	*ΔenvR* pET20b *envR*	*ΔacrR ΔenvR* pET20b *acrR*	*ΔacrR ΔenvR* pET20b *envR*
Ethidium bromide	>1024	**64**	>1024	>1024	>1024	**16**	**16**	>1024	>1024
Ciprofloxacin	0.03	0.03	**<0.008**	0.03	0.03	**<0.008**	**<0.008**	0.03	0.03
Nalidixic acid	4	**2**	4	4	**8**	**1**	**2**	**8**	4
Crystal violet	64	**4**	64	64	64	**16**	64	64	64
Methylene blue	>1024	**32**	>1024	>1024	>1024	**256**	**16**	>1024	>1024
Novobiocin	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5	>0.5
SDS	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024
Erythromycin	64	**4**	64	64	64	64	**16**	64	64
Oxacillin	512	**8**	512	512	**>1024**	**>1024**	**>1024**	**>1024**	**>1024**
Chloramphenicol	2	**0.25**	2	2	2	2	**1**	2	2
Tetracycline	2	2	2	2	2	2	**0.5**	2	2

Table 4.4: Minimum inhibitory concentration (MIC, in μg/mL) of antimicrobial agents to test strains was determined using the agar doubling dilution method. The MIC is the lowest concentration required to inhibit the growth of the test strain. **Red** = increased susceptibility , **Blue** = decreased susceptibility, **bold black** = increased/decreased but not significant.

4.5.4 Curli and cellulose production in strains lacking or overexpressing *acrR* or *envR*

Red and rough colonies indicate both curli and cellulose production. The *Salmonella* strain 14082s produces both curli and cellulose and therefore acts as a positive control. It has been reported that the inhibition of efflux pumps, including AcrB, reduces curli and cellulose production in *S.* Typhimurium (Baugh, Ekanayaka, Piddock *et al.*, 2012). The overexpression of *envR* and *acrR* produced colonies that were clear and smooth, indicating a lack of both cellulose and curli compared to WT (Figure 4.14).

4.5.5 Biofilm formation

Interrupting *acrB* is known to reduce the biofilm-forming capabilities of *S.* Typhimurium (Baugh *et al.*, 2012). Therefore, the effects of overexpressing *acrR* or *envR* on the biofilm formation of *S.* Typhimurium was investigated. There were no significant differences in the ability of WT or strains lacking or overexpressing *acrR* or *envR* to form a biofilm (Figure 4.15).

4.5.6 Altered motility of strains lacking or overexpressing *acrR* or *envR*

Interrupting *acrB* has been reported to cause decreased motility and the downregulation of motility genes (Webber *et al.*, 2009). Therefore, the overexpression of *acrR* and *envR* should also reduce motility via reducing *acrB* expression. Swimming was measured directly by recording the diameter of the area containing bacteria after incubation. The *acrR*/*envR* overexpressing strains had significantly smaller swimming diameters compared to WT (Figure 4.16). The *acrR* and *envR* overexpressing strains were unable to swarm, with agar plates showing that no swarming was observed (Figure 4.17). Taken together, these results suggest that increasing the expression of *acrR* and *envR* has caused these strains to become non-motile.

Figure 4.14: Curli and cellulose production by test strains

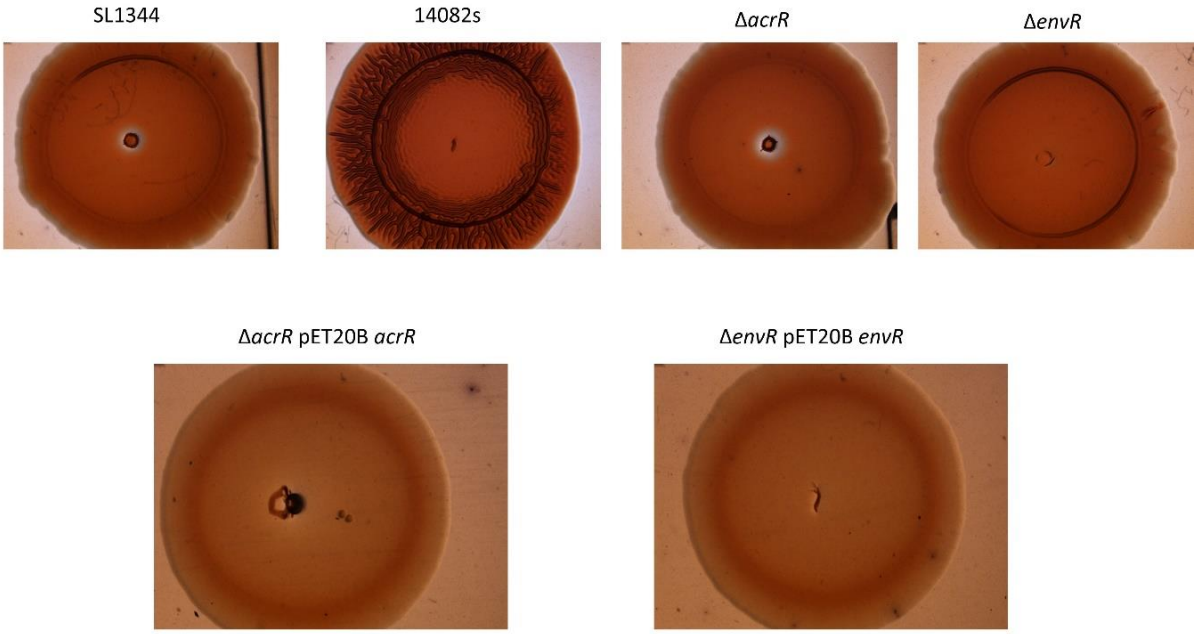


Figure 4.14: The production of curli and cellulose after interruption or overexpression of *acrR* and *envR*. Cultures diluted 1:10,000 were spotted (5 µl) on agar containing no salt and Congo red to a final concentration of 40 µg/ml. After 48hr incubation at 30°C, the plates were visualised using light microscopy. Red and rough colonies indicate both curli and cellulose production. The *Salmonella* strain 14082s produces both curli and cellulose and therefore acts as a positive control. Smooth red colonies produce cellulose but not curli, whereas pale smooth colonies lack both curli and cellulose.

Figure 4.15: Biofilm formation

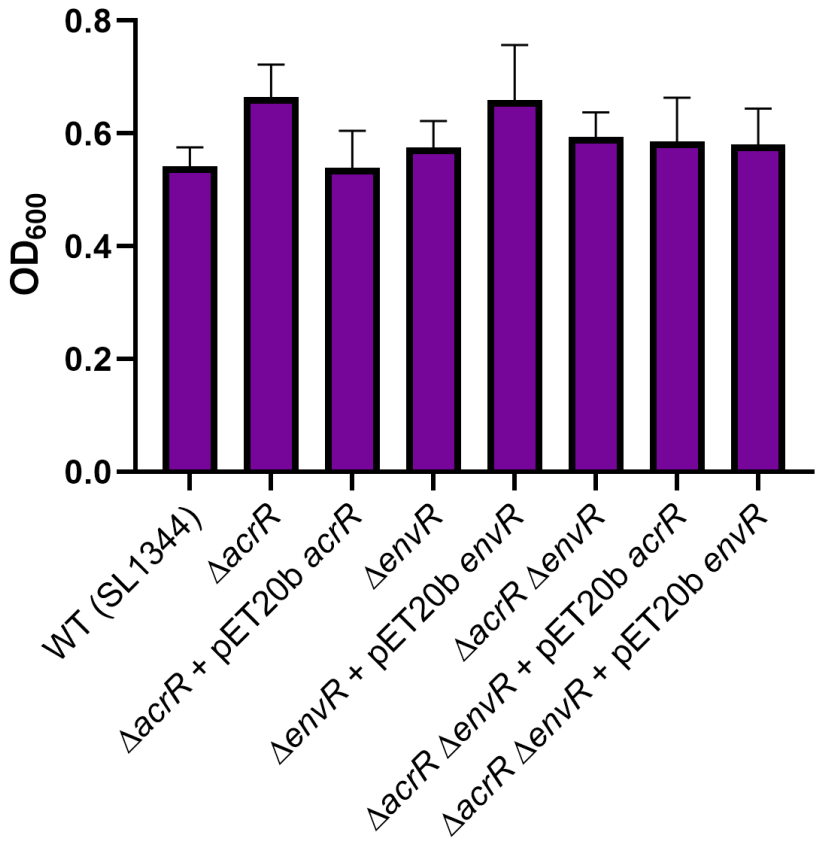


Figure 4.15: Biofilm formation was determined via crystal violet staining. Cells incubated in a microtiter plate with gentle agitation in salt-free LB media were stained with crystal violet (0.1%), staining any formed biofilms. The absorbance at 600 nm was measured to infer density of formed biofilms. Three independent experiments were conducted, shown above is the average absorbance of all 3 experiments, each of which contain n = 3 technical replicates. Error bars represent ± standard deviation of the mean.

Figure 4.16: Effect of low/high *envR* or *acrR* expression on swimming motility

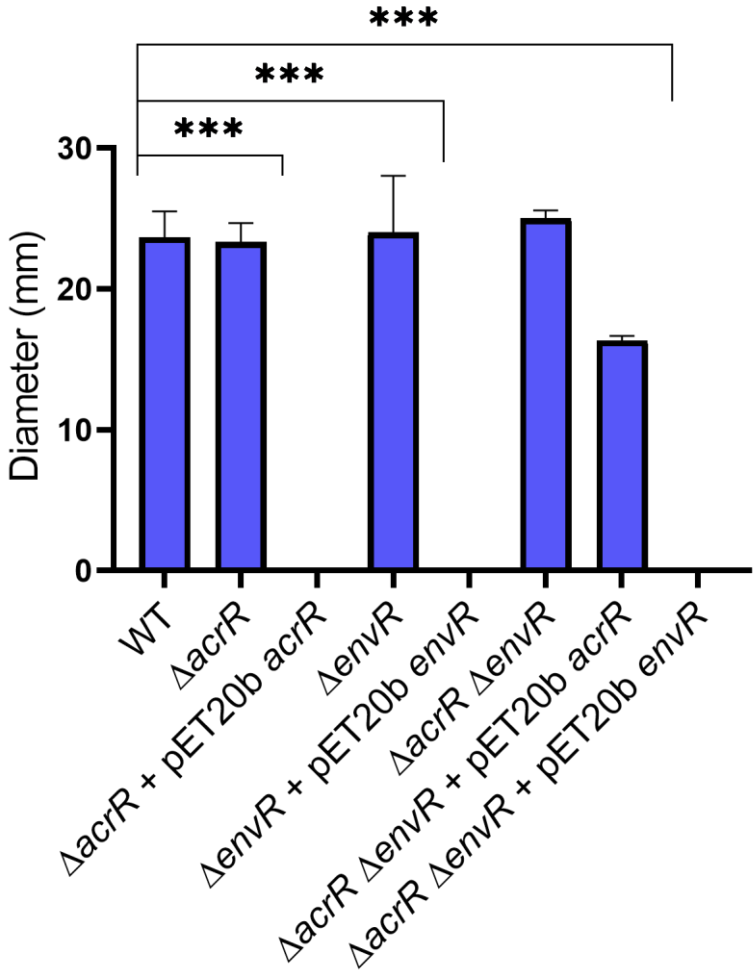


Figure 4.16: The effects of interrupting or overexpressing *acrR* and *envR* genes on swimming motility. The diameter of swimming motility in semi-solid 0.3% agar was measured after 7 hours post-inoculation (n=3). Shown is the average from three biological replicates. Student's T- test was performed * = $P < 0.05$, *** = $P < 0.001$.

Figure 4.17: Effect of low/high *envR* or *acrR* on swarming motility

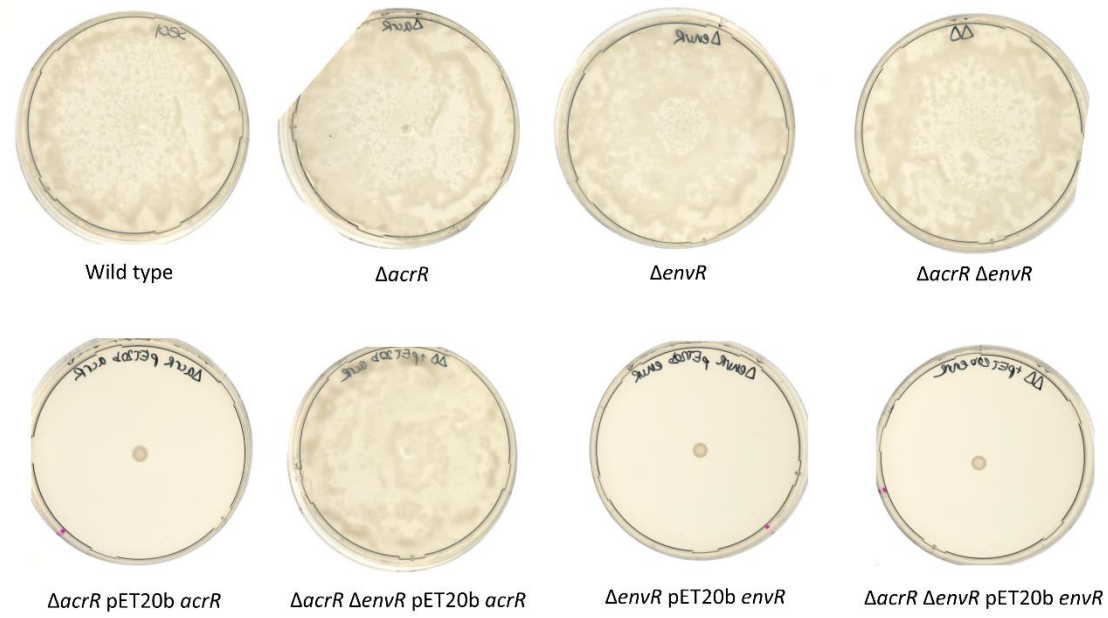


Figure 4.17: The effect of interrupting or overexpressing *acrR* and *envR* on swarming motility. Swarming agar plates (0.6% agar, supplemented with glucose) were inoculated with OD_{600} = 0.5 test strains. Swarming plates were imaged 20 hours post inoculation (n=3).

4.6.0 Discussion

4.6.1 AcrR and EnvR bind a palindrome upstream *acrAB* and regulate *acrAB* expression

It was shown in *E. coli* that AcrR binds a 24-bp palindromic sequence upstream of *acrAB* (Ma *et al.*, 1996a). EnvR has since been shown to bind upstream of and regulate the expression of *acrAB* in *E. coli* by binding this same palindromic sequence (Hirakawa, Takumi-Kobayashi*, et al.*, 2008). However, EnvR binds this region with higher affinity than AcrR, despite AcrR being the locally transcribed regulator (Hirakawa, Takumi-Kobayashi*, et al.*, 2008). As this same palindromic site is found upstream *acrAB* in *Salmonella*, it was hypothesised that AcrR and EnvR would bind this same sequence. There is evidence here that purified AcrR and EnvR protein could bind weakly upstream of *acrAB* in *Salmonella* (Figure 4.10) but EnvR could not bind when the palindromic binding site was mutated (Figure 4.11). Therefore, there is preliminary evidence that both AcrR and EnvR bind the 24-bp palindrome upstream *acrAB* in *Salmonella* and this palindrome is required for binding of EnvR to *acrAB* (Hirakawa, Takumi-Kobayashi*, et al.*, 2008; Ma *et al.*, 1996a).

Although the interaction was weak, EnvR produced a more clearly visible band of protein-bound DNA, indicating that more EnvR than AcrR was able to bind the promoter (Figure 4.10). However, as these interactions were weak and the positive control showed varied binding, these assays need to be repeated for clarity.

It is known that EnvR binds the promoter of *acrAB* in *E. coli* with higher affinity than AcrR (Hirakawa, Takumi-Kobayashi*, et al.*, 2008). Although the data presented here is weak due to low protein concentrations, this apparent visible difference in binding should be investigated further. There are also ways in which these assays could be

improved, for example by introducing competing DNA, or using radiolabelling instead of chemical EMSAs which would improve the robustness of this data. The limitations of the EMSAs and proposed changes are discussed in Chapter 7.

The co-regulation of AcrAB and AcrEF (i.e. EnvR acting as a “switch”), could exist to prevent energy waste. As efflux is an active process, it would be more efficient to only switch on the efflux system which is the most efficient at extruding the substrate. This would be especially important for efflux systems which share substrates such as AcrAB and AcrEF (Nishino & Yamaguchi, 2001). It is not known as to whether the AcrEF and AcrAB systems are ever induced at the same time in response to the same signal. However, it seems unlikely that *acrR* and *envR* gene expression can co-occur. Presumably, in conditions which induce *envR* expression, EnvR protein would preferentially bind and repress the *acrRAB* operon. It is not known whether EnvR could directly displace bound AcrR, or *vice versa*. However, other regulators of the *acrAB* operon, such as MarA/SoxS are known to behave this way, competing for a shared binding site upstream *acrAB* (Duval & Lister, 2013; Martin, Gillette, & Rosner, 2000; McMurry & Levy, 2010).

In addition to binding upstream of *acrAB,* the transcription of *acrB* was potently repressed when these regulators were overexpressed, showing that they negatively regulate the expression of *acrB* (Figure 4.12). Previous work by this lab (currently unpublished) has shown that overexpressing these regulators in this way completely abolishes AcrB protein production, as determined by western blot.

The significance of this is that it is now known that high enough expression of *acrR* or *envR* is sufficient to completely repress *acrB* expression. This could inform drug

discovery, as potential compounds which could alter AcrR/EnvR binding to *acrAB* could be used in combination therapy with antimicrobials to increase the susceptibility of MDR strains. For example, a ligand which could bind these proteins but cause a conformational change which causes irreversible binding of AcrR/EnvR could be a possibility.

Therefore, these results show that AcrR and EnvR bind the 24-bp palindrome upstream of *acrAB* and that both AcrR and EnvR can regulate *acrAB* transcription in *Salmonella.* However, whilst binding of AcrR and EnvR upstream of *acrAB* can be inferred from this data, the conditions which allow AcrR or EnvR to bind are unclear and relate to the conditions which induce the expression of *acrAB* and *acrEF*. This is because the regulators of efflux systems commonly interact directly with substrates of efflux pumps as ligands. These ligands then bind to the regulator and cause a conformational change which prevents the regulator from binding. For example, AcrR binds ethidium bromide as a ligand and ethidium bromide is also a substrate of AcrAB-TolC (Gu *et al.*, 2008; Li *et al.*, 2007).

4.6.2 The phenotypic effects of varying *acrR* and *envR* expression

After showing that AcrR and EnvR were able to decrease expression of *Salmonella acrAB*, the next steps were to identify potential phenotypic effects of this regulation. The first of these was an increased susceptibility to multiple substrates of AcrAB, including antimicrobials, dyes and detergents (Table 4.4). This was expected, as AcrR and EnvR are negative regulators of *acrAB*. Therefore, overexpression of these regulators causes a decreased expression of *acrAB* and consequently, increased susceptibility to substrates of AcrAB-TolC.

The single mutants (*ΔacrR* or *ΔenvR*) and the double knockout had the same susceptibility as WT. This shows that a lack of these genes is being compensated for by another mechanism. However, when the *acrR* or *envR* gene is complemented on a pET20b plasmid, susceptibility to the tested drugs is increased compared to WT. The overexpression of these genes on the pET20b plasmid likely leads to higher levels of AcrR and EnvR than in the WT strain, leading to repression of the *acrAB* genes.

However, complementation of the *ΔacrR ΔenvR* background strain with either pET20b *acrR* or pET20b *envR* did not change the susceptibility to the substrates of AcrAB (Table 4.4). A hypothesis is that there are wider effects resulting from the interruption of both *acrR* and *envR* which is altering the MIC values of these strains. To evaluate this further, western blotting of the single and double mutants for the AcrR and EnvR should be done to determine the protein levels. The expression levels of *acrR* and *envR* from the pET20b vector should also be determined. If no differences in regulator expression of protein levels, RNA-seq on the double mutant strain could be done to begin to unpick the off-target effects of interrupting *acrR* and *envR*.

It is known that interrupting *acrB* in *Salmonella* reduces motility and biofilm formation (Baugh *et al.*, 2012; Webber *et al.*, 2009). Overexpressing *acrR* and *envR* also significantly reduced both swimming and swarming motility (Figures 4.16 - 4.17). Swimming is the movement of a single bacterium, using flagella, through a liquid or semi-liquid substance. Whereas swarming describes the movement of bacteria as a collective, over a solid surface. The regulation of swimming and swarming is complex and hierarchical, with the master genes *flhDC* controlling the downstream expression of many regulators of motility (Bogomolnaya, Aldrich, Ragoza *et al.*, 2014). In *S.*

Typhimurium, while there are some genes which reduce both swimming and swarming motility, this is not always the case (Table 4.5).

Table 4.5: *S.* Typhimurium genes implicated in motility, adapted from (Bogomolnaya *et al.*, 2014)

Reduced swimming	Reduced swarming	Both reduced
flgE, ssaV, fliH and *stjC.*	*ssaU, pefD* and *pefC.*	*flgF, flgG, flhA, flhB, motA, motB, fliD* and *fliM.*

Interrupting the *acrB* in *Salmonella* also reduces the expression of motility genes, including *flgF* and *flgG* (Webber *et al.*, 2009). Reduced expression of *flgF and flgG* in *Salmonella* reduces both swimming and swarming motility (Table 4.5, (Bogomolnaya *et al.*, 2014). Therefore, as overexpressing *acrR* or *envR* reduces the expression of *acrB*, the reduction in motility may be solely due to changes in *acrB* expression. This would also explain why the overexpression of *envR* has a more profound effect on motility. As EnvR binds the promoter of *acrAB* with a higher affinity than AcrR (Hirakawa, Takumi-Kobayashi*, et al.*, 2008), resulting in lower *acrB* expression, this could explain why *envR* overexpression reduces motility more than *acrR* overexpression.

In *E. coli*, it was reported that the expression of motility genes is actually increased in a Δ*acrB* background, including the flagella master operon *flhDC* (Ruiz & Levy, 2014). While this is in contrast to what has been documented in *Salmonella*, it is important to remember that experimental conditions will also play a large role in determining the expression of motility genes. Furthermore, it could also be that the additional

interactions and roles of the AcrAB-TolC system could be different between bacterial species.

There is evidence in other species that interrupting *acrR* has consequences for motility. Multiple binding sites for AcrR adjacent to the *flhDC* operon have been described in *E.* coli and interruption of *acrR* reduced the motility (Kim, Im, Lee *et al.*, 2016).. While these authors propose a direct binding and interaction of AcrR and the *flhDC* operon, the binding sites suggested are not palindromic and are not similar to known AcrR binding sites. As no binding or affinity studies were done, it is not possible to ascertain how likely these are to be true binding sites.

In *A. citrulli,* inactivation of the *acrR* gene inhibits swimming motility and differentially expressed genes for motility, virulence and metabolism compared to a wild-type strain (Guan, Wang, Huang *et al.*, 2020). Similarly, in *E. coli*, swimming, but not swarming, was reduced in an Δ*acrR* background strain (Kim *et al.*, 2016). The interruption of *acrR* in *A. nosocomialis*, on the other hand, increased the expression of motility genes (Subhadra *et al.*, 2018). Therefore, while AcrR seems to be linked to motility in multiple species, these interactions are highly variable, both increasing and decreased motility in different species. Also, many of these studies do not disclose any testing of motility in an *acrB* mutant. This would have to be investigated to better understand if these results are due to changes in *acrB* expression or potentially caused by the regulatory effect of AcrR on other genes.

Another consideration is that the strains containing plasmid may have a fitness disadvantage. Given that motility is an active, energy-consuming process, any

reductions in fitness would likely reduce motility. However, there was no observed growth defect in the pET20b+ strains (Figure 4.13).

Here, the interruption of *acrR* did not reduce swimming or swarming of *Salmonella* (Figures 4.16 and 4.17). Instead, it was the overexpression of either *acrR* or *envR* which showed significantly reduced motility. Interrupting both *acrR* and *envR,* but overexpressing *envR* significantly reduced swimming and swarming motility (Figures 4.16 and 4.17). However, overexpressing *acrR* in the same Δ*acrR* Δ *envR* background did not reduce motility to the same extent. Therefore EnvR, not AcrR, causes more significant changes to motility. This could be due to a decrease in *acrB* expression, as discussed previously or, this could be due to direct regulation of EnvR to relevant target genes, such as those listed in Table 4.5 or the master regulatory genes *flhDC*. However, the more likely answer is that the overexpression of *envR* causes motility changes via repression of *acrB.*

It must also be considered that these results could be because of something other than AcrR/EnvR-mediated changes in *acrB* expression. If this is the case, then perhaps EnvR is binding more strongly to it's target gene(s), producing the bigger reduction in motility seen here. To test this hypothesis, the overexpression of *acrR/envR* in the corresponding opposite background (i.e. Δ*acrR* overexpressing *envR*) should be tested. If the overexpression of *acrR* in a Δ*envR* background confers less of a reduction in motility than in a Δ*acrR* Δ*envR* background, then this hints that EnvR is a more potent repressor of the gene(s) involved in this phenotype. However, this must be tested in both a Δ*acrB* background and Δ*acrB* complemented with a plasmid expressing *acrB*. This will start to unpick the involvement (if any) of these regulators in motility.

Interrupting *acrB* has previously been shown to reduce biofilm formation (Baugh *et al.*, 2012) and interrupting *acrR* has been linked to increased biofilm formation in *A. nosocomialis* (Subhadra *et al.*, 2018), *A. citrulli (Guan et al., 2020)* and *A. baumanii* (Rumbo-Feal, Gomez, Gayoso *et al.*, 2013). Here, neither the interruption of the *acrR* or *envR* genes, or their overexpression, caused any significant changes in biofilm production in *Salmonella* (Figure 4.15). This suggests that AcrR and EnvR may not influence biofilm formation in SL1344. However, this should be interpreted with caution as SL1344 is notoriously poor at forming biofilms (Garcia, Latasa, Solano *et al.*, 2004). Different strains of *Salmonella* should therefore be tested in case this is a strain-specific phenomena. In *S.* Typhimurium (14028-1s), one study reports that *acrAB* strains produce the same levels of biofilm as wild type (Schlisselberg, Kler, Kisluk *et al.*, 2015). However, other authors report that the interruption of any efflux system in *S.* Typhimurium 14028s causes a reduction in biofilm formation, which can be recovered via complementation with the missing gene on a plasmid (Baugh *et al.*, 2012). The relationship between biofilm formation and efflux is therefore complex and heavily reliant on experimental conditions.

A lack of *acrB* is known to alter curli and cellulose production (Baugh *et al.*, 2012). Overexpressing *acrR* or *envR* also had implications for curli and cellulose production. Curli and cellulose are components of the extracellular matrix, enabling the formation of biofilms and adherence to surfaces. The wild type SL1344 does not produce high amounts of curli or cellulose (Garcia *et al.*, 2004), but differences can be seen between different genotypes (Figure 4.14). Interrupting either *acrR* or *envR* did not influence curli or cellulose production, but overexpressing these genes decreased cellulose, seen by the translucency of the colonies (Figure 4.14). Curli and cellulose production

in Δ*acrB* mutants of *S.* Typhimurium have been reported to be similar to wild-type (Schlisselberg *et al.*, 2015). Therefore, the effects seen here may be independent of AcrB.

4.6.3 Problem solving the purification of active AcrR and EnvR protein

Some of the variation in protein activity during the beginning of this work could be due to the strain that was being used. After the construction of the vectors, they were transformed into TOP10 *E. coli* and this strain was then used to express protein. However, this strain provides no protection for premature protein expression or cleavage. Incorrect protein folding or cleavage could cause a purified protein to be non-functional. The pTrc expression plasmids were then transformed in to competent BL21 pLysS cells to try and improve the quality of the expressed protein. The pLysS plasmid expresses low levels of T7 lysozyme, which inhibits T7 RNA polymerase, therefore preventing non-induced expression. However, the DNA contamination was not completely eradicated after switching to the recommended BL21 pLysS expression strain, meaning that the reasons for non-functional protein were multifaceted.

Therefore, those attempting to purify these proteins in the future should focus on removing any DNA during purification. During the lysis of the cells during purification, the proteins would have been exposed to a high concentration of free DNA. It was therefore not surprising that many of the protein samples were DNA-bound. The effect was also concentration-dependent, with AcrR (cloned correctly, high-expression) containing more DNA contamination than EnvR (+ native start codon, lower expression). Moreover, when these protein samples were diluted, it was possible to see free DNA migrate through the acrylamide gel during electrophoresis. During the size exclusion chromatography undertaken at the PEF, high salt washes were used to

try and remove contaminating DNA from the protein sample. However, during this process, almost all the purified protein was lost. There were also numerous issues with column failure during protein concentration and buffer exchange. This could have been due to the presence of DNA:protein aggregates which could have pierced the spin column membrane during concentration.

Due to their nature as DNA-binding proteins, transcription factors can be difficult to purify. A possible solution would be to co-purify the protein with DNA or to denature the protein prior to treatments to remove DNA. However, due to the method used here (DNA fluorescent probe EMSA), it was not possible to do these methods. Switching to another EMSA method, such as radiolabelling, may enable other to overcome these issues. However, whether the DNA is visible or not in the assays, it is still important to ascertain whether contaminant DNA has any influence on the binding of the protein being studied. Despite the issues surrounding protein expression and purification, enough active protein from multiple preparations was obtained to fulfil the aims of this and other chapters of this thesis.

To summarise, the overexpression of *acrR* and *envR* using the pET20b vector showed multiple phenotypic changes, some of which are explainable using our understanding of the roles of AcrB. Specifically, AcrR and EnvR may be involved in motility and production of the extracellular matrix. The overexpression of *acrR* and *envR* cause cells to become non-motile and therefore may have consequences for virulence. However, further work is required to unpick whether these interactions are independent of AcrB or not. Additional characterisation of whether interactions between AcrR/EnvR and potential targets are direct or indirect would also be needed.

4.7 Future directions

- Quantify the expression of *acrR* and *envR* from the pET20b vector to check that expression levels are similar.
- Investigate whether the overexpression of *acrR* or *envR* could be exploited to increase the antimicrobial susceptibility of MDR strains.
- Continue to optimise the purification of AcrR and EnvR to obtain more consistently active protein yields.
- Investigate whether these phenotypes are AcrR or EnvR concentration dependent. For example, by using a vector which can be induced to express at different levels.
- Study the effect of altering *acrB* expression alongside *acrR* and *envR* expression, to try and further identify which of these phenotypes occur independently of changes in *acrB* expression.
- RNA and ChIP-sequencing to identify any potential additional targets of AcrR and EnvR, to understand whether any of the phenotypic effects seen here may be independent of interactions via AcrB. Performing the RNA/ChIP-seq in tandem with an Δ*acrB* strain and comparing this to that of an *acrR/envR* interrupted background would start to explore this.
- Further binding experiments to study the competition between AcrR and EnvR for *acrAB* and other targets should be evaluated to test whether EnvR binds all targets with a higher affinity than AcrR.

4.8 Key findings

- AcrR and EnvR bind upstream of *acrAB* and regulate the expression of *acrAB.*
- There are multiple phenotypic effects which result from increasing *acrR* and *envR* expression and some of these phenotypes are known to occur in *acrB*-null strains.
- AcrR and EnvR may be indirect or direct regulators of other targets and there is existing evidence of binding upstream of motility genes. Therefore, not all of the phenotypes seen here may be attributable wholly to changes in *acrB* expression.

Chapter Five

“Off-target” roles of AcrR and EnvR

5.0 “Off-target” roles of AcrR and EnvR

5.1 Background

While the binding and regulation of *acrAB* is important to characterise, there is also evidence that AcrR and EnvR may have other targets. The majority of TFTR regulators are not single-target regulators, with some even able to repress or activate the expression of multiple targets (Chapter 3). In addition to efflux regulation, AcrR has been implicated in the regulation of motility, organic solvent tolerance and biofilm formation (Baugh *et al.*, 2012; Kim *et al.*, 2016; Watanabe & Doukyu, 2012; Webber *et al.*, 2009). Given the similarity of AcrR and EnvR, there is a possibility that EnvR may share some of these regulatory functions. The interruption and overexpression of *acrR* and *envR* may therefore reveal phenotypes which hint at the wider roles of these proteins. TetR-family regulators are predominantly considered to be local, negative repressors of their targets (Ahn *et al.*, 2012; Cuthbertson & Nodwell, 2013; Ramos *et al.*, 2005). However, there are many examples of these regulators having “off-target” roles (Chapter 3). Recent literature suggests that single-target regulation is likely to be the exception, not the rule (Shimada *et al.*, 2018), and that the roles of TetR-family regulators are currently underestimated. AcrR has also been implicated in the regulation of other targets (Table 5.1) but EnvR has only one confirmed target (the promoter of *acrAB* (Hirakawa, Takumi-Kobayashi*, et al.*, 2008) and one presumed, but unconfirmed target (the promoter of *acrEF* (Anes *et al.*, 2015; Hirakawa, Takumi-Kobayashi*, et al.*, 2008)). Given the similarity of AcrR and EnvR, any additional targets of AcrR could be considered potential targets of EnvR.

In addition, the ability of a TFTR to bind target DNA depends on both the interaction between the DNA-binding (N-terminal) domain and the target DNA, but also the interaction between the TFTR and its ligand(s). AcrR is predicted to bind different ligands with differing affinities (Manjasetty, Halavaty, Luan *et al.*, 2016b), meaning that the time that AcrR is bound to a ligand could vary. Presumably, this could mean that the time that AcrR is bound to a ligand (and therefore unable to bind target DNA) could have implications on the levels of *acrAB* expression in response to a substrate/ligand signal. Therefore, the effect of different ligands on the binding of EnvR to the upstream regions of *acrA* and *acrE* were also studied.

5.2 Aims

- Identify potential targets of AcrR and EnvR regulation.
- Demonstrate whether AcrR or EnvR can bind upstream of these target genes.
- Measure any changes in target gene expression by RT-PCR.
- Identify the conserved residues upstream of AcrR/EnvR targets to determine the required binding motif.
- Study the effect of potential ligands on the binding of EnvR to the promoter of *acrA* and *acrE.*

5.3 Identification of targets of AcrR and EnvR

Here, known targets of AcrR and EnvR were identified by a literature search (Table 5.1). A gene was considered to be a target of the transcription factor if evidence existed of either direct binding (i.e. EMSA/ChIP-seq) or if regulation had been inferred indirectly by measuring gene expression changes following inactivation of either *acrR*/*envR* (i.e. RT-PCR). Other indirect evidence was also considered, for example

phenotypic effects of interrupting *acrR* or *envR.* Six previously identified targets of AcrR were found (*acrA*, *marA*, *soxS*, *micF*, *flhC* and *flhD*), although the only target for which there was evidence of direct binding was *acrA* (Ma *et al.*, 1996a). For EnvR there was only direct evidence of binding for *acrA* (Hirakawa, Takumi-Kobayashi*, et al.*, 2008). Ideally, ChIP and RNA-Sequencing would be done to confirm and identify new binding sites and regulatory pathways involving AcrR and EnvR. However, due to issues with protein purification and ChIP-Seq, targets were tested using the EMSA method. Targets to be investigated were initially those identified in Table 5.1. TetR regulators are known to be important for regulation of efflux gene expression (Colclough *et al.*, 2019). Therefore, other genes considered to be important for efflux regulation (*marA, soxS, ramA* and *rob*) and other RND genes (*mdtA* and *mdsA*) were also included for testing in addition to those listed in Table 5.1.

5.4 Testing whether AcrR and EnvR regulate expression of other genes.

If AcrR/EnvR are direct regulators of these additional targets, they should (a) bind the promoter of the target and (b) cause a change in target gene expression. To evaluate this, the binding of AcrR and EnvR was evaluated by EMSA. The expression of these targets was measured (via RT-PCR) in a variety of background strains, including strains with interrupted *acrR*/*envR* and strains which overexpress *acrR/envR* using the pET20b vector. The upstream regions of genes listed in Table 5.1 were amplified and EMSA binding assays performed. The primers used to amplify all the genes used in this chapter are listed in Chapter 2, Materials and Methods.

Table 5.1: Identification of potential regulatory targets of AcrR and EnvR

Target promoter	Evidence of regulation by AcrR	Evidence of regulation by EnvR
acrA	Direct binding (Ma *et al.*, 1996a)	Direct binding (Hirakawa, Takumi-Kobayashi*, et al.*, 2008)
acrE	-	Presumed, but no evidence
acrD		Indirect phenotypic evidence (Emami, 2014)
marA	Indirect via qPCR (Lee *et al.*, 2014)	-
soxS	Indirect via qPCR (Lee *et al.*, 2014)	-
micF	Indirect via putative binding site (Rodionov *et al.*, 2001)	-
flhC	Indirect via phenotypic evidence and putative binding site (Kim *et al.*, 2016)	-
flhD	Indirect via phenotypic evidence and putative binding site (Kim *et al.*, 2016)	-

Table 5.1: Evidence for the inclusion of targets for testing. Evidence was identified from searching the literature. Direct (i.e. EMSA binding assays) or indirect (i.e. RT-PCR or phenotypic evidence), were also included.

5.4.1 Issues with EMSA assays

Before discussing the results of this chapter, it is important to note that there were several issues with EMSA assays in both this and the previous chapter. Only a small volume of protein was able to be purified which was able to bind upstream *acrAB.* As *acrAB* is a known target of AcrR/EnvR, failure to bind upstream this promoter indicated problems with the protein preparation. Due to the limited protein stocks available and the EMSA method used (SYBR green DNA stain detection, not radiolabelled DNA), the type and number of controls that could be added was limited. As this method involved detecting all DNA in the gel after electrophoresis, it was not possible to introduce other controls such as competing DNA. A change of method to use radiolabelled DNA probes is therefore recommended to permit the adding of further controls. Additionally, the optimisation of the protein purification of these proteins is recommended to improve the quality of the assay.

Using the new method, the following controls should be added:

(1) A non-specific promoter, such as *rrsA*. This was added as a control in Figure 5.9 to check that AcrR/EnvR were not binding all available DNA, but should have been present on all gels. Unfortunately, this was not possible due to lack of protein.

(2) Competing, non-labelled DNA can be used to quench any other proteins which have been co-purified with the AcrR/EnvR and prevent non-specific binding to target DNA.

(3) A concentration titre of protein should be done with each promoter. This would enable the binding relationship to be more clearly understood, for example, to see whether binding is concentration-dependent.

(4) Positive control of the labelled promoter of *acrAB* with protein. To determine the correct running of the gel, etc. Again, this is present on some but not all gels due to a lack of available protein.

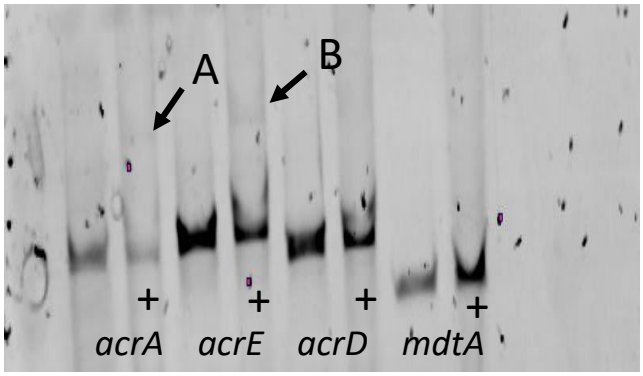
5.4.2 The binding of purified AcrR and EnvR upstream efflux genes

The first set of targets investigated were the upstream regions of other efflux systems. This is because it is already known that *acrR* and *envR* both bind upstream and regulate *acrAB* gene expression (Hirakawa, Takumi-Kobyashi*, et al.*, 2008). Both AcrR and EnvR showed weak binding upstream efflux genes *acrA* and *acrE* (Figure 5.1) and reduce the transcription of *acrB* (Figure 5.2). EnvR could bind upstream *acrD* but was unable to bind upstream *mdtA* or *mdsA*. AcrR could also not bind upstream *mdtA.* Unfortunately, there was not enough purified AcrR protein to test for binding upstream *mdsA*. Binding was weak and this was likely due to the low protein concentration used.

Neither regulator was able to bind upstream *mdtA* or *mdsA*. However, the overexpression of these regulators reduced transcript levels of *mdtA* and *mdsA,* along with all other efflux genes tested (Figure 5.2). This indicates that even if no direct interactions are involved, there may be indirect regulation by AcrR or EnvR.

Figure 5.1: The binding of purified AcrR and EnvR upstream efflux genes

(1) EMSA AcrR



(2) EMSA EnvR

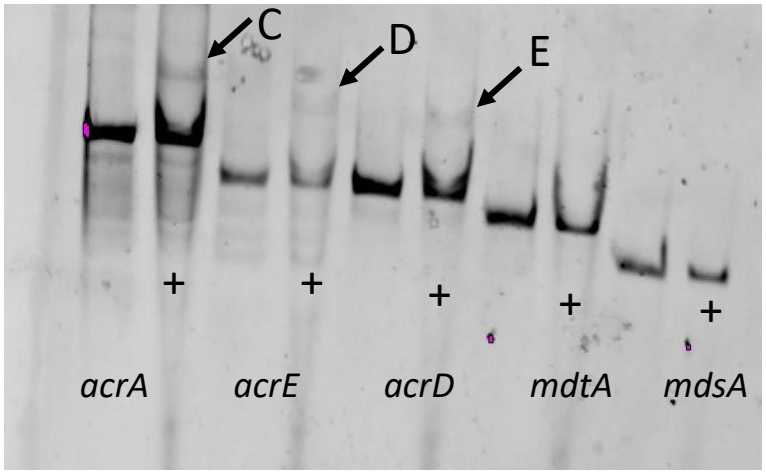


Figure 5.1: EMSA gels stained for DNA with SYBR green showing (1) The binding of AcrR (0.6 µg/mL) to the purified region upstream genes coding for RND efflux pump components *acrA, acrE, acrD* and *mdtA* (15 ng/µl). (2) The binding of EnvR (0.6 µg/ml) to the purified region upstream genes coding for RND efflux pump components *acrA, acrE, acrD, mdtA* and *mdsA* (15 ng/µl). There are 2 x lanes per purified gene tested, the first of which is DNA-only control and the second of which contains DNA and purified protein, indicated by a +. Any shifts to indicate binding are labelled with arrows and annotated with a letter.

Figure 5.2: Transcription of efflux genes with high/low *acrR* or *envR* expression

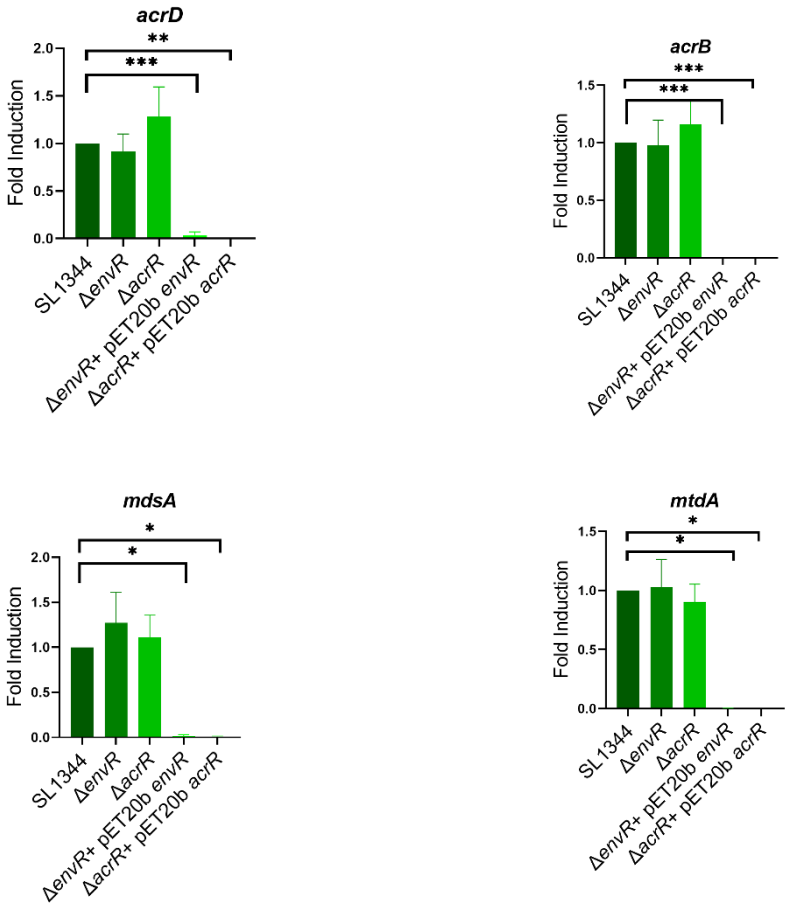


Figure 5.2: Fold changes in transcription of *acrB, acrD, mdtA* and *mdsA* genes in different backgrounds (SL1344, *ΔenvR, ΔacrR* and *ΔenvR* + pET20b *envR* and *ΔacrR* + pET20b *acrR*) was determined by RT-PCR. Efficiency was calculated using a 16S housekeeping gene and normalised to wild-type (SL1344) levels to calculate fold induction (y axis). The error bars represent the standard error of the mean of n = 4 biological replicates. A student's T-test (unpaired) was used to determine whether the differences in mean fold induction of the tested genes were statistically significant in the *ΔenvR, ΔacrR* and *ΔenvR* + pET20b *envR* and *ΔacrR* + pET20b *acrR* backgrounds compared to SL1344, with **** = $P \leq 0.0001$, *** = $P \leq 0.001$, ** = $P \leq 0.005$ and * = $P \leq 0.05$. HM performed the RT-PCR.

5.4.3 The binding of purified AcrR and EnvR upstream efflux regulators

Regulators of efflux systems, such as MarA, SoxS and Rob are known to bind at multiple promoters, and do so by recognising and binding a degenerate sequence (Chubiz *et al.*, 2012). In *E. coli*, MarA, SoxS and Rob are known to activate expression of over 40 promoters and are therefore global regulators (Martin & Rosner, 2011). These regulators are also known to bind and influence the expression of *acrAB.* For example RamA binds a region which overlaps both the promoter and the binding site of AcrR in *Salmonella* (Nikaido *et al.*, 2008). Therefore, the binding of RamA prevents AcrR-mediated repression, promoting the expression of *acrAB*. However, it was not known whether the negative regulators of *acrAB* expression AcrR and EnvR could also influence the expression of other efflux regulators. In this study, both AcrR and EnvR bound upstream *marA* and *rob* but did not bind upstream *soxS* or *ramA* (Figure 5.3). However, the transcription of *marA, soxS, ramA* and *rob* was decreased when *acrR* and *envR* were overexpressed (Figure 5.4). The transcription of *rob* in *acrR* and *envR*-interrupted backgrounds was significantly higher than in WT (Figure 5.4), indicating that AcrR and EnvR may indirectly or directly repress *rob* transcription in the WT background.

Figure 5.3: Binding of AcrR and EnvR upstream of genes that regulate efflux

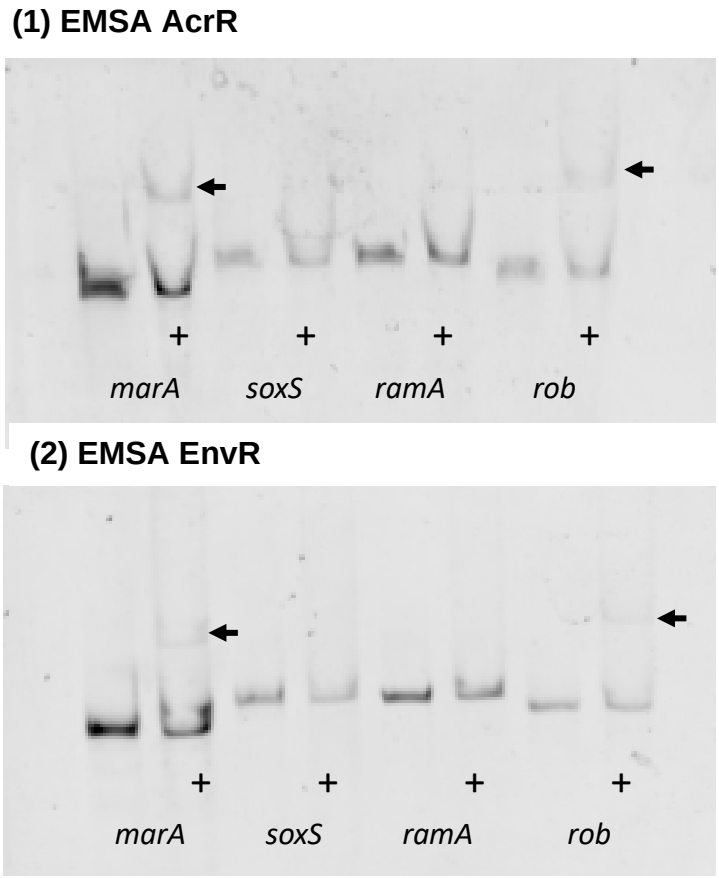


Figure 5.3: EMSA gels stained for DNA with SYBR green showing (1) The binding of AcrR (0.6 µg/ml) to the purified region upstream genes coding for efflux pump regulators *marA, soxS, ramA* and *rob* (15 ng/µl). (2) The binding of EnvR (0.6 µg/ml) to the purified region upstream genes coding for RND efflux pump regulators *marA, soxS, ramA* and *rob* (15 ng/µl). There are 2 x lanes per purified gene tested, the first of which is DNA-only control and the second of which contains DNA and purified protein, indicated by a +. Any shifts to indicate binding are labelled with arrows.

Figure 5.4: Transcription of efflux regulators in backgrounds with high/low *acrR* or *envR* expression

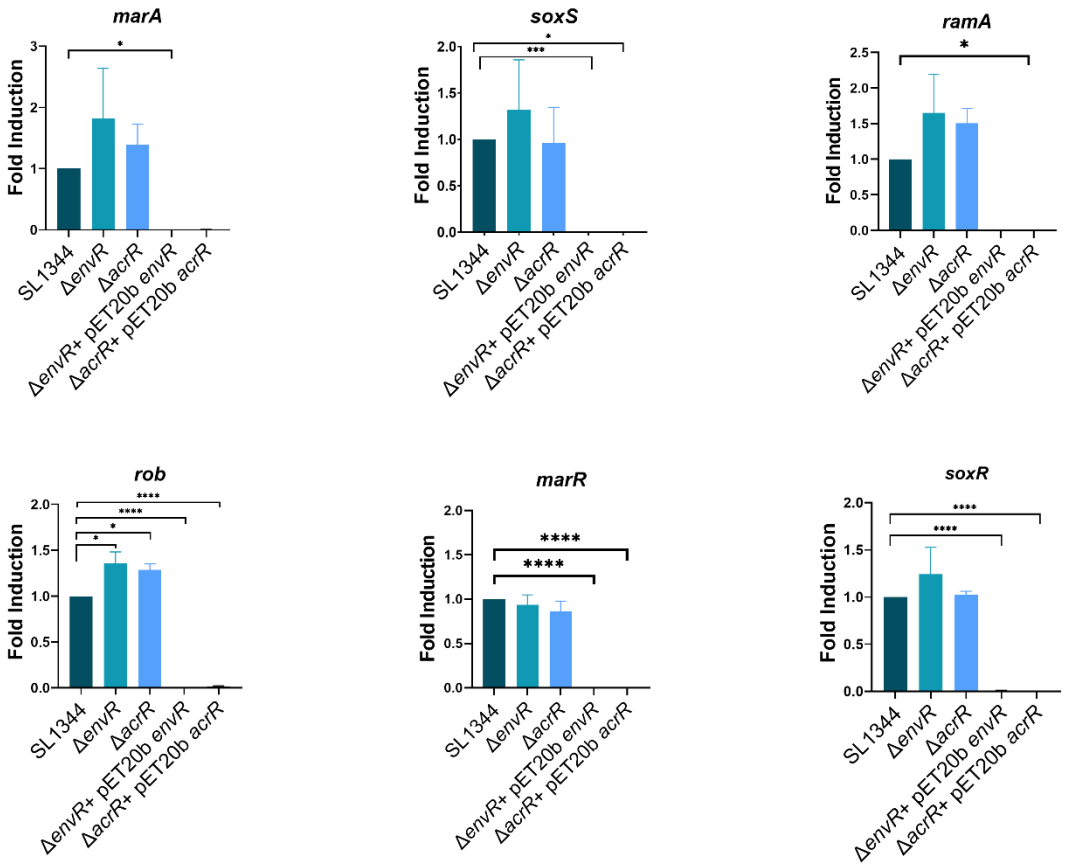


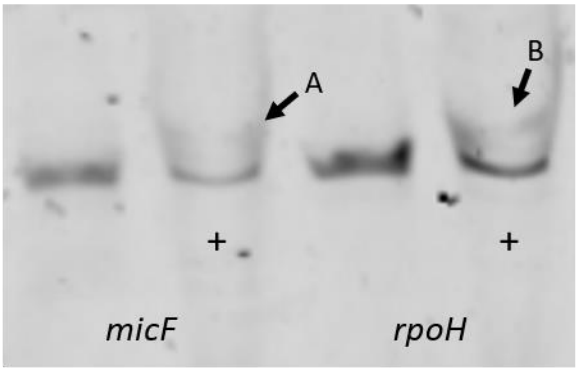
Figure 5.4: Fold changes in transcription of *marA, soxS, ramA, rob, marR* and *soxR* genes in different backgrounds (SL1344, *ΔenvR, ΔacrR* and *ΔenvR* + pET20b *envR* and *ΔacrR* + pET20b *acrR*) was determined by RT-PCR. Efficiency was calculated using a 16S housekeeping gene and normalised to wild-type (SL1344) levels to calculate fold induction (y axis). The error bars represent the standard error of the mean of n = 4 biological replicates. A student's T-test (unpaired) was used to determine whether the differences in mean fold induction of the tested genes were statistically significant in the *ΔenvR, ΔacrR* and *ΔenvR* + pET20b *envR* and *ΔacrR* + pET20b *acrR* backgrounds compared to SL1344, with **** = $P \leq 0.0001$, *** = $P \leq 0.001$, ** = $P \leq 0.005$ and * = $P \leq 0.05$. HM performed the RT-PCR.

5.4.4 The binding of purified AcrR and EnvR upstream *micF* and *rpoH*

MicF is a small RNA which regulates *ompF* expression which in turn influences the insertion of porins into the membrane and the influx of substrates of AcrAB-TolC (Mahendran, Kreir, Weingart *et al.*, 2010). The expression of *micF* is controlled by marA, soxS and rob in *E. coli* (Chubiz & Rao, 2011). RpoH plays a regulatory role in the response to heat and antibiotic stress (Pinto, Torres, Gil *et al.*, 2019). In *Neisseria* the expression of *rpoH* is regulated, in part, by an efflux TFTR MtrR (Folster, Johnson, Jackson *et al.*, 2009). There was very weak binding of AcrR and EnvR upstream of *micF* and *rpoH* (Figure 5.5). Overexpression of *acrR* or *envR* also significantly reduced the transcription of *micF* and *rpoH* (Figure 5.6) indicating that there is either direct or indirect regulation of these genes by AcrR and EnvR.

Figure 5.5: Binding of purified AcrR protein upstream of *micF* and *rpoH*

(1) EMSA AcrR



(2) EMSA EnvR

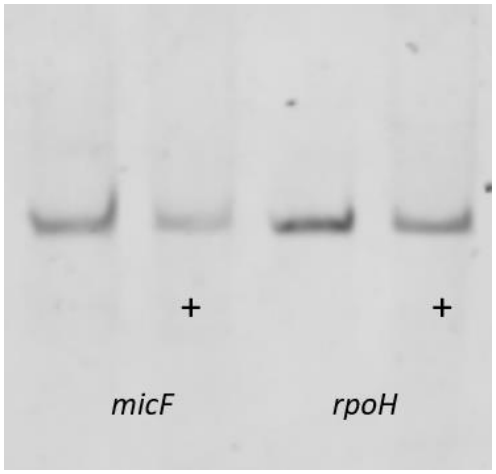


Figure 5.5: EMSA gels stained for DNA with SYBR green showing (1) The binding of AcrR (0.6 µg/ml) to the purified region upstream genes coding for *micF* or *rpoH* (15 ng/µl). (2) The binding of EnvR (0.6 µg/ml) to the purified region upstream genes coding for *micF* or *rpoH* (15 ng/µl). There are 2 x lanes per purified gene tested, the first of which is DNA-only control and the second of which contains DNA and purified protein, indicated by a +. Any shifts to indicate binding are labelled with arrows.

Figure 5.6: Transcription of *micF* and *rpoH* in strains with high/low *acrR* or *envR* expression

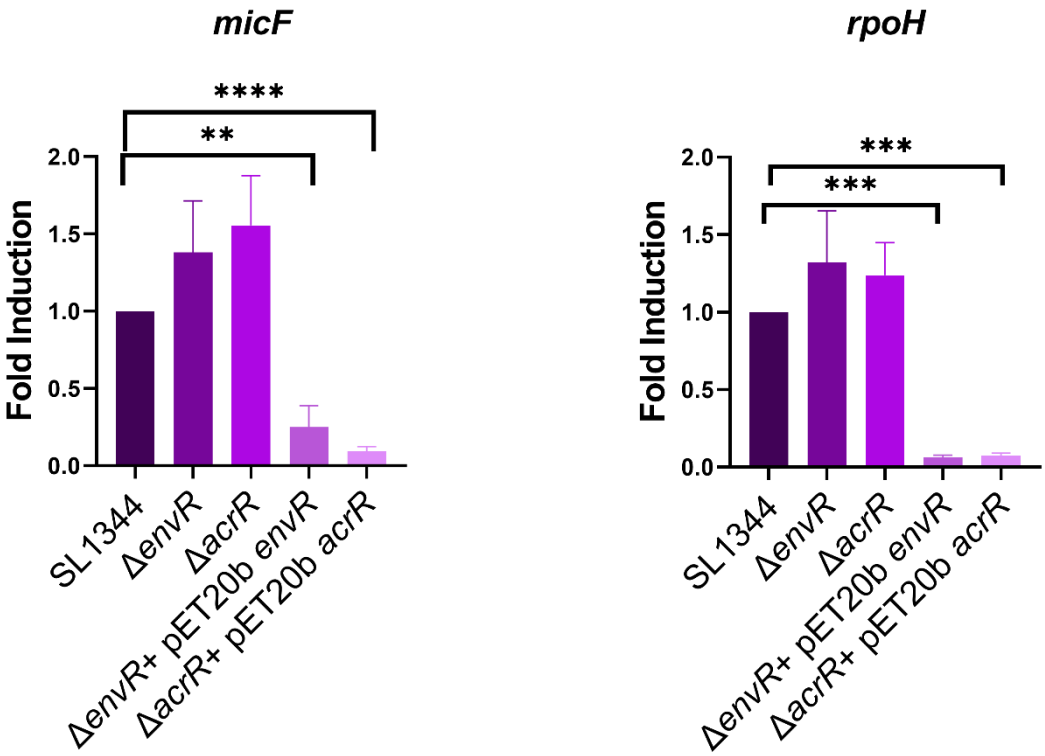


Figure 5.6: Fold changes in transcription of *micF* and *rpoH* genes in different backgrounds (SL1344, *ΔenvR, ΔacrR* and *ΔenvR* + pET20b *envR* and *ΔacrR* + pET20b *acrR*) was determined by RT-PCR. Efficiency was calculated using a 16S housekeeping gene and normalised to wild-type (SL1344) levels to calculate fold induction (y axis). The error bars represent the standard error of the mean of n = 4 biological replicates. A student's T-test (unpaired) was used to determine whether the differences in mean fold induction of the tested genes were statistically significant in the *ΔenvR, ΔacrR* and *ΔenvR* + pET20b *envR* and *ΔacrR* + pET20b *acrR* backgrounds compared to SL1344, with **** = $P \leq 0.0001$, *** = $P \leq 0.001$, ** = $P \leq 0.005$ and * = $P \leq 0.05$. HM performed the RT-PCR.

5.4.5 The binding of purified AcrR and EnvR upstream master flagella genes

There is limited evidence of a putative AcrR binding site upstream flagella genes *flhC/flhD* in *E. coli* (Kim *et al.*, 2016), so these genes were also included for testing. However, the authors propose a site which is not palindromic. As TFTRs are known to preferentially bind palindromic DNA sequences, it is not clear as to whether binding at this site would be possible, or whether any binding would be weak. Previous data in this thesis also demonstrated that AcrR/EnvR overexpression alters motility (Chapter 4), However, while purified AcrR and EnvR protein could not bind upstream *flhC* or *flhD* (Figure 5.7), the expression of both *flhC* and *flhD* was significantly decreased when *acrR* or *envR* was overexpressed (Figure 5.8). As the regulatory proteins didn't bind to the promoter regions this is likely to be an indirect effect of over-expressing acrR or envR.

5.4.6 The binding of purified AcrR and EnvR upstream 16S control gene *rrsA*

As a negative control, the binding of purified AcrR and EnvR upstream 16S gene *rrsA* was also tested. Neither AcrR or EnvR could bind upstream *rrsA* (Figure 5.9). This, accompanied by the fact that AcrR and EnvR did not bind all of the DNA tested, indicates that the binding seen in these results is not an artefact of the experimental design.

Figure 5.7: Binding of AcrR and EnvR upstream *flhC* and *flhD*

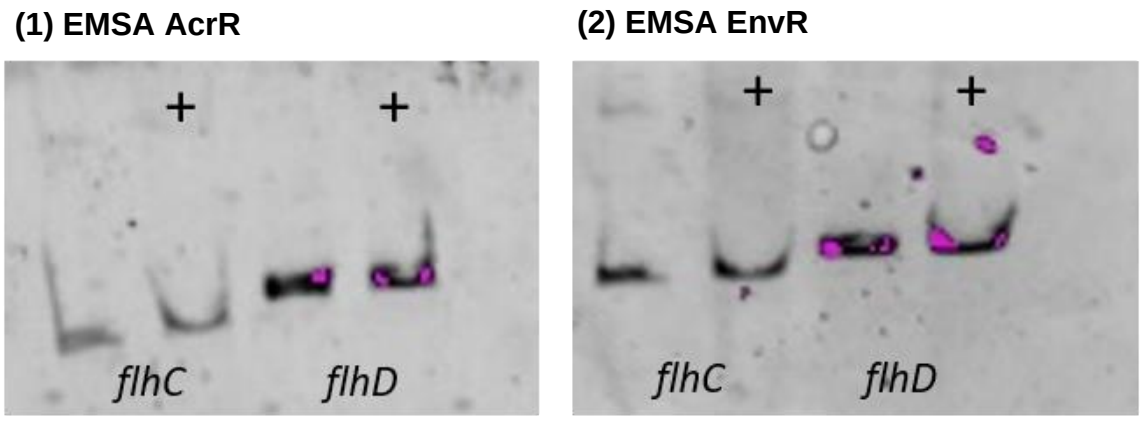


Figure 5.7: EMSA gels stained for DNA with SYBR green showing (1) The binding of AcrR (0.6 µg/ml) to the purified region upstream genes coding for *flhC* or *flhD* (15 ng/µl). (2) The binding of EnvR (0.6 µg/ml) to the purified region upstream master flagella genes *flhC* or *flhD* (15 ng/µl). There are 2 x lanes per purified gene tested, the first of which is DNA-only control and the second of which contains DNA and purified protein, indicated by a +.

Figure 5.8: Transcription of flagella genes *flhC* and *flhD* in strains with high/low *acrR* or *envR* expression

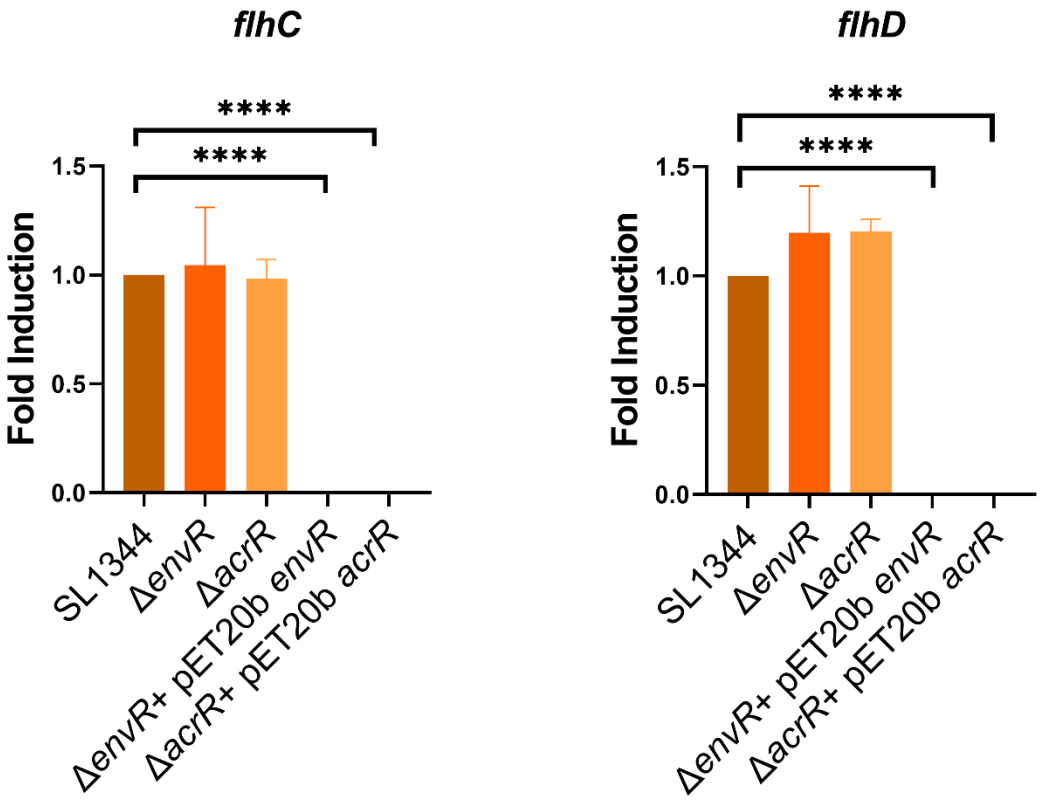


Figure 5.8: Fold changes in transcription of *flhC* and *flhD* genes in different backgrounds (SL1344, *ΔenvR, ΔacrR* and *ΔenvR* + pET20b *envR* and *ΔacrR* + pET20b *acrR*) was determined by RT-PCR. Efficiency was calculated using a 16S housekeeping gene and normalised to wild-type (SL1344) levels to calculate fold induction (y axis). The error bars represent the standard error of the mean of n = 4 biological replicates. A student's T-test (unpaired) was used to determine whether the differences in mean fold induction of the tested genes were statistically significant in the *ΔenvR, ΔacrR* and *ΔenvR* + pET20b *envR* and *ΔacrR* + pET20b *acrR* backgrounds compared to SL1344, with **** = $P \leq 0.0001$, *** = $P \leq 0.001$, ** = $P \leq 0.005$ and * = $P \leq 0.05$. HM performed the RT-PCR.

Figure 5.9: Binding of AcrR and EnvR upstream *rrsA* 16S gene

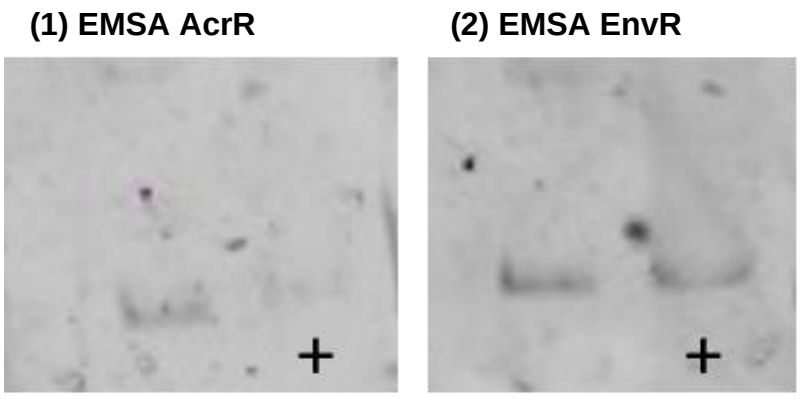


Figure 5.9: EMSA gels stained for DNA with SYBR green showing (1) The binding of AcrR (0.6 µg/ml) to the purified region upstream genes coding for *rrsA* (15 ng/µl). (2) The binding of EnvR (0.6 µg/ml) to the purified region upstream *rrsA* (15 ng/µl). There are 2 x lanes per purified gene tested, the first of which is DNA-only control and the second of which contains DNA and purified protein.

5.4.7 Generating a consensus binding logo

As AcrR and EnvR were found to bind some of the same binding sites, searching for conserved regions in those sites can give an indication of the AcrR-EnvR shared binding site. The MEME suite (Bailey, Boden, Buske *et al.*, 2009) was used to search for short palindromic repeats in the DNA sequences of the upstream regions of the *acrA, marA* and *rob* genes. The search for palindromic sequences was limited to sequences no longer than 30 bp, as the binding site of AcrR upstream of *acrAB* is a 24-bp palindrome (Ma *et al.*, 1996a). As AcrR and EnvR were both able to bind these sequences, this would indicate which residues were required for the binding of these proteins. There were 6 positions in the identified palindromes which were 100% conserved between the sequences inputted, indicating that the amino acids at these positions may be required for AcrR and EnvR to bind (Figure 5.10).

Figure 5.10: Consensus logo of AcrR and EnvR targets

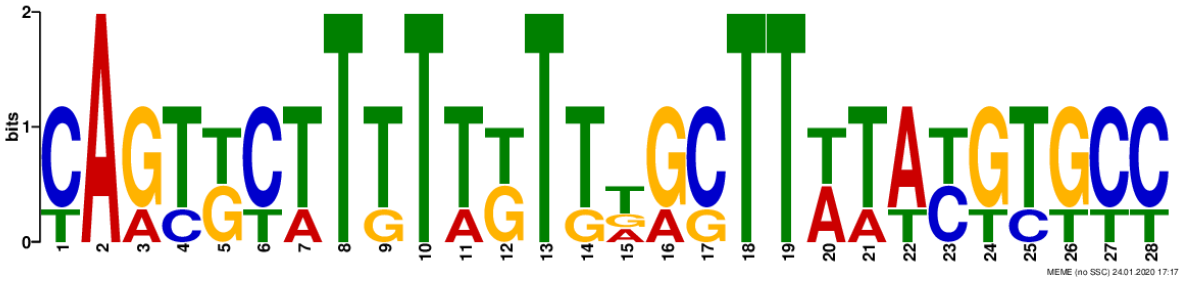


Figure 5.10: Consensus logo of AcrR and EnvR binding sites generated using Meme Suite (Bailey *et al.*, 2009) to search for short palindromic repeats. Targets of both AcrR and EnvR (the upstream regions of *acrA, acrE*, *rob* and *marA* were inputted into the MEME software. The size of the nucleotide represents the conservation of each nucleotide in the consensus logo in bits.

5.5 The binding of EnvR upstream *acrAB* and *acrEF* in the presence of various compounds

As the AcrAB and AcrEF efflux systems are similar, it was hypothesised that AcrEF may share some of the same substrates as AcrAB. The regulators (AcrR/EnvR) are thought to bind these substrates as ligands, which causes a conformational change that promotes *acrAB* or *acrEF* expression. Rhodamine 6g and proflavine have been shown to interact with AcrR (Su *et al.*, 2007) and are also substrates of AcrAB. Therefore, the presence of these substrates may prevent the binding of these regulators to the promoter of *acrA* and perhaps also *acrE.*

Therefore, the binding of EnvR upstream *acrA* and *acrE* in the presence of rhodamine 6g and proflavine was tested (Figure 5.11). Rhodamine 6g or proflavine did not prevent the binding of EnvR upstream of *acrAB* (Figure 5.11, panel A). Rhodamine 6g prevented the binding of EnvR upstream *acrA* at a concentration of 770 ug/ml (Figure 5.11, panel B).

Kanamycin is known to not be a substrate of AcrAB-TolC and was also tested and was not therefore predicted to influence binding of AcrR/EnvR to the promoters. As expected, the addition of kanamycin did not influence the binding of EnvR upstream *acrAB* (Figure 5.12).The binding of EnvR upstream *acrAB* in the presence of AcrAB-TolC substrates erythromycin, tetracycline and ciprofloxacin was also tested. Ciprofloxacin and erythromycin did not influence the binding of EnvR upstream *acrA* or *acrE* (Figures 5.14 – 5.15). Low (0.077, 0.77 and 3.8 µg/ml), but not high (7.7 and 77 µg/ml) concentrations of tetracycline inhibited the binding of EnvR upstream *acrAB* (Figure 5.13).

Figure 5.11: EMSA of EnvR binding *acrAB/acrEF* promoter with R6G/proflavine

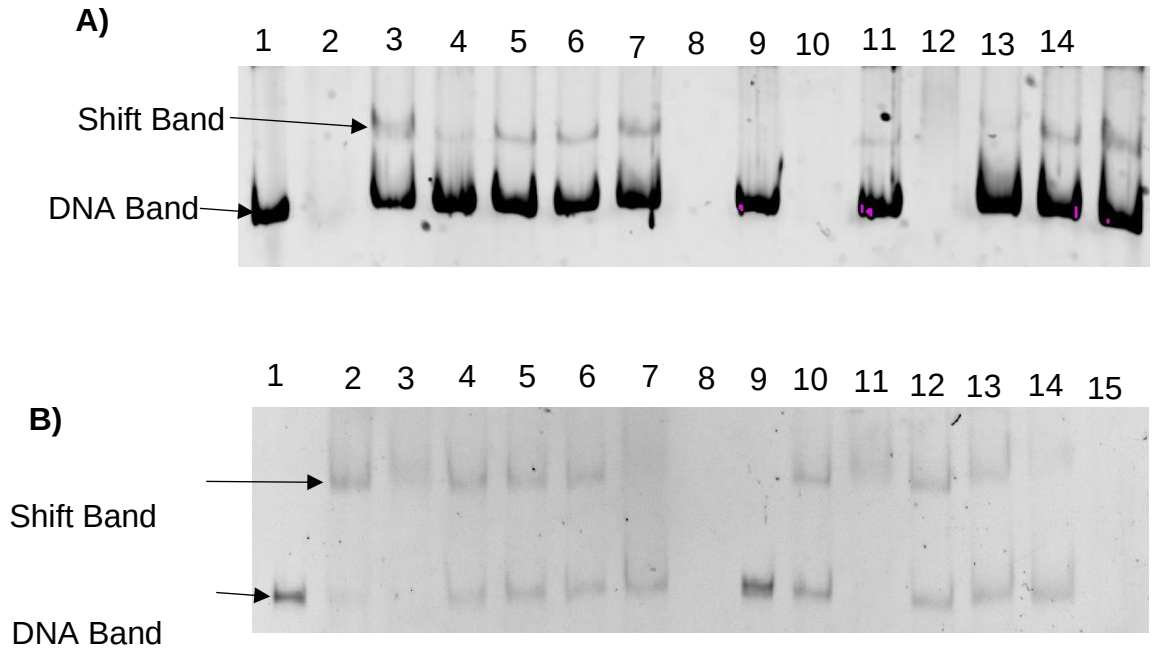


Figure 5.11: **A)** EMSA gel using the promoter *acrAB* with EnvR with the addition of rhodamine 6G (Lanes 4-7) and proflavine (Lanes 9-15) dilutions. Lane 8 was left blank. The DNA only control, EnvR only control and DNA with EnvR (Lanes 1-3, respectively). Shift bands were seen in the DNA and EnvR control and at all rhodamine 6G concentrations (770, 77, 7.7 and 0.77 µg/ml, Lanes 4-7). For proflavine dilutions the controls were observed (Lanes 9-11). Shift bands were seen at both 7.7 µg/ml and 0.77 µg/ml (Lanes 14-15). **B)** EMSA gel using promoter of *acrEF* with EnvR with the addition of rhodamine 6G (Lanes 4-7) and proflavine (Lanes 9-15) dilutions. The DNA only control, DNA with EnvR and EnvR only control (Lanes 1-3, respectively). Shift bands were seen in the DNA and EnvR control and at rhodamine 6G concentrations 0.77, 7.7 and 77 µg/ml (Lanes 4-6, respectively). No shift band was seen at 770 µg/ml. For proflavine dilutions the controls were observed (Lanes 9-11). However, proflavine dilutions showed no shift bands at 770 µg/ml, yet no DNA band was seen either (Lane 15). At 77 µg/ml no shift band was observed (Lane 14). Shift bands were seen at both 7.7 µg/ml and 0.77 µg/ml (Lanes 12-13). Lane 8 was left blank. Presented in *"Understanding the regulation of Efflux pumps AcrAB and AcrEF in Salmonella Typhimurium" MSc Thesis* by JS (Scadden, 2019).

Figure 5.12 EMSA of EnvR binding *acrAB* promoter with kanamycin

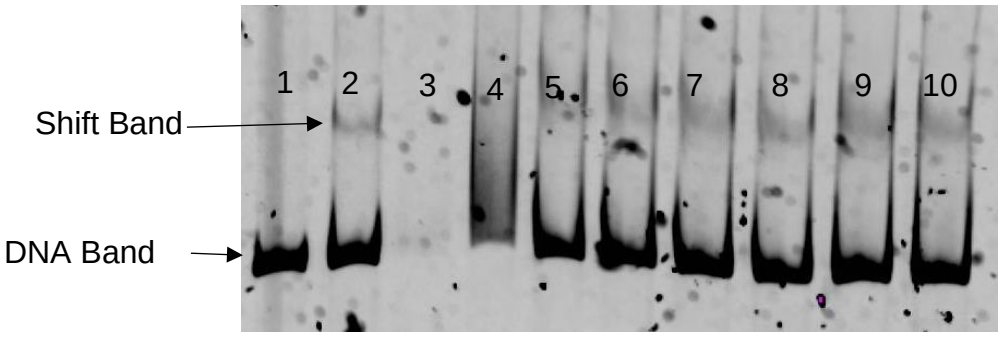


Figure 5.12: EMSA gel using *acrAB* with EnvR along with the addition of kanamycin dilutions. The DNA only control, DNA with EnvR, EnvR only control and 77 µg/ml proflavine showed expected results (Lanes 1-4, respectively). Shift bands were seen in the DNA and EnvR control and at all kanamycin concentrations (0.077, 0.77, 3.8, 7.7, 38 and 77 µg/ml) (Lanes 5-10). originally presented in *"Understanding the regulation of Efflux pumps AcrAB and AcrEF in Salmonella Typhimurium" MSc Thesis* by JS (Scadden, 2019).

Figure 5.13 EMSA of EnvR binding *acrAB* promoter with tetracycline

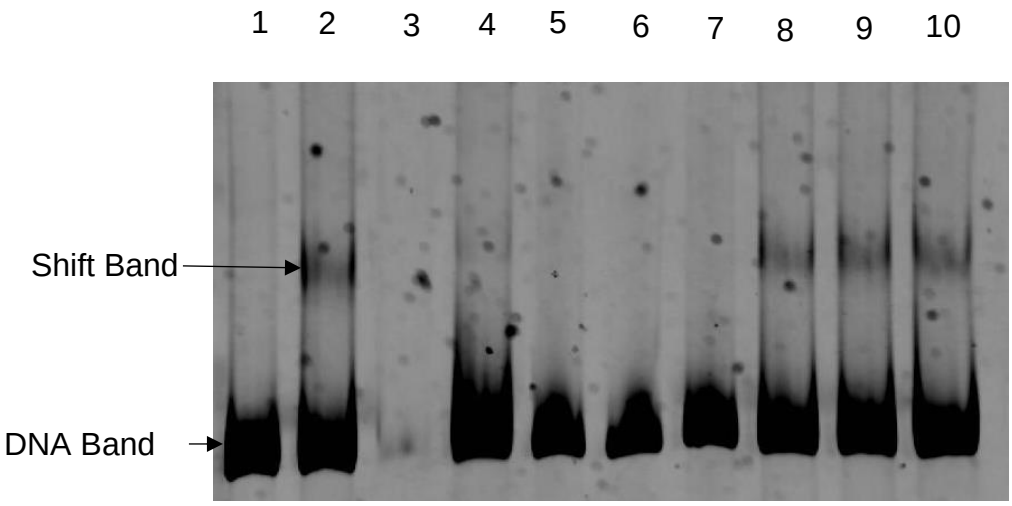


Figure 5.13: EMSA using *acrAB* with EnvR along with the addition of tetracycline dilutions. The DNA only control, DNA with EnvR, EnvR only control and 77 µg/ml proflavine showed expected results (Lanes 1-4, respectively). Shift bands were seen in the DNA and EnvR control and at all tetracycline concentrations 7.7, 38 and 77 µg/ml (Lanes 8-10). However, at lower concentrations (0.077, 0.77 and 3.8 µg/ml) (Lanes 5-7) no shift bands were observed. originally presented in *"Understanding the regulation of Efflux pumps AcrAB and AcrEF in Salmonella Typhimurium" MSc Thesis* by JS (Scadden, 2019).

Figure 5.14: EMSA of EnvR binding *acrAB/acrEF* promoter with erythromycin

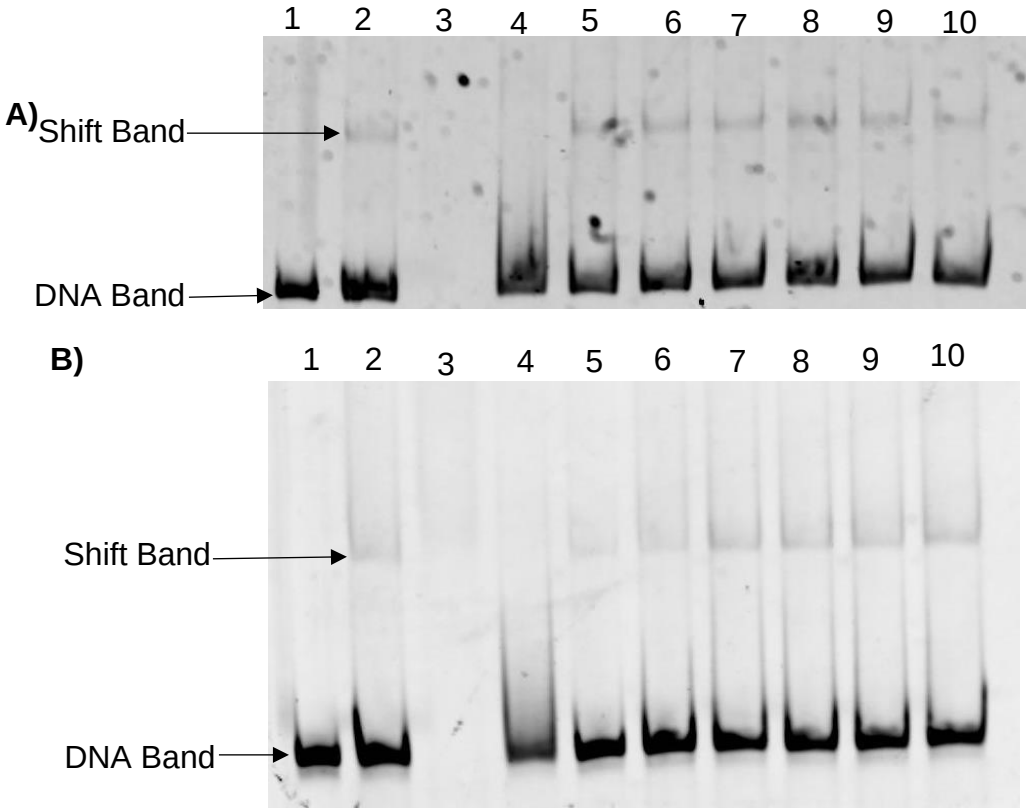


Figure 5.14: **A)** EMSA gel using acrAB with EnvR along with the addition of erythromycin dilutions. The DNA only control, DNA with EnvR, EnvR only control and 77 µg/ml proflavine showed expected results (Lanes 1-4, respectively). Shift bands were seen in the DNA and EnvR control and at all erythromycin concentrations (0.077, 0.77, 3.8, 7.7, 38 and 77 µg/ml) (Lanes 5-10). **B)** EMSA gel using acrEF with EnvR along with the addition of erythromycin dilutions. The DNA only control, DNA with EnvR, EnvR only control and 77 µg/ml proflavine showed expected results (Lanes 1-4, respectively). Shift bands were seen in the DNA and EnvR control, as expected, and at all erythromycin concentrations (0.077, 0.77, 3.8, 7.7, 38 and 77 µg/ml) (Lanes 5-10). originally presented in *"Understanding the regulation of Efflux pumps AcrAB and AcrEF in Salmonella Typhimurium" MSc Thesis* by JS (Scadden, 2019).

Figure 5.15: EMSA of EnvR binding *acrAB/acrEF* promoter with ciprofloxacin

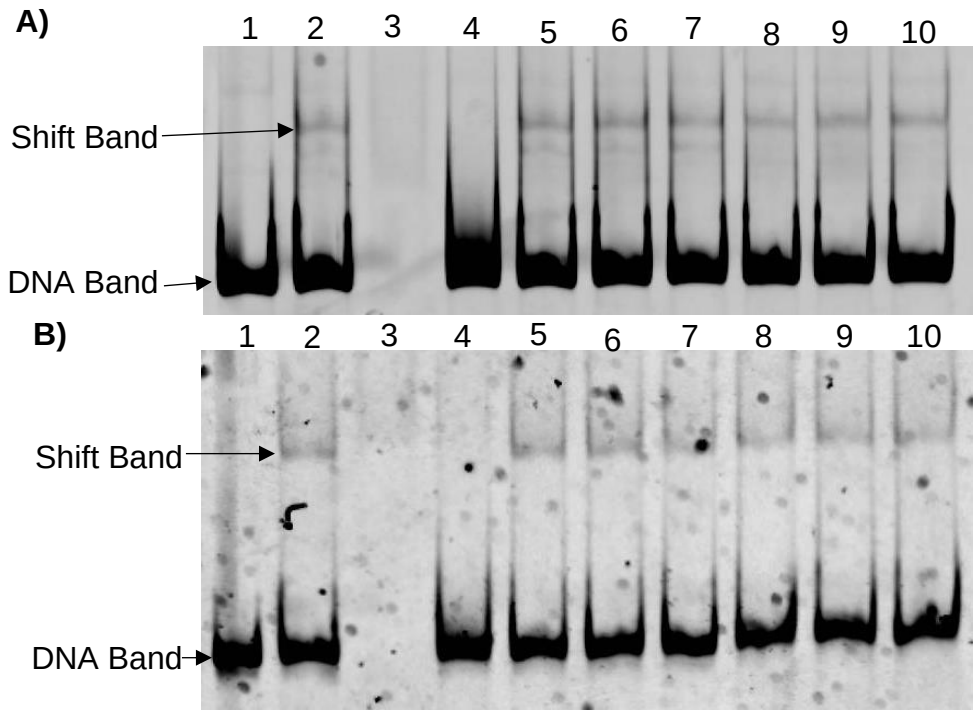


Figure 5.15: **A)** EMSA gel using acrAB with EnvR along with the addition of ciprofloxacin dilutions. The DNA only control, DNA with EnvR, EnvR only control and 77 µg/ml proflavine showed expected results (Lanes 1-4, respectively). Shift bands were seen in the DNA and EnvR control and at all ciprofloxacin concentrations (0.077, 0.77, 3.8, 7.7, 38 and 77 µg/ml) (Lanes 5-10). **B)** EMSA gel using acrEF with EnvR along with the addition of ciprofloxacin dilutions. The DNA only control, DNA with EnvR, EnvR only control and 77 µg/ml proflavine showed expected results (Lanes 1-4, respectively). Shift bands were seen in the DNA and EnvR control, as expected, and at all ciprofloxacin concentrations (0.077, 0.77, 3.8, 7.7, 38 and 77 µg/ml) (Lanes 5-10). originally presented in *"Understanding the regulation of Efflux pumps AcrAB and AcrEF in Salmonella Typhimurium" MSc Thesis* by JS (Scadden, 2019).

5.6 Discussion

Regulators are often quoted as having ‘off-target’ effects if they bind upstream of or regulate an unexpected or previously uncharacterised target. This is particularly true for TFTRs, which are thought to predominantly bind targets within 200 bp of the TFTR gene itself (Balhana *et al.*, 2015). However, it is now known that some TFTRs can bind multiple targets and can both repress and activate the expression of targets. For example, the efflux regulator MtrR which locally represses *mtrCDE* efflux gene expression also regulates “off-target” loci (P. J. Johnson & W. M. Shafer, 2015).

AcrR and EnvR are known for their roles as local regulators of the AcrAB and AcrEF efflux systems, respectively. However, it is also known that EnvR also regulates the expression of *acrAB*, meaning that EnvR already has a role in addition to the local regulation of *acrEF* expression. There is also evidence in the literature that AcrR has wider roles, able to bind upstream and influence the expression of multiple genes (Table 5.1). Moreover, previous data in this thesis (Chapter 4) shows that overexpressing AcrR and EnvR has phenotypic consequences which are not, to our current knowledge, caused by a lack of AcrAB. Taken together, this hints that these proteins may have wider regulatory roles.

As AcrR and EnvR share a target, the promoter of *acrAB* (Hirakawa, Takumi-Kobayashi*, et al.*, 2008), any potential or known targets of AcrR were tested as potential targets of EnvR. The focus of this chapter was to determine whether AcrR and EnvR can bind or influence the expression of genes and whether this binding is altered in the presence of potential AcrR/EnvR ligands.

To investigate this, the binding of AcrR and EnvR upstream a range of genes was tested. A literature search was undertaken to discover any potential targets of AcrR or EnvR. For some targets there was evidence in the literature of binding, either by direct binding assays or via binding site prediction. Other evidence was indirect, for example interrupting *acrR* altered motility (Kim *et al.*, 2016). As the regulation of these potential targets could be direct or indirect, a lack of binding is not sufficient to rule out a regulatory role. For this reason, all evidence, both direct (i.e. binding) and indirect (i.e. phenotypic consequences to gene interruption) was considered during selection of promoters to test.

Some targets were added even though there was no evidence in the literature of binding or regulation. The first group of additional targets were the promoters of other RND efflux systems *mdsA* and *mdtA,* which were tested in addition to the promoters of *acrD, acrE* and *acrA.* Given that the regulation of *acrAB* and *acrEF* is interlinked, it also made sense to test whether AcrR or EnvR could bind upstream or influence the expression of any other efflux genes. Both EnvR and AcrR could bind upstream *acrAB,* but EnvR bound with higher affinity in these experiments using *Salmonella* DNA, which is the same as what has been shown in *E. coli* (Hirakawa, Takumi-Kobayashi*, et al.*, 2008) (Figure 5.1). AcrR could bind the promoter of *acrAB* and *acrEF,* with no visible, qualitative difference in the band intensity between the two bands (Figure 5.1). Therefore, while EnvR seems to bind the *acrAB* promoter better than the locally-encoded *acrEF* promoter, this is not true of AcrR. However, it is important to note that (as discussed in both Chapter 4 and 5), the data here is limited and therefore these assays must be improved and repeated. Doing a concentration titre of AcrR/EnvR

protein with these targets would start to unpick the binding affinities of these proteins to their targets.

EnvR also bound upstream *acrD* and there is preliminary evidence that EnvR may upregulate *acrD* expression (Emami, 2014). However, here overexpressing *envR* reduced *acrD* transcription, which disagrees with these authors. These different conclusions could be due to the experimental conditions used. As the role of AcrD is largely unknown, it could be that the expression of this system only occurs in specific circumstances. Neither regulator was able to bind upstream *mdtA* or *mdsA*. However, the overexpression of these regulators reduced transcript levels of *mdtA* and *mdsA,* along with all other efflux genes tested (Figure 5.2). This indicates that even if no direct interactions are involved, there may be indirect regulation by AcrR or EnvR. Alternatively, it could be that the overexpression of AcrR and EnvR has multiple global effects which indirectly influence the expression of these genes via other regulators.

AcrR and EnvR were also able to bind upstream other efflux regulators. Both AcrR and EnvR could bind upstream *marA* and *rob,* but not *soxS* or *ramA*. However, the protein concentration here was low and it could be that these differences are not significant. Moreover, if *acrR* and *envR* were overexpressed using the pET20b plasmid then the transcript level for not only *marA* and *rob* but also for *soxS* and *ramA* was reduced to almost zero (Figure 5.4). Interrupting either *acrR* or *envR* increased the expression of *rob* by 1.4 and 1.3 fold for Δ*acrR* and Δ*envR*, respectively. The drastic differences when overexpressing or interrupting *acrR* or *envR* could hint at whether these regulators could be activating or repressing the expression of these targets. A combination of ChIP and RNA-Seq could be used to determine the regulatory networks surrounding AcrR and EnvR, and this is recommended for future work.

As the global regulators of *acrAB* share targets and can regulate one another, it would not be surprising that if AcrR/EnvR can bind upstream all of these targets. If AcrR and EnvR bind a degenerate sequence similar to the marsox box then this is plausible explanation for these results. There is also evidence that AcrR regulates the expression of both *soxS* and *marA*, influencing solvent tolerance in *E. coli* (Lee *et al.*, 2014). Work to determine if and when AcrR binds these promoters could help us to understand the relationships between efflux and other processes such as solvent tolerance.

Some of these genes, including *marA* and *soxS* are known to be expressing divergently of other regulators (in this case, *marR* and *soxR),* the expression changes in these genes were also investigated. Generally, the same patterns were seen for *marR* and *soxR* as for *marA* and *soxS.* This could mean that the binding of AcrR and EnvR to these regions is bi-directional, influencing the expression of genes in both directions. This is a common mechanism of TFTR binding and regulation. For example, AcrR binds a region which prevents the expression of both *acrAB* and, in the opposite direction, *acrR.* This autoregulation allows TFTRs to work as regulatory switches. Presumably, in the case of *acrAB*, the whole operon remains inactive until a signal to remove the AcrR-mediated repression arises. This could be in the form of a ligand binding to AcrR, or through interactions with other regulators.

If the preliminary evidence of binding seen here is real and happens *in vivo,* then it is possible that AcrR and EnvR may employ a similar binding technique to the regulators tested here as targets. Regulators of efflux systems, such as MarA, SoxS and Rob are known to bind at multiple promoters and do so by recognising and binding a degenerate sequence (Jair *et al.*, 1996; Martin *et al.*, 1999; Martin *et al.*, 2000; Martin

& Rosner, 2011). Their ability to bind with differing affinities to targets allows for a variable response, and to allow different regulators to outcompete one another at a target. For example, RamA binds a region which overlaps both the promoter and the binding site of AcrR in *Salmonella* (Nikaido *et al.*, 2008). Therefore, the binding of RamA prevents AcrR-mediated repression, promoting the expression of *acrAB*. In E. coli, MarA, SoxS and Rob are known to activate expression of over 40 promoters (Martin & Rosner, 2011). Although these regulators bind different sites with different affinities, there is not a direct correlation between binding affinity and promoter activation (Martin *et al.*, 2000). Therefore, a stronger binding affinity does not necessarily equal stronger regulation. There are also complicated relationships between these proteins. For example, MarA activates numerous genes (including *acrAB*), but can also repress *rob* expression (Schneiders & Levy, 2006). These networks are therefore complex, and whilst understanding individual connections is important, it is also key to remember that the regulatory networks are much larger and much more complex. This could also be true of AcrR and EnvR. Similarly, while overexpression of *ramA* can override AcrR-mediated regulation of *acrAB* in *Klebsiella*, overexpression of *marA* does not, hinting that the affinity of these regulators versus the local repressor is also important. RamA is also thought to be the primary activator of *acrAB* expression in *Salmonella* (Nikaido *et al.*, 2008). Therefore, some of the regulatory 'hierarchy' seen in the networks of efflux regulators may be conserved in some bacterial species.

A putative binding site for AcrR has also been found upstream the *micF* gene in an automated search based on the known binding site of AcrR upstream *acrAB* (Ma *et al.*, 1996a). Here, AcrR was able to bind upstream the *micF* gene and the overexpression

of *acrR* and *envR* reduced the transcription of *micF* (Figure 5.6). MicF is an antisense RNA which negatively regulates the expression of *ompF*, which encodes the porin OmpF. OmpF is responsible for the influx of numerous substrates of AcrAB, including various antimicrobials (Mahendran *et al.*, 2010). The expression of *micF* is known to be decreased in a RamA knockout (Zheng, Tian, Cui *et al.*, 2011) and is also regulated by SoxS and MarA (Chubiz *et al.*, 2012). The expression of *acrAB* and *micF* are also increased in response to some of the same inducers (Hartog, Ben-Shalom, Shachar *et al.*, 2008). This co-expression enables increased efflux (via AcrAB) to be coupled with decreased influx (via MicF-mediated downregulation of *ompF* expression). This led to the hypothesis of one author that AcrR may play a modulating role in determining *micF* expression (Hartog *et al.*, 2008). The same authors demonstrate that the *micF* promoter is stronger than the *acrA* promoter. It could be possible, therefore, that AcrR binds the promoter of *micF* to negatively regulate *micF* and upregulate *ompF* expression. This coupling of influx and efflux makes sense, although it is not clear if AcrR would bind this target *in vivo*. To evaluate this further, ChIP and RNA-seq should be performed.

AcrR has also been implicated in motility, with *acrR*-null mutants show motility defects (Kim *et al.*, 2016). However, there was no direct binding of AcrR or EnvR to the flagella gene promoters (Figure 5.7), but overexpression of *acrR* or *envR* decreased the transcript level of both *flhC* and *flhD* (Figure 5.8). This does not mean that AcrR/EnvR do not regulate these genes, but only that if they do, this is not direct. AcrR also bound upstream *rpoH* (Figure 5.5) and overexpressing either *acrR* or *envR* reduced *rpoH* expression (Figure 5.6). It could be that EnvR is also able to bind upstream *rpoH* but may do so with a lesser affinity than AcrR.

It is important to note that the binding seen in the EMSAs in this study were weak and require further verification. However, EnvR and AcrR were able to weakly bind upstream multiple targets. They also did not bind upstream all targets tested, with no binding upstream the flagella genes *flhC*, *flhD* or 16S gene *rrsA.* It is difficult to interpret these data due to the variation seen in the binding of the positive control (AcrR/EnvR with the promoter *acrAB*). Therefore, while these initial findings are interesting, they require further follow up with repeated EMSAs, ChIP and RNA-Sequencing.

An interesting finding of these data was that not all targets of one regulator were targets of the other. Given the similarity of the DNA binding regions of these proteins, it was hypothesised that EnvR may bind all targets with a higher binding affinity than AcrR, as is seen for the binding of EnvR to *acrAB.* However, AcrR bound 8 targets whereas EnvR bound 5. There were also four genes (*soxS*, *ramA*, *micF*, *rpoH*) which AcrR could bind that EnvR could not, whereas EnvR could only bind one target (*acrD*) that AcrR could not bind. Therefore, although similarities in the DNA binding region can (and should) be used to predict a regulator's targets, this approach is too simplistic, with even small (~25 kDa) and similar regulators such as these potentially having differences in binding ability.

There are also multiple factors which can influence the binding of a regulator to a target. The presence of other AcrR/EnvR ligands within the cell may bias the binding of AcrR/EnvR, for example. Or, perhaps the overexpression of these regulators would need to be reduced to reveal differences between AcrR/EnvR regulation. A final consideration is that the binding assay uses linear, small fragments of DNA. We know that regulator binding relies on the availability of the DNA for binding, which is regulated by either physical (i.e. supercoiling) or regulatory (i.e. global regulators, sigma factors,

etc.). Therefore, while the DNA used in the EMSA is fully available for binding, this may not be the case *in vivo*, or there may be select circumstances which allow for binding. There are many suggestions for further experiments to better understand these mechanisms listed at the end of this chapter.

In addition to the N-terminal DNA binding region, the C-terminal region, and the target promoter itself are also important in determining whether a regulator can bind. The sequence required for AcrR binding to the *acrAB* promoter is known and was shown to also be important for EnvR binding in a previous chapter (Chapter 4). Studying the conservation of these residues in all shared AcrR/EnvR targets, in addition to studying the consensus sequence of targets of the regulators as individuals, can start to separate the binding requirements of these regulators.

Several residues known to be important for AcrR/EnvR binding to the promoter of *acrAB* were present in the promoters of other genes identified as targets here (Figure 5.10). The same authors who originally proposed that AcrR could regulate the *flhC*/*flhD* believe they identified a binding site upstream these genes (Kim *et al.*, 2016). However, the proposed binding site is not palindromic and it is not clear how AcrR would bind. Possibly, weak binding could occur, with half of each AcrR monomer able to bind target DNA. However, whether such weak binding would be strong enough to cause any significant phenotypic is not clear.

The ability of AcrR and EnvR to bind some of the same targets with differing affinities could be explained by the presence of a degenerate, shared binding site. MarA, SoxS and Rob are known to upregulate the expression of *acrAB* and bind the degenerate sequence known as the marbox in *E. coli*. The variability of the marbox allows the

relative binding affinity of MarA, SoxS and Rob to be altered to fine-tune the regulatory response. For example, SoxS binds the marbox next to genes involved in the superoxide response with a higher affinity than MarA (Martin *et al.*, 2000). These global regulators can also activate or repress targets depending on the orientation of the marbox (Schneiders, Barbosa, McMurry *et al.*, 2004), allowing a further layer of regulation. Some of the conserved residues present in all the shared targets of AcrR and EnvR (Figure 5.10) are present in the known binding site of AcrR upstream of *acrAB* in *E. coli*. Mutating some of these residues in the *acrA* promoter reduced or abolished the binding of AcrR and EnvR to the promoter, respectively (Chapter 4). Targets with a higher affinity for either AcrR or EnvR show a different panel of conserved residues, highlighting how variations in these sequences may promote the binding of either of these similar regulators. Although TFTRs are often quoted to be local, negative regulators, there are numerous examples where this is not the case. As previously described (Chapter 3), the TFTR family of regulators have diverse and often, global roles. It is unclear as to whether there is a 'consensus' binding sequence for TFTRs. This seems unlikely, due to the many roles and binding locations of these regulators. However, certain residues seem to be important for the binding to specific targets. For example, the binding site for AcrR upstream of *acrAB* in *E. coli* is required for binding (Li *et al.*, 2007). In Chapter 4, the same region was shown to be vital for AcrR binding upstream of *acrAB* in *Salmonella.*

The C-terminal region also plays a major role in binding. The binding of a ligand to the C-terminus of a TFTR usually confers a conformational change which alters the TFTR-DNA binding affinity. For the majority of studied TFTRs, the binding of a ligand causes the TFTR to be unable to bind target DNA (i.e. relieved repression). For example,

rhodamine 6g is a substrate of AcrAB-TolC (Anes *et al.*, 2015) which also binds AcrR (Li *et al.*, 2007; Manjasetty *et al.*, 2016b). Therefore, under conditions where *acrAB* transcription is required, such as when intracellular concentrations of rhodamine 6g are high, AcrR is bound by rhodamine 6g and AcrAB-TolC can then actively extrude rhodamine 6g. This therefore allows the regulation of *acrAB* to be targeted in response to specific signals. It was hypothesised that all substrates of AcrAB-TolC would also be ligands of AcrR and EnvR and influence their binding to the *acrAB* promoter. However, this was not the case. It could be that some substances activate the expression of *acrAB* or *acrEF* independently of AcrR/EnvR, or that they activate one of the positive regulators (such as RamA).

Binding was inhibited at high concentrations (i.e. classical TFTR repressor model) by rhodamine 6g and proflavine (Figure 5.11). However, low, but not high, concentrations of tetracycline inhibited the binding of EnvR to the promoters in a concentration-dependent manner (Figure 5.13). The reason for this phenotype is not clear. Two polymorphisms in the sequence of TetR (the local negative regulator of *tetA*), cause 'reversed' functionality of TetR (Kamionka, Bogdanska-Urbaniak, Scholz *et al.*, 2004). This 'reversed TetR' requires tetracycline for binding to *tetA,* instead of tetracycline binding causing TetR to dissociate from the *tetA* promoter. Therefore, the functionality and interaction of TFTRs with their ligands may be reliant on only a few residues. It may also be possible for ligands to compete for binding, or for multiple ligands to bind simultaneously.

The binding of EnvR to the promoters of *acrA* and *acrE* was shown to be influenced by the presence of different ligands (Figures 5.11 – 5.15). It was not possible to test the effect of these ligands on the binding of AcrR due to a lack of active protein during the

time of these experiments. However, the fact that EnvR responded so variably to these ligands further supports the hypothesis that EnvR is a global regulator, able to be influenced by the binding of multiple ligands. Interestingly, there was no difference between the effect of a ligand on binding to the *acrA* or *acrE* promoters. This may be because the promoters of these genes are so similar, meaning that any conformational changes to EnvR caused by a ligand binding have the same effect at these targets.

Here, there is preliminary evidence to show that AcrR and EnvR can bind and influence the expression of multiple targets. There was also variation in the targets of AcrR and EnvR. To validate these results, the EMSA method should be changed and followed up with ChIP and RNA-Seq to confirm the binding and regulation of targets.

Although it is likely from these data that the roles of AcrR and EnvR have been underestimated, it is also important to state that this is not always the case. For example, MarR was recently shown to only bind the promoter of *marA* and is therefore presumed to be a single-target regulator (Sharma *et al.*, 2017). Therefore, although some regulators of efflux have wider roles, this cannot be assumed to be true of all efflux regulators.

5.7 Future directions

- ChIP and RNA-seq to confirm and search for more targets of AcrR and EnvR. ChIP-seq would indicate which gene(s) are directly bound by AcrR/EnvR whereas RNA-seq would show which gene(s) are influenced by changing levels of AcrR/EnvR.

- Repeating the EMSA data using a new method (i.e., radiolabelling DNA probes), for a more targeted approach with additional controls, making sure that the molar ratios of DNA:Protein are optimised in the assays.
- Modelling of the ligand binding domains of AcrR/EnvR to identify any differences and therefore ligand-specificity of these proteins.
- Co-crystalize AcrR/EnvR with ligands.
- Test whether the overexpression of *acrR* or *envR* changes the expression of the *acrEF* genes.
- Search for AcrR/EnvR binding sites in the SL1344 genome using the consensus sequence identified in Figure 5.10.

5.8 Key findings

- AcrR and EnvR are global regulators as purified AcrR and EnvR could bind upstream many genes and did so with differing affinities.
- AcrR and EnvR do not share all targets, which hints at specific roles for EnvR and the AcrEF efflux system.
- The overexpression of *acrR* and *envR* alters the expression of these target genes.
- The regulation of these novel targets by AcrR and EnvR is likely to be both direct and indirect, as although the expression of all targets was altered if *acrR* or *envR* were overexpressed, the purified AcrR and EnvR proteins did not necessarily bind upstream the tested genes.

Chapter Six

Condition and drug-dependent induction of *acrAB* and *acrEF* expression

6.0 Condition and drug induction of *acrAB* and *acrEF* expression

6.1 Background

AcrEF-TolC is an RND efflux system proposed to have a very similar substrate specificity to AcrAB-TolC (Anes *et al.*, 2015). However, very little is known about which conditions or compounds can induce *acrEF* transcription and whether there are any conditions or compounds which are AcrEF-specific. Understanding whether there are any inducers which are specific to the AcrEF-TolC system may also uncover ligands which are specific to the local regulator EnvR.

To study the induction of transcription of the AcrAB and AcrEF systems, a suite of strains containing the pMW82 GFP reporter constructs pMW82 *acrA*, pMW82 *acrE* and pMW82 *ramA* were transduced into multiple backgrounds. The GFP constructs were kindly donated by Dr. Mark Webber of the Quadram institute and comprise of the promoter of the genes of interest fused to the *gfp* gene. Therefore, when transcription of the target promoter is initiated on the plasmid, GFP protein is produced and the relative fluorescence of this compared to a non-induced and no-plasmid controls can be measured. This chapter therefore refers to the proportional increase in GFP fluorescence as 'induction' of these genes.

As the *acrEF* genes are H-NS silenced in *Salmonella* (Nishino *et al.*, 2009), it was not clear whether induction of the *acrE* promoter could be achieved only through the addition of an inducer, or whether H-NS repression would first need to be relieved.

First, the induction of *acrAB* and *acrEF* was measured over time in the WT-background in response to the presence of indole in the media, or changing glucose, phosphate, $MgCl_2$, or pH. These conditions were selected because they are a) known inducers of

acrAB (i.e. Indole), (b) conditions which have been previously shown to relieve H-NS repression or (c) conditions which more closely reflect infection environments. *Salmonella* lack *tnaA* and therefore does not produce indole from tryptophan metabolism. However, multiple genes including *acrAB* are induced in the presence of indole in the environment (Nikaido, Giraud, Baucheron *et al.*, 2012). This is important, as *Salmonella* encounter indole during the infection of the gastrointestinal tract and the induction of virulence genes in these environments may promote survival and proliferation (Khan, 2014).

It has been shown that a lack of functional AcrB results in reduced virulence of *Salmonella* (Wang-Kan *et al.*, 2017) and reduced ability of *Salmonella* to adhere to human epithelial cells and invade mouse macrophages (Buckley, Webber, Cooles *et al.*, 2006). Therefore, conditions experienced by *Salmonella* during the infection cycle such as variations in pH and magnesium and phosphate limitation (*Salmonella*-containing vacuole) were mimicked using variations of minimal MOPS media. Moreover, there is evidence that these conditions, such as magnesium limitation may relieve H-NS repression of target genes (Will, Whitham, Reid *et al.*, 2018), which could lead to changes in *acrEF* expression.

Secondly, induction of *acrAB* and *acrEF* in an *hns*-interrupted strain was measured in a set of single time point experiments. These experiments were designed to allow optimal growth of the *hns*-interrupted strain. Strains were challenged with substrates of AcrAB (indole, rhodamine 6g, proflavine, tetracycline, ciprofloxacin and novobiocin) and the induction was measured after exposure. Kanamycin is not a substrate of AcrAB and was used as a control.

Previous work in this study has shown that AcrR and EnvR bind upstream of multiple genes and either directly or indirectly, influence their expression (Chapter 5). Potential ligands of AcrR and EnvR can also influence their binding to these targets (Chapter 5). Some of the ligands of AcrR and EnvR are also substrates of the AcrAB-TolC efflux system. For example, rhodamine 6g, a substrate of AcrAB-TolC, can bind AcrR (Su *et al.*, 2007). Consequently, in circumstances where increased *acrAB* expression is needed (i.e. during intracellular accumulation of a toxic substrate of AcrAB-TolC), the TFTR regulator is presumably bound by the substrate/ligand and is not repressing the expression of *acrAB,* allowing the extrusion of the substrate. Conversely, when no substrate/ligand signal is present, the TFTR reduces the expression of *acrAB.* It makes sense that the substrates of AcrAB-TolC should also interact with the regulators of this system to achieve a fine-tuned, specific regulatory response.

In addition to the role of AcrR and EnvR as negative regulators, other positive regulators such as RamA may be involved in increasing the expression of *acrAB* and *acrEF.* It is known that RamR, the negative regulator of *ramA* expression, binds some of substrates of AcrAB and AcrEF systems, including ethidium bromide, rhodamine 6g and bile salts (Yamasaki *et al.*, 2019; Yamasaki *et al.*, 2013). Therefore, the same ligands which bind AcrR/EnvR may bind other TFTRs such as RamR to simultaneously increase *acrAB* or *acrEF* expression. This work therefore also sought to measure the induction of *ramA* expression in response to ligands tested to induce *acrAB* and *acrEF*, to begin to understand whether any of these inductions may involve RamA.

6.2 Aims

- Using pMW82 GFP reporter constructs, test to see if conditions thought to relieve H-NS repression or those that mimic the infection life cycle of *Salmonella* can induce AcrAB or AcrEF.
- Develop protocols which are suitable for measuring induction of H-NS-silenced system AcrEF.
- Test for induction of AcrAB and AcrEF using known substrates of AcrAB-TolC.
- Identify the involvement of RamA, an activator of AcrAB and AcrEF, in the activation of these systems in response to inducers.

6.3.0 Continuous measurement of induction of *acrA*, *acrE* and *ramA* expression

6.3.1 Selection of conditions and methodology

Initially, this work aimed to quantify the induction of *acrA, acrE* and *ramA* expression over 16 hours using a 96-well plate method (Chapter 2, Materials and Methods). The aim of these experiments was to see if either (a) Indole, a known inducer of AcrAB or (b) conditions either believed to relieve H-NS expression or conditions which mimic those experienced by *Salmonella* during the infection cycle could induce *acrAB* or *acrEF* transcription.

Briefly, strains containing the pMW82 reporter plasmid were diluted in minimal media (MOPS) and incubated for 16 hours in a 96-well plate reader (FLUOstar, BMG BIOTECH, Germany). These strains have the pMW82 reporter plasmid, which contains the promoter of the gene of interest (i.e. *acrA* or *acrE*) fused to the *gfp* gene. Therefore, if transcription on the promoter is initiated, then GFP protein is produced and fluorescence increases. The amount of fluorescence is therefore representative of the

activity on the promoter of interest- herein referred to as the ‘induction’. To vary constituents, minimal media was produced using the protocol by Neidhardt *et al.,* (Neidhardt *et al.*, 1974). Minimal media (MOPS) was selected as MOPS is a well-defined media with constituents which could easily be varied. Details of media composition are in Chapter 2.

6.3.2 The induction of *acrA, ramA* and *acrE* transcription in response to indole

Indole is a known inducer of *acrAB* expression in both *E. coli* and *Salmonella* and this induction is mediated by RamA (Nikaido *et al.*, 2012). Here, the addition of indole to the MOPS media increased the activity of the *acrA* and *ramA* promoters in a concentration-dependent manner (Figure 6.1 - 6.2).

However. there was no induction of *acrE* in response to the addition of indole and the overall levels of *acrE* were much lower than that for *acrA* (Figure 6.3). The Fl/OD of the autofluorescence of the WT (no plasmid) strain were similar to that of the WT + pMW82 *acrE* strain, so that there was no *gfp* fluorescence to indicate *acrE* gene promoter activity. This could mean that *acrE* expression is not induced by either direct (i.e., indole interactions with EnvR) or indirect (i.e., indole interacting with RamA, leading to *acrE* expression) regulation after indole treatment. Alternatively, it could be that H-NS silencing cannot be sequestered due to the addition of indole alone.

Figure 6.1: Peak *acrA* and *ramA* induction in response to indole.

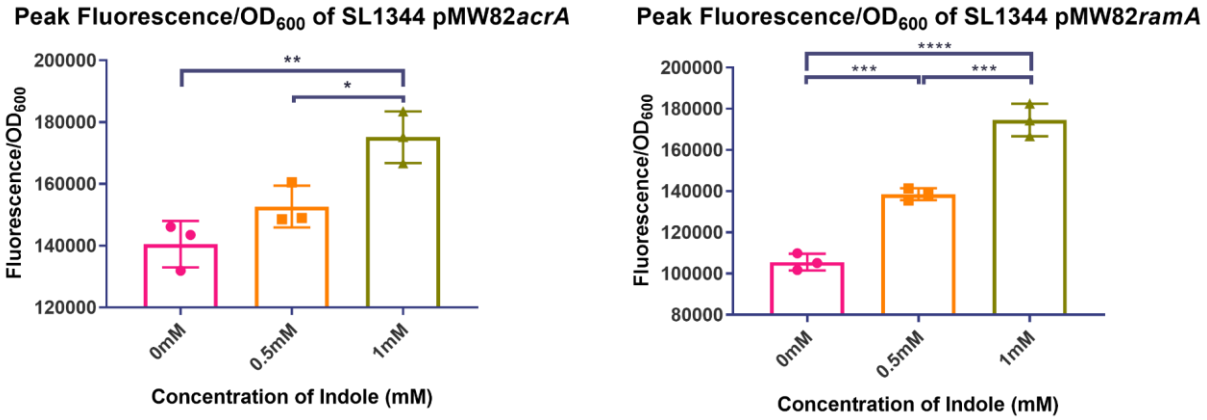


Figure 6.1: The effect of indole (0, 0.5 or 1 mM) on the maximum peak fluorescence/OD_{600} of the WT + pMW82 *acrA* and WT + pMW82 *ramA* strains was measured in triplicate and the mean of n = 3 biological replicates was calculated. Fluorescence/optical density at 600 nm (Fl/OD) was calculated as the mean endpoint fluorescence in minimal MOPS media (excitation 485 nm, emission 520 nm) divided by the OD_{600}. Both the Fl and OD_{600} were blank corrected for no-bacteria and no-plasmid controls. Error bars are shown (± standard deviation of the mean). A one-way ANOVA was used to determine whether there were any statistically significant differences between the treatment concentration groups, with ****= *P* <0.0001, ***= *P* <0.001, ** = *P* <0.005, * = *P* <0.05. Figure initially presented in the MSci thesis of HD (Doherty, 2019).

Figure 6.2: Induction of *acrA* and *ramA* in the presence of indole over time

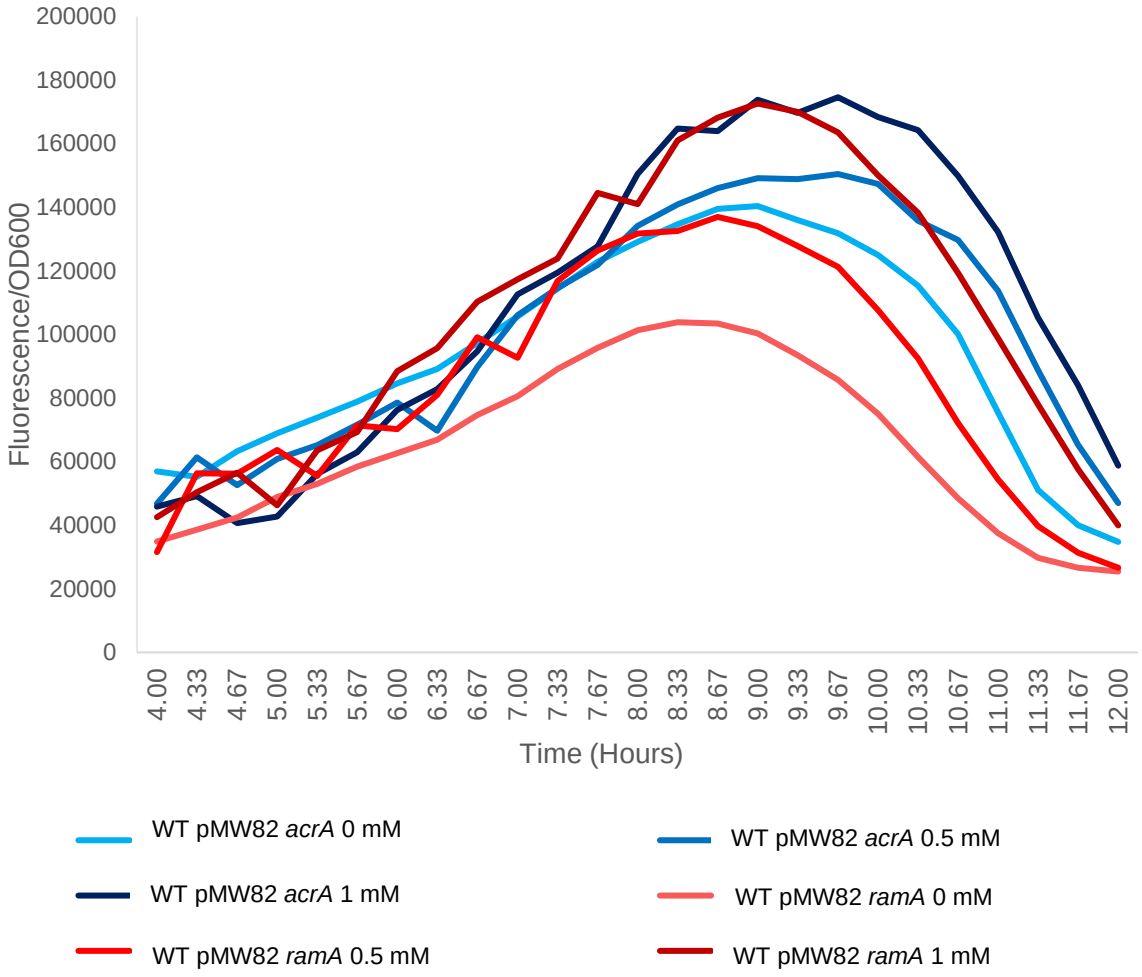


Figure 6.2: The fluorescence/OD_{600} of WT + pMW82 *acrA* and WT + pMW82 *ramA* was measured over time (4 – 12 hours after inoculation of 92 well plate), in minimal MOPS media supplemented with either 0, 0.5 or 1 mM indole. Fluorescence/optical density at 600 nm (Fl/OD) was calculated as the mean endpoint fluorescence in minimal MOPS media (excitation 485 nm, emission 520 nm) divided by the OD_{600}. An average Fl/OD for each strain was calculated from n = 3 technical replicates. The experiment was repeated on three separate occasions to give n = 3 biological replicates. Both the Fl and OD_{600} were blank corrected to account for the autofluorescence of *Salmonella* and any fluorescence/absorbance of the minimal MOPS containing either 0, 0.5 or 1 mM indole. Figure initially presented in the MSci thesis of HD (Doherty, 2019).

Figure 6.3: Induction of *acrE* in the presence of indole over time

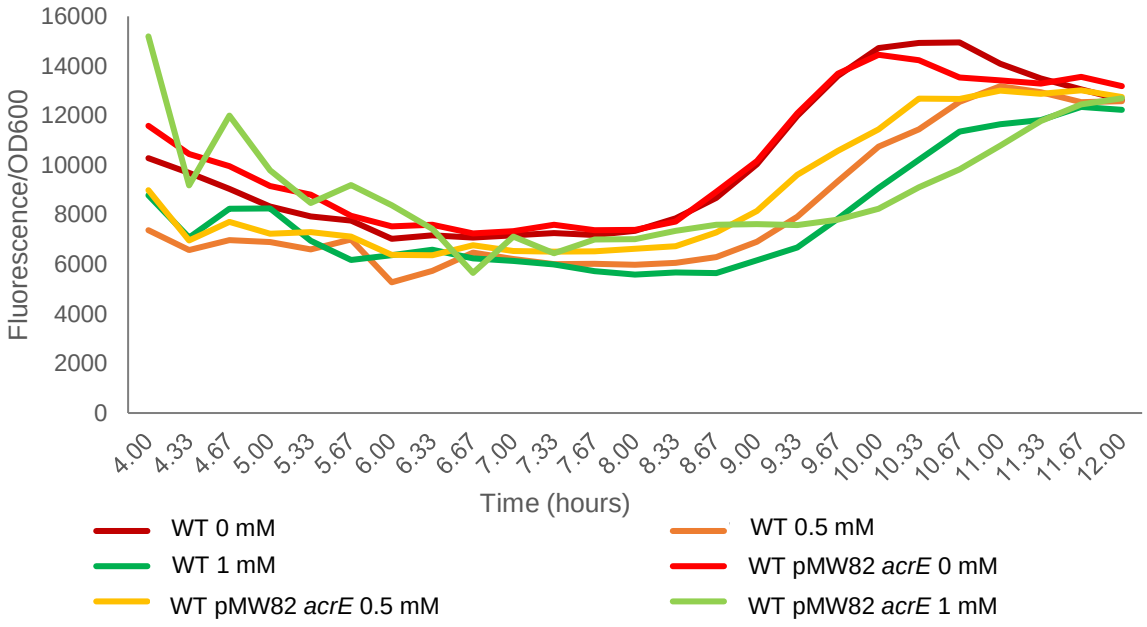


Figure 6.3: The fluorescence/OD_{600} of WT (no plasmid) and WT + pMW82 *acrE* measured over time (4 – 12 hours after inoculation of 92 well plate), in minimal MOPS media supplemented with either 0, 0.5 or 1 mM indole. Fluorescence/optical density at 600 nm (Fl/OD) was calculated as the mean endpoint fluorescence in minimal MOPS media (excitation 485 nm, emission 520 nm) divided by the OD_{600}. An average Fl/OD for each strain was calculated from n = 3 technical replicates. The experiment was repeated on three separate occasions to give n = 3 biological replicates. Both the Fl and OD_{600} were blank corrected to account for any fluorescence/absorbance of the minimal MOPS containing either 0, 0.5 or 1 mM indole. Figure initially present in the MSci thesis of HD (Doherty, 2019).

6.3.3 The induction of *acrA* and *acrE* in various concentrations of glucose

Salmonella experience nutrient limitation once inside the *Salmonella*-containing vacuole (Dandekar, Astrid, Jasmin *et al.*, 2012). The expression of *acrA* and *acrE* was measured in response to varying the concentration of glucose in the minimal MOPS media (0.025, 0.05, 0.075, 0.1 or 0.2%). The standard concentration of glucose in minimal MOPS media is 0.2% glucose. At the lowest concentration of glucose (0.025%), the peak of *acrA* induction was lower and occurred earlier (Figure 6.4). In intermediate glucose concentrations (0.05 – 1%), the *acrA* induction did not reduce as is seen in the 0.025 and 0.2% glucose treatments (Figure 6.4). The peak in *acrA* induction was significantly lower in 0.025% glucose compared to all other conditions (Figure 6.5).

The expression of *acrE* did not change and was not induced in response to varying the concentration of the glucose in the media (Figure 6.6). Figure 6.6 shows that the autofluorescence of *Salmonella* (bacteria only, no reporter plasmid) was similar to the fluorescence of the WT + pMW82 *acrE* strain in all concentrations of glucose, indicating that there was no *gfp* fluorescence to signal transcription at the *acrE* promoter.

Figure 6.4: Induction of *acrA* in varying glucose concentrations over time

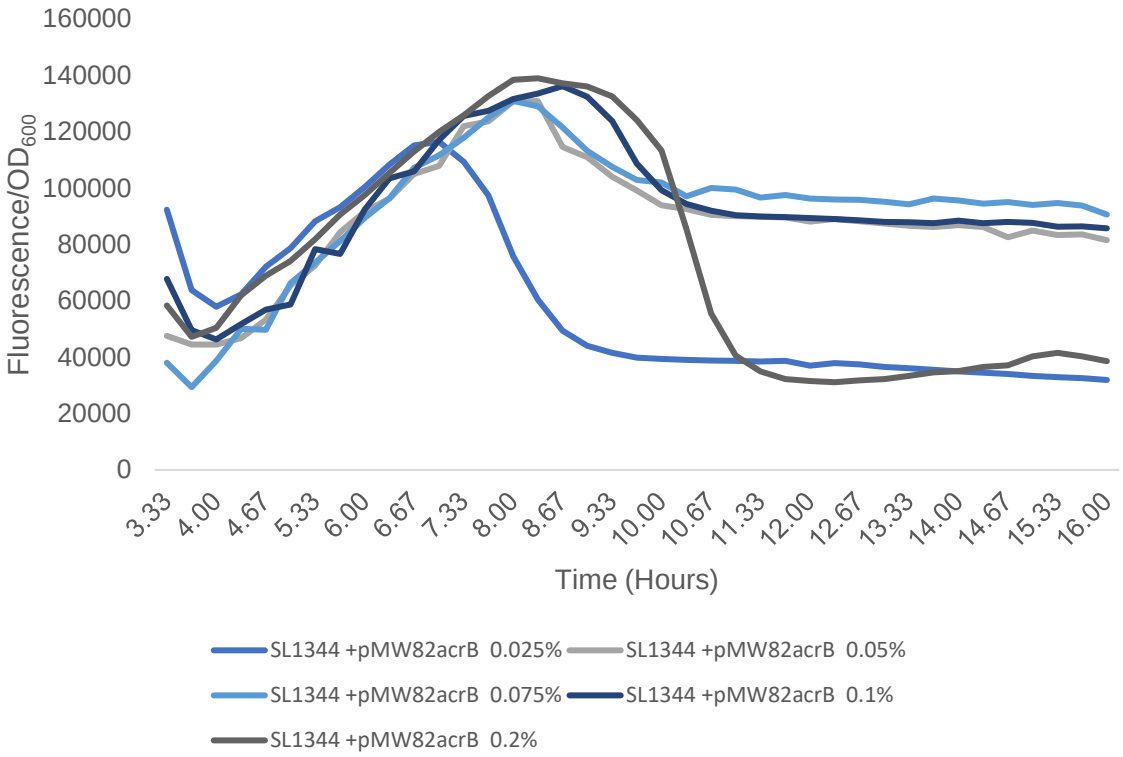


Figure 6.4: The fluorescence/OD_{600} of WT + pMW82 *acrA* measured over time (4 – 12 hours after inoculation of 92 well plate), in minimal MOPS media supplemented with either 0.025, 0.05, 0.075, 0.1 or 0.2% glucose (w/v). Fluorescence/optical density at 600 nm (Fl/OD) was calculated as the mean endpoint fluorescence in minimal MOPS media (excitation 485 nm, emission 520 nm) divided by the OD_{600}. An average Fl/OD for each strain was calculated from n = 3 technical replicates. The experiment was repeated on three separate occasions to give n = 3 biological replicates. Both the Fl and OD_{600} were blank corrected to account for the autofluorescence of *Salmonella* and any fluorescence/absorbance of the minimal MOPS containing different concentrations of glucose. Figure initially presented in the MSci thesis of HD (Doherty, 2019).

Figure 6.5: Peak *acrA* induction in varying concentrations of glucose

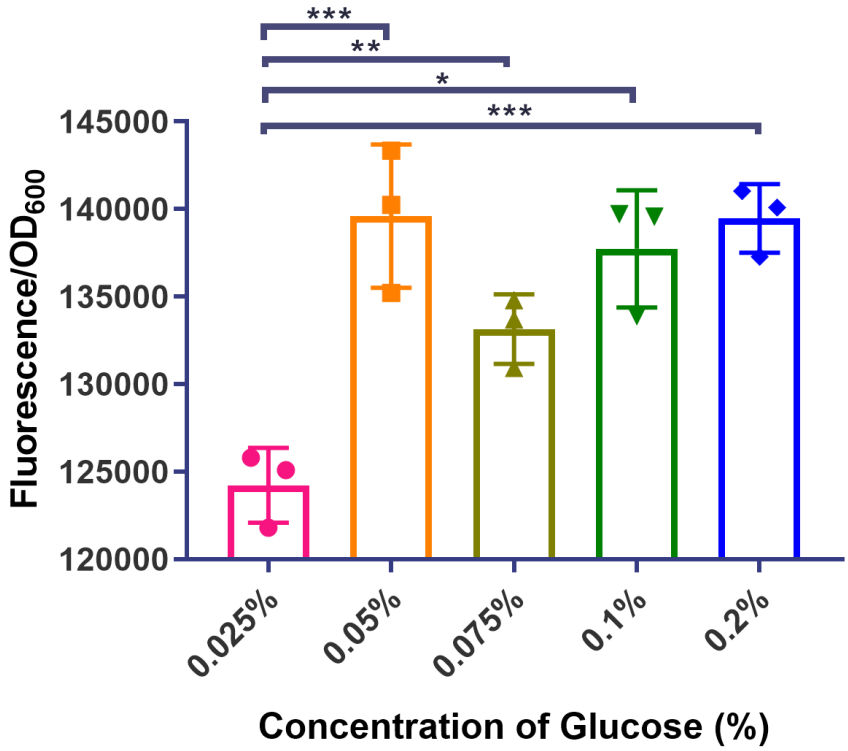


Figure 6.5: The effect of varying the concentration of glucose (0.025, 0.05, 0.075, 0.1 and 0.2% w/v) in the minimal MOPS media on the maximum peak fluorescence/OD_{600} of the WT + pMW82 *acrA* was measured in triplicate and the mean of n = 3 biological replicates was calculated. Fluorescence/optical density at 600 nm (Fl/OD) was calculated as the mean endpoint fluorescence in minimal MOPS media (excitation 485 nm, emission 520 nm) divided by the OD_{600}. Both the Fl and OD_{600} were blank corrected for no-bacteria and no-plasmid controls. Error bars are shown (± standard deviation of the mean). A one-way ANOVA was used to determine whether there were any statistically significant differences between the treatment concentration groups, with ****= p<0.0001, ***= p<0.001, ** = p<0.005, * = p <0.05. Figure initially presented in the MSci thesis of HD (Doherty, 2019).

Figure 6.6: Induction of *acrE* in varying concentrations of glucose over time

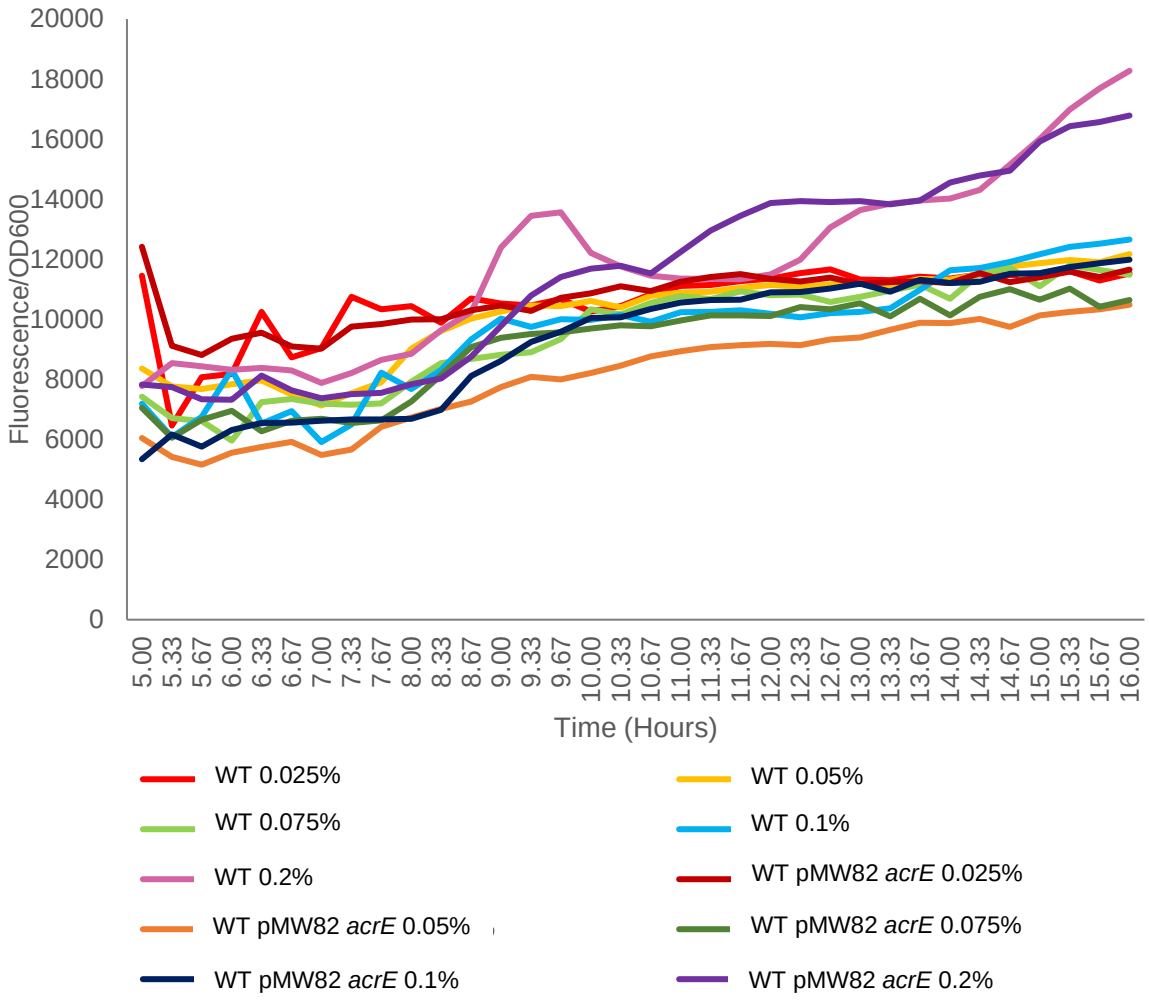


Figure 6.6: The fluorescence/OD_{600} of WT (no plasmid) and WT + pMW82 *acrE* measured over time (4 – 12 hours after inoculation of 92 well plate), in minimal MOPS media supplemented with either 0.025, 0.05, 0.075, 0.1 or 0.2% glucose (w/v). Fluorescence/optical density at 600 nm (Fl/OD) was calculated as the mean endpoint fluorescence in minimal MOPS media (excitation 485 nm, emission 520 nm) divided by the OD_{600}. An average Fl/OD for each strain was calculated from n = 3 technical replicates. The experiment was repeated on three separate occasions to give n = 3 biological replicates. Both the Fl and OD_{600} were blank corrected to account for any fluorescence/absorbance of the minimal MOPS containing either 0.025, 0.05, 0.075, 0.1 or 0.2% glucose. Figure initially presented in the MSci thesis of HD (Doherty, 2019).

6.3.4 The induction of *acrA* and *acrE* in different pH minimal MOPS media

During the infection cycle, *Salmonella* encounters environments which range from pH 4 – 8 (Fallingborg, 1999; Rathman, Sjaastad, & Falkow, 1996; Srikumar, Kroger, Hebrard *et al.*, 2015). The expression of both *acrA* and *acrE* were measured in minimal media adjusted to pH4, pH5, pH6, pH7.3 and pH8. While altering the pH of the media to pH 8 appeared to increase the induction of *acrA* over time (Figure 6.7), this was not statistically significant (Figure 6.8). However, the induction of *acrA* in pH 4, pH 5 and pH 6 was significantly lower than that of pH 7.3 (the standard pH of minimal MOPS), Figure 6.8.

The expression of *acrE* did not change and was not induced in response to varying the pH of the media (Figure 6.9). Figure 6.9 shows that the autofluorescence of *Salmonella* (bacteria only, no reporter plasmid) was similar to the fluorescence of the WT + pMW82 *acrE* strain in all pH conditions, indicating that there was no *gfp* fluorescence to signal transcription at the *acrE* promoter.

Figure 6.7: Induction of *acrA* in different pH minimal MOPS over time

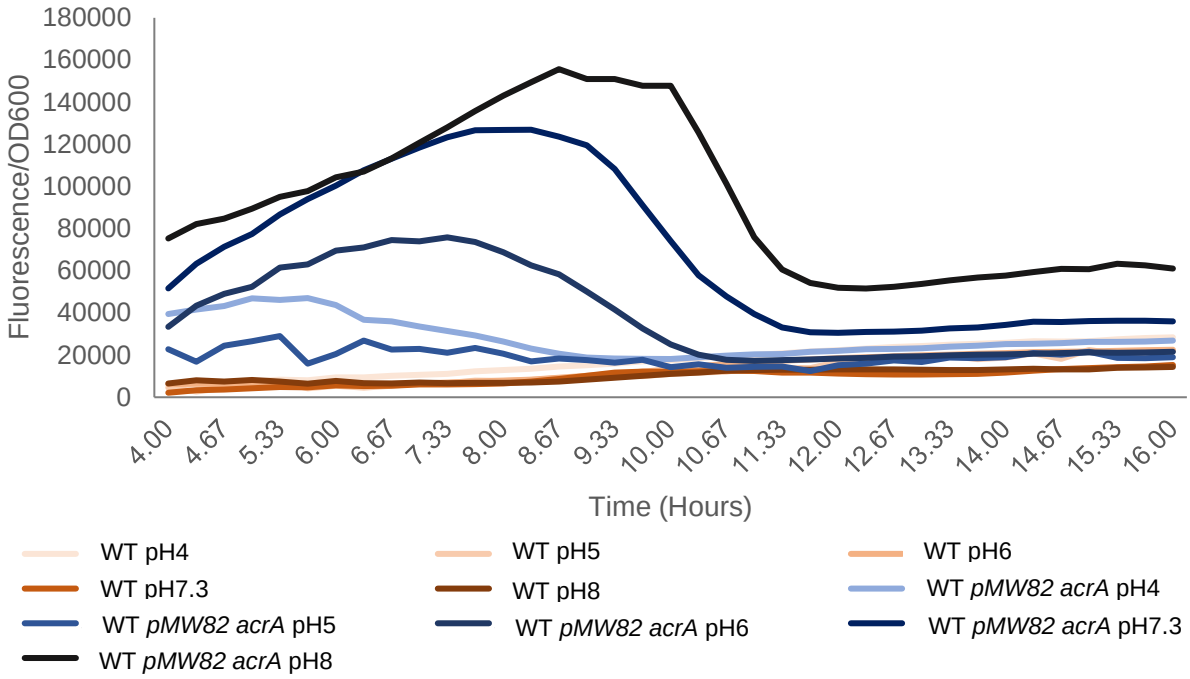


Figure 6.7: The fluorescence/OD_{600} of WT + pMW82 *acrA* measured over time (4 – 12 hours after inoculation of 92 well plate), in minimal MOPS media (pH 4, pH 5, pH 6, pH 7.3 and pH 8). Fluorescence/optical density at 600 nm (Fl/OD) was calculated as the mean endpoint fluorescence in minimal MOPS media (excitation 485 nm, emission 520 nm) divided by the OD_{600}. An average Fl/OD for each strain was calculated from n = 3 technical replicates. The experiment was repeated on three separate occasions to give n = 3 biological replicates. Both the Fl and OD_{600} were blank corrected to account for the autofluorescence of *Salmonella* and any fluorescence/absorbance of the minimal MOPS of each pH. Figure initially presented in the MSci thesis of HD (Doherty, 2019).

Figure 6.8: Peak *acrA* induction in varying pH minimal MOPS

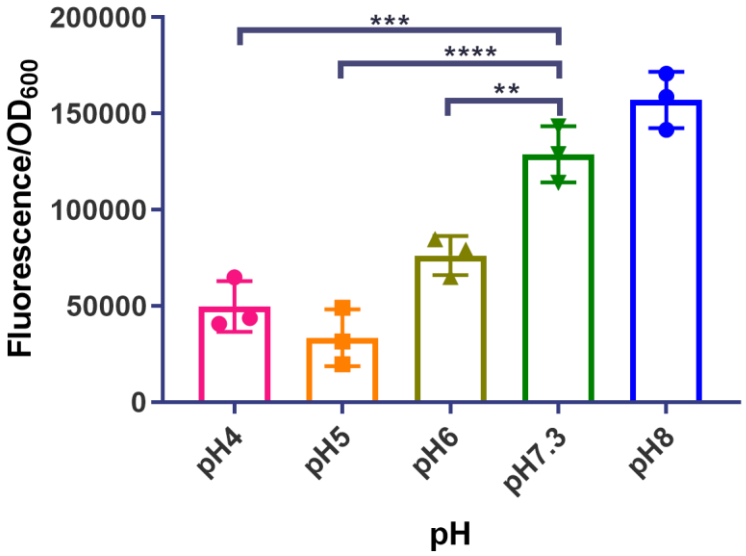


Figure 6.8: The effect of varying the pH (4, 5, 6, 7.3 and 8) of the minimal MOPS media on the maximum peak fluorescence/OD_{600} of the WT + pMW82 *acrA* was measured in triplicate and the mean of n = 3 biological replicates was calculated. Fluorescence/optical density at 600 nm (Fl/OD) was calculated as the mean endpoint fluorescence in minimal MOPS media (excitation 485 nm, emission 520 nm) divided by the OD_{600}. Both the Fl and OD_{600} were blank corrected for no-bacteria and no-plasmid controls. Error bars are shown (± standard deviation of the mean). A one-way ANOVA was used to determine whether there were any statistically significant differences between the treatment concentration groups, with ****= P<0.0001, ***= P<0.001, ** = P<0.005, * = P <0.05. Figure initially presented in the MSci thesis of HD (Doherty, 2019).

Figure 6.9: Induction of *acrE* in varying pH minimal MOPS over time

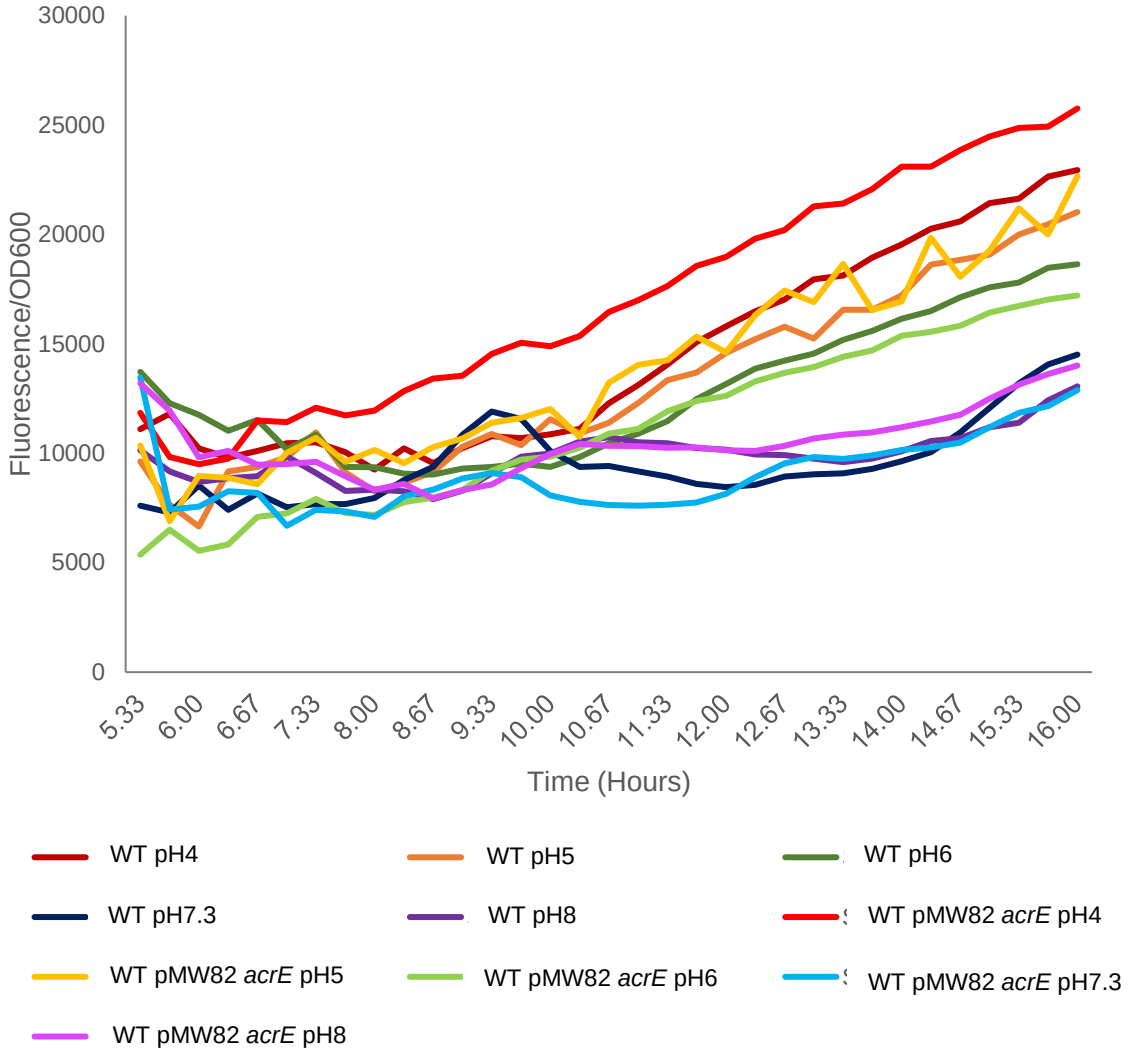


Figure 6.9: The fluorescence/OD_{600} of WT (no plasmid) and WT + pMW82 *acrE* measured over time (4 – 12 hours after inoculation of 92 well plate), in minimal MOPS media (pH 4, pH 5, pH 6, pH 7.3 or pH 8). Fluorescence/optical density at 600 nm (Fl/OD) was calculated as the mean endpoint fluorescence in minimal MOPS media (excitation 485 nm, emission 520 nm) divided by the OD_{600}. An average Fl/OD for each strain was calculated from n = 3 technical replicates. The experiment was repeated on three separate occasions to give n = 3 biological replicates. Both the Fl and OD_{600} were blank corrected to account for any fluorescence/absorbance of the minimal MOPS at each pH. Figure initially presented in the MSci thesis of HD (Doherty, 2019).

6.3.5 The induction of *acrA* and *acrE* in various magnesium chloride concentrations

Magnesium limitation decreased the induction of *acrA* in a concentration-dependent manner (Figure 6.10). Magnesium concentrations of 10 µM and 5 µM had significantly lower peaks of *acrA* induction compared to strains grown in 5.25 mM magnesium chloride (P =0.0015 and P =0.0015, respectively), Figure 6.11.

The expression of *acrE* did not change and was not induced in response to varying the magnesium chloride concentration of the media (Figure 6.12). Figure 6.12 shows that the autofluorescence of *Salmonella* (bacteria only, no reporter plasmid) was similar to the fluorescence of the WT + pMW82 *acrE* strain in all concentrations of magnesium chloride, indicating that there was no *gfp* fluorescence to signal transcription at the *acrE* promoter.

Figure 6.10: Induction of *acrA* in varying $MgCl_2$ concentrations over time

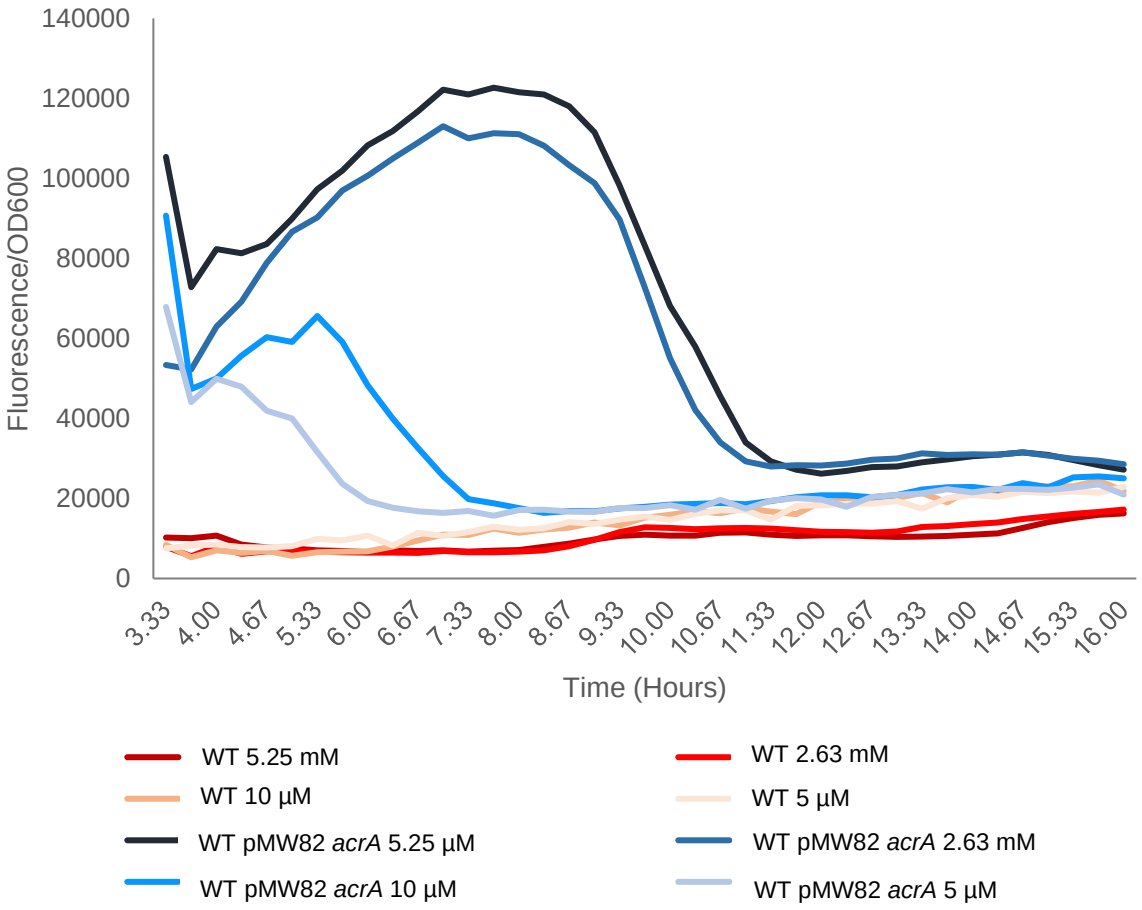


Figure 6.10: The fluorescence/OD_{600} of WT + pMW82 *acrA* measured over time (4 – 12 hours after inoculation of 92 well plate), in minimal MOPS media supplemented with either 5 µM, 10 µM, 2.63 mM or 5.25 mM magnesium chloride. Fluorescence/optical density at 600 nm (Fl/OD) was calculated as the mean endpoint fluorescence in minimal MOPS media (excitation 485 nm, emission 520 nm) divided by the OD_{600}. An average Fl/OD for each strain was calculated from n = 3 technical replicates. The experiment was repeated on three separate occasions to give n = 3 biological replicates. Both the Fl and OD_{600} were blank corrected to account for the autofluorescence of *Salmonella* and any fluorescence/absorbance of the minimal MOPS supplemented with either 5 µM, 10 µM, 2.63 mM or 5.25 mM magnesium chloride. Figure initially presented in the MSci thesis of HD (Doherty, 2019).

Figure 6.11: Peak *acrA* induction in varying concentrations of $MgCl_2$

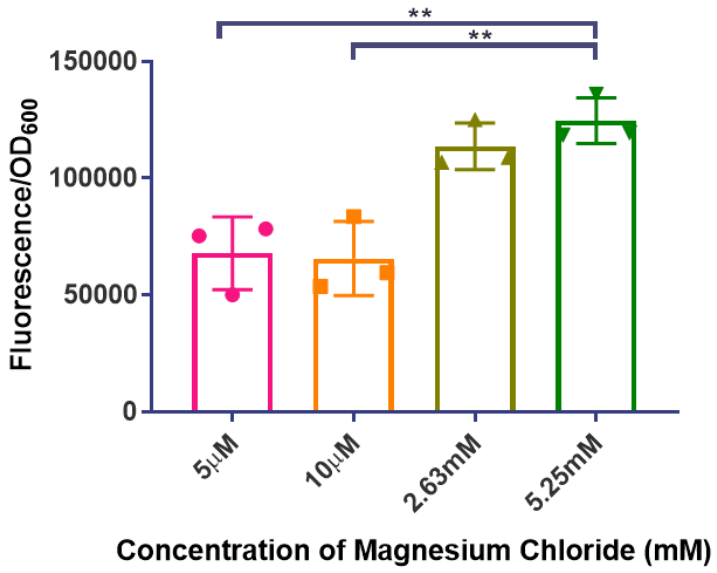


Figure 6.11:: The effect of varying the concentration of magnesium chloride (5 µM, 10 µM, 2.63 mM or 5.25 mM) of the minimal MOPS media on the maximum peak fluorescence/OD_{600} of the WT + pMW82 *acrA* was measured in triplicate and the mean of n = 3 biological replicates was calculated. Fluorescence/optical density at 600 nm (Fl/OD) was calculated as the mean endpoint fluorescence in minimal MOPS media (excitation 485 nm, emission 520 nm) divided by the OD_{600}. Both the Fl and OD_{600} were blank corrected for no-bacteria and no-plasmid controls. Error bars are shown (± standard deviation of the mean). A one-way ANOVA was used to determine whether there were any statistically significant differences between the treatment concentration groups, with ****= *P*<0.0001, ***= *P*<0.001, ** = *P*<0.005, * = *P* <0.05. Figure initially presented in the MSci thesis of HD (Doherty, 2019).

Figure 6.12 Induction of *acrE* in varying concentrations of $MgCl_2$ over time

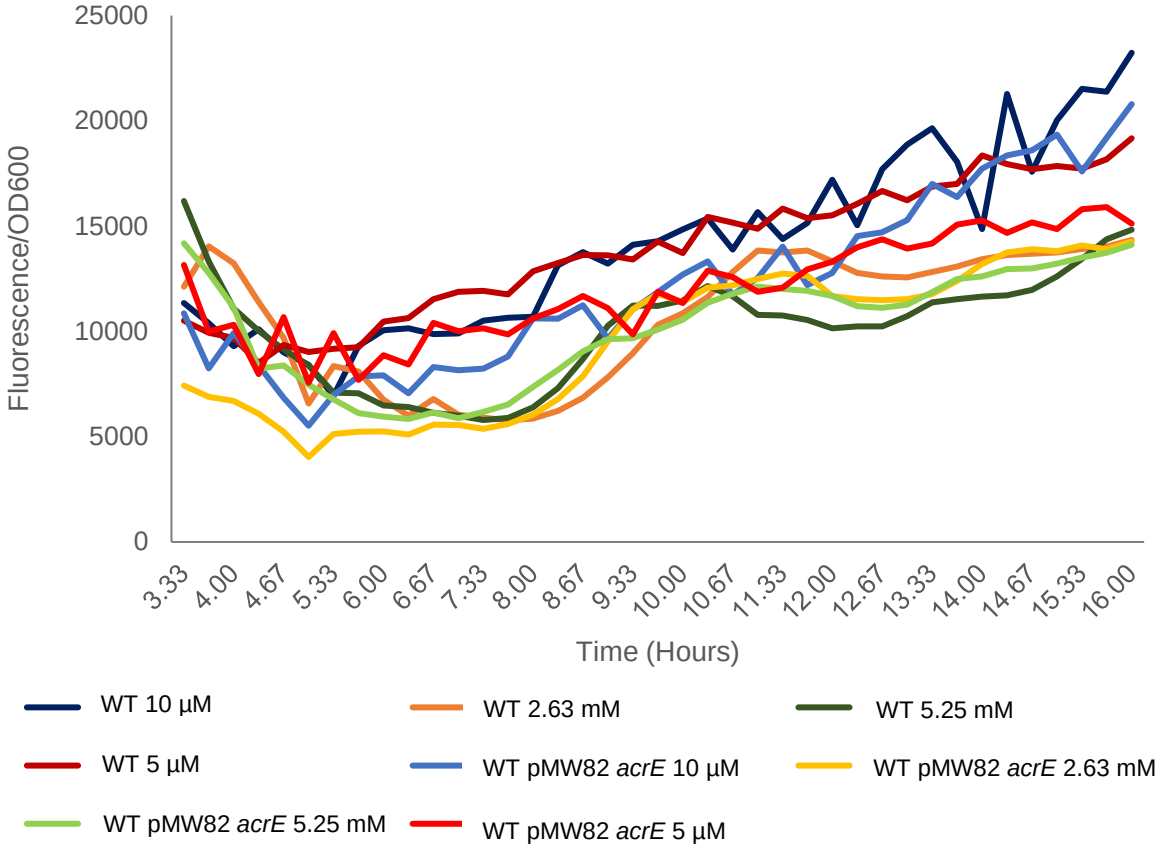


Figure 6.12: The fluorescence/OD_{600} of WT (no plasmid) and WT + pMW82 *acrE* measured over time (4 – 12 hours after inoculation of 92 well plate), in minimal MOPS media supplemented with various concentrations of magnesium chloride (5 µM, 10 µM, 2.63 mM or 5.25 mM). Fluorescence/optical density at 600 nm (Fl/OD) was calculated as the mean endpoint fluorescence in minimal MOPS media (excitation 485 nm, emission 520 nm) divided by the OD_{600}. An average Fl/OD for each strain was calculated from n = 3 technical replicates. The experiment was repeated on three separate occasions to give n = 3 biological replicates. Both the Fl and OD_{600} were blank corrected to account for any fluorescence/absorbance of the minimal MOPS supplemented with 5 µM, 10 µM, 2.63 mM or 5.25 mM of magnesium chloride. Figure initially presented in the MSci thesis of HD (Doherty, 2019).

6.3.6 Effects of phosphate limitation on induction of *acrA* and *acrE* transcription

The *Salmonella*-containing vacuole is a phosphate limited environment so phosphate limitation was studied to determine if this induced the expression of *acrA* or *acrEF* (Dandekar *et al.*, 2012). Varying the phosphate concentration of the minimal MOPS media did not alter the induction of *acrA* (Figure 6.13) and any differences in the means of the peak Fl/OD of the different treatments were not statistically significant (Figure 6.14). Therefore, phosphate limitation did not increase or decrease *acrA* expression in these experiments.

The expression of *acrE* did not change and was not induced in phosphate limited media (Figure 6.15). Figure 6.15 shows that the autofluorescence of *Salmonella* (bacteria only, no reporter plasmid) was similar to the fluorescence of the WT + pMW82 *acrE* strain in all concentrations of phosphate, indicating that there was no *gfp* fluorescence to signal transcription at the *acrE* promoter.

Figure 6.13: Induction of *acrA* in varying phosphate concentrations over time

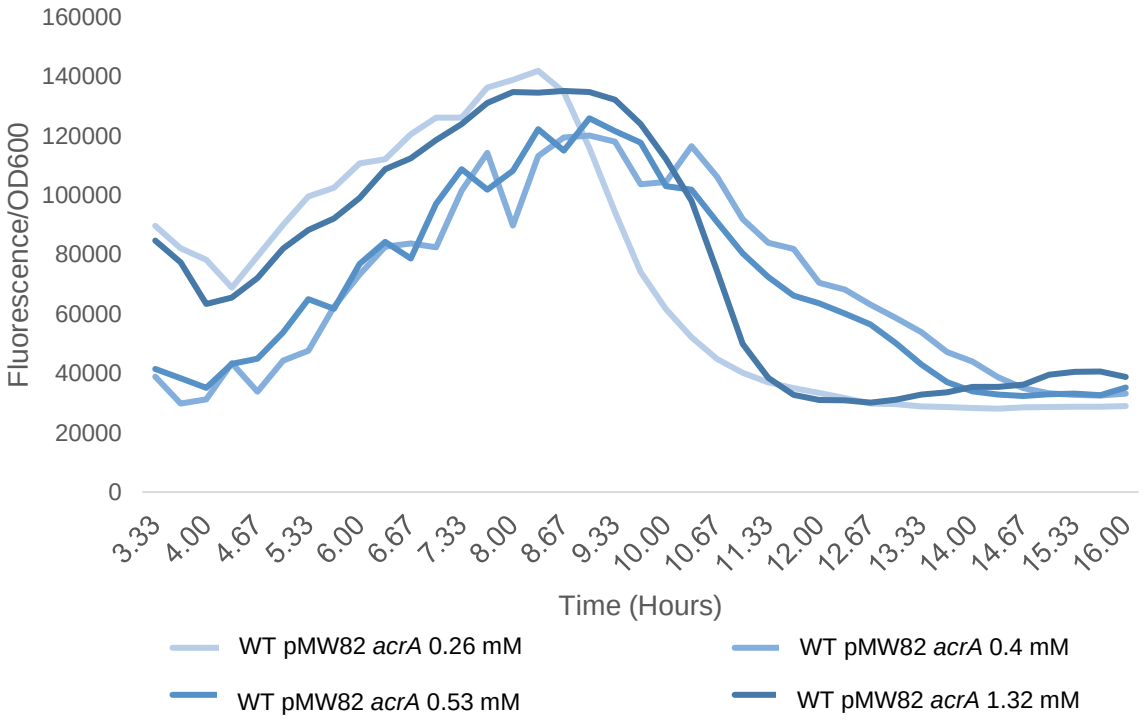


Figure 6.13: The fluorescence/OD_{600} of WT + pMW82 *acrA* measured over time (4 – 12 hours after inoculation of 92 well plate), in minimal MOPS media supplemented with either 0.26, 0.4, 0.53 and 1.32 mM phosphate. Fluorescence/optical density at 600 nm (Fl/OD) was calculated as the mean endpoint fluorescence in minimal MOPS media (excitation 485 nm, emission 520 nm) divided by the OD_{600}. An average Fl/OD for each strain was calculated from n = 3 technical replicates. The experiment was repeated on three separate occasions to give n = 3 biological replicates. Both the Fl and OD_{600} were blank corrected to account for the autofluorescence of *Salmonella* and any fluorescence/absorbance of the minimal MOPS supplemented with either 0.26, 0.4, 0.53 and 1.32 mM phosphate. Figure initially presented in the MSci thesis of HD (Doherty, 2019).

Figure 6.14: Peak *acrA* induction in varying concentrations of phosphate

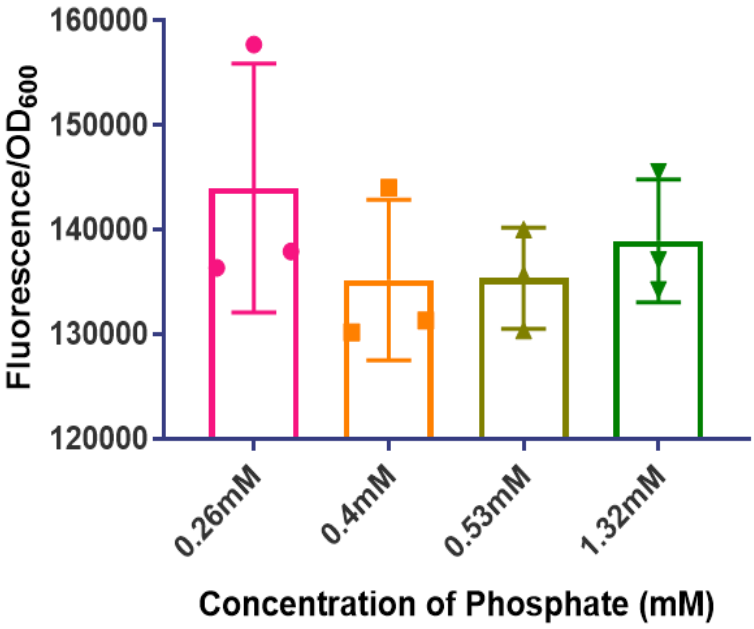


Figure 6.14:: The effect of varying the concentration of phosphate (0.26, 0.4, 0.53 and 1.32 mM) of the minimal MOPS media on the maximum peak fluorescence/OD_{600} of the WT + pMW82 *acrA* was measured in triplicate and the mean of n = 3 biological replicates was calculated. Fluorescence/optical density at 600 nm (Fl/OD) was calculated as the mean endpoint fluorescence in minimal MOPS media (excitation 485 nm, emission 520 nm) divided by the OD_{600}. Both the Fl and OD_{600} were blank corrected for no-bacteria and no-plasmid controls. Error bars are shown (± standard deviation of the mean). A one-way ANOVA was used to determine whether there were any statistically significant differences between the treatment concentration groups, with ****= P<0.0001, ***= P<0.001, ** = P<0.005, * = P <0.05. Figure initially presented in the MSci thesis of HD (Doherty, 2019).

Figure 6.15: Induction of *acrE* in varying concentrations of phosphate over time

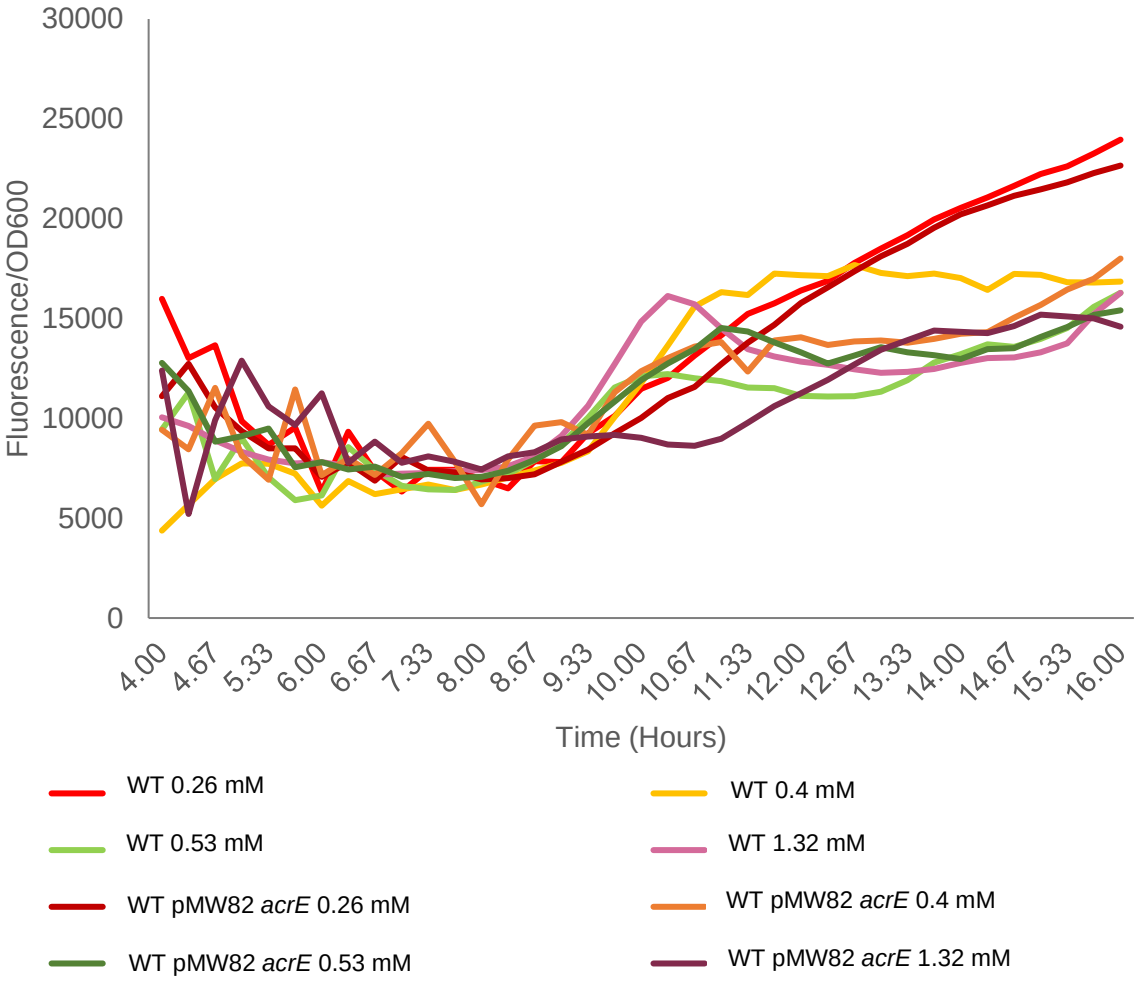


Figure 6.15: The fluorescence/OD_{600} of WT (no plasmid) and WT + pMW82 *acrE* measured over time (4 – 12 hours after inoculation of 92 well plate), in minimal MOPS media supplemented with various concentrations of phosphate (0.26, 0.4, 0.53 and 1.32 mM). Fluorescence/optical density at 600 nm (Fl/OD) was calculated as the mean endpoint fluorescence in minimal MOPS media (excitation 485 nm, emission 520 nm) divided by the OD_{600}. An average Fl/OD for each strain was calculated from n = 3 technical replicates. The experiment was repeated on three separate occasions to give n = 3 biological replicates. Both the Fl and OD_{600} were blank corrected to account for any fluorescence/absorbance of the minimal MOPS supplemented with 0.26, 0.4, 0.53 and 1.32 mM of phosphate. Figure initially presented in the MSci thesis of HD (Doherty, 2019).

6.4 Attempting to induce *acrEF* in *hns* and *acrB*-interrupted backgrounds

The *acrA* gene was differentially induced in response to changes in indole (Figure 6.1), glucose % (Figure 6.5), pH (Figure 6.8) and $MgCl_2$ (Figure 6.11). However, induction of *acrE* was not seen in any of the infection relevant conditions tested (Figures 6.3, 6.6, 6.9, 6.12, 6.15). Additionally, no induction of *acrE* was observed in response to indole, despite indole being a suspected substrate of AcrEF (Kawamura-Sato *et al.*, 1999).

Under laboratory conditions the *acrEF* genes in *Salmonella* are known to be silenced by the DNA binding protein H-NS (Nishino *et al.*, 2009) which could explain why no induction was seen in these conditions. Therefore, it was hypothesised that *acrEF* may be expressed or become inducible is the absence of functional H-NS. A strain which produced non-functional H-NS was kindly donated by Prof Jay Hinton (Falconi, McGovern, Gualerzi *et al.*, 1991; Hinton *et al.*, 1992). The *hns* gene interruption was transduced into SL1344 wild-type Salmonella using P22 phage transduction and the interruption of the *hns* gene was verified by PCR. This strain produces a truncated, non-functional H-NS protein. However, the *hns*::*aph* strain had a significant growth defect when compared to wild-type SL1344 (Figure 6.16). Due to this, it was not possible to determine *acrE* induction using the method used thus far. The growth conditions of this experiment (96-well plate, minimal media, growth in a plate reader) likely restricted the growth of this strain further.

Figure 6.16: Growth of *hns*-interrupted strain in standard minimal media

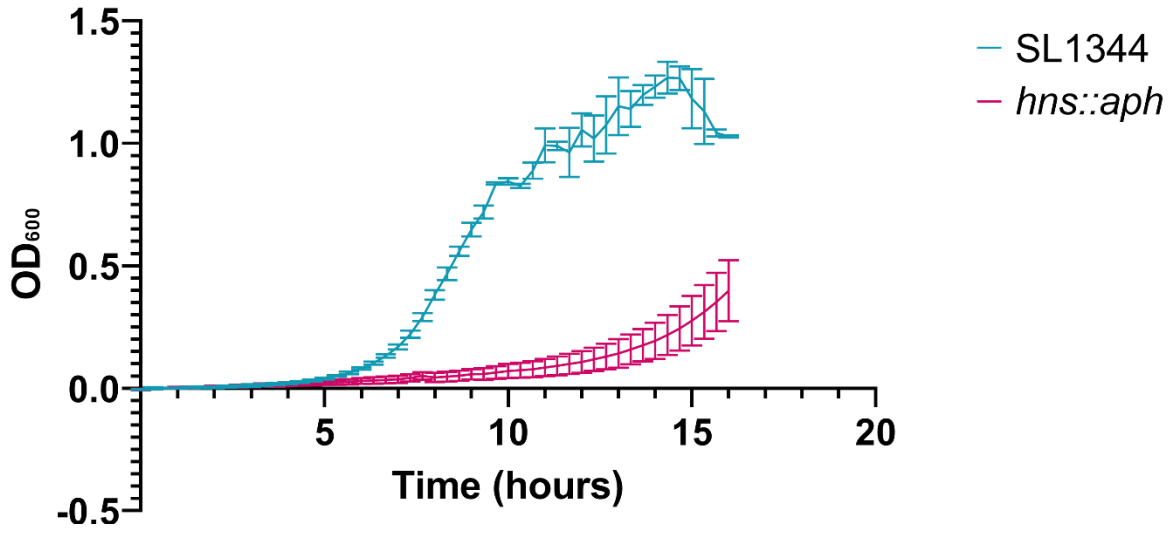


Figure 6.16: The growth of SL1344 (blue) compared to the *hns*-interrupted strain (pink) in MOPS minimal media (no inducer). Optical density (OD) at 600 nm was recorded over time to measure bacterial growth. The OD_{600} values for each time point were blank corrected using wells containing only minimal media MOPS (no inducer). Data shown is the mean of three biological replicates ± standard deviation of the mean.

6.5 Development of a single timepoint induction experiment

Due to the limited growth of the *hns*::*aph* strain in the minimal media MOPS 96-well plate assay, a new method was developed based on a previously designed method (Lawler *et al.*, 2013). Strains were grown in LB media in flasks at 37°C with aeration until an OD_{600} of 0.6 was reached. They were then incubated with an inducer compound for an hour before being resuspended in PBS for fluorescence and OD_{600} measurements in a 96-well plate. Instead of attempting to mimic infection environments, induction was attempted using concentrations of suspected inducers of AcrAB. A negative control (kanamycin) not believed to induce or be a substrate of AcrAB or AcrEF was also included.

6.6 Non-induced levels of *acrA* and *acrE*

The average *acrA* and *acrE* expression in all experiments was collated to study the consistency of *acrA* and *acrE* expression in the absence of inducer. Expression of *acrA* and *acrE* was consistent across all experiments (Figure 6.17). The expression of *acrA* was lower in the *hns*-interrupted strain and *acrE* expression was higher in both the *hns*-interrupted and hns-*ΔacrB* backgrounds. The expression of *acrA* and *acrE* were not statistically significant when comparing WT to Δ*acrR,* Δ*acrR or* Δ*acrR* Δ*envR* backgrounds (Figures 6.18 – 6.19).

Figure 6.17: Average *acrA* and *acrE* expression across all experiments

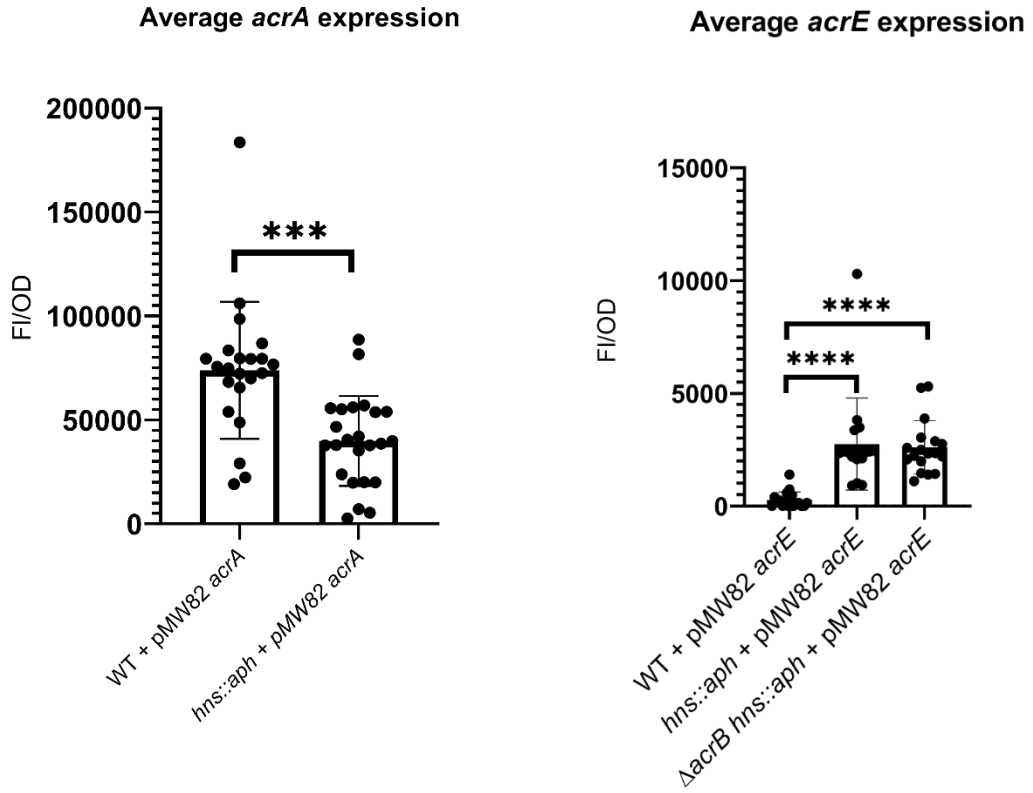


Figure 6.17: The average *acrA* and *acrE* expression levels of WT + pMW82 *acrA/acrE, hns::aph* + pMW82 *acrA/acrE* and Δ*acrB hns::aph* + pMW82 *acrE* measured across all experiments. This graph was produced by combining the data of all controls (Minimal media MOPS, no inducer) of figures 6.20 - 6.27 and therefore represents the non-induced expression levels of *acrE* and *acrA* in these genotypes. Induction (Fl/OD) was calculated as the mean endpoint fluorescence in PBS (excitation 485 nm, emission 520 nm) divided by the OD_{600}. Both the Fl and OD_{600} were blank corrected for no-bacteria and no-plasmid controls. Error bars represent the standard deviation of the mean. Note that the scales on these graphs are different due to the large difference between *acrA* and *acrE* induction levels.

Figure 6.18: Expression of *acrA* in Δ*acrR*, Δ*envR* and Δ*acrR* Δ*envR* backgrounds

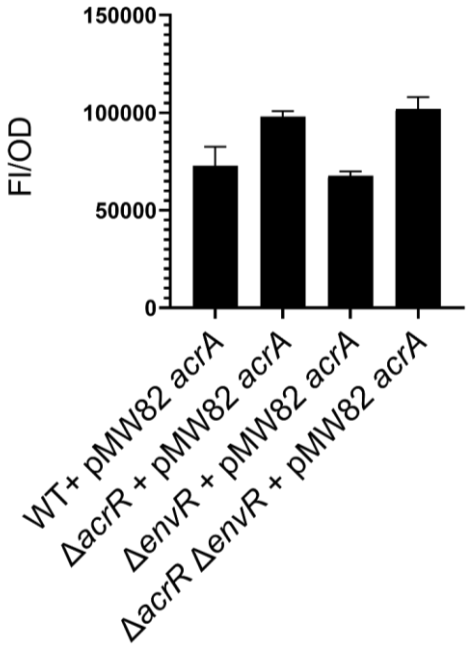


Figure 6.18: The expression of *acrA* was measured in WT (SL1344), Δ*acrR*, Δ*envR* and Δ*acrR* Δ*envR* backgrounds (minimal media MOPS, no inducer). The average endpoint fluorescence (excitation 485 nm, emission 520 nm) and OD_{600} in PBS was measured for three technical replicates and was blank corrected using no-bacteria and no-plasmid controls. This was then repeated 3 times to give n = 3 biological replicates. Shown is the mean of these biological replicates, including error bars (± standard deviation of the mean).

Figure 6.19: Expression of *acrE* in Δ*acrR*, Δ*envR* and Δ*acrR* Δ*envR* backgrounds

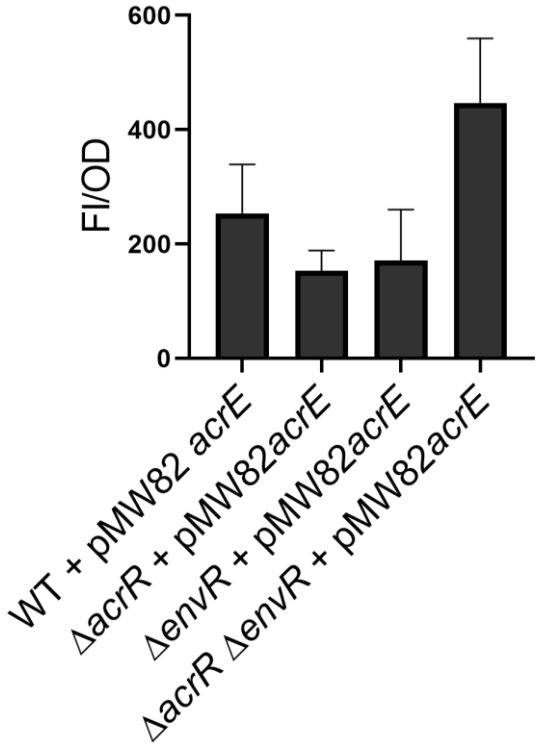


Figure 6.19: The expression of *acrE* was measured in WT (SL1344), Δ*acrR*, Δ*envR* and Δ*acrR* Δ*envR* backgrounds (minimal media MOPS, no inducer). The average endpoint fluorescence (excitation 485 nm, emission 520 nm) and OD_{600} in PBS was measured for three technical replicates and was blank corrected using no-bacteria and no-plasmid controls. This was then repeated 3 times to give n = 3 biological replicates. Shown is the mean of these biological replicates, including error bars (± standard deviation of the mean).

6.7 Varying effects of inducer challenge on *acrA* and *acrE* induction

Seven potential inducers were selected which are either (a) known inducers of *acrAB* expression and known substrates of AcrAB-TolC (Indole (Hirakawa, Inazumi, Masaki *et al.*, 2005; Kawamura-Sato *et al.*, 1999; Nikaido *et al.*, 2008)), (b) known substrates of AcrAB-TolC (rhodamine 6g, proflavine, tetracycline, ciprofloxacin and novobiocin (Eicher *et al.*, 2012; Yu, Aires, & Nikaido, 2003; Zwama *et al.*, 2018)) or (c) Compounds *not* thought to be substrates of AcrAB (Kanamycin (Eicher *et al.*, 2012)).

Of the 7 potential inducers tested, only rhodamine 6G was able to induce both *acrA* and *acrE* expression (Figures 6.20 – 6.21). Proflavine appeared to increase *acrE* expression in all backgrounds, but this was not statistically significant (Figure 6.27). Conversely, tetracycline was able to reduce the expression of *acrA* and *acrE* in a concentration-dependant manner (Figures 6.22 – 6.23). Novobiocin did not alter the expression of *acrA* (Figure 6.24) but decreased the expression of *acrE* (Figure 6.25). Indole, proflavine, ciprofloxacin and kanamycin and had no significant effect on *acrA* (Figure 6.26) or *acrE* (Figure 6.27) expression.

Figure 6.20: Induction of *acrA* expression after addition of rhodamine 6g in WT and *hns*::*aph* backgrounds

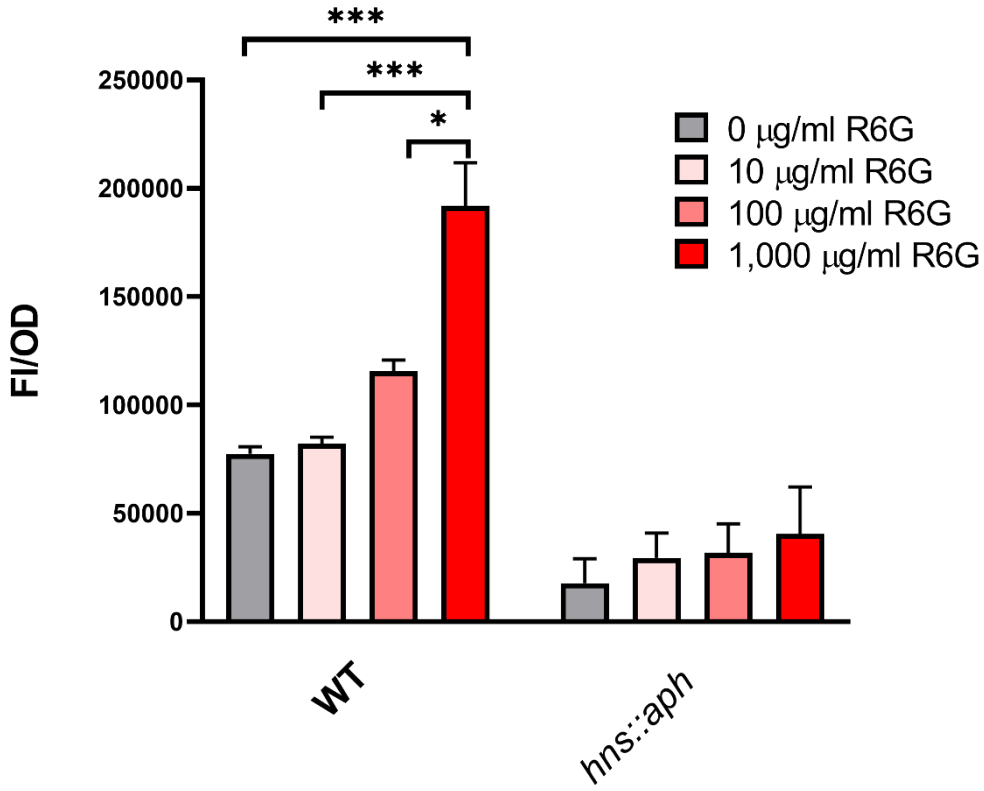


Figure 6.20: The induction of *acrA* was measured in WT (SL1344) and *hns*::*aph* backgrounds. The average endpoint fluorescence (excitation 485 nm, emission 520 nm) and OD_{600} in PBS was measured after addition of rhodamine 6G (final concentrations 0, 10, 100 or 1,000 µg/ml) for three technical replicates. The averages of these replicates were blank corrected using no-bacteria and no-plasmid controls. This was repeated for n = 3 biological replicates. Shown is the mean of these biological replicates, including error bars (standard deviation of the mean). An ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare the means of all genotypes and inducer concentrations. Statistical significance is represented by: * = $P < 0.05$, ** = $P< 0.001$, *** = $P < 0.0005$ and **** = $P <0.0001$.

Figure 6.21: Induction of *acrE* expression in response to the addition of rhodamine 6g in WT, *hns*::*aph* and Δ*acrB hns*::*aph* backgrounds

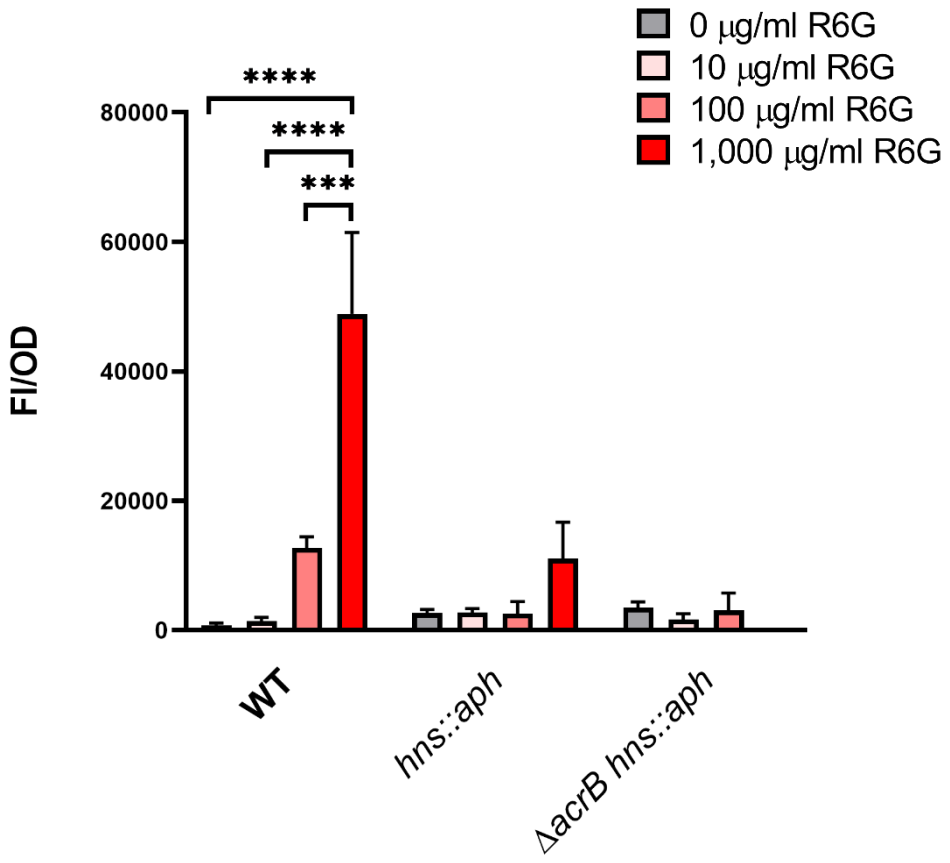


Figure 6.21: The induction of *acrE* was measured in WT (SL1344), *hns*::*aph* and *ΔacrB hns::aph* backgrounds. The average endpoint fluorescence (excitation 485 nm, emission 520 nm) and OD_{600} in PBS was measured after addition of rhodamine 6G (final concentrations 0, 10, 100 or 1,000 μg/ml) for three technical replicates. The averages of these replicates were blank corrected using no-bacteria and no-plasmid controls. This was repeated for n = 3 biological replicates. Shown is the mean of these biological replicates, including error bars (standard deviation of the mean). An ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare the means of all genotypes and inducer concentrations. Statistical significance is represented by: * = $P < 0.05$, ** = $P < 0.001$, *** = $P < 0.0005$ and **** = $P < 0.0001$.

Figure 6.22: Induction of *acrA* expression in response to the addition of tetracycline in WT, *hns*:: *aph* backgrounds

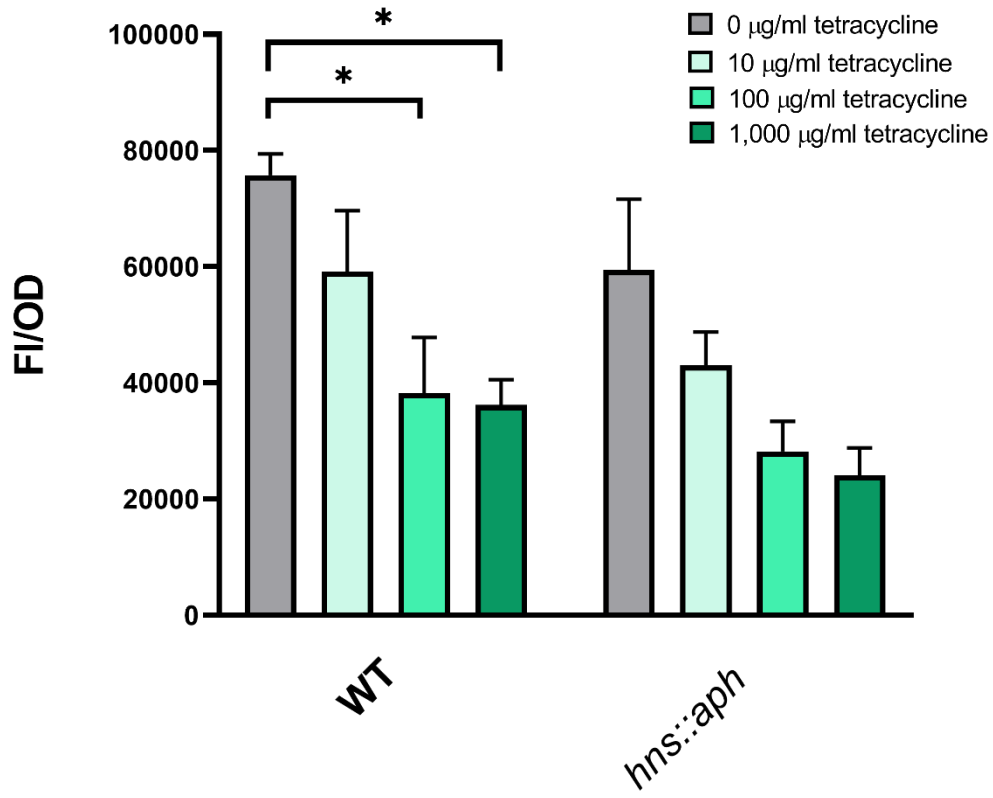


Figure 6.22: The induction of *acrA* was measured in WT (SL1344) and *hns*::*aph* backgrounds. The average endpoint fluorescence (excitation 485 nm, emission 520 nm) and OD_{600} in PBS was measured after addition of tetracycline (final concentrations 0, 10, 100 or 1,000 µg/ml) for three technical replicates. The averages of these replicates were blank corrected using no-bacteria and no-plasmid controls. This was repeated for n = 3 biological replicates. Shown is the mean of these biological replicates, including error bars (standard deviation of the mean). An ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare the means of all genotypes and inducer concentrations. Statistical significance is represented by: * = $P < 0.05$, ** = $P < 0.001$, *** = $P < 0.0005$ and **** = $P < 0.0001$.

Figure 6.23: Induction of *acrE* expression in response to the addition of tetracycline in WT, *hns*:: *aph* and Δ*acrB hns*::kn backgrounds

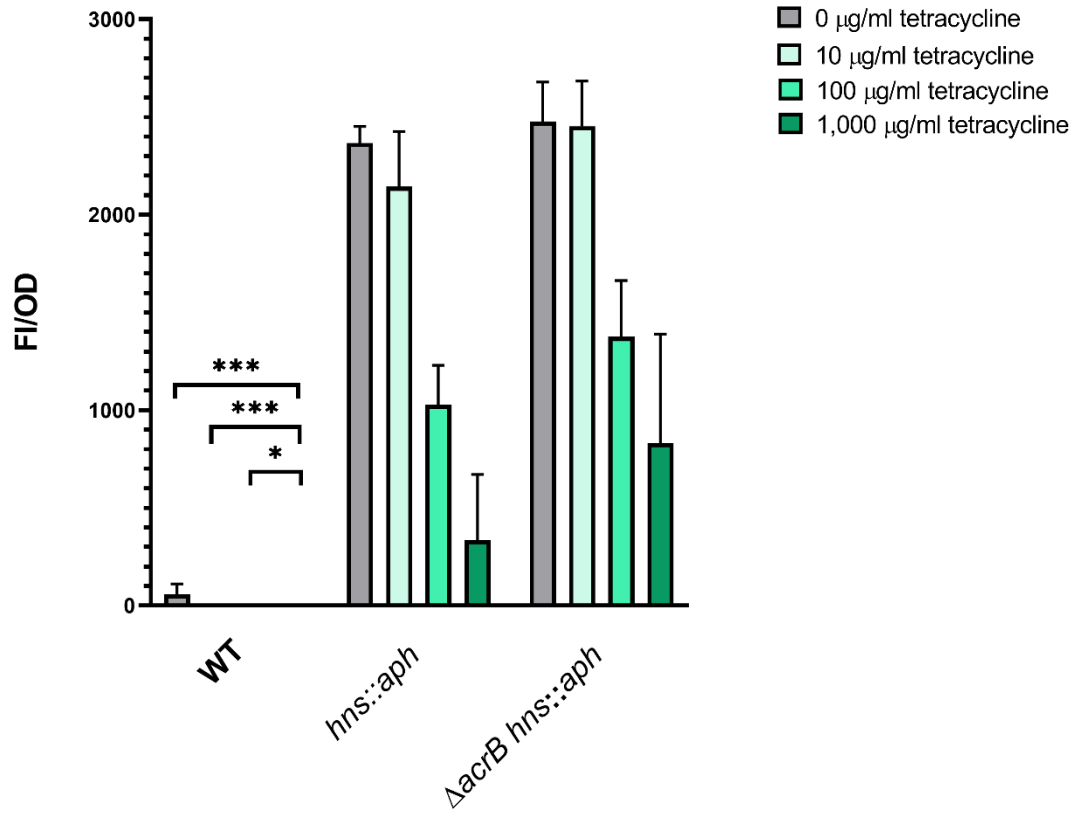


Figure 6.23: The induction of *acrE* was measured in WT (SL1344), *hns*::*aph* and *ΔacrB hns::aph* backgrounds. The average endpoint fluorescence (excitation 485 nm, emission 520 nm) and OD_{600} in PBS was measured after addition of tetracycline (final concentrations 0, 10, 100 or 1,000 µg/ml) for three technical replicates. The averages of these replicates were blank corrected using no-bacteria and no-plasmid controls. This was repeated for n = 3 biological replicates. Shown is the mean of these biological replicates, including error bars (standard deviation of the mean). An ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare the means of all genotypes and inducer concentrations. Statistical significance is represented by: * = $P < 0.05$, ** = $P < 0.001$, *** = $P < 0.0005$ and **** = $P < 0.0001$.

Figure 6.24: Induction of *acrA* expression in response to the addition of novobiocin in WT and *hns*:: *aph* backgrounds

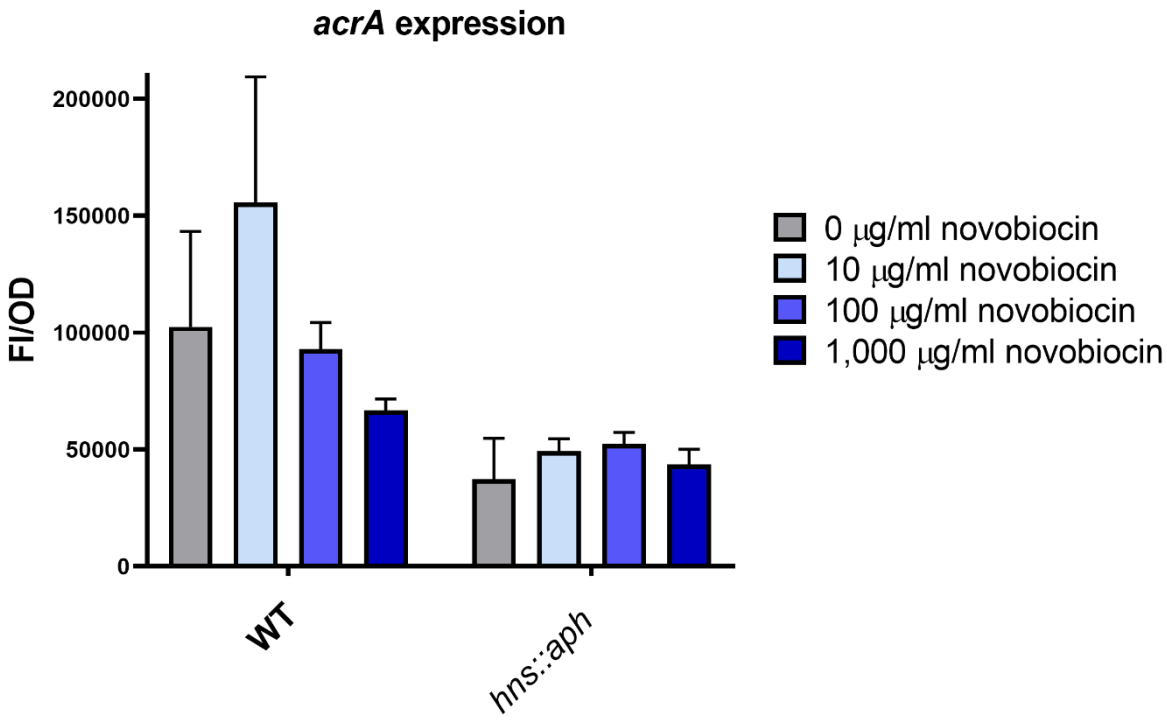


Figure 6.24: The induction of *acrA* was measured in WT (SL1344) and *hns*::*aph* backgrounds. The average endpoint fluorescence (excitation 485 nm, emission 520 nm) and OD_{600} in PBS was measured after addition of novobiocin (final concentrations 0, 10, 100 or 1,000 μg/ml) for three technical replicates. The averages of these replicates were blank corrected using no-bacteria and no-plasmid controls. This was repeated for n = 3 biological replicates. Shown is the mean of these biological replicates, including error bars (standard deviation of the mean). An ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare the means of all genotypes and inducer concentrations. Statistical significance is represented by: * = $P < 0.05$, ** = $P < 0.001$, *** = $P < 0.0005$ and **** = $P < 0.0001$.

Figure 6.25: Induction of *acrE* expression in response to the addition of novobiocin in WT, *hns*:: *aph* and ΔacrB *hns*::*aph* backgrounds

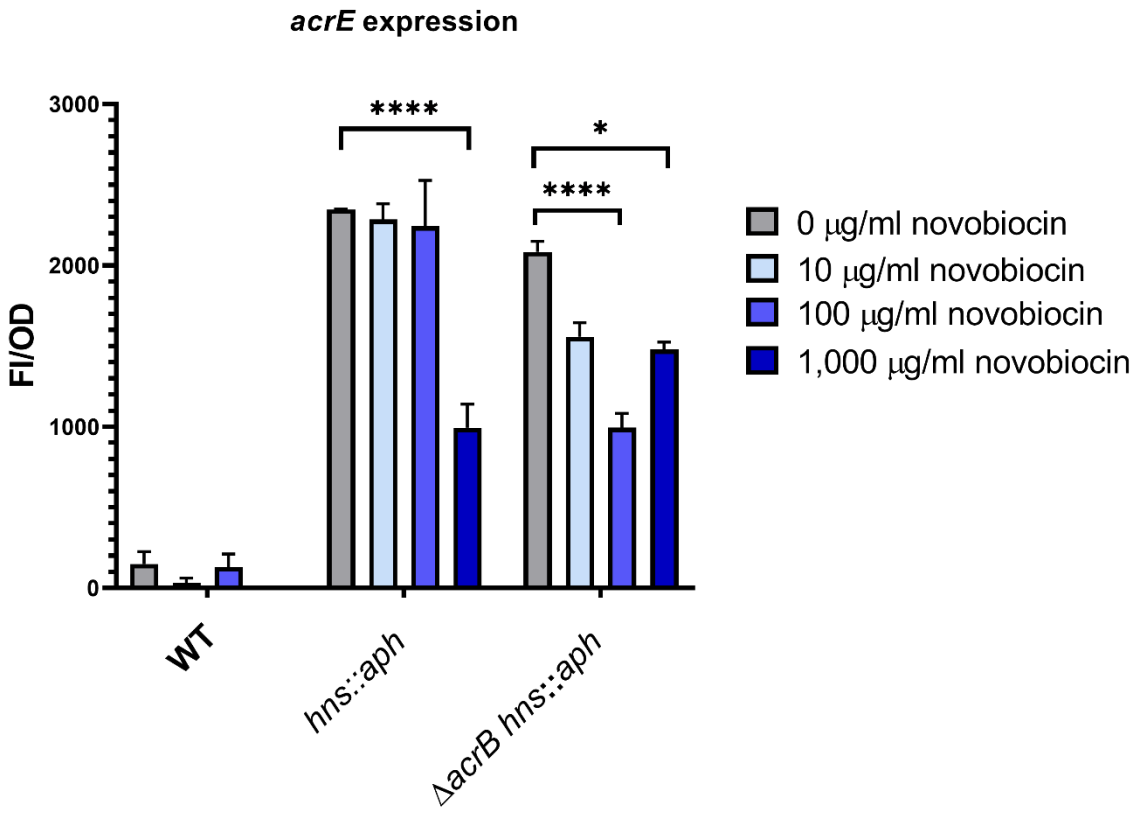


Figure 6.25: The induction of *acrE* was measured in WT (SL1344), *hns*::*aph* and *ΔacrB hns::aph* backgrounds. The average endpoint fluorescence (excitation 485 nm, emission 520 nm) and OD_{600} in PBS was measured after addition of novobiocin (final concentrations 0, 10, 100 or 1,000 μg/ml) for three technical replicates. The averages of these replicates were blank corrected using no-bacteria and no-plasmid controls. This was repeated for n = 3 biological replicates. Shown is the mean of these biological replicates, including error bars (standard deviation of the mean). An ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare the means of all genotypes and inducer concentrations. Statistical significance is represented by: * = $P < 0.05$, ** = $P < 0.001$, *** = $P < 0.0005$ and **** = $P < 0.0001$.

Figure 6.26: Panel of compounds which did not alter *acrA* expression

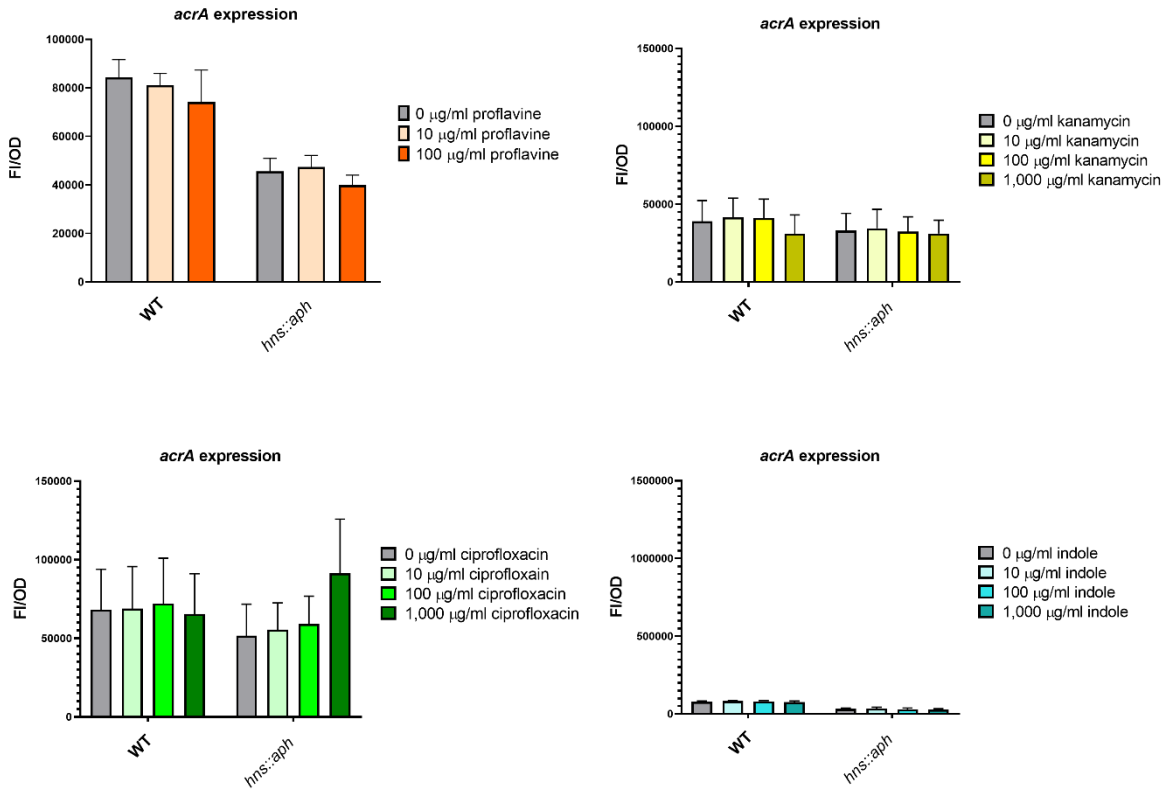


Figure 6.26**:** The induction of *acrA* by proflavine, kanamycin, ciprofloxacin or indole was measured in WT (SL1344) and *hns*::*aph* backgrounds. The average endpoint fluorescence (excitation 485 nm, emission 520 nm) and OD_{600} in PBS was measured after addition of inducers (final concentrations 0, 10, 100 or 1,000 µg/ml) for three technical replicates. The averages of these replicates were blank corrected using no-bacteria and no-plasmid controls. This was repeated for n = 3 biological replicates. Shown is the mean of these biological replicates, including error bars (standard deviation of the mean). An ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare the means of all genotypes and inducer concentrations. Statistical significance is represented by: * = $P < 0.05$, ** = $P < 0.001$, *** = $P < 0.0005$ and **** = $P < 0.0001$.

Figure 6.27: Panel of compounds which did not alter *acrE* expression

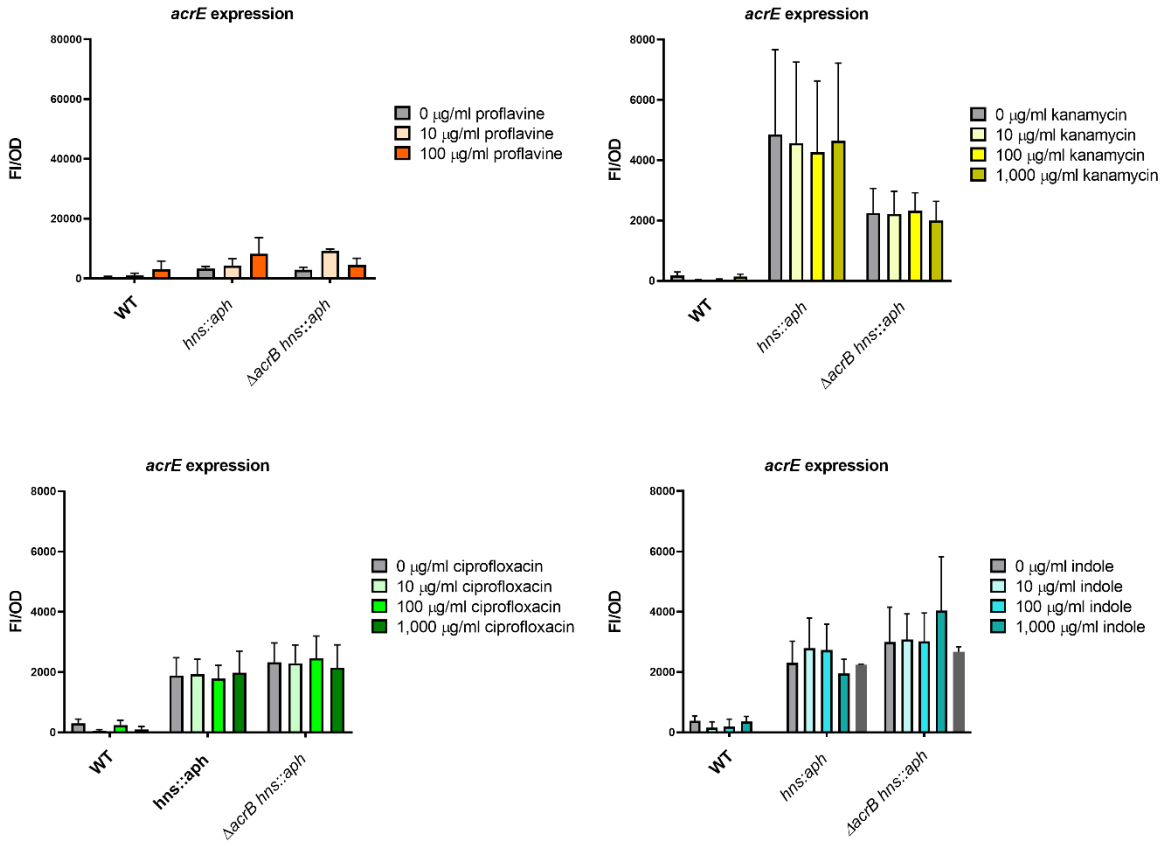


Figure 6.27: The induction of *acrE* by novobiocin, kanamycin, ciprofloxacin or indole was measured in WT (SL1344), *hns::aph* and *ΔacrB hns*::*aph* backgrounds. The average endpoint fluorescence (excitation 485 nm, emission 520 nm) and OD_{600} in PBS was measured after addition of inducers (final concentrations 0, 10, 100 or 1,000 µg/ml) for three technical replicates. The averages of these replicates were blank corrected using no-bacteria and no-plasmid controls. This was repeated for n = 3 biological replicates. Shown is the mean of these biological replicates, including error bars (standard deviation of the mean). An ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare the means of all genotypes and inducer concentrations. Statistical significance is represented by: * = $P < 0.05$, ** = $P < 0.001$, *** = $P < 0.0005$ and **** = $P < 0.0001$.

6.8 RamA-mediated changes in *acrA* and *acrE* expression

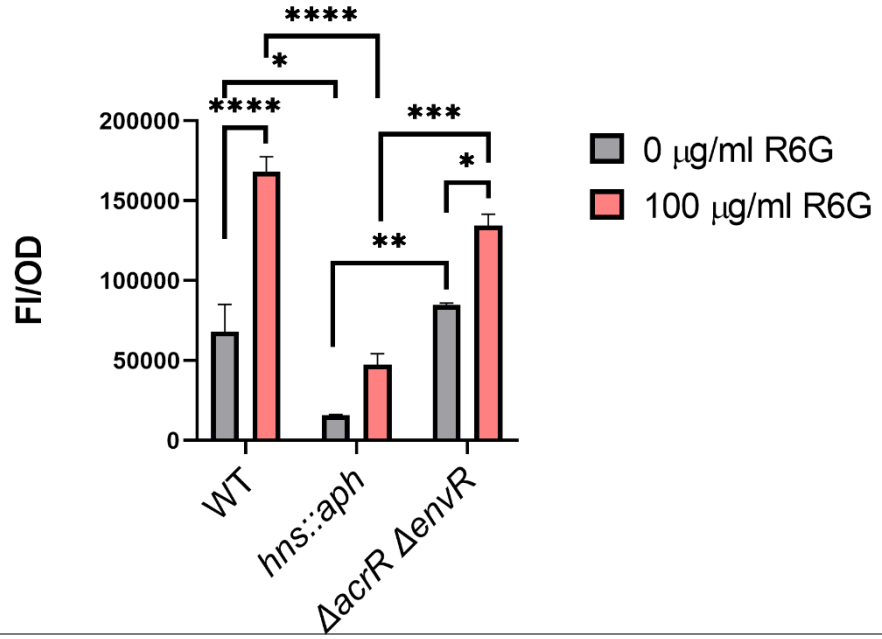
The expression of *ramA* in response to the inducers found to increase (Rhodamine 6g) or reduce (tetracycline and novobiocin) the expression of *acrA* or *acrE* were tested, to see if these effects were *ramA*-mediated. The expression of *ramA* in response to proflavine was also measured because of the small, (but not statistically significant) increase in *acrE* expression after addition of proflavine in the *hns::aph* background (Figure 6.27).

The expression of *ramA* was significantly increased after induction with rhodamine 6g in all backgrounds (Figure 6.28). Proflavine, an inducer of *acrE* expression, also increased *ramA* expression, but this was only statistically significant in the *hns*-interrupted background (Figure 6.29). Expression of *ramA* was significantly reduced in the presence of tetracycline (Figure 6.30) and novobiocin (Figure 6.31).

6.9 Proflavine increased the rate of ethidium bromide efflux

If an inducer increased the expression of *acrA* or *acrE,* this should lead to the increased efflux of substrates. However, due to the fluorescence of rhodamine 6g, it was not possible to measure the effect of the inducer rhodamine 6g on the efflux of ethidium bromide. The addition of proflavine increased the rate of ethidium bromide efflux in both a wild type and a Δ*acrB* background, decreasing the time taken to efflux pre-loaded ethidium bromide (Figure 6.32). This suggests that proflavine can be transported by AcrEF if AcrAB is not present.

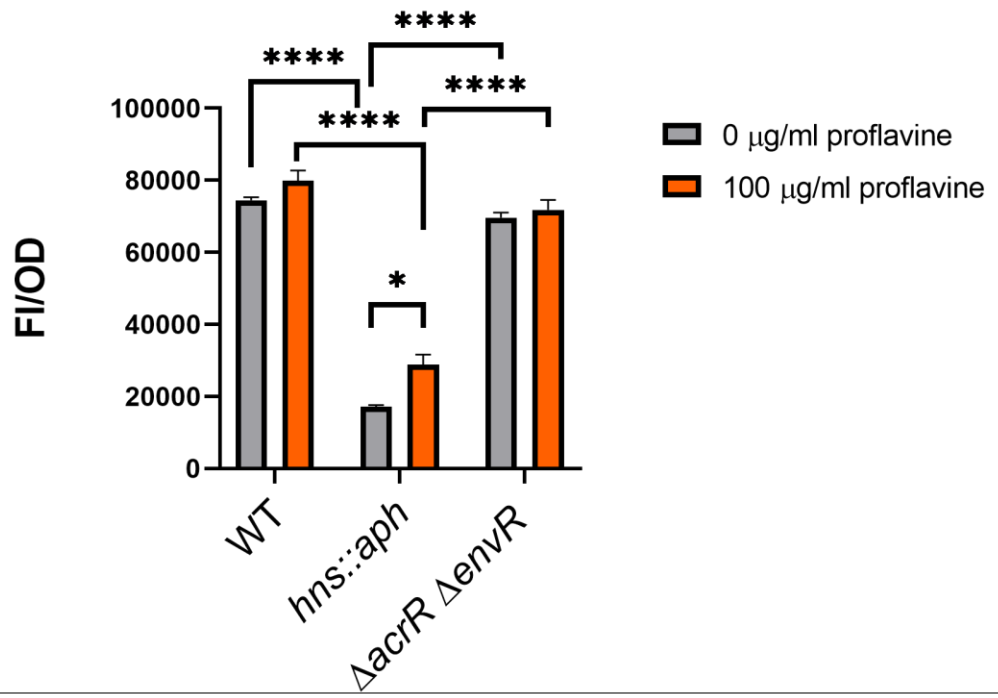
Figure 6.28: Induction of *ramA* in response to addition of rhodamine 6g



Multiple comparison ANOVA	***p*** value (significance)
WT 0 µg/ml vs. *hns::aph* 0 µg/ml	0.0129 (*)
WT 0 µg/ml vs. WT 100 µg/ml	<0.0001 (****)
hns::aph 0 µg/ml vs. Δ*acrR* Δ*envR* 0 µg/ml	0.0015 (**)
Δ*acrR* Δ*envR* 0 µg/ml vs. Δ*acrR* Δ*envR* 100 µg/ml	0.0182 (*)
WT 100 µg/ml vs *hns::aph* 100 µg/ml	<0.0001 (****)
hns::aph 100 µg/ml vs. Δ*acrR* Δ*envR* 100 µg/ml	0.0002 (***)

Figure 6.28: The induction of *ramA* was measured in WT, *hns::aph* and Δ*acrR* Δ*envR* backgrounds. The average endpoint fluorescence (excitation 485 nm, emission 520 nm) and OD_{600} in PBS was measured after addition of tetracycline (final concentrations 0, 10, 100 or 1,000 µg/ml) for three technical replicates. The averages of these replicates were blank corrected using no-bacteria and no-plasmid controls. This was repeated for n = 3 biological replicates. Shown is the mean of these biological replicates, including error bars (± standard deviation of the mean). An ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare the means of all genotypes and inducer concentrations, shown in the table. Statistical significance is represented by: * = $P < 0.05$, ** = $P< 0.001$, *** = $P < 0.0005$ and **** = $P <0.0001$.

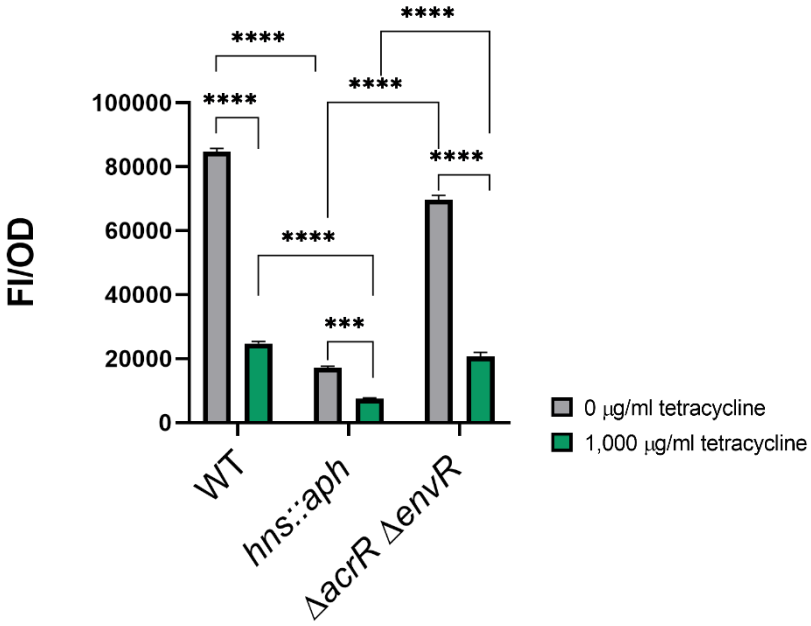
Figure 6.29: Induction of *ramA* in response to addition of proflavine



Multiple comparison ANOVA	***p*** value (significance)
WT 0 µg/ml vs. *hns::aph* 0 µg/ml	<0.0001 (****)
hns::aph 0 µg/ml vs. Δ*acrR* Δ*envR* 0 µg/ml	<0.0001 (****)
hns::aph 0 µg/ml vs. *hns::aph* 100 µg/ml	0.0205 (*)
WT 100 µg/ml vs. *hns::aph* 100 µg/ml	<0.0001 (****)
hns::aph 100 µg/ml vs. Δ*acrR* Δ*envR* 100 µg/ml	<0.0001(****)

Figure 6.29: The induction of *ramA* was measured in WT, *hns::aph* and Δ*acrR* Δ*envR* backgrounds. The average endpoint fluorescence (excitation 485 nm, emission 520 nm) and OD_{600} in PBS was measured after addition of proflavine (final concentrations 0, 10, 100 or 1,000 µg/ml) for three technical replicates. The averages of these replicates were blank corrected using no-bacteria and no-plasmid controls. This was repeated for n = 3 biological replicates. Shown is the mean of these biological replicates, including error bars (± standard deviation of the mean). An ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare the means of all genotypes and inducer concentrations, shown in the table. Statistical significance is represented by: * = $P < 0.05$, ** = $P< 0.001$, *** = $P < 0.0005$ and **** = $P <0.0001$.

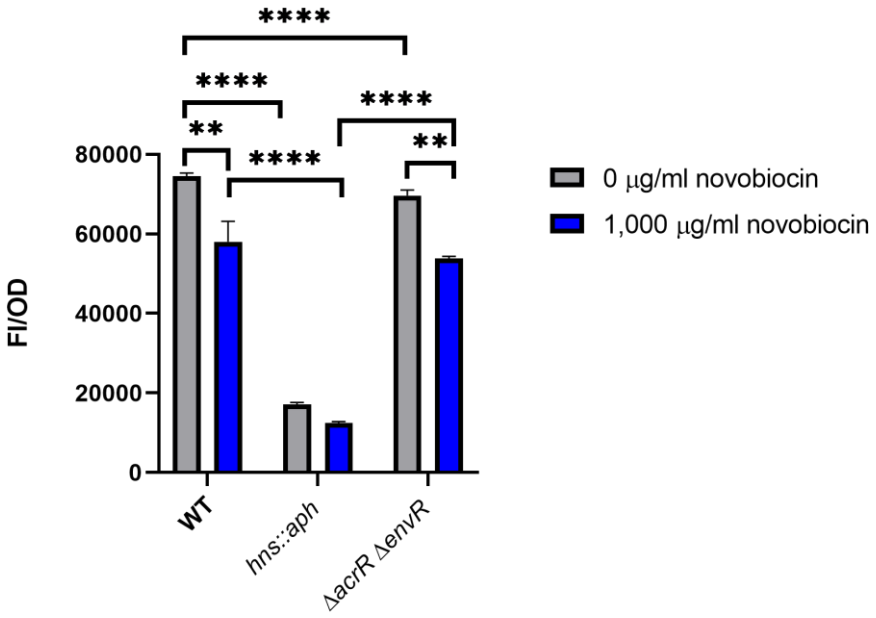
Figure 6.30: Induction of *ramA* in response to addition of tetracycline



Multiple comparison ANOVA	***p*** value (significance)
WT 0 μg/ml vs. *hns::aph* 0 μg/ml	<0.0001 (****)
WT 0 μg/ml vs. Δ*acrR* Δ*envR* 0 μg/ml	<0.0001 (****)
WT 0 μg/ml vs. WT 100 μg/ml	<0.0001 (****)
hns::aph 0 μg/ml vs. Δ*acrR* Δ*envR* 0 μg/ml	<0.0001 (****)
hns::aph 0 μg/ml vs. *hns::aph* 100 μg/ml	0.0003 (***)
Δ*acrR* Δ*envR* 0 μg/ml vs. Δ*acrR* Δ*envR* 100 μg/ml	<0.0001 (****)
WT 100 μg/ml vs. *hns::aph* 100 μg/ml	<0.0001 (****)
. *hns::aph* 100 μg/ml vs. Δ*acrR* Δ*envR* 100 μg/ml	<0.0001 (****)

Figure 6.30: The induction of *ramA* was measured in WT, *hns::aph* and Δ*acrR* Δ*envR* backgrounds. The average endpoint fluorescence (excitation 485 nm, emission 520 nm) and OD_{600} in PBS was measured after addition of tetracycline (final concentrations 0, 10, 100 or 1,000 μg/ml) for three technical replicates. The averages of these replicates were blank corrected using no-bacteria and no-plasmid controls. This was repeated for n = 3 biological replicates. Shown is the mean of these biological replicates, including error bars (± standard deviation of the mean). An ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare the means of all genotypes and inducer concentrations, shown in the table. Statistical significance is represented by: * = $P < 0.05$, ** = $P< 0.001$, *** = $P < 0.0005$ and **** = $P <0.0001$.

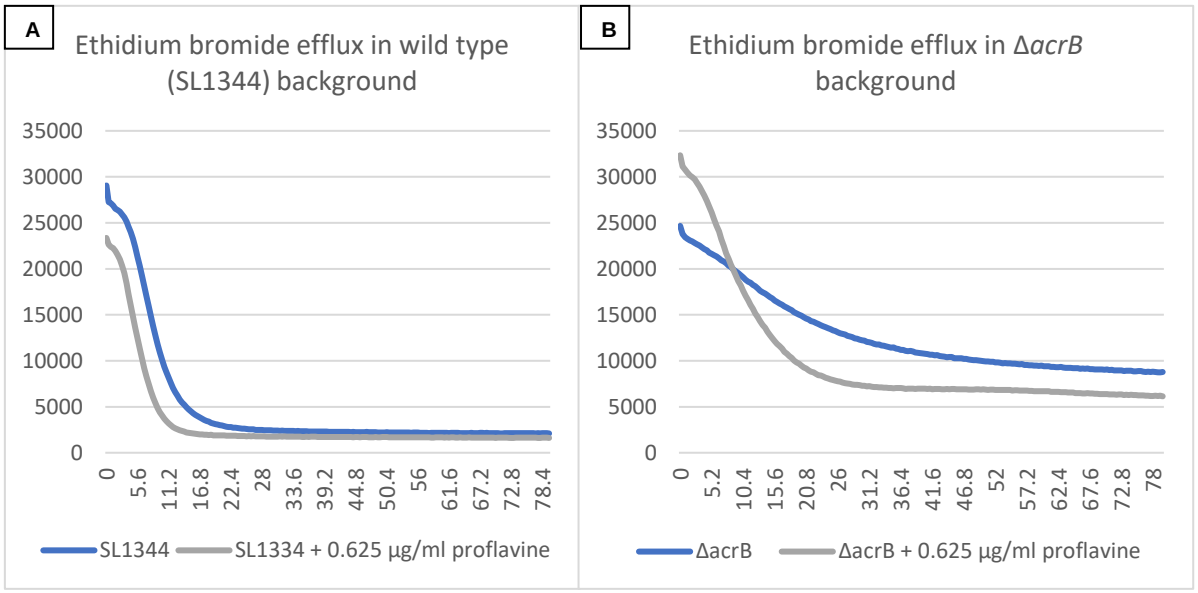
Figure 6.31: Induction of *ramA* in response to addition of novobiocin



Multiple comparison ANOVA	***p*** value (significance)
WT 0 µg/ml vs. *hns::aph* 0 µg/ml	<0.0001 (****)
WT 0 µg/ml vs. WT 100 µg/ml	0.022 (**)
hns::aph 0 µg/ml vs. Δ*acrR* Δ*envR* 0 µg/ml	<0.0001 (****)
Δ*acrR* Δ*envR* 0 µg/ml vs. Δ*acrR* Δ*envR* 100 µg/ml	0.0031 (**)
WT 100 µg/ml vs. *hns::aph* 100 µg/ml	<0.0001 (****)
hns::aph 100 µg/ml vs. Δ*acrR* Δ*envR* 100 µg/ml	<0.0001 (****)

Figure 6.31: The induction of *ramA* was measured in WT, *hns::aph* and Δ*acrR* Δ*envR* backgrounds. The average endpoint fluorescence (excitation 485 nm, emission 520 nm) and OD_{600} in PBS was measured after addition of novobiocin (final concentrations 0, 10, 100 or 1,000 µg/ml) for three technical replicates. The averages of these replicates were blank corrected using no-bacteria and no-plasmid controls. This was repeated for n = 3 biological replicates. Shown is the mean of these biological replicates, including error bars (± standard deviation of the mean). An ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare the means of all genotypes and inducer concentrations, shown in the table. Statistical significance is represented by: * = $P < 0.05$, ** = $P< 0.001$, *** = $P < 0.0005$ and **** = $P <0.0001$.

Figure 6.32: Proflavine increases the efficiency of ethidium bromide efflux.



C

Strain	Average time for ethidium bromide fluorescence to reduce (minutes)		
	10%	**25%**	**50%**
SL1344, 0 µg/ml proflavine	1.28	3.32	5.52
SL1344, 0.625 µg/ml proflavine	1.07	2.08	2.61
SL1344 Δ*acrB*, 0 µg/ml proflavine	3.46	7.47	14.82
SL1344 Δ*acrB*, 0.625 µg/ml proflavine	2.09	4.76	6.05

Figure 6.32: The rate of ethidium bromide efflux by WT (panel A) and Δ*acrB* (panel B) strains after the addition of proflavine (0 or 0.625 µg/ml). The average time for ethidium bromide fluorescence to reduce for each strain is listed in panel C. Fluorescence (excitation 544, emission 590) was measured for 200 cycles. The average fluorescence was blank corrected. Originally presented in the MSci thesis of HD (Doherty, 2019).

6.10 Discussion

The RND efflux systems AcrAB and AcrEF share sequence and structural homology, with the AcrB and AcrF proteins sharing 80% similarity in *Salmonella* (Eaves *et al.*, 2004). However, the 'natural' substrates of both systems remain unknown. It is unclear if there are any AcrEF-specific substrates, or whether the primary function of AcrEF is to act as a "back-up" system to AcrAB. This view of AcrEF as a "back-up" is perpetuated by other findings in the literature. For example, in the absence of functional AcrB, the overexpression of *acrEF* can restore WT-pattern susceptibility to known substrates of AcrB, or even confer resistance to these substrates (Zhang *et al.*, 2018). In an *ΔacrB* background mutations are selected which enable overexpression of *acrEF* in response to fluoroquinolone stress (McNeil *et al.*, 2019). Increased *acrEF* expression also confers resistance to tigecycline and tetracyclines (Hirata, Saito, Nishino *et al.*, 2004). However, a lack of functional AcrB is not necessarily a prerequisite for *acrEF* overexpression, with *acrEF* overexpression also reported independently in clinical isolates (Chetri *et al.*, 2018), even when functional AcrB is present.

The regulation of *acrAB* and *acrEF* expression is also interconnected, with both positive (e.g. RamA) and negative (e.g. EnvR) regulators known to target and influence the expression of both systems (Bailey *et al.*, 2010; Zheng *et al.*, 2009). As EnvR has a higher affinity for the promoter of *acrAB*, it is possible that the expression of *acrEF* specifically switches off expression of the *acrAB* genes, thereby preventing the co-transcription of these two similar systems (Hirakawa, Takumi-Kobayashi*, et al.*, 2008).

If the conditions which induce *acrAB* or *acrEF* transcription can be identified, they may hint at what the 'natural' roles or substrates of these efflux systems are. To investigate

whether there are any conditions that can induce the transcription of these systems, a 96-well plate method was developed using minimal media designed to mimic *Salmonella* infection environments. These conditions were selected because it is known that efflux in *Salmonella* is linked to virulence (Wang-Kan *et al.*, 2017). It is also generally accepted that minimal media better represents conditions experienced by bacteria when colonising and infecting a host, as richer media such as LB do not represent the nutrient-limited conditions present during colonisation and infection of a host (Bailey, Webber, & Piddock, 2006; Neidhardt *et al.*, 1974). For this reason, initial experiments were designed using minimal media MOPS. Minimal media MOPS is a well-defined media and the constituents of this media could be altered to mimic different environments encountered by *Salmonella.* It was hypothesised that by altering the minimal media to represent conditions found in the host during infection (e.g. nutrient starvation / pH challenge), it would be possible to quantify *acrA* and *acrE* expression in response to individual conditions.

Glucose was selected as the carbon source because *Salmonella* requires glucose in order to survive and cause infection within macrophages in mouse models (Bowden, Rowley, Hinton *et al.*, 2009). Decreasing the glucose concentration of the media decreased the expression of *acrA* (Figures 6.4 – 6.5). As efflux is an active process requiring cellular energy, this likely prevents energy wastage when carbon sources are limited. However, intermediate concentrations of glucose (0.05 - 0.1%) permitted an extended period of *acrA* expression, with expression of *acrA* remaining high and not decreasing as was seen in the low (0.025%) and higher (0.2%) glucose treatments (Figure 6.4). In *E. coli, acrAB* expression has been shown to increase when growth rate is slower due to restricted carbon source (Rand, Danby, Greenway *et al.*, 2002).

These authors attribute this to the promoter of *acrA* being a 'gearbox' promoter, which is alternatively targeted by RpoS-encoding sigma factor σS, which is the sigma factor used to selectively target stress response genes for transcription. Gearbox promoters are promoters which are said to be more active when bacterial growth is slow (Vicente, Kushner, Garrido *et al.*, 1991). The consequences of this is that under stressful conditions, *acrAB* expression would be increased by this kind of promoter. However, there are no other reports of increased *acrB* expression in nutrient-starved conditions. Lower concentrations of glucose decreased *acrA* induction (Figures 6.4 – 6.5), which disagrees with the findings of these authors. Moreover, the promoter of *acrAB* in *Salmonella* does not have homology to the -10 element which identifies a gearbox promoter (CGGCNAGT). Therefore, results reported by these authors may be *E. coli*-specific as it is unlikely that the promoter of *acrAB* in SL1344 is a promoter of this type. Instead, the proposed promoter of *acrA* is shown in Figure 6.33.

Figure 6.33: The promoter of *acrAB* in *Salmonella* Typhimurium

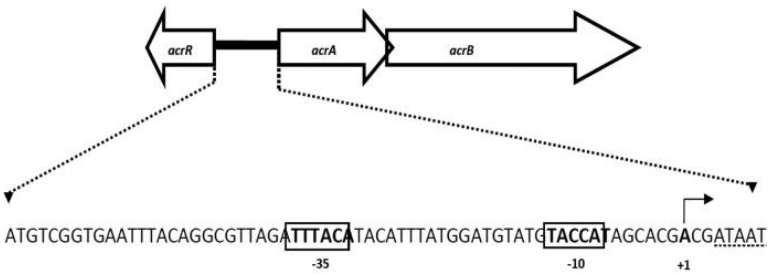


Figure 6.33: Promoter of *acrAB* adapted from (Ricci, Attah, Overton *et al.*, 2017). The -35 and -10 elements are shown. The -10 element shown does not resemble the gearbox promoter CGGCNAGT.

Altering pH also influenced *acrA* induction, with more acidic pH decreasing *acrA* expression (Figures 6.7 – 6.8). The expression of *acrAB* in *S.* Typhimurium has previously been shown to decrease in response to low pH (Kroger, Colgan, Srikumar *et al.*, 2013). Although the activity on the *acrAB* promoter was increased in response to low pH, this does not necessarily equate to an increase in *acrAB* transcription or translation. Therefore, these data must be interpreted with caution in the absence of further transcription/protein data.

One report in *E. coli* states that AcrAB-mediated efflux of ethidium bromide is not energy dependant at pH 5 but becomes energy dependant at pH 8 (Martins, Spengler, Rodrigues *et al.*, 2009). It is not known whether the same is true in *Salmonella.* However, *Salmonella* must survive in both the stomach/macrophage and large intestine (pH range 4 – 8). Therefore, it is likely that the transcriptional response to these environments and the resulting changes in efflux gene expression reflect the requirements in each environment. During infection, *Salmonella* invade macrophages and form a *Salmonella*-containing vacuole (SCV). When inside the acidic environment of the SCV, *Salmonella* actively lower the pH of the cytoplasm in order to induce the expression of SPI-2 virulence genes (Richardson, 2015). It is possible that decreasing the intracellular pH also has consequences for wider gene expression. For example, in *Y. enterocolitica,* OmpR is thought to bind upstream and induce *acrAB* transcription in response to multiple stress-inducing conditions, including low pH (Raczkowska, Trzos, Lewandowska *et al.*, 2015).

The final two conditions tested to try and induce *acrEF* expression are magnesium and phosphate limitation, both of which occur in the SCV. Low magnesium is known to relieve H-NS repression via activation of SlyA by the PhoPQ two component system

(Perez, Latifi, & Groisman, 2008). Low concentrations of magnesium (5 – 10 µM) significantly reduced *acrA* induction (Figures 6.10 – 6.11). Phosphate limitation also leads to H-NS re-repression via SsrB, with the *ssrB* gene upregulated in low phosphate conditions (Jiang, Yu, Qi *et al.*, 2018). Although these conditions were predicted to relieve H-NS and therefore induce *acrEF* expression, no significant changes in *acrE* expression were observed (Figures 6.12 and 6.15).

Although it was possible to measure changes in *acrA* expression using this method, no induction of *acrE* expression was observed in any condition tested. The conditions tested were predicted to relieve H-NS repression and allow the induction of *acrE* expression. The fact that this could not be measured could be due to several factors. The first is that regulation is inherently complex- perhaps a combination of the tested conditions (as is the case in the host environment), are required to induce *acrEF*. It is also possible that H-NS was relieved under some of these conditions, but that the expression of *acrEF* was repressed by either local (e.g. EnvR) or global transcription factors. As the strain producing non-functional H-NS did not grow well in this experiment (Figure 6.16), it was not possible to detect induction of the *acrE* promoter due to poor growth. As the host conditions are so variable and complex, it was decided that the best way to proceed was to design a new induction experiment which would focus on the optimal growth of test strains, measuring the effect of single inducer compounds.

In the single time point experiments, increased *acrE* expression was observed in both wild type and *hns*-interrupted backgrounds after addition of rhodamine 6g, indicating that the *acrE* promoter can be activated even if functional AcrB is present (Figure 6.21). As the *acrEF* genes are H-NS silenced in *Salmonella* (Nishino *et al.*, 2009), it was

hypothesised that *acrE* expression would be higher in a strain which could not produce functional H-NS. It was also expected that in the absence of *acrB,* the induction of *acrE* would increase to compensate for the loss of *acrB.*

While the absence of functional H-NS did increase the induction of *acrE* transcription, the effect of interrupting *acrB* did not have a significant additive effect (Figure 6.17). However, the level of *acrE* expression in a *ΔacrB* background, with functional H-NS, should be investigated to better understand the effects of these genotypes.

The expression of *acrA* was consistently higher than *acrE* in the wild type background, even if *hns* and *acrB* were interrupted (Figure 6.17). This agrees with the literature which reports that *acrAB* is expressed at higher levels than the H-NS silenced AcrEF system (Nishino & Yamaguchi, 2001). The expression of *acrA* was also decreased in the strain lacking functional H-NS (Figure 6.17). This could be because EnvR is known to bind the promoter of *acrAB* with higher affinity than *acrEF* (Hirakawa, Takumi-Kobyashi*, et al.*, 2008), meaning that the same conditions with induce *acrEF* also induce *envR* expression (as these genes are divergently transcribed from the same promoter region) and therefore, the downregulation of *acrAB* expression.

The expression of *acrE* was lower than *acrA* in all backgrounds and was highest in the *hns*-interrupted strains (Figure 6.17). This is unsurprising as the *acrEF* genes are H-NS silenced in *E. coli* (Nishino & Yamaguchi, 2004) and *S.* Typhimurium (Nishino *et al.*, 2009). Therefore, this data agrees with the literature that H-NS is the main negative regulator of *acrEF* in these species.

The expression of *acrEF* is also known to be upregulated by LeuO, a protein which acts as an H-NS antagonist to allow the transient expression of H-NS silenced genes

(Shimada *et al.*, 2011). Given the complexity of bacterial regulation, it is possible that inactivation of *acrR* and *envR* may directly or indirectly activate LeuO or induce *acrE* expression via interactions with other regulators. There are also many other possible H-NS:DNA interactions, each of which is relieved via a different mechanism (Grainger, 2016). Therefore, it is difficult to predict both the interactions between H-NS with *acrEF*, but also the mechanisms by which H-NS is relieved at this location.

The compounds used to induce *acrA* and *acrE* expression were selected based on the premise that they are substrates of AcrAB or AcrEF. Kanamycin was also included as it is not a known substrate of either AcrAB or AcrEF, thereby acting as a negative control (Eicher *et al.*, 2012). Some of this same panel of potential inducers were previously shown to prevent EnvR binding to the promoters *acrA* or *acrE* in binding assays (Chapter 5), suggesting that these compounds act as ligands of AcrR or EnvR. Hypothetically, these inducers would act to bind and sequester AcrR/EnvR away from the promoter of *acrA* or *acrE*, allowing transcription of the efflux genes. Therefore, the expected pattern was that after the addition of an inducer, expression of *acrA*/*acrE* would either increase in response to an inducer or remain the same in response to a non-inducer. Although the substrates of AcrAB and AcrEF are expected to be identical, it was expected that *acrA* and *acrE* would differentially induced by some compounds. This would also support the hypothesis of a unique role for AcrEF, as opposed to existing purely as a backup to AcrAB.

Proflavine (77 µg/ml) was shown to prevent binding of EnvR to the promoter of *acrA* and *acrE* (Chapter 5). Proflavine also increased the induction of *acrE,* but this was not statistically significant (Figure 6.27). Proflavine also did not induce an increase in *acrA* expression (Figure 6.26). However, proflavine is known to bind AcrR (Su *et al.*, 2007),

and was proposed to be a ligand of AcrR, promoting the expression of AcrR-regulated genes such as *acrAB.* Moreover, proflavine increased the expression of *ramA* (Figure 6.29), indicating that it could still cause indirect activation of the AcrAB or AcrEF efflux systems. The ethidium bromide efflux assay was used to investigate whether the addition of proflavine changes the efficiency of ethidium bromide efflux. Proflavine increased the efficiency of ethidium bromide efflux in both a wild type and a Δ*acrB* background (Figure 6.32). As proflavine altered the efficiency of ethidium bromide efflux in a Δ*acrB* background, it is possible that proflavine can induce the expression of multiple efflux genes, not just *acrAB.* This could suggest that AcrEF is involved in the transport of proflavine, but as there are many other efflux systems in *Salmonella* in addition to AcrAB and AcrEF, these experiments should be repeated using strains which lack all but one efflux system. This would start to unpick the involvement of individual efflux systems in the extrusion of different substrates. Although induction with proflavine caused small, non-significant increases in *acrE* transcription, such small increases may have more drastic effects on AcrEF protein production or efflux phenotype. Due to the many factors involved, it is not possible to directly correlate increased *acrE* promoter activity with protein production or efflux efficiency.

Rhodamine 6g is also a known ligand of AcrR (Su *et al.*, 2007). It was not possible to test the effects of rhodamine 6g on ethidium bromide efflux as the absorption and emission spectra for the two compounds overlap. However, rhodamine 6g was able to increase the transcription of both *acrA* and *acrE* (Figures 6.20 – 6.21). Interestingly, rhodamine 6g was able to significantly induce *acrE* expression in a wild type background, even though functional H-NS is present in this strain (Figure 6.21). Interrupting *hns* increased the induction of *acrE* in the no-drug control compared to

wild type and this was further inducible with the addition of rhodamine 6g. However, increases in *acrE* induction in the *hns*-interrupted strain were not statistically significant and could not achieve the same high level of *acrE* expression in response to rhodamine 6g as seen in the wild type background. This indicates that AcrEF may be able to respond to some known AcrAB substrates even when functional AcrAB is present. Moreover, the induction with rhodamine 6g was presumably able to overcome both global (H-NS) and then local (AcrR/EnvR) repression to induce *acrE* expression. If increased *acrEF* expression relied only on the absence of H-NS repression, then the levels of *acrE* induction in response to rhodamine 6g in the wild type and *hns*-interrupted backgrounds should be comparable. The fact that they are not indicates that there is an additional requirement to achieving higher-level *acrEF* expression, for example, the involvement of activator proteins.

In *S.* Typhimurium, RamA is thought to be the primary activator of *acrAB* expression (Nikaido *et al.*, 2008). Moreover, overexpression of *ramA* is known to increase the expression of both *acrAB* and *acrEF* (Bailey *et al.*, 2010; Nikaido, Shirosaka, Yamaguchi *et al.*, 2011). Induction of *ramA* was increased after treatment with rhodamine 6g (Figure 6.28), which also increased *acrE* expression (Figure 6.21). It is possible therefore, that this induction of *acrE* expression is at least partially RamA-mediated. It is known that some substrates of AcrAB induce *acrAB* expression via RamA. For example, indole is known to induce *ramA* and *acrA* expression in *Salmonella* (Nikaido *et al.*, 2012; Nikaido *et al.*, 2011; Nikaido *et al.*, 2008). This was also seen in the 96-well plate induction experiments, with each incremental increase in *ramA* being followed by an increase in *acrA* expression (Figure 6.1 – 6.2). Rhodamine 6g and proflavine are known to bind RamR as a ligand, removing RamR

from the *ramA* promoter and allowing *ramA* expression (Yu, Aires*, et al.*, 2003). Therefore, it is possible that rhodamine 6g may also act as a ligand to local negative regulators (i.e. AcrR/EnvR, Chapter 5), whilst simultaneously promoting activation of *acrAB* by interacting with RamR to increase *ramA* expression. Interrupting *ramA* increases susceptibility to a range of compounds, including proflavine (Zheng *et al.*, 2011). However, RamA is known to be a global regulator which influences the expression of over 200 genes in *Salmonella,* so it is not possible to exclude the possibility that these effects are caused by indirect interactions (Bailey *et al.*, 2010). Therefore, as RamA has many roles (and is also not the only activator of *acrAB*/*acrEF*), it is difficult to directly prove the interactions between RamA and the expression of these efflux systems without further experimental evidence.

Many compounds tested did not increase *acrA* or *acrE* expression (Figures 6.26 – 6.27). However, even if a compound did not alter *acrA* or *acrE* expression, it is not possible to exclude these as potential activators of these efflux systems *in vivo*. For example, indole induces *acrAB* expression during the 96-well induction experiment over 12 hours (Figure 6.1 – 6.2) but not in the second shorter induction experiment (Figures 6.26 – 6.27). Therefore, if a compound/condition does not induce the expression of *acrAB* or *acrEF*, it cannot be excluded as a potential inducer on this evidence alone. On the contrary, if a given compound induces in an experiment, this effect cannot be assumed to occur *in vivo* or without the involvement of other regulators or efflux systems. Although indole is considered to be a substrate of both AcrAB and AcrEF, it has been shown previously that indole induces *acrAB,* but not *acrEF* expression (Nikaido *et al.*, 2008). It is also not possible to correlate the activity on the promoter (as per these promoter fusion assays) with transcript levels or protein

productions. It is also known that a combination of factors contributes towards the induction of efflux systems. It is also important to note that when discussing these results, the GFP fluorescence reports only on the activity on the promoter. Although we propose that activity on the promoter would be correlated to the transcription and translation of the AcrAB and AcrEF proteins, we cannot prove this without further experiments (e.g. western blots). Moreover, it is important to consider that there may be other factors which influence the final protein level of AcrAB and AcrEF such as post-transcriptional modification.

Surprisingly, some compounds significantly decreased the expression of *acrA* or *acrE*. Tetracycline decreased the expression of both *acrA* and *acrE* (Figures 6.22 – 6.23) and novobiocin decreased *acrE,* but not *acrA,* expression (Figures 6.24 - 6.25). Novobiocin was also able to prevent EnvR binding to the promoter of *acrAB* and *acrEF* (Chapter 5), abolishing binding in the way expected of a classical TetR repressor protein. The finding that novobiocin decreased *acrE* expression is therefore more likely to be due to indirect interactions with novobiocin, not direct interactions between novobiocin and AcrR/EnvR.

Previous binding assay data (Chapter 5), also showed that high, but not low, concentrations of tetracycline allowed EnvR to bind the promoters of *acrA* and *acrE*. As the binding assay contains only protein and DNA in isolation of all other regulators/cellular processes, it was hypothesised that tetracycline may directly interact with AcrR/EnvR in order to either (a) increase the binding affinity of AcrR/EnvR to their targets or (b) increase the strength of binding enabling stronger repression of targets. It is known that TetR, the regulator of the tetracycline pump efflux genes, can undergo a single amino-acid change which results in a complete switch of function.

This “reverse TetR” requires tetracycline for binding to the promoter of *tetA*, instead of requiring tetracycline to become detached from the promoter of *tetA.* There is one report that tetracycline increases the efficacy of other antibiotics in the treatment of Gram-negative infections (Mawabo, Noumedem, Kuiate *et al.*, 2015). Given that the predominant role of these efflux systems is unknown, it is possible that tetracycline resembles a natural substrate of an uncharacterised efflux system which is more efficient at extruding tetracycline-like molecules than AcrAB or AcrEF, thereby resulting in a targeted downregulation of *acrA* and *acrE,* similar to the downregulation of *acrA* seen during higher *acrE* expression. It could also be that tetracycline is interacting with other regulators of efflux, not just AcrR and EnvR. As *ramA* expression is controlled locally by a TetR-family protein RamR, it is possible that tetracycline could also be binding RamR and increasing the affinity of RamR for the *ramA* promoter. The expression of *ramA* also decreased with the addition of tetracycline (Figure 6.30) indicating that this is one possible explanation for decreased *acrA* and *acrE* expression. It is important to note that this is a hypothesis based on the direct interaction between tetracycline and these regulators. This hypothesis would need to be tested experimentally, initially by repeating these experiments in a *ΔramA* mutant.

6.11 Future directions

- Quantify AcrB and AcrF protein production using western blotting before and after induction to check that induction of promoter activity translates to protein production.
- Study the expression of *acrA* and *acrE* in response to tetracycline in a Δ*ramA* background to see if the tetracycline effects are RamRA-dependent.

- Use protein docking models to study potential interactions between inducers (e.g. rhodamine 6g) and non-inducers (e.g. tetracycline) of with regulators AcrR and EnvR. This will start to investigate whether these compounds directly interact with AcrR or EnvR
- Follow the docking models with crystal structures of AcrR/EnvR/RamR in the presence of tetracycline to elucidate any direct interactions.

6.12 Key findings

- Substrates of AcrAB can increase (rhodamine 6g) but also decrease (tetracycline, novobiocin) the induction of *acrA* and *acrE* transcription.
- Interrupting the *hns* gene increases the transcription of *acrEF* but decreased expression of acrAB. The addition of an *acrB* interruption does not further increase the induction of *acrEF*, so loss of functional AcrB is not necessarily a pre-requisite for *acrEF* transcription.
- Transcription of H-NS silenced efflux genes *acrEF* can be induced in a WT background after induction with rhodamine 6g.

Chapter 7

Overall Discussion and Conclusions

7.0: Overall Discussion and Conclusions

Introduction

The TetR-family transcription factor AcrR is best-known as a negative regulator of *acrAB* gene expression in *E. coli* (Ma *et al.*, 1996a). However, a second regulator, EnvR can also bind the same region upstream *acrAB* in *E. coli* (Hirakawa, Takumi-Kobayashi*, et al.*, 2008). The *acrR* gene is transcribed divergently of *acrAB* and is therefore the local regulator of these efflux genes (Ma *et al.*, 1996a), whereas EnvR is a global regulator of *acrAB*. However, despite not being the local regulator, EnvR binds the promoter of *acrAB* with higher affinity than local regulator AcrR in *E. coli* (Hirakawa, Takumi-Kobayashi*, et al.*, 2008).

The majority of TFTRs are thought to be single-target, local regulators (Ramos *et al.*, 2005). However, by regulating *acrAB*, EnvR is an example of a global TFTR. Despite single-target regulation being the assumed default for this family of transcription factors, other TFTRs have been classified as multi-target (e.g. RutR (Shimada *et al.*, 2007; Shimada *et al.*, 2008)) or multi-functional (i.e. both repressors and activators of multiple targets), such as MtrR (P. J. Johnson & W. M. Shafer, 2015). Recent work suggests single-target regulators form only a minority of transcription factors in *E. coli* (Shimada *et al.*, 2018)

Bioinformatic analysis of AcrR and EnvR

Here, the majority of TFTR regulators in *Escherichia* and *Salmonella* were identified as having more than one target. Moreover, the number, sequence, and presence/absence of TFTRs varied, even between strains of the same species (Chapter 3). The TFTR gene *eefR* and target efflux genes *eefABC* were also found in

some *Escherichia* strains for the first time. This data supports the notion that there are more TFTRs than have been characterised and that the roles of some TFTRs that have been characterised is underestimated.

Limitations of the EMSA data in this study

In addition to the bioinformatic analyses, this study set out to characterise the binding of AcrR and EnvR to the promoter of *acrAB* and to other potential target promoters (Chapters 4 and 5). EMSA data suggested weak binding of purified AcrR and EnvR protein upstream of *acrAB* and other target genes (Chapters 4 and 5). However, the binding strength of the positive control varied throughout this study and was a weak interaction, making the results of the weak binding of the test DNA and protein in the EMSAs difficult to interpret. It was also not always possible to include sufficient negative controls (protein-only) due to a lack of available protein.

As an EMSA involves the mixing and incubation of DNA, protein and binding buffer as an isolated, *in vitro* event, where other biological molecules and physiologically relevant conditions are absent, even strong binding interactions seen in these experiments should be supported by other assays such as RNA and ChIP Sequencing (RNA-Seq and ChIP-Seq). The conditions of an EMSA are also not comparable to conditions *in vivo* and must therefore always be interpreted alongside other data. The weak binding reported in this study should therefore be interpreted cautiously, with further assays (e.g., Radiolabelled EMSA/ChIP-Seq/RNA-Seq) required to build on these results.

A positive EMSA assay showing binding of protein to target DNA demonstrates that this event can happen as an isolated interaction with only protein, DNA and binding

buffers present. Concentration titres of protein and/or target DNA increase the confidence in the EMSA assay, as this shows that the effect is concentration-dependent. While it was possible to get this data for some EMSAs in this study, it was not possible to do a concentration titre for every assay due to the limited amount of purified protein. Improving the protein purification method and performing protein concentration titres would be an important addition to future EMSAs.

In fact, the original intention of this study was not to search for new targets using EMSA, but to follow-up ChIP and RNA sequencing targets with EMSA to confirm binding and study binding affinity. However, due to the temporary cessation of ChIP-Seq at our institution due to significant technical issues, it was not possible to complete ChIP-Seq or RNA-Seq within the timeframe of this study. Therefore, the EMSA assay was used more than initially intended when this study was originally designed.

To follow-up the EMSAs in this study, a new approach is recommended. The current method used SYBR green nucleic gel stain to detect DNA after separation by electrophoresis. Using radiolabelled DNA probes would increase the sensitivity of this assay and allow the addition of controls such as non-specific and specific DNA competitors. Non-specific competitors would help to sequester any co-purified proteins in a sample, which is recommended due to the co-purification of a doublet during AcrR and EnvR purification (Chapter 4, Figure 4.4). Specific probes could also be added to help determine the specificity of the purified protein to the target DNA. These controls are important because transcription factors are DNA-binding and the concentration of these proteins is tightly controlled (and varied) in cells (Ishihama, Kori, Koshio *et al.*, 2014). Furthermore, multiple factors including promoter strength, affinity of a given regulator for a target and the concentration of the transcription factor (local to the

target) and presence/absence cofactors all determine whether binding occurs or not (Alon, 2007; Balleza, Lopez-Bojorquez, Martinez-Antonio *et al.*, 2009)It is therefore possible that higher concentrations of protein, or the presence (or absence) of other normally-occurring factors can influence the binding of AcrR and EnvR to their targets. Using radiolabelled probes would increase the confidence in this data, as some of these additional controls such as competitor DNA, could then be added. An example of an EMSA using radiolabelled DNA probes is shown in Figure 7.1.

Firstly, the interruption or overexpression of *acrR* or *envR* influenced the transcription of multiple target genes (Chapter 5). Overexpressing *acrR* or *envR* also influenced the transcription of some genes which they could not bind upstream of. To better understand whether these are true targets of AcrR and EnvR, ChIP and RNA-sequencing in strains which lack or overexpress *acrR* and *envR* should be done. The combination of ChIP and RNA-Seq data would provide information on the number of locations that AcrR and EnvR regulate in the genome but also the total changes in all gene transcription in these backgrounds. This should also be investigated in an Δ*acrB* background, as this would explore which of these regulatory effects are due to *acrR* and *envR* in isolation of their known target *acrB*. In combination with further EMSA data to confirm binding of AcrR/EnvR to the potential targets identified in this study, RNA-Seq and ChIP-Seq could explore whether the binding of AcrR/EnvR to these targets has any transcriptional effect. The advantages and limitations of these methods are discussed in Table 7.1. Although the binding data in this study is weak, there is some evidence provided by expression and phenotypic data to suggest that some of the weak binding observed warrants further investigation.

Figure 7.1: A set of example EMSA assays taken from Shimada *et al.*, 2008

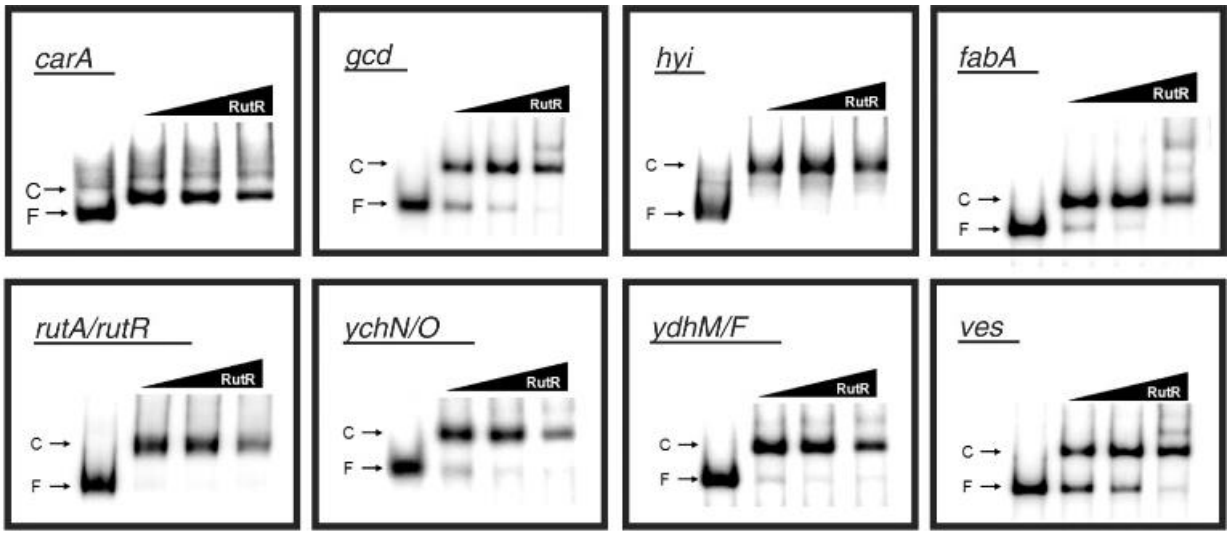


Figure 7.1. A selection of EMSA assays presented in by Shimada *et al.* using ^{32}P labelled DNA probes. This is an excerpt of Figure 3 in "*The Escherichia coli RutR transcription factor binds at targets within genes as well as intergenic regions"* (Shimada *et al.*, 2008). In this example, the EMSA is used to determine binding of RutR to multiple targets. The first lane on each gel is a negative control, showing free DNA, indicated by the arrow labelled F. If RutR has bound the target DNA, the band is shifted up, indicated by the arrow labelled C. For *gcd*, *fabA*, *rutA/rutR*, *ychN/O*, *ydhM/F* and *ves*, increasing concentrations of RutR decrease the amount of free DNA, indicating that more of the free DNA is bound to RutR. The samples were also incubated with 12.5 µg/ml herring sperm DNA to prevent binding to the target DNA of any additional proteins present, despite the RutR protein purification being over 95% pure (Shimada *et al.*, 2008).

Table 7.1: Comparison of EMSA, ChIP and RNA-Seq

Method	Advantages	Limitations	References
EMSA	• If using radiolabelled probes, can be highly sensitive and show specific binding of protein to target DNA. • Quick and relatively simple once optimised. • Binding affinity can be calculated.	• Requires knowledge of potential targets to generate target DNA, so not able to detect unknown targets. • Results can vary depending on gel composition and running conditions, which must be optimised before assays are started. • Can not give information on binding sites or residues important for binding. • Binding does not equal biological role – more assays are required to determine this.	(Garner, 1986; Hellman & Fried, 2007)
ChIP-Seq	• Reveals binding sites on a genome-wide level. • Does not require prior knowledge of targets, so is unbiased. • High resolution. • Can compare data between different background strains or treatments.	• Expensive and technically challenging. • Binding alone does not demonstrate a biological role or confirm that binding can happen in vivo.	(Myers, Park, Beauchene *et al.*, 2015; Park, 2009)

RNA-Seq	• Does not require prior knowledge of targets, so is unbiased. • Can show gene expression differences between different background strains or treatments. • High resolution.	• Expensive • Similar to ChIP-seq, the presence of a transcript does not equal biological role. • Changes in transcription do not infer whether the regulation is direct or indirect.	(Croucher & Thomson, 2010)

Table 7.1: The advantages and limitations of EMSA, ChIP and RNA-sequencing for determining binding and regulation by transcription factors.

Induction of *acrAB* and *acrEF*

The *acrEF* genes are silenced in *Salmonella* by H-NS (Nishino *et al.*, 2009) but mutations conferring *acrEF* overexpression are selected in Δ*acrB* backgrounds (Jellen-Ritter & Kern, 2001). As the AcrEF-TolC system is believed to export some of the same substrates as AcrAB-TolC, overexpression of *acrEF* can cause multi-drug resistance (Nishino & Yamaguchi, 2001). The fact that the *acrEF* and *envR* genes are H-NS silenced is likely due to the high GC% content of these genes. Genes with a higher GC% content are silenced in *Salmonella* and are thought to have been acquired by another species with higher GC% content, such as *Shigella* spp. (Ali *et al.*, 2014; Navarre, Porwollik, Wang *et al.*, 2006). The origins of the *acrEF-envR* genes may have been a duplication event of the *acrRAB* operon- or vice versa, which would account for the broad substrate overlap of these systems (Anes *et al.*, 2015). The AcrEF system may also act as a 'spare parts' system for AcrAB. This is in part supported by evidence that the periplasmic adapter protein AcrE can replace AcrA to complex with AcrB and TolC (McNeil *et al.*, 2019; Smith & Blair, 2014). If either the *acrAB* and *acrEF* genes exist due to a duplication event, this would explain both the similarity of the proteins and the redundancy between their components. As *acrEF* overexpression can be seen in isolates even when AcrAB is functional, this suggests a distinct role for AcrEF (Chetri *et al.*, 2018).

Before this study, it was also not clear whether H-NS repression would need to be overcome prior to the initiation of *acrEF* transcription. Interrupting *hns* did increase the induction of *acrE* transcription, but this was not further increased by interrupting *acrB* and the levels of the induction of *acrE* transcription were much lower than that for *acrA* in all genotypes. Even though interrupting *hns* increased induction of *acrE*

transcription, induction was also possible in a wild-type background with the addition of rhodamine 6g. However, the induction of *acrE* transcription in the *hns::aph* background was also not inducible by rhodamine 6g to the same levels of wild-type. Rhodamine 6g also increased induction of *ramA* transcription and prevented the binding of EnvR upstream of *acrEF.* Rhodamine 6g can bind RamR, the negative regulator of *acrAB* activator RamA (Yamasaki *et al.*, 2013). Therefore, the induction of *acrEF* transcription may involve not only the removal of H-NS and local regulators, but also interactions with RamA.

The test for inducers of *acrA* and *acrE* transcription revealed that not all substrates of AcrAB-TolC could induce transcription of these genes. This data also did not always match up with the ligand binding data. For example, proflavine prevented the binding of EnvR to the promoter of *acrAB* and *acrEF* at a concentration of 77 µg/ml, but proflavine induced transcription of *acrE* but not *acrA.* Proflavine also reduced the time taken for ethidium bromide to be extruded from both wild-type and Δ*acrB* cells, indicating that proflavine had by some mechanism, increased the rate of ethidium efflux. There were also some differences in the induction data depending on the method used. Indole is a known substate of AcrAB-TolC and induced *acrA* transcription in a continuous-measurement 96-well plate method, but not in the single time point experiments. Therefore, although much can be taken from these experiments, the induction of these systems is complex and is likely to vary depending on multiple factors. For this reason, a lack of induction by a condition or compound does not categorically rule it out as an inducer of these systems. Some of the differences seen may have also been due to the actions of other efflux systems in addition to AcrAB and AcrEF. To continue to investigate the roles of AcrAB and AcrEF, these experiments

could be repeated in a background strain which did not have any other RND efflux genes. In this way, the effects of each inducer could be attributed to an individual RND system. The induction of *acrA* varied when testing conditions which mimic *Salmonella* infection environments, such as glucose % and magnesium and phosphate limitation. Although the induction of *acrE* was not possible in these experiments, testing more of these conditions in combination may induce *acrA/acrE* and may start to unpick the natural roles of these efflux systems.

Some, but not all, of the substrates of AcrAB-TolC were able to prevent the binding of EnvR upstream *acrAB* or *acrEF* (Chapter 5). However, some substrates did not influence binding and others (tetracycline and novobiocin) only inhibited binding at high concentrations. Tetracycline and novobiocin also decreased the induction of *acrA*, *acrE* and *ramA* transcription. It is possible that at high concentrations, the binding of these compounds to AcrR/EnvR is altered in a way which changes the confirmation of the proteins. Single mutations of TetR have been shown to cause the functionality of this protein to switch and require tetracycline for binding to the *tetA* promoter (Kamionka *et al.*, 2004). However, the fact that *ramA* was also decreased indicates a further interaction between RamR and these compounds.

The data presented in this study shows that the roles of AcrR and EnvR may extend beyond the regulation of *acrAB* and that single-target regulation by TFTRs should be confirmed, not assumed. The EMSA data in this study is weak and should be repeated using a new method such as radiolabelling DNA. Further follow-up of these results with RNA and ChIP-sequencing will verify whether the DNA:protein interactions are likely to be real. Understanding the full roles of TFTRs is especially critical for understanding efflux-mediated resistance. For example, our understanding of the positive regulation

of *acrAB*, involving a multitude of regulators which bind the same degenerate sequence, should be considered for negative regulators. If AcrR and EnvR can bind multiple targets, the orchestration of RND efflux gene expression may be considerably more complex than is already known.

8.0: Publications resulting from this study

AL. Colclough, J. Scadden and J. M. A. Blair. TetR-family transcription factors in Gram-negative bacteria: conservation, variation and implications for efflux-mediated antimicrobial resistance. BMC Genomics, October 2019, issue 20.

AL Colclough[1], I. Alav[1], E. Whittle[1], H. Pugh[1], E. Darby[1], S. Legood[1], H. McNeil and J. M. A. Blair. RND efflux pumps in Gram-negative bacteria; regulation, structure and role in antibiotic resistance. Future Microbiol., January 2020, issue 15.

H. Pugh, E. Darby, **AL. Colclough**, S. Dunn, C.Connor, V.Bavro, A.McNally, J.M.A. Blair. *Escherichia coli* possesses a seventh RND efflux pump system. *In preparation.*

9.0: Appendix 1

1A Strains used in Chapter 3

Supplementary Table 1:

Organism taxid	Strain	Size (Mb)	Number TFTRs
K. pneumoniae			
507522	342	5.92025	27
1420013	30684/NJST258_2	5.41722	22
1432561	IS39	4.51898	14
1432553	IS46	4.67912	15
1432558	ISC21	4.35834	14
272620	ATCC 700721 / MGH 78578	5.69489	23
667127	ATCC 13884	5.45003	21
1269006	909957	5.63755	22
1432552	IS43	4.84219	13
P. aeruginosa			
381754	PA7	6.58834	36
1262663	VRFPA01	6.99164	31
208963	UCBPP-PA14	6.53765	40
1350465	VRFPA03	7.03773	39
1402545	BL04	7.33338	45
E. coli			
585055	55989	4.98988	14
566546	ATCC 9637	4.90096	13
469008	BL21-DE3	4.55895	15
749527	MS 21-1	5.30899	15
409438	SE11	5.15563	15
439855	SMS-3-5	5.21538	16
1281200	3162-1	5.2556	15
364106	UTI89	5.17997	12
1444132	1-110-08_S3_C1	5.52006	14
511145	k-12	4.64165	13
E. albertii			
502347	TW07627	4.74659	10
550693	B156	5.02305	11
1440052	KF1	4.70188	10
E. fergusonii			
585054	ATCC 35469	4.64386	10
981367	ECD227	4.86611	9
***S.* Typhimurium**			
85569	DT104	5.02766	13

1218144	STm2	4.95114	13
909946	4_74	4.95138	13
588858	14028s	4.9641	13
216597	SL1344	5.06745	13
Other *S. enterica* serovars			
1192586	2009K0958	4.70517	12
1192688	UC16	4.85203	12
476213	RKS4594	4.88849	13
1395108	CVM N6509	4.59701	12
321314	SC-B67	4.944	14
54736	ATCC 43975	4.46011	13
1079477	BAA1594	5.58808	13
1439843	CVM N32599PS	4.58901	13
423368	SL254	4.71746	13
295319	ATCC 9150	4.58523	13

Supplementary Table 1: Strains used for bioinformatic analyses in Chapter 3.

1B Strains lacking *eefR* also lacked *eefAB* efflux genes

Supplementary Table 2:

Strain ID of strains lacking *eefR*	Sequence >80% identical or positive to *eefA*	Sequence >80% identical or positive to *eefB*
585055	No	No
566546	No	No
469008	No	No
409438	No	No
1444132	No	No
511145	No	No
585054	No	No
981367	No	No

Supplementary Table 2: Strains lacking *eefR* also lacked the *eefA* and *eefB* genes.

Sequences of *eefA* and *eefB* from *E. cloacae* used to identify the above sequences:

>tr|A0A0H3CCT9|A0A0H3CCT9_ENTCC Multidrug efflux periplasmic linker protein EefA OS=Enterobacter cloacae subsp. cloacae (strain ATCC 13047 / DSM 30054 / NBRC 13535 / NCDC 279-56) OX=716541 GN=eefA PE=3 SV=1

MMKKITTSIAALLLLTGCDNAQTSAPQRPLPEVGIVTLMSQPVSVVSELTGRTTAAM
SAEVRPQVGGIIQKRLFTEGDTVKAGQALYQIDPSSYRAAFDEAAAALKQAQALVQA
DCQKARRYAQLVKDDGVSRQDAEDAKSTCAQDKASVESKKAAQESARINLNWTTV
TAPIAGRIGISSVTPGALVTAQQDTALATIRGLDTMYVDLTRSSADLLRLRKQTLASN
SDTLSVTLQLEDGSTYSEKGRLALTEVAVDESTGSVTLRAVFPNPQHQLLPGMFVR
ARVDEGIMNDAILAPQQGITRDAKGKATALVVNASNKVEQRQLETGDTYGDKWLVL
SGLKAGDRLIVEGT DKVTAGQQVKAEEMKSSGGNA

>tr|A0A0H3CGE3|A0A0H3CGE3_ENTCC Efflux pump membrane transporter OS=Enterobacter cloacae subsp. cloacae (strain ATCC 13047 / DSM 30054 / NBRC 13535 / NCDC 279-56) OX=716541 GN=eefB PE=3 SV=1

MFSRFFVRRPVFAWVIAILIMLAGILAIRTLPVAQYPDVAPPSIKISATYTGASAQTLEN
SVTQVIEQQLTGLDNLLYFTSTSSSDGSVSITVTFEQGTDPDTAQVQVQNKVQQAE
SRLPTEVQQSGITVEKSQSNFLLIMGVYDKTDTASSSDIADWLVSNMQDPLARVDG
VGSLQVFGAEYAMRIWLDPAKLASYSLMPSDVQSAIEAQNVQVSAGKIGALPSSNA
QQLTATVRAQSRLQTVDQFKNIIVKSQSNGAVVHISDVARVEMGSEDYTSTAKLNG
HPAAGMAVMLSPGANALNTATAVKEKIAEFKKSMPEGYDVAYPKDSTEFIKISVEDV
IQTLFEAIILVVVVMYLFLQNIRATLIPALAVPVVLLGTFGVLALFGYSINTLTLFAMVLAI
GLLVDDAIVVVENVERIMRDEGLPAREATEKSMGEISGALVAIALVLSAVFLPMAFFG
GSTGVIYRQFSVTIISAMLLSVVVALTLTPALCGSILSHTAPHKKGFFGAFNRFYSKTE
HGYQNKVLRALRRSGGMLVIYVLLCGAMGFAMLKLPGSFLPTEDQGEIMVQYTLPA
GATSTRTAEVSRQVREWFLTKEKANTNVIFTIEGFSFSGSGQNAGMAFVSLKNWSE
RKGDENTAQAIALRATQELSTIRDATIFAMTPPAVDGLGQSNGFTFELMASGGTDR
DTLLKLRNQLIGEANQDASLHAVRANDLPQMPQLQVDIDNNKAVSLGLSLSDVTDTL
SSAWGGTYVNDFIDRGRVKKVYIQGDSDYRAVPSDLNKWYVRGSDSTMTPFSAFA
TTRWEYGPESLVRYNGSAAYEIQGENASGASSGTAMSKMEQLANNLPSGSTWAW
SGLSLQEKLASGQAMSLYALSILVVFLCLAALYESWSVPISVILVIPLGVLGAAIAASL
RGLNNDVYFQVALLTTIGLSSKNAILIVEFAEAKVAEGYSLTRAALRAAQTRLRPIIMT
SLAFIAGVTPLAIATGAGANSRVAIGTGIIGGTLAATLLAIFFVPLFFVLVKRLFSGKHS
NRRS

1C Non-functional or missing genes identified in *Escherichia* and *Salmonella*

- The *acrF* gene was not present in *E. coli* 9 (taxid:439855) and *E. albertii* (*taxid:502347).*

- A truncated *acrE* gene was present in *S.* Dublin (taxid:1192688) gene accession: EMR51234.1

- *S.* Choleraesuis had both a truncated *acrR* (AAX67232.1) *and acrE* (AAX67233.1). *S.* Typhimurium (*taxid:*85569) had truncated *envR.*

10.0: References

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