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Biomedical Sciences

MScR in Pathway Medicine

Aishwarya Saxena

S1461865

Principal Investigator: Dr Thamarai Schneiders

Project Title:

To establish the extended regulon of the RamA regulator by performing ChIPseq.

Declaration

The thesis has been composed by the candidate, Aishwarya Saxena. The work is the candidate's as a member of the research group, and it has been made to contribute to the field of chromosomal antibiotic resistance regulation in *Klebsiella pneumoniae*.

The work has been submitted in full requirement for the MRes in Biomedical Science (Pathway Medicine) and not for any other degree or professional qualification within or outside the University

Name: Aishwarya Saxena

Date : 07 August 2019

Signature

Abstract

RamA is a transcriptional regulator that has shown to regulate antimicrobial resistance in *Klebsiella pneumoniae*. Upregulation of RamA has been associated with resistance towards a broad range of antibiotics. Efflux pump assembly AcrAB and the outer membrane porin, OmpF are key determinants of antimicrobial resistance and their expression is modulated by transcriptional regulator, RamA. MarA, a better-known homologue of this AraC/XylS family member, have also shown decreased susceptibility to antibiotics via control of expression of AcrAB, OmpF in addition to genes controlling other cell functions such as DNA repair. As RamA is a key regulator that aid the nosocomial pathogen, *K pneumoniae* in developing resistance there is interest in determining cell functions other than membrane permeability that are under RamA's control and are responsible for the pathogen's ability to resist broad spectrum of antibiotic classes. This project aims to perform a transcriptomic study on RamA to unravel new DNA binding sites of this regulator across the genome of *K pneumoniae* utilising ChIPseq.

Lay Summary

Resistance to clinically available antibiotics is a growing concern all across the globe. The rise of infectious pathogens that are not easily treatable make routine surgical procedures dangerous. *Klebsiella pneumoniae* is one of the most common infectious pathogens. Like other bacterial pathogens, it is able to resist the effect of antibiotics by either acquiring foreign DNA material or by mutation of its own. Many of it's clinical isolates have shown a mutation that leads to upregulation of a transcriptional regulator, RamA.

In addition to *K pneumoniae*, RamA, a global transcriptional regulator, has shown to regulate antimicrobial resistance in other gram-negative pathogens such as *Enterobacter aerogenes, Enterobacter cloacae, Salmonella enterica Paratyphi B* etc.

Transcriptional regulators like RamA function by associating with the promoter regions of genes and recruiting the transcriptional machinery to activate transcription. Genes that are known to be under the control of RamA are the efflux pump assembly AcrAB that actively pump out antibiotics and micF, a negative regulator of outer membrane Porin, OmpF which acts as a channel for antibiotic entry. Hence, RamA mediates antibiotic resistance by increasing efflux pump assembly and decreasing Outer Membrane Porin F, OmpF.

However, homologues of RamA, such as MarA and SoxS have demonstrated that they are able to confer decreased susceptibility to antibiotics and it is not limited to regulation of outer membrane permeability.

Therefore, there is keen interest in determining similar RamA regulated genetic determinants that are possibly responsible for making infectious pathogen such

as *K pneumoniae* key in disseminating resistance to other gram-negative pathogens.

ChIPseq (Chromatin Immunoprecipitation followed by sequencing) is a platform used to study Protein-DNA binding associations. This project aims to perform a transcriptomic study on RamA to unravel new DNA binding sites of this regulator across the genome of *K pneumoniae* utilising ChIPseq. Further studies into these DNA targets and their effect on antimicrobial resistane will underline the gene regulatory network regulated by transcriptional factor, that results in decreased susceptibility.

This technique relies on antibody against the protein of interest. Since RamA doesn't have an antibody that can aid in its immunoprecipitation, this project attempts to make an expression plasmid expressing RamA with a suitable tag. This study also attempts to validate two targets that were identified in the previous RamA ChIPseq analysis by using qPCR and Green Fluorescence Protein (GFP) gene fusion assay (Bharathwaj, M.A., 2018).

Section 1: Acknowledgements

I would like to begin by extending my gratitude to the University of Westminster for entrusting me with the Ken Bird Memorial Scholarship (2011). I owe my career and educational achievements to the educational, pastoral and financial support provided by the University and International Student's House. I would like to thank the staff members of Scindia Kanya Vidyalaya (Scindia Girl's School) who are responsible for nurturing my curiosity in biology since a very early age.

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Section 2: Content

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Section 3: Background

3.1. History of Antibiotics and Resistance

Penicillin produced by *Penicillium* was first discovered by Sir Alexander Fleming in 1928. Produced as a secondary metabolite, it was responsible for a zone of growth inhibition of *Staphylococci* (Fleming, 1929) . Advances in biotechnological processes made large-scale production of penicillin possible revolutionizing treatment of infection during World War II (Figure 1). Many antimicrobial agents have since been discovered to treat infections that help eradicate or inhibit growth of pathogens. The use of such antimicrobial agents such as antibiotics (against bacteria) or antifungals (against fungi), to treat infections is one of the major advances in the field of medicine that has made surgical procedures and transplants a routine procedure. Other than the field of healthcare, antibiotics are also widely used as prophylaxis treatment in animal husbandry.

The introduction of a new antibiotic in the clinical or agricultural field has in parallel followed the evolution and dissemination of pathogens resistant to its action (Figure 2). The constant race between the discovery and improvement of new antibiotic classes against emerging resistant pathogens is further fueled by the unnecessary, indiscriminate or incorrect use of antibiotics.

Drug resistance is the mechanism by which pathogens evolve or acquire pathways to evade the effect of an antibiotic. Emergence of resistance towards multiple antibiotics is a common phenomenon due to the high selective pressure faced by these pathogens in hospital settings. Development of multi drug resistant (MDR) pathogens that can evade the effect of broad range of antibiotic classes makes routine medical procedures life threatening. There are limited treatment options for such infections, with fewer antibiotics available and the antibiotics which are available being more toxic, ultimately resulting in a less efficacious treatment. Therefore, multiple drug resistance bacteria is a major

public health concern that raises questions about the future of healthcare and also about national security and international political stability in the post-antibiotic era due to its rapid dissemination.

Figure 1: Life Magazine advertisement of Schenley Laboratories, Inc from August 1944. This advertisement highlights how penicillin was considered a "wonder" drug at the time of its introduction. It highlights the challenge faced in the post-antibiotic era and how the development of antibiotics steered healthcare in the as place we know today (Schenley Laboratories, 1944)

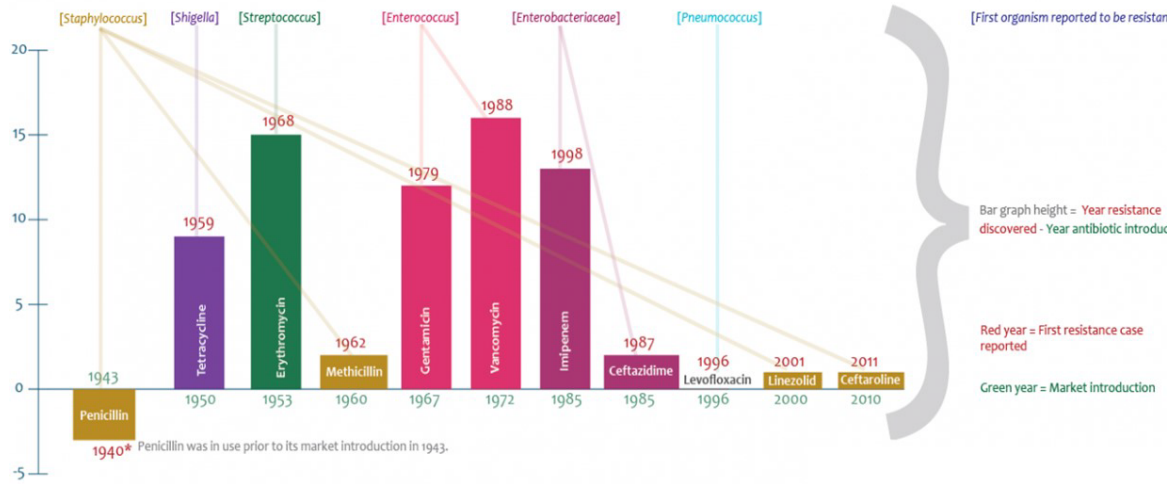


Figure 2: Development Antibiotic resistance: Image illustrating the early reports of resistance in literature compared to the introduction of the drug. Note: In 1943, Penicillin become widely used, even though it was first discovered in 1929 followed by which it was used limitedly until the World War II. Image taken from The Centre for Disease Dynamics Economics & Policy (CDDEP), First reported cases of bacterial resistance against key antibiotics (CDDEP, 2018).

3.2 Multi Drug Resistant - Hospital Acquired Infections

Hospital Acquired or Healthcare Associated Infections (HAI) are often caused by opportunistic pathogens which when confronted with a host that has weakened immunity, manifests into an infection. In hospitals, patients that are more susceptible to such pathogens are those suffering immunodeficiency due to diabetes or HIV, those undergoing routine healthcare interventions like gut surgery, caesarean sections, treatment for cancer, organ transplantation etc. or individuals with disrupted microbiota. Due to their exposure to clinically relevant

antibiotics, these pathogens are routinely under selective pressure to evolve and often result in multiple drug resistant – hospital acquired infections (MDR-HAI). These infections can be so rampant that in many occasions they have led to force closures of hospitals and intensive care unit (Macrae *et al.*, 2001; Wilks *et al.*, 2006; Brahmi *et al.*, 2007; Laurent *et al.*, 2008). The economic cost of HAI was reported to be €1.5 billion in the EU and $5 billion in the USA in 2015 (Pendleton, Gorman and Gilmore, 2013) and this has been estimated to rise to $100 trillion by 2050 (O'Neill, 2016). In 2011, the Centres for Disease Control and Prevention (CDC) reported death of 75,000 patients due to HAI in the US (Magill *et al.*, 2014). The O'Neil commission has estimated that if this current situation persists, the number of deaths caused by these resistant pathogens could rise to 10 million by 2050 (O'Neill, 2016).

World Health Organization (WHO) (WHO, 2001) , the European Centre for Disease Prevention and Control (ECDC/EMEA Joint, 2009) and the Infectious Diseases Society of America (IDSA) (Talbot *et al.*, 2006) have identified ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes* and the newly identified *Stenotrophomonas maltophilia*) as a group of bacterial pathogens responsible for the majority of the MDR HAI. These Gram-positive and Gram-negative nosocomial pathogens are exposed to therapeutic doses of antibiotics in hospitals. Under this selective pressure they spontaneously develop or acquire resistance for their survival under sub-therapeutic dosages of antibiotics. Hence, these bacteria are able to escape the biocidal action of antibiotics by acquiring or evolving new modes of resistance and pathogenesis (Rice, 2008; Boucher *et al.*, 2009; Pendleton, Gorman and Gilmore, 2013). Of these, the Gram-negative members of *Enterobacteriaceae* family, *Klebsiella pneumoniae* and *Acinetobacter baumannii*, are well known for their notoriety in developing multiple resistance to most commonly used antibiotics (Navon-

Venezia, Leavitt and Carmeli, 2007; Hussein *et al.*, 2009). Isolates from *Acinetobacter spp* and *K. pneumoniae* species that are resistant to all available antibiotics, rely on treatment options that are suboptimal, toxic and use abandoned ancient drugs such as polymyxins and colistin.

3.3 *Klebsiella pneumoniae*

Amidst the *Enterobacteriaceae, K. pneumoniae* is one of the leading causative pathogens of MDR-HAI. Infection caused by this pathogen can lead to liver abscesses, pneumonia, bloodstream and urinary tract-infections in new-borns and intensive-care unit patients (Gorrie *et al.*, 2017). Between 2011 to 2014, the European Centre for Disease Prevention and Control reported that *K. pneumoniae* displayed a significant increase of individual and combined resistance to fluoroquinolones, third-generation cephalosporins and aminoglycosides (European Centre for Disease Prevention and Control., 2014). WHO advised carbapenems as the next in line treatment but in some parts of the world (India, Greece, Belarus and Egypt) more than half of the pathogenic *K pneumoniae* isolates are carbapenem resistant [Figure 3 (Geneva: World Health Organization, 2017; The Center for Disease Dynamics Economics & Policy., 2018; World Health Organization, 2018)]. The last resorts for these Carbapenem Resistant *K. pneumoniae* are Tigecycline and Colistin. However, resistance to both these drugs have already been reported in *K pneumoniae* (Sheng *et al.*, 2014; Halaby *et al.*, 2016; de Man *et al.*, 2018).

MDR conferring genetic elements to carbapenems KPC, OXA-48 and NDM-1 first emerged in the *K. pneumoniae* before disseminating to other pathogens (Wyres and Holt, 2018) . Due to this *K. pneumoniae* has been regarded as the key amplifier and spreader of MDR genes from environmental sources to pathogens (Wyres and Holt, 2018).

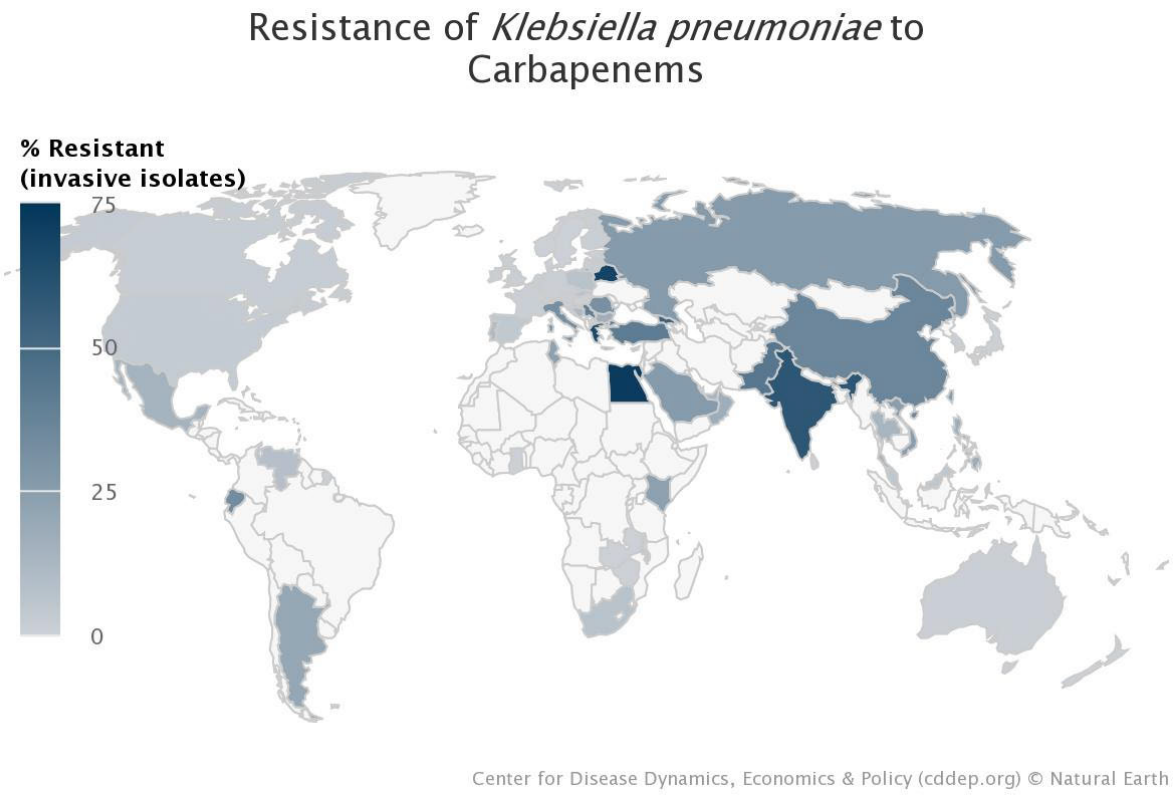


Figure 3: Figure showing the prevalence of Carbapenem resistant *K pneumoniae* isolates across the world. Image taken from CDDEP, The Centre for Disease Dynamics Economics & Policy, Washington New Delhi. Image take from (CDDEP (1), 2018)(The Center for Disease Dynamics Economics & Policy., 2018)

3.4 Mechanisms of MDR

In order to resist the effect of antibiotics, bacteria have evolved many mechanisms including modification of the drug target, inactivation of the drug itself, reduction in uptake and active efflux of the toxic compound (Figure 4) (Blair *et al.*, 2015). Resistant determinants conferring these phenotypes are either a result of endogenous chromosomal point mutations and/or are exogenously acquired in the form of foreign DNA. Acquired resistance mechanisms rely on acquisition of DNA by horizontal gene transfer by either conjugation, transduction or transformation whereas chromosomal mutations can occur spontaneously due to point mutations, gene duplications or gene deletion.

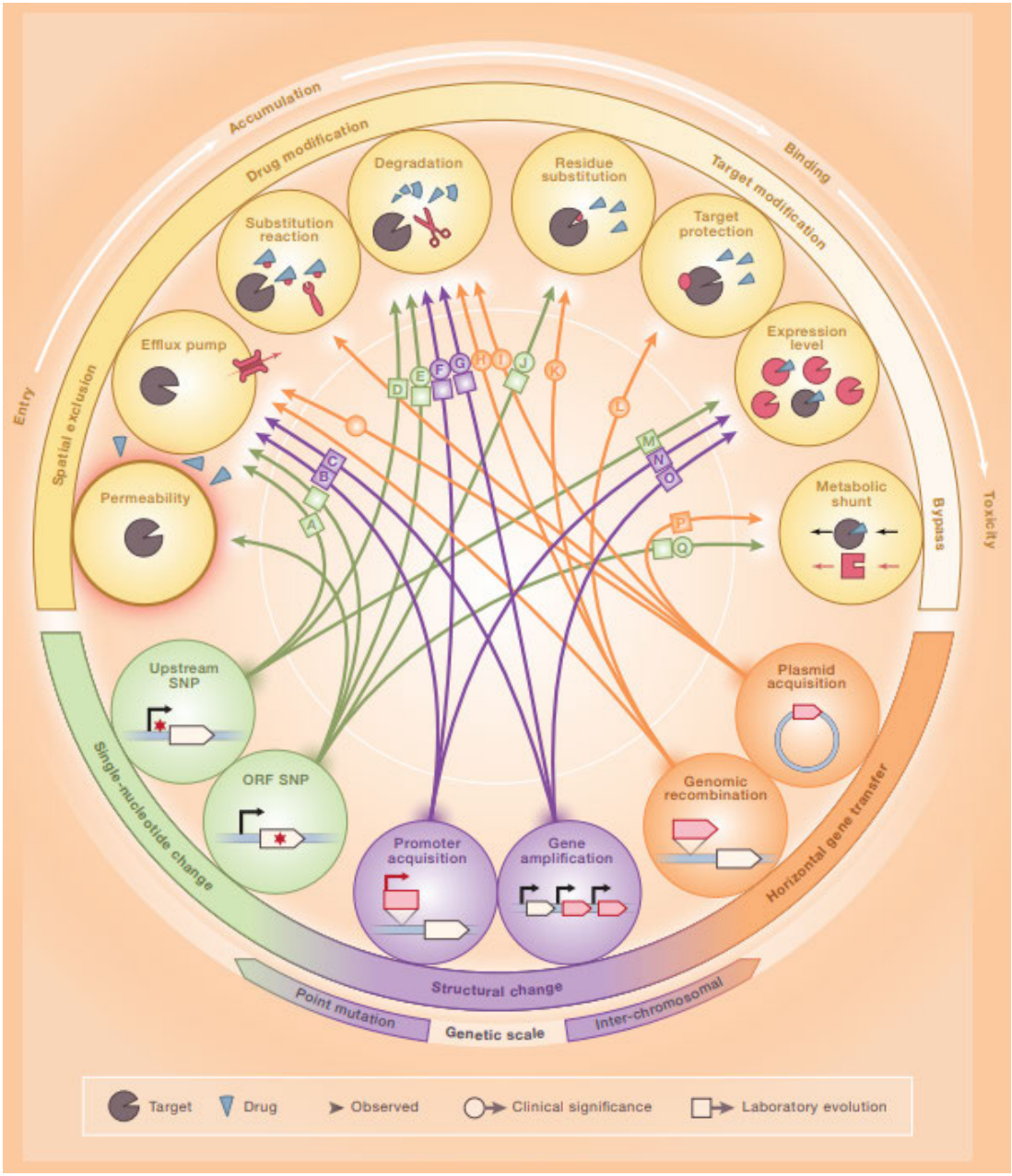


Figure 4: The image is taken from Cell Snapshot: Antibiotic Resistance (Yelin and Kishony, 2018). It)shows the genomic changes responsible for antibiotic resistance are Structural changes such as, i.e. point mutations (such as Single Nucleotide Polymorphisms, SNP's in the upstream promoter regions or in the gene (Open Reading Frame, ORF) itself, reassembly of genetic elements (by promoter acquisition or gene amplification). Foreign DNA material by acquired Horizontal Gene Transfer (HGT), by genomic recombination through integration in chromosome or maintaining them as extrachromosomal expression vectors such as Plasmids.

All these changes can lead to multiple mechanism of infliction resistance such as, inhibition of entry (Spatial exclusion by inhibition of entry), accumulation (decrease of Permeability by porins and efflux pumps, Drug modification by substitution or degradation reactions), target binding (by target residue substitution, target protection, change in expression levels or metabolic shunt that changes the functionality of the cell), inactivating proteins or downstream toxicity(circumventing the need of the pathway, Bypass). A: point mutations in promoter regions of a gene encoding permeability such as a pump, B: Promoter acquisition in front of a gene e.g. pump, C: gene amplification of the gene determining permeability, i.e. pump, D: point mutations leading to an increased transcription of a deactivating enzyme such as β lactamases present in the chromosome; E: point mutation in the inactivating gene itself , F: promoter acquisition in front of an inactive deactivating enzyme, G: gene amplification of such enzyme to increase its expression. H: Acquiring foreign DNAs encoded deactivating enzyme that integrates in the genome, I: Plasmid acquisition of such a gene, J: point mutation leading to changes in the residues of the target, K: Genomic recombination leading to changes in affinity of the drug to the target, L: horizontally acquired gene accessory protein reduces affinity of the drug, M: SNP's leading to increased expression of the target, N: genetic recombination in the promoter again leading to the target's overexpression, O: gene amplification of the target, P: acquisition of new metabolic pathway by HGT, Q: de novo changes in metabolic pathway.

The image above displays chromosomal mutations like *de novo* point mutations (in green) and rearranging of genetic materials (in purple) that appear frequently in laboratory conditions (squares) and Horizontal Gene Transfers (HGT) (orange) is responsible for acquisition of rapid resistance in hospitals and other communal environments (circles).

3.4.1. Transcriptional Regulation of Membrane Permeability

Reduced membrane permeability leads to reduced entry of toxic compounds into the bacterial cell and so is associated with MDR phenotypes (Cohen *et al.*, 1989; Abouzeed, Baucheron and Cloeckaert, 2008; Bailey *et al.*, 2010; Fernández and Hancock, 2012). There are several mechanisms by which bacterial can reduce

their membrane permeability. Reduction of membrane permeability caused by an overexpression of efflux pumps (e.g. AcrAB-TolC from the resistance nodulation division, RND) or downregulation of porins (such as OmpF) is one way of escaping the effect of antibiotics. Pathogens can acquire an additional efflux pump assembly encoded on a plasmid. Intrinsically, reduced permeability can also result from either mutation and/or loss of function in their respective local transcriptional repressor (AcrR, OmpR) or activation by global transcriptional regulator (Figure 5).

A few members of ESKAPE pathogens boast intrinsic regulation of their permeability by global transcriptional regulators such as MarA, SoxS, Rob, RarA, RamA (Pomposiello and Demple, 2000; Webber and Piddock, 2001; Martin and Rosner, 2002; De Majumdar *et al.*, 2013). Intrinsic resistance mechanisms like these, gives bacteria an innate ability to transiently resist antibiotics under sub-therapeutic concentrations. This increases the selective pressure tolerated by them, hence buys them more time to either acquire foreign resistant mechanism or promote chromosomal mutability, leading to higher level of resistance. Therefore, the expression of these global transcriptional regulators, induced or constitutive due to chromosomal mutations, assists in quick adaptability and survival under clinically used therapeutic levels of antibiotics that later assist in development of MDR (Oz *et al.*, 2014).

The expression of these can either be inducible under stress or is constitutive due to mutations in their locally repressor proteins (RamR, MarR). Upregulation of these transcriptional regulators results in low-level resistance to structurally unrelated compounds (Jacoby and Archer, 1991; Alekshun and Levy, 1997). Constitutive upregulation of these global transcriptional regulator has been observed in many clinical isolates.

RamA is one such transcriptional regulator first identified in *K. pneumoniae,* which when overexpressed, reduces antimicrobial susceptibility to structurally

diverse compounds (George, Hall and Stokes, 1995). It is also found in other Gram-negative bacteria like *E. aerogenes, Enterobacter cloacae, Salmonella enterica Paratyphi B, Citrobacter* (Yassien *et al.*, 2002; Chollet *et al.*, 2004; Ruzin *et al.*, 2005) but is absent in *E coli* and *Shigella* species.

3.5 Transcriptional Regulators

Transcriptional factors (TF) help bacterial cells to adapt and survive in different environments by coordinating changes in its transcriptional landscape under an environmental signal. Likewise, in enteric pathogens MarA like global transcriptional regulator belonging to the AraC/XylS family of TF are activated on exposure to stress agents such as antibiotics and illicit high level of resistance in these microbes.

Each transcriptional regulator has its own gene regulatory network which interconnects various cellular processes. TF does so by recognising a highly specific DNA sequence and depending on the signals and cofactors present will regulate the level of expression. Transcriptional factors would hence regulate expression of genes by recognition, binding followed by either activating or downregulating their expression.

3.5.1 AraC type regulators

Transcriptional regulators are accessory proteins that either activate or repress transcription from promoter region of a gene or a set of genes by either recruiting or interfering with the transcriptional machinery. AraC/XylS is a large family of such transcriptional regulators known for their ability to control expression of resistance, virulence, adherence and colonization by regulating transcription from their corresponding regulons (Gallegos *et al.*, 1997).

A subset of this family of regulators have demonstrated regulation of innate resistance mechanisms that is inducible under stress such as antibiotics, reactive oxygen species or toxic metal. MarA, SoxS, Rob, RarA, and RamA constitute a subset of these AraC regulators that are relatively smaller in size (100-160

residues) and are known for inflicting the inducible mar phenotype (multiple antibiotic resistance)(Hächler, Cohen and Levy, 1991; Cohen *et al.*, 1993; Cohen, Hächler and Levy, 1993; Cohen, Yan and Levy, 1993; Gambino, Gracheck and Miller, 1993). RamA like regulators are related to the N terminus region of AraC family of regulatory proteins (George, Hall and Stokes, 1995) and they bind to the DNA as monomers (Dangi *et al.*, 2001) to promote transcription. It has been suggested that they activate transcription from dispersed loci by assisting in the pre-recruitment of RNA polymerase that binds to the upstream of the promoters they regulate (Barbosa and Levy, 2000; Pomposiello, Bennik and Demple, 2001; Griffith *et al.*, 2002, 2009).

These closely related inducible regulators pleiotropically activate efflux and reduce outer membrane porin expression, conferring reduced susceptibility (Cohen, McMurry and Levy, 1988; Viveiros *et al.*, 2007; Fernández and Hancock, 2012). Their inducible nature assist the bacteria in responding to various environmental stress and stimuli e.g. oxidative stress by SoxS in *Escherichia coli* and *Salmonella* Typhimurium; antibiotics, organic solvents and heavy metals by MarA and Rob in *E coli* and RamA in *K. pneumoniae* (George and Levy, 1983; George, Hall and Stokes, 1995; Webber and Piddock, 2003).The resulting increase in survival of the pathogen under environmental stress such as antibiotics buys time for pathogens to accumulate other genetic determinants leading to high level of resistance.

The basal expression of these global regulators is maintained through the release of repression (activation in case of SoxS) by their corresponding repressor protein (Figure 5). Upon induction under antibiotics, reactive oxidative species or toxic metal stress, release of repression by their corresponding repressor RamR or MarR, or activator in case of SoxS by SoxR is observed(Gallegos *et al.*, 1997). In the absence of an environmental signal, these respective TetR type of repressors e.g. RamR or MarR would bind to the operator region of the AraC/XylS regulator,

i.e. RamA or MarA and inhibit its expression. Hence, induction or inactivation mutation in respective repressors of these RamA like regulators leads to their overexpression which activates the transcription of their regulon manifesting decreased susceptibility towards antibiotics.

MarA, RamA, Rob and SoxS have all demonstrated an overlapping regulatory effect, resulting in similar phenotypes and many genes are shared between their regulons. This has been explained by their ability to recognise a degenerate and asymmetric 20 bp region present in the promoters of genes called "*marbox/soxbox*" but with varied affinity (Martin *et al.*, 1999). Of these MarA in *E. coli* is the most well studied regulator that inflicts the mar phenotype by increasing expression of efflux and porin expression via *acrAB, tolC* and *micF* (down-regulator of OmpF) (Ruiz and Levy, 2010). Even though *in-silico* analysis of *marbox/soxbox* estimates about 10,000 potential binding sites for MarA, very few have been validated experimentally(Griffith *et al.*, 2002; Martin *et al.*, 2002). Various transcriptomic analysis of MarA in *E coli* has been attempted (Barbosa and Levy, 2000; Pomposiello, Bennik and Demple, 2001) but it was only recently that the role of MarA in regulating DNA repair and lipid trafficking was underlined(Sharma *et al.*, 2017). The regulation of these phenotypes explains the reduced susceptibility of MarA overexpressor to quinolones, tetracyclines and β lactams (Sharma *et al.*, 2017) and highlights their importance of these regulators in mediating MDR.

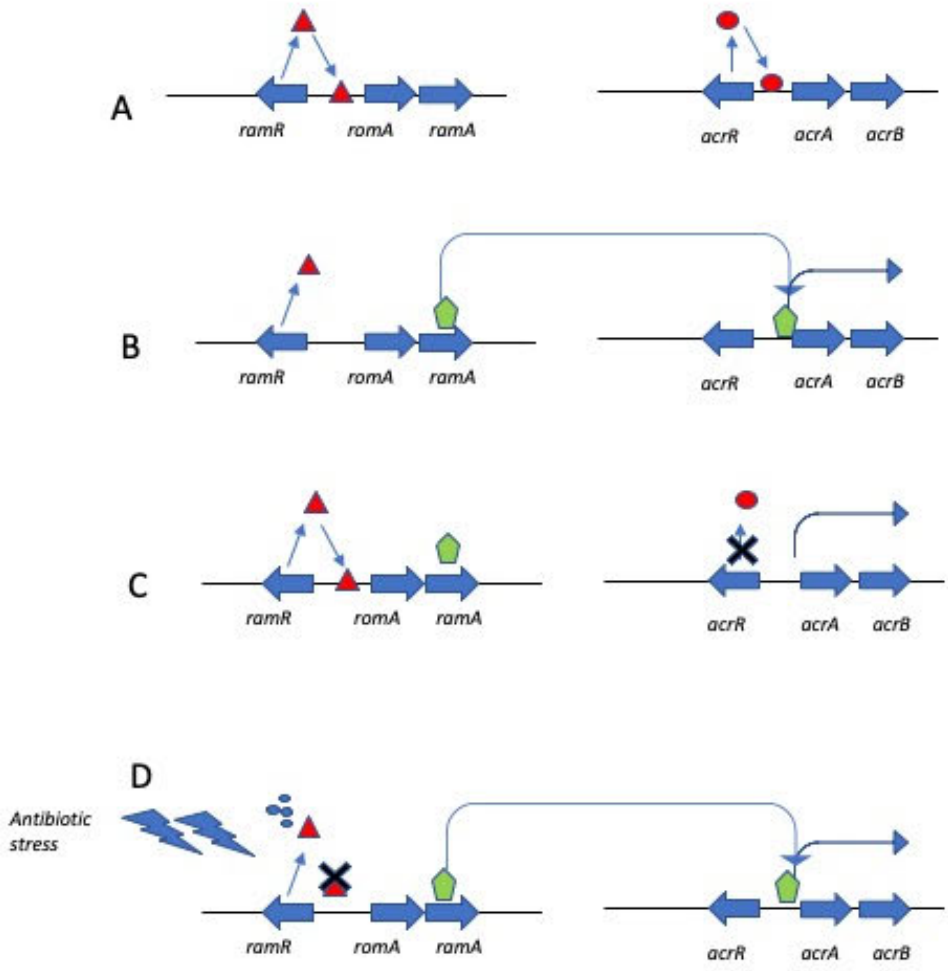


Figure 5: Regulation of Expression of RamA, an AraC type global regulator. Basal expression of AcrAB is maintained by its locally encoded repressor i.e. AcrR for *acrAB* (Red spheres). The basal expression of RamA (Green pentagon) is maintained by its cognate Tet-R type regulator, RamR(Red triangle) which further assists in regulating expressions of genes under the *ram* regulon, like the *acrAB*(A). Overexpression of RamA caused by the mutations or deletion of RamR will lead to an increased transcription of the efflux pumps (B). Activation of transcription from the *acrAB* operon will also occur due to mutations in AcrR (C). Section D shows the response of RamA under antibiotic stress which results in its upregulation

causing it to activate expression from its regulon, in this example, the *acrAB* operon. Only activated pathways are indicated. Adapted from (Blair *et al.*, 2015).

3.5.2 RamA as a regulator of MDR

RamA is an important AraC type regulator that plays a role in developing resistance in *K* pneumoniae, a gram-negative pathogen that is considered key disseminators of resistance. The ram locus has been identified in *K pneumoniae Ecl8,* where mutants of this locus have displayed decreased susceptibility to tetracycline, chloramphenicol, nalidixic acid, puromycin and trimethoprim (George, Hall and Stokes, 1995). Besides *K. pnuemoniae*, RamA is also found in other gram-negative bacteria such as *Salmonella enterica* Serovar Paratyphi B (rma gene)(Yassien *et al.*, 2002), *Salmonella enterica* Serovar Typhimurium(van der Straaten *et al.*, 2004), *Enterobacter aerogenes* and *Enterobacter cloacae* (Veleba *et al.*, 2013). In these gram-negative, RamA is a key regulator of MDR as compared to its other homologues such as MarA, SoxS, Rob and RarA (Nikaido, Yamaguchi and Nishino, 2008; Bailey *et al.*, 2010; Jiménez-Castellanos *et al.*, 2016).

Overexpression of this positive transcriptional activator from its locus has long been identified for its MDR phenotype. RamA renders *K. pneumoniae* and *S. enterica* less susceptible to tetracyclines, macrolides and β-lactams (Schneiders, Amyes and Levy, 2003; Ruzin *et al.*, 2005; Nikaido, Yamaguchi and Nishino, 2008; Bialek-Davenet *et al.*, 2011). More specifically, the role of RamA has been confirmed with conferring low-level resistance towards quinolones and β lactams in *K. pneumoniae*. RamA's overexpression has also been associated with reduce susceptibility to polymyxin and cyclic antimicrobial peptides, CAMPS (De Majumdar *et al.*, 2015). A recent study has further reconfirmed that RamA plays a role in regulating MDR giving rise to resistance against widely used antibiotics; cefotaxime, ertapenem, nalidixic acid and chloramphenicol in *K. pneumoniae*

(Zgurskaya *et al.*, 2018). Tigecycline resistant MDR clinical isolates of not only *K. pneumoniae* but also *E. cloacae* and *E. aero genes* (R Rosenblum *et al.*, 2011; Ricci, Busby and Piddock, 2012; Veleba *et al.*, 2013) have been reported with constitutive RamA overexpression.

In *K. pneumoniae* and *S. enterica.,* RamA is considered a primary regulator of MDR as compared to its homologue MarA which is more likely to be overexpressed in *E coli*. Furthermore, in *S. enterica* serovar Typhimurium, the role of RamA in inducing *acrAB* transcription is known to takes place independently of its counterparts such as MarA, Rob and SoxS (Nikaido, Yamaguchi and Nishino, 2008).

RamA regulates transcription like its well-studied homologue MarA in *E coli*, by activating the expression of dispersed loci in its genome. It promotes transcription (Nikaido, Yamaguchi and Nishino, 2008; Padilla *et al.*, 2010) in the promoter region of genes such as *acrA* . Upregulation of AcrA is a well known resistance mechanism and is also know to affect virulence (Padilla *et al.*, 2009). In addition to efflux pumps, RamA further regulates cell permeability by reducing the expression of outer membrane porin F, OmpF, via direct upregulation of *micF*, a small regulatory RNA that represses the transcription of *ompF* (Bailey *et al.*, 2010; De Majumdar *et al.*, 2015; Jiménez-Castellanos *et al.*, 2018). Therefore, RamA enables decreased antibiotic susceptibility in *K. pneumoniae* by reducing the permeability of the membrane through increased efflux by AcrAB and decreased influx due to lower OmpF (Figure 6).

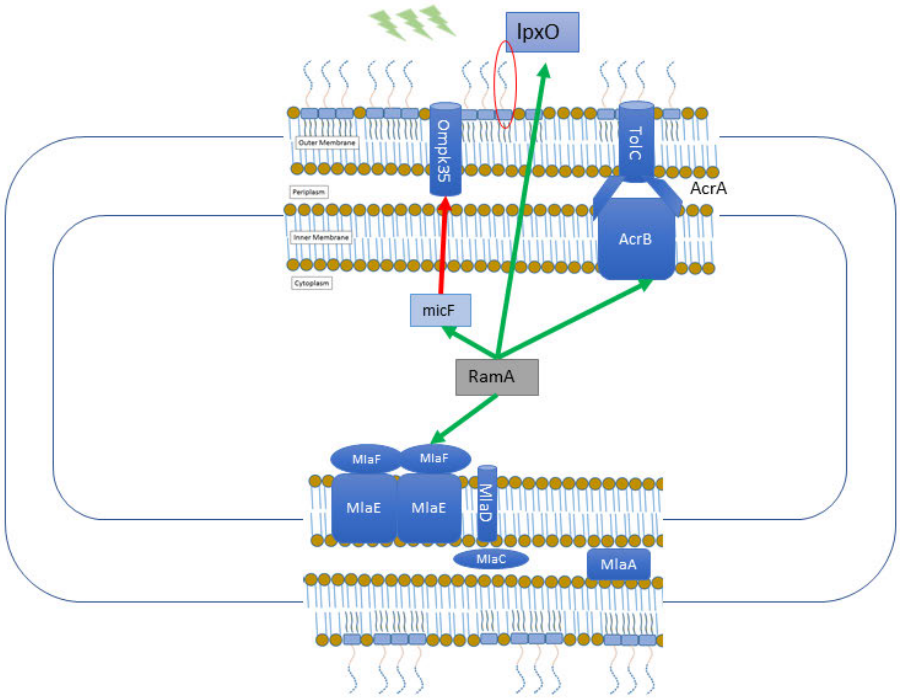


Figure 6: Image showing RamA controlling known elements of the Outer Membrane. RamA regulates the expression of Ompk35 (OmpF) by upregulation the expression of its repressor, *micF*, hence decreasing the expression of this outer membrane porin. RamA positively regulates efflux due to its well know activation of AcrAB, AcrZ. There is evidence of RamA regulation of lpxO, which modulates the lipid A moiety of the LPS membrane and has been suggested to be responsible for pathoadaptive response of this pathogen. Unpublished data from Schneiders lab also suggested that RamA is responsible for positively regulating the *mla* operon which is responsible for maintain OM asymmetry.

Other than regulation of OM permeability by directly activating expression of *micF* and AcrAB-tolC, there is evidence of RamA dependent regulation of another small inner membrane component, *ybhT* or *acrZ* (De Majumdar *et al.*, 2015). Mutants of *ybhT* in *E coli* are sensitive to a subset of antibiotics that are substrates for AcrAB-tolC, hence this gene enhances the ability of AcrAB-TolC in MDR (Hobbs *et al.*, 2012).

These findings emphasize the importance of RamA in conferring MDR in hospital and community acquired infections. However, the regulation of only outer membrane permeability by RamA is a limited explanation of this observed broad range resistance.

3.5.3 Extended regulon of RamA

The above section highlights the well-established role of RamA regulator in modulating various determinants in the outer membrane permeability. It does so by decreasing the expression of OmpF via micF, activating efflux by transcriptional activation of acrAB, and differentially expressing *acrZ* (*ybhT*) (Majumdar 2015). AcrZ is a 49-residue small protein that is a part of the periplasmic AcrAB complex that affects the substrate preferences(Du *et al.*, 2014). Other genes that are proposed to be regulated by RamA that overlap with the regulon of other AraC regulator like SoxS, MarA and Rob are yhbW, *nfnB* and *mlaB-F* (De Majumdar et al., 2015).

The *mla* operon has also been suggested to come under the direct regulation of RamA's homologue, MarA (De Majumdar *et al.*, 2015; Sharma *et al.*, 2017). The *mla* operon is composed of *mlaFEDCB* encoding an ABC transport system involved in lipid trafficking. This operon assists to maintain the characteristic asymmetric nature of the outer membrane (OM) in gram-negative bacteria that comprises of Lipopolysaccharide (LPS) in its outer leaflet and PL in the inner leaflet. The *mla* operon maintains this lipid asymmetry by removing phospholipid (PL) from the outer leaflet of the outer membrane (OM) (Malinverni and Silhavy, 2009). Disruption of this operon increases the permeability of the bilayer due to accumulation of PL's in the outer leaflet leading to a defective barrier function. *mla* mutants have shown increased

susceptibility to hydrophobic antibiotics like minocycline and doxycycline due to an increase in surface hydrophobicity (Sharma *et al.*, 2017).

NfnB is a minor oxygen insensitive nitroreductase and has been shown to be regulated by other members of the AraC family of regulators (Barbosa and Levy, 2002; Amábile-Cuevas and Arredondo-García, 2013). Its role in conferring resistance is not clear. However, a recent study has analysed the increased activity of the drug Nitrofurantoin in very short term under soxRS activation due to *nfnB* highlights this genes importance in MDR.

In *S eneterica* servoar Typhimurium RamA has also been suggested to be involved in regulation of genes belonging to Salmonella pathogenicity island 2(SPI-2) (Bailey *et al.*, 2010). However, De Majumdar et al., 2015, demonstrated that RamA mediated increased virulence in *K. pneumoniae* by lipid A modifications.

These changes manifests via lipid-A modulation that have been suggested because of recent established of regulating genes associates with lipid A biosynthesis (*lpxL2,* lipid A biosynthesis lauryl acyltransferase; *lpxC,* metalloamidase and *lpxO,* dioxygenas e protein encoding gene*)* by RamA. Therefore, other than reduced susceptibility to various classes of antibiotics such as polymyxin and colistin could be due to alteration in LPS moiety by RamA. In vivo studies have furthermore suggested that Rama also displays evasion from host immune system by reduced adhesion and uptake by macrophages (De Majumdar *et al.*, 2015). To conclude, in *K. pneumoniae* RamA seems to be controlling a plethora of factors such as like lipid A, pumps, porins and PL asymmetry that have been identified as key in the modulation of resistance, colonization, infection, and virulence (Martin and Bachman, 2018). Hence it outlines the major role played by this transcriptional

factor in regulating resistance, virulence and pathoadaptive responses in *K pneumoniae*.

Above mentioned findings have reiterated the importance of RamA in regulating genes that are key in survival of *K pneumoniae* under antibiotics stress and against host immune response. To fully comprehend how RamA employs other genes in the cell to survive antimicrobial stress, a detailed transcriptomic analysis of this regulator needs to be undertaken. RamA is a unique transcriptional regulator that can communicate with many cell functions and regulate their expression to promote cell survival making these pathogens highly resistant and in case of *K pneumoniae,* virulent. Furthermore, the effect of these genetic determinants under antibiotics will highlight the potential pathways that could be targeted for future antibiotic therapy.

3.5.4. Genetic Organisation RamA in *K pneumoniae*

The genetic organization of the *ram* locus in *K. pneumoniae* consists of *ramR* and *romA*-*ramA* genes that are divergently transcribed from the promoter, PI (Figure 7). Like other AraC/XylS family members, transcription of *ramA*-*romA* is negatively regulated by a TetR-type family regulator, RamR (De Majumdar *et al.*, 2015). RamR binds to a highly conserved 28 bp palindromic sequence (ATGAGTGcgtactCACTCAT) overlapping the promoter region of *ramA* and *romA,* IR in Figure 7 (Baucheron *et al.*, 2012) . Basal levels of RamA is maintained by the interaction of the 60nt sRamA5 interacting with RamR. sRamA5 is co-transcribed as a primary *romA* transcript and is cleaved to produce this small regulatory RNA which competes the binding of RamR to the pI promoter. RamA binding site is also present in the promoter region of *ramA,* demonstrating that it is capable of autoregulation (R Rosenblum *et al.*, 2011). Ligand-mediated or functional inactivation of RamR results in derepression of the transcriptional activator, RamA overexpression and regulates transcription from cognate promoters

(Yamasaki *et al.*, 2013). Constitutive overexpression of RamA due to loss of function of RamR or changes in the promoter region of RamA has been observed in clinical isolates (Hentschke *et al.*, 2010; R Rosenblum *et al.*, 2011).

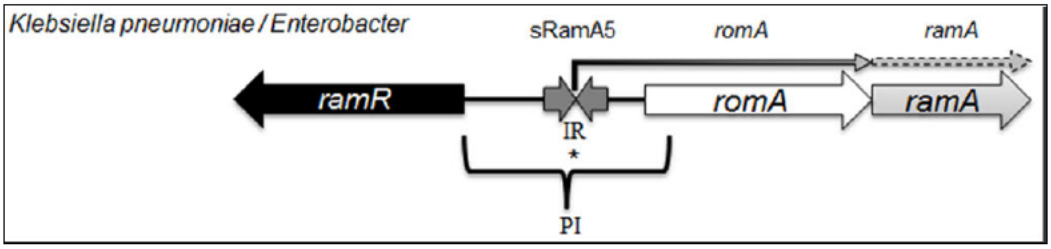


Figure 7: Genetic organization of the ram locus in K. pneumoniae / Enterobacter spp. *romA* and *ramA* are co-transcribed from the PI promoter. *ramR* is divergently transcribed from this promoter and its protein product binds to palindromic sequence, repressing transcription of *romA* and *ramA*. sRamA5 co-transcribed as a primary construct with *romA* helps in maintaining basal levels of RamA by elevating repression by interaction with RamR. (De Majumdar *et al.*, 2015)

3.6 Study of Transcription factors

Numerous experimental and computationally methods are available to undertake the study transcription factors. Experimental based methods can be *in vivo* and *in vitro* based.

These *in vitro* based methods are based on incubating purified TF with a pool of random DNA oligos. Earlier methods of studying the effect of transcriptional factors involved *in vitro* DNA binding analysis such as EMSA, Electrophoretic Mobility Shift Assay or SELEX, Systematic evolution of ligands by exponential enrichment (Geertz and Maerkl, 2010). Other relative mutational analysis was undertaken to observe the resulting quantitative change in expression level of a single transcript (Kukurba and Montgomery, 2015).

Microarray studies made it possible to analyse the binding of regulators to multiple transcripts by hybridisation. These studies have facilitated the

determination of genome wide regulatory potential of transcriptional factors (Zheng, Cui and Meng, 2009; Bailey *et al.*, 2010). There are a few limitations of hybridisation techniques, including requiring prior knowledge of the genome for creation of expression libraries, cross hybridization of similar sequences and the limited number of transcripts that can be analysed (Kukurba and Montgomery, 2015).

Next Generation Sequencing (NGS) has greatly developed the study of transcriptomic analyses by greatly reducing the cost of sequencing and facilitating the determination of genome wide recognition and binding sites.

RNAseq is transcriptomic profiling using NGS that involves extraction of small amount of RNA from cells under the absence or abundance of regulator or under an environmental stress. This is followed by conversion of RNA to cDNA by reverse transcriptase which by the introduction of NGS help sequence this large amount of complementary cDNA that are generated by the extracted RNA. The data on analysis then assists in identification and quantification of relative abundance of transcripts along with precise determination of binding and recognition motifs. Unlike microarray hybridisation or cDNA sequencing, RNAseq can detect a dynamic range of gene expression levels and result in high throughput data with single base pair resolution (Wang, Gerstein and Snyder, 2009). Hence, it is a precise and sensitive transcriptomic profiling technique that helps identify genome wide expression profile of a transcriptional factor.

Often RNAseq can be performed in combination with *in vivo* based ChIPseq or DIPseq techniques that are genome level hybridisation assays (Gossett and Lieb, 2008; Gordân, Hartemink and Bulyk, 2009). ChIPseq exploits the specific binding interaction between proteins and the target genes by precipitating *in-vivo* DNA and protein complexes that are crosslinked using formaldehyde (Kukurba and Montgomery, 2015). To be able to perform this technique an antibody against the transcriptional factor or against the epitope tagged to the transcriptional

factor which is either expressed chromosomally or using an expression plasmid. After the formaldehyde has been used to make DNA-protein crosslinks, the bacterial cells are lysed, and the specific antibody is used to immunoprecipitated these crosslinks. The DNA is then purified from the crosslinks, sequenced and mapped against a reference genome (Figure 8) (Grainger, Lee and Busby, 2009). This technique greatly reduces the number of steps required in the generation of the library and hence the generation of false positives. As data from ChIPseq can only determine an interaction and not the nature of its regulation, it is often combined with transcriptomic studies, like RNAseq, qPCR or RT-PCR to elucidate the nature of regulation, i.e. repression or overexpression(Seo *et al.*, 2015) and quantifying the change in level of gene expression. ChIPseq provides a higher resolution alternative to this transcriptomic profiling technique with better signal to noise ratio and is used to study direct protein-DNA binding of transcriptional regulators (Wang, Gerstein and Snyder, 2009). ChIPseq combined with whole genome expressional analysis like RNAseq, can not only identify direct or indirect nature of regulation but the RNA levels determined can quantify relative abundance of these transcripts (Myers *et al.*, 2015).

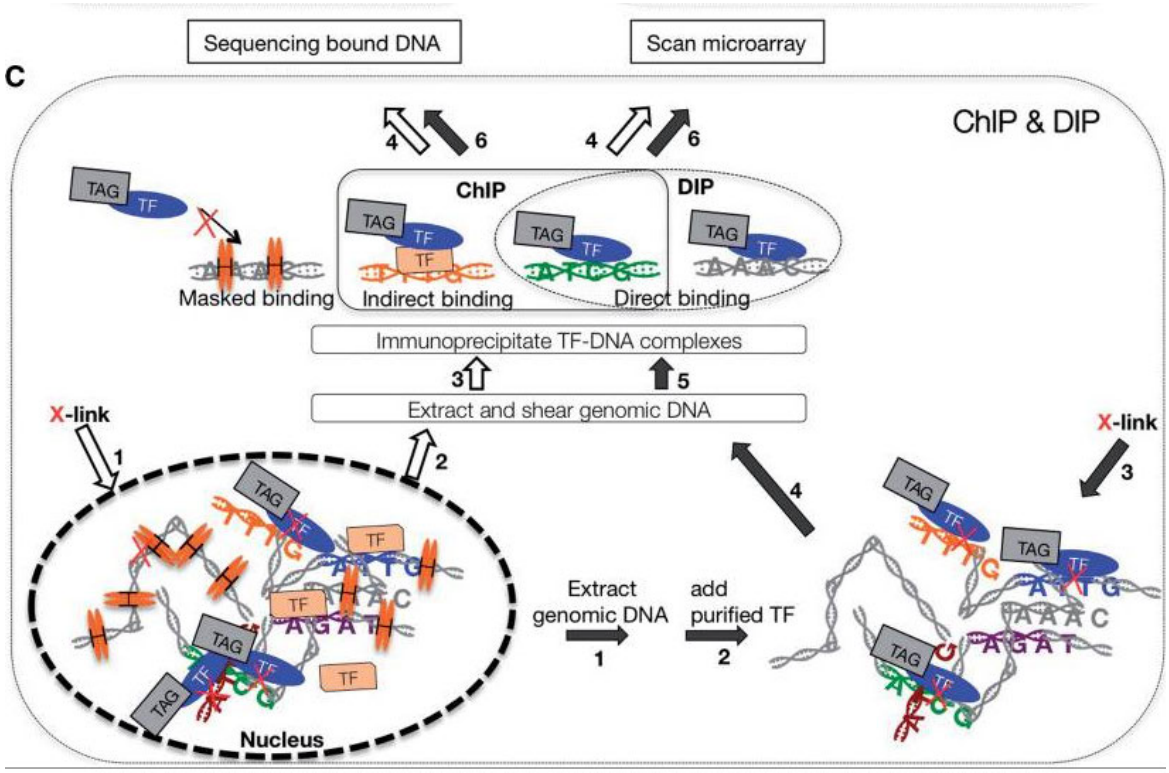


Figure 8: Image taken from Geertz and Maerkl, 2010, displaying the experimental flow chart of immunoprecipitated techniques such as DIP-seq (*in-vitro*) and ChIP-seq (*in-vivo*). Tagged transcriptional factor is cross-linked with loci in the genome, which is preceipitated using an antibody against it. Once these cross-links are purified, the cross-links are reversed and the bound DNA is sequenced. Alternatively, the DNA fragments can be analysed using microarray techniques. The numbers are sequential to the experimental procedure.

3.7 Transcriptional landscape of RamA by RNA-seq

Gene expression analysis of AraC type transcriptional regulators help establish the diverse mechanisms adapted by the gram-negative pathogens towards environmental stresses such as antimicrobial agents. Previously, RNAseq studies have been used to describe the transcriptional regulon of AraC members such as HilC and HilD in *S. typhimurium* (Schechter, Damrauer and Lee, 1999). HilC and HilD are transcriptional regulators that have been reported to affect entry of the pathogen in tissue culture cells and *Salmonella* invasion (Golubeva *et al.*, 2016). A similar expression analysis study has also been performed in *K.*

pneumoniae by De Majumdar *et al.*, 2015 elucidating the expression profile of RamA. This study was pivotal in understanding the role of this antibiotic resistance regulator in *Klebsiella pneumoniae*, a major disseminator of resistance. The study highlighted a total of 103 genes that were differentially regulated by RamA. Other than the confirmation of the previous RamA gene targets, e.g. *acrAB*, novel genes that were found to be differentially expressed due to RamA overexpression were, *mlaB-F, lpxL-2* and *lpxO.*

3.8 Transcriptional landscape of RamA by ChIP-seq

Recently, ChIPseq has been used to map the genome wide regulatory role played by MarA, a well-known AraC family member, that has shown to modulate resistance in *E coli* K-12. This expression analysis study identified the novel role of MarA in DNA repair that might assist in quinolone tolerance by the regulation of *xseA,* encoding Exonuclease VII and maintaining phospholipids symmetry, i.e. *mla* operon (Sharma *et al.*, 2017). A slight variant of ChIPseq, ChIPexo has been used to determine the transcriptional regulatory network under oxidative stress in *E. coli* K-12 MG1655 genome (Seo *et al.*, 2015). Here, three transcriptional factors, OxyR, SoxR and SoxS were used to perform ChIPexo that utilizes exonuclease digestion to refine the resolution of the binding site by degrading unbound double stranded DNA. This study, however, was unable to determine some binding sites recognised by other in vitro methods. A possible explanation of this could be cross regulation of these binding sites by other factors.

A ChIPseq study analysing the binding sites of RamA in *K pneumoniae* could answer important questions regarding the development of resistance in this important pathogen. As addressed earlier, *K pneumoniae* is a key amplifier of resistance and has key intrinsic capabilities that is regulated by a transcriptional regulator like RamA modulating the cell's fate to antibacterial agents with varied targets.

3.9 Preliminary work

This research project was an extension of a RamA ChIPseq study set to determine the said regulator's direct regulon in *Klebsiella pneumoniae* Ecl8. *K pneumoniae* Ecl8 is a non-mucoidal strain of human origin (Forage and Lin, 1982), the whole genome sequence of which has been determined by (Fookes *et al.*, 2013). This isolate has been reported to have only 258 single-nucleotide polymorphisms (SNPs), as compared to KCTC 2242. Therefore, studies performed in *K pneumoniae* Ecl8 are considered applicable to other *K pneumoniae* isolates of veterinary, environmental and clinical importance. The strain can undergo targeted gene mutation relatively easily using homologous recombination method described in (Merlin, McAteer and Masters, 2002). An isogenic derivative of the *K pneumoniae* Ecl8 strain harbouring a *ramR* mutation (overexpressing RamA by release of repression) has also been used in this study (De Majumdar *et al.*, 2015).

In the first phase of the study which was performed by the previous MScR student, ChIPseq was performed using these two isoforms of Ecl8 strains, the wild type and the chromosomal RamA overexpressor due to ramR mutation (Bharathwaj, M.A., 2018). Since RamA doesn't have an antibody against it, RamA ChIPseq was performed using chromosomally Histidine-Tetracycline-FLAG (HTF) tagged RamA and posed some challenges. Ecl8ramA-HTF and Ecl8ΔramRramA-HTF were taken through the ChIPseq procedure where formaldehyde was added to form DNA-protein cross links. Anti-FLAG antibody was used to immunoprecipitated *in vivo* RamA crosslinked with DNA. Illumina platform was used to sequence the fragments and the data obtained was mapped to the genome of *K. pneumoniae* HS11286 (Figure 6) (NCBI accession number: CP003200.1).

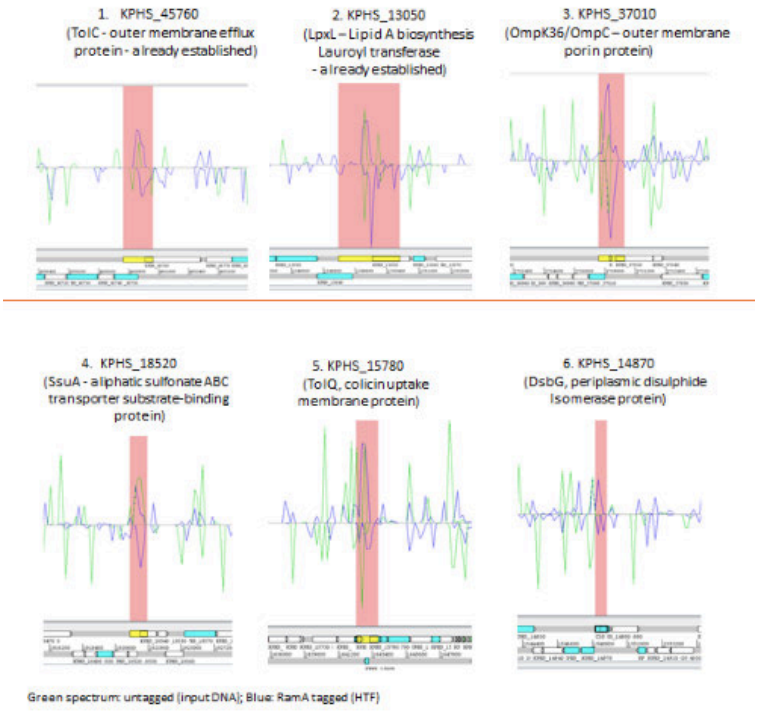


Figure 9: Target genes from first phase of ChIPseq sequencing results. Genes were identified when mapping the sequences amplified in ChIP-seq against the genome of of *K. pneumoniae* HS11286 after mapping. The green spectrum refers to the mapped reads of the input DNA (Ecl8*ΔramR*) and the blue spectrum refers to those of Ecl8*ΔramRramAHTF*. (1) *lpxL* (2) *tolC* (3) *ompK36/ompC* (4) *ssuA* (5) *tolQ* (6) *dsbG* (Bharathwaj, M.A., 2018).

Six targets were initially identified, *tolC, dsbG, ssuA, ompK36* and *tolQ*, (Figure 9) using this data. Further, qPCR analysis was performed, to demonstrated RamA mediated alteration in expression levels of the abovementioned genes in Ecl8 *ΔramR* background (Figure 10). Reporter assay was then used to assess RamA mediated GFP expression from the promoter regions of these potential targets. This phase of the study determined the potential role of RamA in upregulating expression levels of OmpK36. The negative control of outer membrane porin F, Ompk35 by RamA mediated upregulation of *micF* has already been established. The upregulation of OmpK36 has been proposed to promote membrane homeostasis (Schneiders Lab, Unpublished Data). This re-

instate the importance of RamA in modulating the outer membrane permeability by differential regulation of genes such as *tolC, tolQ* and *ompK36*. Other target genes that were investigated were *lpxL*; *ssuA*, a gene coding for an aliphatic sulfonate ABC transporter substrate-binding protein; *dsbG*, a gene coding for a periplasmic protein and is said to confer resistance to DTT.

The ChIP data was re-mapped on to the genome of *K pneumoniae* KCTC 2242 as its more closely related to the sequence of *K pneumoniae* Ecl8. The data from this mapping was found to be more reliable and identified some new potential targets. Some of the new potential targets revealed were *rimP, ybgC and tolQ,* and as part of this project an attempt to validate these as RamA's targets was done(Appendix 5). Further to identifying the change in their expression level under RamA, the potential role of these novel targets in development of resistance in *K. pneumoniae* was addressed.

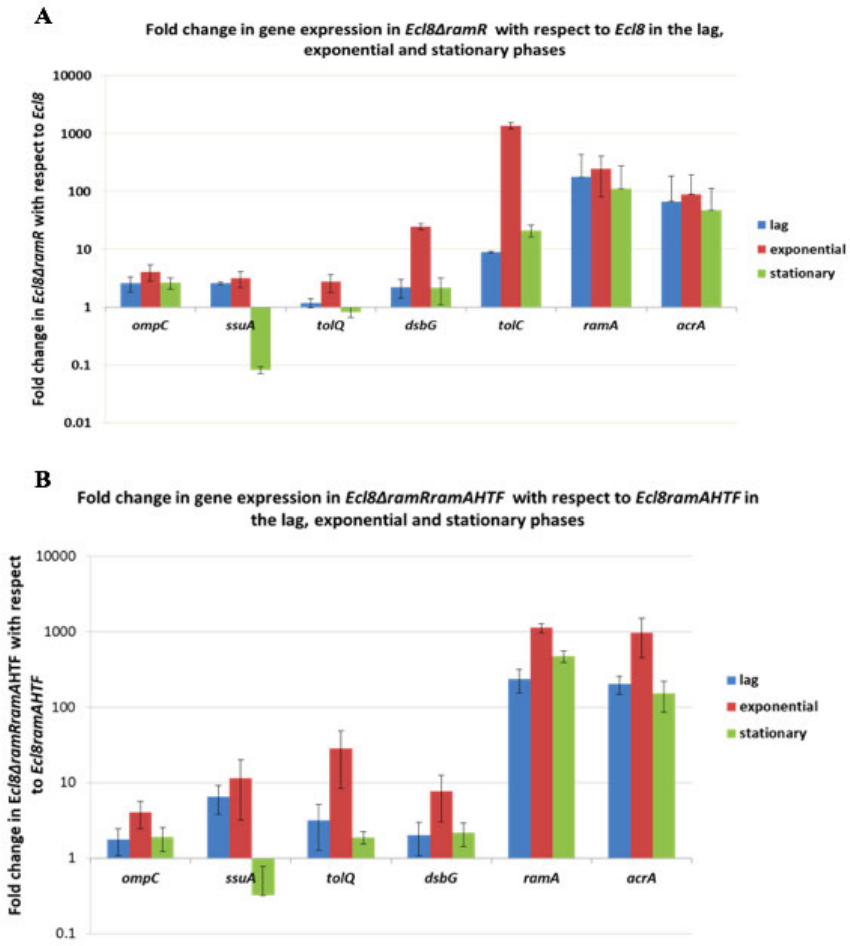


Figure 10: qPCR analysis carried out as part of previous MscR students project. Gene expression was measured via qPCR analysis in the lag, exponential and stationary phases when RamA is overexpressed relative to wild type(A) Relative fold changes in o*mpC, ssuA, tolQ, dsbG, tolC, ramA* and *acrA* in Ecl8 *ΔramR* with respect to Ecl8 (n=4 for all genes except tolC (n=2) and error bars represent standard deviations) (B) Fold changes in *ompC, ssuA, tolQ, dsbG, ramA and acrA* in Ecl8 *ΔramRramAHTF* relative to Ecl8 *ramAHTF* (n=4 and error bars represent standard deviation) (Bharathwaj, M.A., 2018).

3.10 Transcriptional landscape of RamA by ChIP-seq: Phase 2

This project is the second phase of the RamA ChIPseq and was designed and tagged RamA was expressed on plasmid rather than chromosomally. Two types

of tags were used, MYC and FLAG and attempt to tag RamA from both the N-Terminal and the C-Terminal domain (NTD/CTD). In extension of this project a tagged version of RamA with the closest phenotype to wild-type RamA will be used to perform the RamA-DNA cross-linking. Benefits of using these NTD/CTD, MYC and FLAG tags are their relatively smaller size as compared to the HTF tagged used previously. Another advantage will be the absence of a Tetracycline cassette which might have modulated the specific binding of RamA. Another drawback in the chromosomally tagged RamA ChIPseq was the change in MIC of Ecl8 and Ecl8 *ramA-HTF* (Appendix 1), which meant that the presence of the tag had effects on susceptibility of the strain.

Other than using expression vectors for RamA expression, another potential difference in this second phase of ChIPseq can utilize is performing a simultaneous ChIPseq for σ70 subunit of RNA polymerase. As seen in the MarA ChIPseq study, binding sites for σ70 subunits can help identify TF's binding sites by identifying the transcription start sites. Therefore, performing σ70 subunit ChIPseq could help define RamA binding sites closer to the promoter sequences in the entire genome and will greatly in the removal of false positives.

This second phase of RamA ChIPseq project0 will help us identify *in vivo* specific DNA binding interactions with RamA and in *K pneumonaie* and could determine the putative DNA motif recognised by RamA, i.e. "*rambox*". A putative *rambox* has already been described in *Salmonella enterica* serovar Typhimurium (Nikaido, Yamaguchi and Nishino, 2008), but this information is still unknown in *K. pneumonia.* Further to the ChIPseq, techniques like qPCR, eGFP fusion assay and EMSA can be used to verify this proposed motif and direct interaction of RamA with the promoter region of *tolC, acrA, micF* and other novel target genes in *K. pneumoniae*.

Section 4: Aims and Objectives

The main aim of this study was to establish the extended regulon of the RamA regulator by performing ChIPseq.

Hypothesis 1:

This first part of the project was a continuation of preliminary work already performed. It attempts to validate the targets identified from the ChIPseq performed using chromosomally tagged *K pneumoniae* Ecl8 and Ecl8 Δ*ramR (*Ecl8 *ramA-HTF*, Ecl8 Δ*ramRramA-HTF)*. The two putative targets assessed in this study were the TolPal operon and RimP.

Therefore, the first aim of the study is to determine whether RamA transcriptionally regulates the expression TolPal operon and RimP in *K pneumoniae* Ecl8.

Specific aims of the project in order to address the above hypothesis are:

- Determining change in levels of transcription of *tolQ* and *ybgC* when RamA is overexpressed using qPCR
- Determining the nature of RamA regulation from the promoter region of *ybgC* and *rimP* by determining change in level of GFP expression.
    - Construction of GFP expression vectors containing promoter regions of the targets followed by GFP protein.
    - Performing the GFP expression assay

Hypothesis 2:

The main of this study was to constructs vectors expression tagged RamA for performing a second run of RamA ChIPseq. RamA was attempted to be tagged on the N and C Terminal domain with two different tags, MYC and FLAG. The

position of the tag, i.e. N-Terminal and C-terminal along with the type of tag used (MYC or FLAG), will allow to choose for a vector expressing RamA without affecting its function due to its size and nature of the tag.

The aim of this study is to construct NTD or CTD; MYC or FLAG tag on RamA on an expression vector resulting in a detectable and functional RamA suitable to be used in future ChIPseq experiments.

Specific aims of the project in order to address the above hypothesis are:

- Construction of RamA Tagged expression plasmids
- Validation of the tagged RamA expression vectors
    - Validating the expression of these tagged proteins by immunoblotting.
    - Validating the expression of tagged RamA by performing susceptibility assays.

Section 5: Materials and Methods

5.1 Standard laboratory Techniques

5.1.1 Liquid and Solid Media Composition

LB (Lysogeny Broth, Lennox broth or Luria-Bertani Medium) Agar was made by dissolving Yeast extract(7g/L; VWR Chemicals), Sodium Chloride (10g/L; Sigma-Aldrich, UK), Bacteriological Agar(15.4g/L; Sigma-Aldrich, UK), Tryptone (10g/L; Foremedium, UK) in distilled water; **LB Broth** was prepared using LB Broth Lennox powder (20g/l; Formedium, UK) dissolved in distilled water. Both media types were autoclaved before use.

M9 liquid Media was prepared by dissolving sterile 20ml of 5x M9 salts (Sigma-Aldrich, UK), 200µl of 1M Magnesium Sulphate (Sigma-Aldrich, UK), 10µl of 1M Calcium Chloride (Sigma-Aldrich, UK) along with 1ml of 20% filter sterilised Glucose*(Sigma-Aldrich, UK). All solutions were autoclaved before use [*20% Glucose was sterilised using 0.2µM filter (Merck Millipore, UK)]. The M9 media was enriched with 2ml of MEM Amino Acids Solution 50x Essential amino acids (Gibco, ThermoFisher Scientific, UK) and 1ml of MEM Non-Essential Amino Acids Solution 100x (Gibco, ThermoFisher Scientific, UK) and the final volume was made to 100ml using sterile distilled water.

5.1.2 Bacterial strains

Bacterial strain predominantly used in this study is *Klebsiella pneumoniae* Ecl8 and *Klebsiella pneumoniae* Ecl8 dramR. *E coli* K-12 Dh10β is used for cloning purposes. *E coli* K-12 Dh10β cells harbouring NTD 3xFLAG, CTD 3xFLAG, NTD 8xMYC and CTD 8xMYC [ATUM, Newark (Appendix 2 for Plasmid maps)] were a kind gift from Professor David Grainger, School of Biosciences, University of Birmingham, UK. A comprehensive list of all the bacterial strains generated or used as part of this project are listed below in Table 1.

5.1.3 Conditions for growth of bacteria

Bacterial cultures were grown on solid or liquid media at 37°C, unless specified. For growth on LB agar plates, colonies were inoculated using scrapes from the frozen stocks or 5μl of from overnight culture which was then streaked using a flamed metal loop. Liquid broths were inoculated either by a single colony from LB agar plates, directly from stocks or overnight culture and the culture was shaken at 200 RPM using the shaking incubator (INFORS HT, Switzerland). Appropriate antibiotics were added to maintain plasmid selection (Table 2 for stock concentrations of antibiotics used).

Table 1: List of bacterial strains used.

Strain	**Selection**	**Source or Reference**
Klebsiella pneumoniae Ecl8	-	Fookes et al 2013, Forage 1982
Klebsiella pneumoniae Ecl8 *ΔramR*	-	Veleba and Schneiders 2012
Escherichia coli K-12 Dh10β	-	Grant et al 1990
Klebsiella pneumoniae Ecl8 pkco26	Cm^R	This study
Klebsiella pneumoniae Ecl8 *ΔramR* pkco26	Cm^R	This study
Klebsiella pneumoniae Ecl8 pkco26 *rimP*	Cm^R	This study
Klebsiella pneumoniae Ecl8 *ΔramR* pkco26 *rimP*	Cm^R	This study
Klebsiella pneumoniae Ecl8 pkco26 *ybgC*	Cm^R	This study
Klebsiella pneumoniae Ecl8 *ΔramR* pkco26 *ybgC*	Cm^R	This study

Escherichia coli K-12 Dh10β NTD flag *ramA*	Km^R	This study
Escherichia coli K-12 Dh10β NTD myc ramA	Km^R	This study
Escherichia coli K-12 Dh10β CTD flag *ramA*	Km^R	This study
Escherichia coli K-12 Dh10β CTD myc *ramA*	Km^R	This study
Ecl8 NTD flag *ramA*	Km^R	This study
Ecl8 NTD myc *ramA*	Km^R	This study
Ecl8 CTD flag *ramA*	Km^R	This study
Ecl8 CTD myc *ramA*	Km^R	This study

5.1.4 Storage of bacterial strains

5ml of Overnight bacterial cultures were centrifuged using Mega Star 1.6R (VWR, England) at 4000 RPM for 15 minutes at 4°C. These harvested cells were stored at -80°C after resuspension in 10% glycerol in a total volume of 1ml LB broth.

Table 2: Antibiotic used in the lab with their stock and working concentration specified. Stock solutions were made by measuring appropriate quantities and dissolving then in solvent depending on the antibiotic.

Antibiotic	**Stock Concentrations**	**Final concentration**
Kanamycin	50 mg/ml	100 µg/ml
Chloramphenicol	50 mg/ml	50 µg/ml or 30 µg/ml
Ampicillin	50 mg/ml	50 µg/ml
Tetracycline	30 mg/ml	30 µg/ml

Tigecycline	1 mg/ml	Variable

5.1.5 Growth Curve

Liquid broth was inoculated with 10% of overnight culture and growth was monitored by measuring the optical density at 600nm every half an hour using the POLARstar® Omega Microplate Reader (BMG Labtech, Germany).

5.1.6 Determination of Minimum Inhibitory Concentration (MIC)

Agar Dilution Method was used for the determination of MIC of Tigecycline for different strains. Overnight cultures were diluted in 1 x PBS to obtain an optical density (at 600nm) of 0.6. A Multipoint Inoculator (Denley, England) that can apply multiple inocula at the same agar plate was used to inoculate multiple strains on the LB agar plate. For this 600μl of the diluted culture was loaded on a 37 well mold and LB Agar plates of doubling concentrations of tigecycline was prepared (0, 0.5, 2 and 4 μg/ml). Multi-inoculator was then used to inoculate multiple strains on LB agar plates of increasing antibiotic concentrations. Plates were incubated for 24 hours at 37°C before appearance of growth was observed.

5.2 Basic Laboratory Techniques

5.2.1 Cell lysate preparation

Cell lysates were either prepared from pelleted cells obtained from overnight broth culture or through colonies from LB agar plates emulsified in MilliQ water. In case of broth culture, after pelleting [using EppendorfTM MinispinTM Benchtop Centrifuge (Fisher Scientific, UK)] at maximum speed, cells were washed in MilliQ water twice, followed by their resuspension in water. Suspensions from liquid culture or emulsified colonies from agar plates were then heated at 95°C for 10 minutes using a heating block. The cell suspension was later plunged on ice for 5 minutes. The solution was centrifuged using the

benchtop centrifuge at 13400 g for 5 minutes and the supernatant was stored at -20°C for further use.

5.2.2 Plasmid Extraction and Quantification

Plasmids were extracted using the GeneJET Plasmid MiniPrep Kit (Thermo Fisher Scientific, UK) according to the manufacturer's instructions. Briefly, 5ml of overnight culture was spun to obtain pelleted cells which were resuspended in 250μl of Resuspension solution. 250μl of Lysis solution was added, and the Eppendorf was mixed gently by inverting. After adding 350μl of Neutralisation solution, the lysed cells were spun using the benchtop centrifuge at 13400 g for 5 minutes. The cell debris was discarded, and the supernatant was then run through the columns provided in the kit. The columns were washed twice by the Washing buffer, and the column was given an additional spin to get rid of any residual Washing buffer. The plasmid was then eluted in MilliQ water by adding 35μl of MilliQ water and spinning the reaction mixture at top speed after 5 minutes of incubation on bench. The concentration was determined using Nanodrop™ ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, UK).

Table 3: Plasmids used along with their selection marker

Plasmid name	**Marker**	**Reference**
NTD-3 FLAG	Km^R	Grainger Lab
CTD-3 FLAG	Km^R	Grainger Lab
NTD-8 Myc	Km^R	Grainger Lab
CTD-8 Myc	Km^R	Grainger Lab
pJET 1.2	Amp^R	Thermo Fisher Scientific, UK
PkC026	Cm^R	Bharathwaj, M.A., 2018

5.2.3 PCR

PCR reactions were either performed to amplify a fragment used for cloning purposes, in which case the Q5® High-Fidelity PCR Kit (New England Biolabs, USA) was used or for verifying the presence or absence of a gene by using MyTaq™ DNA Polymerases & Kits (Bioline, UK) or OneTaq® Polymerase kit (New England Biolabs, USA). Reaction mixture for each kit was prepared in PCR tubes on ice as described in the Tables of specifications for each PCR kit below. Before placing them on the Veriti™ Thermal Cycler (Applied Biosystems, USA), the tubes were gently mixed and given a quick spin to collect the liquid at the bottom of the tube. Thermal profile used for each reaction has been described in the table of specification depending the corresponding kit used (Table 4 for Q5® High-Fidelity PCR Kit; Table 5 for MyTaq™ DNA Polymeras kit and Table 6 fo OneTaq® Polymerase kit). Annealing temperature for each reaction was determined using the primer sequence (See Table 7 for list and details of Primers used for PCR and qPCR reactions) in the NEB Tm calculator. PCR products obtained were then run on an agarose gel by gel electrophoresis.

Table 4: Specifications for the Q5® High-Fidelity PCR Kit including the reaction volume and the thermocycler conditions used.

Reaction volumes for Q5® High-Fidelity PCR Kit		
	25µl Reaction Volume	**Final Concentration**
Q5 High Fidelity 5x Reaction Buffer	5 µl	1x
10 mM dNTPs	0.5 µl	200 µM
10µM Forward Primer	1.25µl	0.5µM
10µM Reverse Primer	1.25µl	0.5µM
5X Q5 High GC Enhancer(optional)	(5 µl)	(1X)
Q5 High-Fidelity DNA Polymerase	0.25 µl	0.02 U/µl
Template DNA	variable	< 1,000ng

Nuclease Free Water	Upto 25μl	Not Applicable
Thermocycling Conditions of Q5® High-Fidelity PCR reaction		
Step	**Temperature**	**Time**
Initial Denaturation	98°C	30 seconds
Cycles (25-30)	98°C	5-10 seconds
	50-72°C	10-30 seconds
	72°C	20-30 seconds/kb
Final extension	72°C	2 minutes
Hold	4°C	∞

Table 5: Specifications for the MyTaq™ DNA Polymerase kit including the reaction volume and the thermocycler conditions used.

Reaction volumes for MyTaq™ PCR kit		
	12.5μl Reaction Volume	**Final Concentration**
5x MyTaq Reaction Buffer	2.5μl	1x
10μM Forward Primer	0.5μl	0.4μM
10μM Reverse Primer	0.5μl	0.4μM
Template DNA	variable	< 1,000ng
My Taq DNA polymerase	0.06 μl	0.024 U/μl
Nuclease Free Water	Upto 12.5μl	Not Applicable
Thermocycling Conditions of MyTaq™ PCR reaction		
Step	**Temperature**	**Time**
Initial Denaturation	95°C	1 minute
Cycles (25-35)	95°C	15 seconds
	50-72°C	15 seconds

	72°C	10 seconds
Final extension	72°C	2 minutes
Hold	4°C	∞

Table 6: Specifications for the OneTaq® Polymerase kit including the reaction volume and the thermocycler conditions used.

Reaction volumes for OneTaq® Polymerase kit		
	25µl Reaction Volume	**Final Concentration**
10X Standard *Taq* Reaction Buffer	2.5 µl	1x
10 mM dNTPs	0.5 µl	200 µM
10µM Forward Primer	0.5 µl	0.2 µM
10µM Reverse Primer	0.5 µl	0.2 µM
Taq DNA Polymerase	0.125 µl	0.02 U/µl
Template DNA	variable	< 1,000ng
Nuclease Free Water	Upto 25µl	Not Applicable
Thermocycling Conditions of OneTaq® Polymerase kit reaction		
Step	**Temperature**	**Time**
Initial Denaturation	95°C	30 seconds
Cycles (25-30)	95°C	5-10 seconds
	45-68°C	15-60 seconds
	68°C	1 minute /kb
Final extension	68°C	2 minutes
Hold	4°C	∞

Table 7: List of Primers and their corresponding sequence used for cloning and qPCR reactions

Primer name	Sequence
RamATagF_KpnI	GGCGGTACC ATGACGATTTCCGCTCAGGTG
RamATagF_HindIII	GAG AAGCTT TCAGTGGGCGCGACTGTGGTTC
ybgCF_QPCR	CGCAAAATGACGCTGGAGTA
ybgCR_QPCR	CTGCTTAAACTCCGCGACAA
primPR_XbaI	ACATCTAGAAAATTCGATGCCGACCAGCT
primPF_XbaI	ACATCTAGACAATCGGGGGCGCTAAAAAG
pybgCF_XbaI	CACTCTAGAATCGTGCTCGCTTTAGGTCTC
pybgCR_XbaI	CACTCTAGAACCACGAAGGCAACGCGTTCA
TolQF_QPCR	CAAATTCTGGTCCGGGATCG
TolQR_QPCR	GTTTCCAGTTCGCGGTTCAT
KpRamA_F_QPCR	GGAATTCCATATGACGATTTCCGCTCAGG
KpRamA_R_QPCR	CGGGATCCTCAGTGGGCGCGACTGTG
Kp_16SQ_F	GTTACCCGCAGAAGAAGCAC
Kp_16SQ_R	CTACGCATTTCACCGCTACA
GFP Fwd	TGTCACTACTTTGACCTATGG
GFP Rev	CTCGATACGATTAACAAGGG
pJET1.2 Forward	CGACTCACTATAGGGAGAGCGGC
pJET1.2 Reverse	AAGAACATCGATTTTCCATGGCA

5.2.4 Restriction digest (RD)

DNA fragments (in the form of plasmid or PCR product) were digested using 1 Unit, i.e. 1µl of enzyme(s) for 1µg of DNA concentration. Each restriction digest reaction contained buffer corresponding to the enzymes used (see table 8) which was made to a final concentration of 1x, using TE buffer [10 mM Tris-HCl (Sigma-Aldrich, UK); 1 mM EDTA (Sigma- Aldrich, UK) pH=8.0]. The reaction was

gently mixed and spun to collect liquid at the bottom of the tube, before incubating for 30 minutes in a 37°C water bath. Digested fragments were then run on a 2% Agarose gel to confirm the release of correct size. The right sized fragment was then excised from the gel, and gel purification was performed (as described below in 5.2.5.1).

Table 8: List of enzymes used and the manufacturer's details

Enzyme	**Buffer used**	**Manufacturer Details**
Kpn-I	Multicore™ (10x)	Promega, USA
Hind-III	Multicore™ (10x)	Promega, USA
Fast Digest Xba-I	Fast Digest Buffer (10x)	ThermoFisher Scientific, UK
Fast Digest Bgl-II	Fast Digest Buffer (10x)	ThermoFisher Scientific, UK

5.2.5 Agarose Gel Electrophoresis and Gel purification

PCR products and fragments from Restriction Digest reaction were run on an Agarose gel. 2% Agarose gel was prepared by dissolving Agarose (Web Scientific, UK) in 1x TBE [1080g/L Tris (Sigma- Aldrich, UK); 550g/L Boris acid (Sigma-Aldrich, UK); 400ml/L of 0.5M EDTA (Sigma-Aldrich, UK) pH=8.0]. 1x GelRed® Nucleic Acid Gel Stain was added to molten gel before pouring into a cast. Once the gel was solidified, the wells were loaded with either PCR or RD samples along with a ladder [1μl of either GeneRuler 1Kb ladder (Thermo Fisher Scientific, UK) or Hyperladder™ 1kb ladder (Bioline, UK)] and the gel was run at 100V until the dye front reached the end.

5.2.5.1 Gel Purification

Fragments with the correct size were sliced from the gel followed by gel purification using GeneEluteTM Gel Extraction Kit (Sigma-Aldrich, UK)°C for 10 minutes after which the solution was passed through columns provided at a speed of 13,400 g for 1 minute. After discarding the flow through, the column

was washed twice with 500µl of washing buffer. After an additional spun to remove any remaining buffer, the fragment was eluted in 35 MilliQ water. To elute the DNA, the column was incubated on bench for 5 minutes after addition of MilliQ water to the centre of the column before a using the benchtop centrifuge for 2 minutes at top speed.

5.2.6 Ligation of insert into Vectors

0.5 µl of T4 DNA ligase (Thermo Fisher Scientific, UK) was used in each ligation reaction along with its corresponding buffer to perform cloning of a linearized vector and insert. Briefly, to 50ng of vector and the desired insert was mixed such that the final vector to insert molar ratio is 3:1 in a 10 µl reaction. The reaction is then carried on the bench for 1 hour or overnight at 4°C.

5.2.7 Transformation

5.2.7.1 Chemical Transformation

Chemically Competent cells were prepared by harvesting cells from a bacterial culture with an optical density of 0.8-0.9 at 600nm monitored using SmartSpec^{TM} 3000 Spectrophotometer (BioRad, UK). The cells were resuspended in equal volumes of the bacterial culture to ice-cold 0.1 M Calcium chloride ($CaCl_2$) solution. The suspension was centrifuged at 4500 RPM for 15 minutes at 4°C (Mega Star 1.6R, VWR, England) and the pellet was similarly washed two more times with ice-cold 0.1M $CaCl_2$. The final pellet obtained was resuspended in 1ml of ice-cold $CaCl_2$. Aliquots of chemically competent cells stored at -80°C with 10% glycerol up to 2 weeks.

5µl of ligation mixture was added to 40 µl aliquot $CaCl_2$ cells and incubated on ice for 30 minutes. The mixture was subjected to heat shock at 42°C, following which they were again plunged on ice for 1 minute. The cells were recovered by adding 1ml of fresh LB broth and shaken for 1 hour at

37°C. The cells are then spread on LB agar plates with antibiotics to select for the plasmid and incubated at 37°C overnight.

5.2.7.2 Electroporation

Electrically Competent cells were prepared by harvesting cells from a bacterial culture with an optical density of 0.6-0.8 at 600nm monitored using SmartSpec^{TM} 3000 Spectrophotometer (BioRad, UK). The pellet obtained was resuspended in same volumes of ice-cold 10% glycerol. For example, if 50ml of bacterial culture was used to obtain a pellet by centrifuging at 4500 RPM for 15 minutes at 4°C (Mega Star 1.6R, VWR, England). Then 50ml of ice cold 10% glycerol was used to resuspend the cells. This washing step was subsequently repeated two times using half the volume of 10% glycerol from previous step. Finally, the cells are resuspended in 1ml of 10% glycerol and aliquots were stored at -80°C for up to two weeks.

500ng of plasmid or 5 µl ligation mix is added to an aliquot of electrocompetent cells in a pre-chilled electroporation cuvette. Electroporation was performed at 1800V and cells are immediately recovered by incubating in 1ml of LB broth at 37°C shaker for 1 hour. The cells are then spread on LB agar plates containing appropriate antibiotics for selection of the plasmid. After overnight incubation at vector specific temperature, the transformants were then checked by colony PCR using plasmid-specific or insert specific primers.

5.3 Cloning Strategies

5.3.1 Constructing the new strains for ChIPseq

5.3.1.1 Cloning ramA into pJET 1.2

Q5 PCR kit was used to amplify *ramA* from cell lysate of *K pneumoniae* Ecl8 strain using the *ramA* primers with KpnI and HindIII restriction digest overhangs. The *ramA* fragment was then ligated into linearized vector pJET1.2 (Appendix 3) which is chemically transformed into *E coli* DH10β cells. After transformation, MyTaq colony PCR using pJET forward and reverse primers was performed on transformants and *E coli* DH10β pJET *ramA* colony 2 and 8 (Figure 18A) were selected based on the results. Plasmid purification was performed on these two strains. The two plasmids were taken through Bgl-II digestion to confirm the size of the *ramA* fragment and the RD product was run along with the *ramA* PCR product. Additionally, a confirmatory -PCR was performed on these two pJET *ramA* 2 and 8 with pJET forward and reverse primers (Table 7).

5.3.1.2 Cloning ramA into tagged plasmids

To clone *ramA* into tagged plasmids such as CTD/NTD 3x FLAG and CTD/NTD 8x MYC, *ramA* fragment was obtained from pJET ramA 2 and 8 plasmids. This was done by performing large scale restriction digest in 20μl volume on pJET *ramA* 2 and 8 (Figure 10A) using KpnI and HindIII. Restriction Digest products were run on a gel along with *ramA* PCR product and the band aligning to approximately 360bp was excised followed by gel extraction and estimation of its concentration using NanoDrop™.

The *ramA* DNA fragments thus obtained from pJET *ramA* 2 and 8 digest after purification and estimation were ready to be ligated into tagged plasmid that have also been digested with KpnI and HindIII (CTD/NTD 3x

FLAG and CTD/NTD 8x MYC). Ligation was performed and ligated plasmids were transformed into *E coli* K-12 DH10β. Colony PCR was performed using MyTaq PCR kit to verify successful transformants using the *ramA* specific primers. This was followed by validation of the strains using western blotting to confirm the expression of the protein attached with a detectable tag.

5.3.1.3 Western Blotting to confirm the expression of tagged RamA

Total Protein Extraction: Overnight cultures were seeded into fresh LB broth in a 1 in 100 ratios with appropriate antibiotic selection. Bacterial culture was grown until the desired optical density after which it was centrifuged at 4000 RPM for 15 minutes at 4°C (Mega Star 1.6R, VWR, England). The obtained pellet is then resuspended in 30mM Tris HCl buffer and sonicated using Soniprep 150, MSE (Labtech, UK) on ice for 15 seconds at 6 MHz, three times. The lysed cells were centrifuged for 15 minutes at 8000 RPM (Mega Star 1.6R, VWR, England) to remove the unbroken cells. The supernatant was retained, and the protein concentration was estimated using Biorad Protein Assay (Biorad, UK).

Sample Preparation: Equal volumes of protein samples and Sample buffer 2 X Laemmli concentrate (Sigma-Aldrich, UK) were added in an Eppendorf tubes and the solution was boiled for 10 minutes at 98°C.

Crude protein extract: OD_{600} of overnight cultures was adjusted to 0.6 by using 1 X PBS. 5ml of this cell suspension was centrifuged at 4000 RPM for 15 mins at 4°C using Mega Star 1.6R (VWR, England). The harvested cells are washed with 5ml of 1XPBS. The cells were pelleted and were resuspended in 400 µl of Sample buffer 2 X Laemmli concentrate (Sigma-Aldrich, UK). The solution was then boiled at 98°C for 10 mins and plunged on ice for 5 minutes before being stored at -20°C.

SDS PAGE Gel Electrophoresis: Separating gel was prepared as per the Table 9 below and was poured into the gel mould. 100% isopropanol is added on top to remove bubbles and even the top surface. Once the gel was set, the isopropanol was drained and stacking gel (Table 9) was poured on top along with the appropriate gel combs. The gel is then assembled in the Mini Protean Tetra System (Biorad, UK) which is filled with running buffer. 10 µl of samples were loaded in to the wells along with 4 µl of the Spectra™ Multicolor Broad Range Protein Ladder(ThermoFisher Scientific, UK). The gel was run at 100 V until the dye front is at the very bottom of the gel. The gel is carefully removed, and the stacking gel is sliced. Distilled water is used to wash the gel by gently rocking it. This step was repeated thrice. The gel is then either stained with Coomassie blue to confirm the presence of protein or transferred on to a polyvinylidene difluoride (PVDF) membrane for western blotting.

Table 9: Detailed constituents of buffer and gels used in SDS PAGE Gel electrophoresis

SDS Page Gels and Buffers	**Components**
Separating gel	6.9ml of Distilled water; 4.8ml Bis-Acrylamide 29:1 (Fisher Bioreagents, UK), 4ml of 1.5M Tris (pH 8.8, Sigma-Aldrich); 160µl of 10% SDS (Melford, UK); 160µl of 10% Ammonium persulphate (Sigma Aldrich); 16µl of TEMED (Melford, UK).
Stacking gel	5.8ml of Distilled water; 1.5ml Bis-Acrylamide 29:1 (Fisher Bioreagents, UK), 2.5ml of 0.5M Tris (pH 6.8, Sigma-Aldrich); 100µl of 10% SDS (Melford, UK);

	100μl of 10% Ammonium persulphate (Sigma Aldrich); 10μl of TEMED (Melford, UK).
Running Buffer	Tris glycine Buffer

Coomassie Staining: Coomassie stain and Destain was prepared as per the Table 9. The gel was submerged in Coomassie blue solution for 30 minutes or until the bands were visible. The Coomassie blue solution was replaced with the destain solution for another 30 minutes or more for the background stain is cleared. The destain solution is then replaced with water.

Table 10: Detailed constituents of solutions used in Coomassie Blue Staining

Staining Solutions	**Components**
Coomassie Blue Dye	2.5g of R250 Coomassie blue (BioRad), 45ml 100%methanol, 45ml of distilled water and 10ml of acetic acid and filtered
De-stain	45% of methanol, 45% of water and 10% of acetic acid

Transferring gel to a PVDF membrane: The PVDF Amersham Hybond P 0.45 PVDG Blotting Membrane (GE Healthcare, UK) was activated in 100% methanol (Fisher Scientific, UK), following which the gel, 6 Whatman filter paper and the activated membrane were incubated in transfer buffer for 10 minutes. The transfer of the protein from the gel is carried out using the transfer machine set up at 25V for 1 hour. For the transfer, the gel and the membrane were placed in between 3 Whatman filter paper on either side (Figure 11 and Table 11 for buffer solutions).

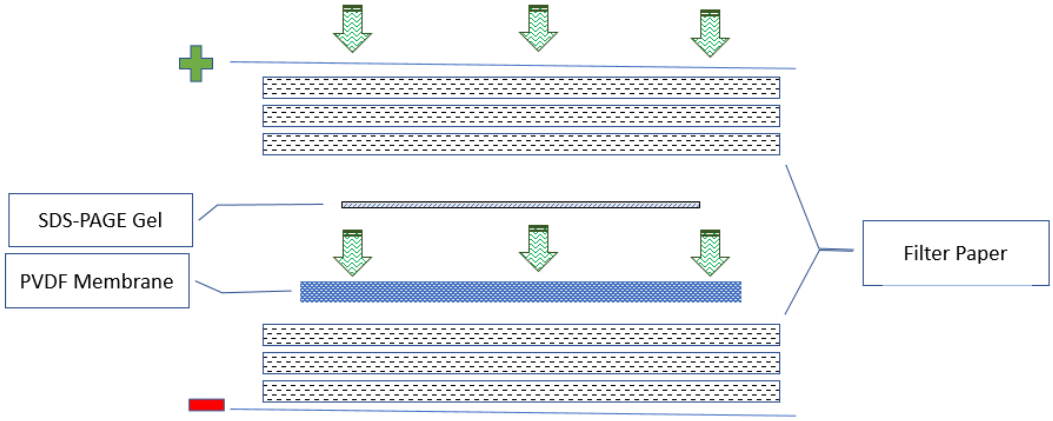


Figure 11: Image displaying the arrangement of the filter papers, the membrane and the SDS-PAGE gel used in the transfer machine.

Table 11: Detailed constituents of the buffer used in the transfer of gel to a membrane and subsequent buffers used to treat the membrane.

Buffers	**Anti-FLAG**	**Anti-MYC**
Transfer Buffer	25 mM Tris, 192 mM glycine, 10% methanol	25 mM Tris, 192 mM glycine, 10% methanol
Blocking buffer	Milk, BSA, 1 X TBS	5% Skimmed Milk in TBS-Tween
Antibody buffer	0.5% SDS, 0.1% BSA, 0.5% milk, 0.05% Triton X 100 in 1X TBS	5% Skimmed Milk in TBS-Tween

Table 12: Antibody titre used for Western blot experiment

Antibody	**Titre**
Anti-FLAG (Sigma)	1: 10000
Anti-MYC (Santa cruz)	1 : 5000
Anti-mouse IgG	1:10000

Blocking and Antibody incubation: Depending on the tag (MYC or FLAG) of the RamA, the buffers and titres specified in Table 12 were prepared. The PVDF Membrane was incubated with 75 ml of blocking buffer overnight at 4°C. The membranes are then washed with 0.1% PBS Tween-20 (PBS-T). 10ml of primary antibody solution for 1 hour and is treated again with 0.1% PBS-T. Secondary antibody incubation was carried out for 3 hours using 10 ml of secondary antibody solution. Membranes were washed as before.

Developing the membrane: Membranes are developed using Amersham ECL Prime Western Blotting Detection Reagents (GE healthcare, USA) reagents (1:1 reagent A: reagent B) and water (1:1, ECL reagents: water) for 2min. Amersham™ Hyperfilm™ ECL is exposed to the membrane at various times of exposure (1min, 30 seconds and 5 seconds). After the exposure, the Hyperfilms are developed using the Mi5 Processor (JET X-RAY, UK).

5.3.2 Construction of vector used in GFP gene expression assay

Promoter regions of genes proposed to be regulated by RamA, *ybgC* and *rimP* were cloned into a GFP gene expression vector, pkC026. This vector is designed such that it will demonstrate higher GFP production when RamA is upregulated, e.g. in the *K pneumoniae Ecl8 ⊿ramR* background. This is due to activation of transcription from the promoter regions of genes cloned into the vector, pkC026. Promoter regions of *ybgC* and *rimP* were amplified from *K pneumoniae Ecl8* cell lysate using primers with XbaI restriction site overhangs (Table 7). PCR products corresponding to these promoter regions of *ybgC* and *rimP* were run on a gel, followed by excision of the correctly sized fragment, gel purification and quantification of its concentration using NanoDrop™ The DNA fragments were then were ligated into the commercially available pJET1.2 vector. The

ligated product was chemically transformed into *E coli* K-12 Dh10β. Post plasmid purification of these cloned pJET plasmids containing promoter regions of *ybgC* and *rimP* (pJET1.2 *ybgC*, pJET1.2 *rimP*), restriction digest was performed using XbaI enzyme on these plasmids. The products from the restriction digest (after gel electrophoresis, gel purification and NanoDrop quantification) was ligated with XbaI digested pkC026 which was transformed into *K pneumoniae Ecl8* and *K pneumoniae Ecl8 ΔramR.*

5.4 RNA extraction

RNA extraction was performed on *K pneumoniae Ecl8* and *K pneumoniae Ecl8 ΔramR* to compare the levels of expression of a gene. Bacterial cells were grown to an optical density of 0.6-0.8 by inoculating a overnight seed culture in a 1 in 100 dilution. The cells were then obtained by centrifuging the culture at 8000xg for 10 minutes at 4°C. The pellet was resuspended in 1ml of RNAprotect Bacteria Reagent (QIAGEN). At this point the cells can be stored at 4°C. Briefly, the cells were centrifuged for 10 minutes at 11,000 RPM using the benchtop centrifuge. Post this step, all steps were performed in a Laminar hood using filtered tips. 200µl of TE buffer containing 1mg/ml lysozyme was used to resuspend the obtained pellet. The suspension was vortexed, after which 700µl of RLT buffer from the RNeasy Mini Kit (Qiagen, UK) containing 10µl/1ml of β-mercaptoethanol was added to the solution and mixed vigorously. 500µl of 100% ethanol was added to this and gently mixed by pipetting. The solution was transferred to an RNeasy Mini spin columns and spun for 30 seconds at 11,000 RPM after which the supernatant was discarded. 300µl of RW1 buffer was then added to wash the column and the column was centrifuged at 11,000 RPM for 30 seconds. Next, 500µl of RPE buffer was added and the final centrifuge was for 2 minutes at 11,000 RPM. 35-45µl of Nuclease Free Water was then used to elute RNA in a fresh Eppendorf.

To get rid of any DNA contamination from the RNA samples, TURBO DNA-free^{tm} kit (thermofisher Scientific, UK) . The samples were checked for any DNA contamination by performing mytaq DNA Polymerase PCR using 16S primers (table) where presence of a band after gel electrophoresis would confirm the presence of DNA contamination and hence a repeat of TURBO DNA free™ digest. Estimation of RNA concentration was done using nanodrop^{tm}.

5.4.1 cDNA Synthesis

AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, USA) was used to synthesize complementary DNA from RNA samples obtained from RNA extraction. Briefly, In a microcentrifuge tube, in a total volume of 20µl of reaction volume made up using Nuclease free water, 10µl of first strand master mix (2x), 3 µl of Random primers (0.1 µg/ µl) 1 µl of AffinityScript RT/RNase Block enzyme mixture and volume of RNA corresponding to 2 µg of concentration was added. The tubes were then placed in a thermocycler with the following thermal profile: 5 minutes at 25°C, 15 minutes for 42°C, 5 minutes at 95°C, followed by storage at 4°C.

5.5 Gene expression analysis

5.5.1 GFP Gene Fusion Assay

GFP gene fusion assay was used to determine the RamA dependent regulation of genes which were proposed to be targets of RamA in the previous ChIPseq results. GFP expression vectors containing promoter regions of these genes were assessed in *K pneumoniae Ecl8 (Klebsiella pneumoniae* Ecl8 pkco26 *rimP, Klebsiella pneumoniae* Ecl8 pkco26 ybgC*)* and *K pneumoniae Ecl8 ΔramR (K pneumoniae* Ecl8 dramR pkco26 *rimP, K pneumoniae* Ecl8 dramR pkco26 ybgC *)* along with their respective vector only control (*K pneumoniae* Ecl8 pkco26, *K pneumoniae* Ecl8 *dramR* pKC026) using the POLARstar® Omega Microplate

Reader (BMG Labtech, Germany). 96 well plate was used to set up an experiment where M9 media with appropriate concentration of Chloramphenicol was inoculated with 10 µl of overnight culture of each of these strains. Every half an hour, optical density at 600nm and fluorescence at 509 nm was measured using POLARstar® Omega Microplate Reader. The data obtained was analyzed to calculate the fold change of GFP expression in *K pneumoniae Ecl8 ⊿ramR* relative to *K pneumoniae Ecl8*.

5.5.2 qPCR

In order to quantify the relative fold changes of gene expression from one strain to another, qPCR was performed using Brilliant II SYBR® Green QPCR Master Mix (Agilent Technolgies, USA). For each reaction, 5µl of 2X Brilliant II SYBR Green QPCR master mix, 0.5µl of upstream and downstream primer, 0.15µl of 1:500 diluted ROX dye in a total volume of 10µl where 1µl of cDNA of the respective samples. For each sample, duplicate reactions were prepared in a 96 well plate.

Before determining the relative RNA expression levels of a gene, standard curve was obtained to determine the percentage efficiency for each set corresponding primers. This was done so by using the same reaction mix as for standard qPCR reaction described above, except for the addition of cDNA. In place of cDNA, gDNA of *K pneumoniae Ecl8*, obtained from a member of laboratory using the Wizard® Genomic DNA Purification Kit (Promega, UK) was added at 200ng, 100ng, 10ng, 1ng and 0.1ng concentration. Primer sets with an efficiency percentage of 100%±10% were considered appropriate for use. To determine whether the gene under investigation was upregulated or downregulated, a qPCR reaction for a housing gene, 16S ribosomal subunit was used in duplicate. Hence the relative Ct values helped to identify whether there was an increase of decrease of the gene in question.

The thermal profile setting used in the machine are specified below. Output from each reaction was store in forms of a table of Ct values, amplification and disassociation plots. For primers look at Table 7.

Table 13: Details of the qPCR setting used for the qPCR reactions

Temperature	Time	No of Cycle
95°C	3 min	1 cycle
95°C	20 sec	40 cycles
60°C	20 sec	
95°C	1 min	1 cycle
55°C	30 sec	
95°C	30 sec	

The calculation of relative fold change was done by the formula specified below:

$\Delta Ct = \Delta Ct_{gene} - \Delta Ct_{16S}$

$\Delta\Delta Ct = \Delta Ct_{ramR\ mutant} - \Delta Ct_{WT}$

RQ (Relative fold change) = $2^{-(\Delta\Delta Ct)}$

5.6 Image J

Image J software was used to quantify the expression of the bands after immunoblotting.

Section 6: Results

6.1 Validation of the Targets by qPCR

The results of the first phase of ChIPseq identified RamA binding at the *tolpal* operon. The operon has two transcriptional start sites, upstream of *ybgC* and *tolQ* respectively. In order to determine whether TolQRA operon is under the regulatory control of RamA, gene expression levels of *ybgC* and *tolQ* was estimated using qPCR. *K pneumoniae* Ecl8's isoform, *K pneumoniae* Ecl8 *ΔramR* was used to assess the relative increase in activity of the protein under increased production of RamA due to release of its repression. QPCR was performed on RNA samples from Ecl8, Ecl8 *ΔramR*. The gene expression level of *ramA* was also measured as positive control.

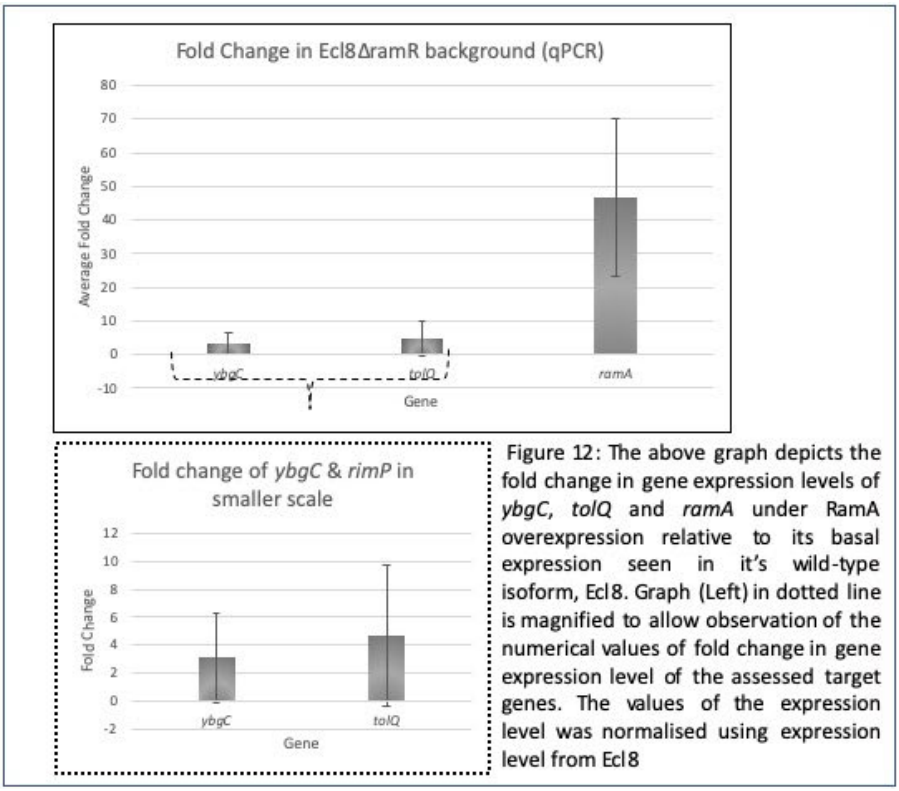


Figure 12: The above graph depicts the fold change in gene expression levels of *ybgC*, *tolQ* and *ramA* under RamA overexpression relative to its basal expression seen in it's wild-type isoform, Ecl8. Graph (Left) in dotted line is magnified to allow observation of the numerical values of fold change in gene expression level of the assessed target genes. The values of the expression level was normalised using expression level from Ecl8

Results from the qPCR analysis, showed an average increase in RamA expression levels of 46 folds, which is consistent with previous findings that in a Δ*ramR*

background, RamA is overexpressed. The expression of the target genes, *ybgC* and *tolQ* has shown 3-4.7-fold positive expression albeit with a large standard deviation. The previous results of expression level of *tolQ* from the Schneiders lab showed a similar fold change, but the standard deviation observed was far less (see Figure 10). Hence, it is difficult to decipher from these results whether RamA overexpression plays any role of in changing the expression level of the TolPal operon.

6.2 Validation of the Targets by GFP fusion assay

Another way to assess the regulation of potential target by a transcriptional factor is by reporter gene assay. In case of this study, GFP (green fluorescent protein) produced by expression vector, pkC026 was the reporter used to see activation of transcription from the promoter regions of putative target genes. Promoter regions from *ybgC* and *rimP* was cloned upstream to the reporter protein in the GFP vector.

6.2.1 Constructing the GFP plasmid

Upstream region of *ybgC* and *rimP* were first amplified using primers containing XbaI site overhangs and this amplified product was cloned into pJET1.2 plasmid that was transformed into *E coli* DH10β. After plasmid purification, pJET clones containing promoter regions of *ybgC* and *rimP* were digested with XbaI and these sticky ended inserts were cloned into XbaI digested pkCO26. The ligation product was transformed into *E coli* DH10β. To confirm whether the promoter region was cloned in the correct orientation confirmatory PCR was performed. This PCR utilized the forward primer corresponding to the upstream region of the promoter and the GFP specific reverse primer (Figure 13). The constructed vectors were transformed into *K pneumoniae* Ecl8 and *K pneumoniae* Ecl8*ΔramR*

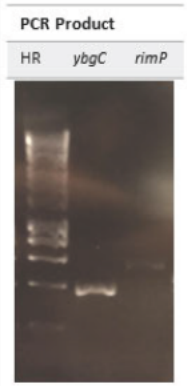
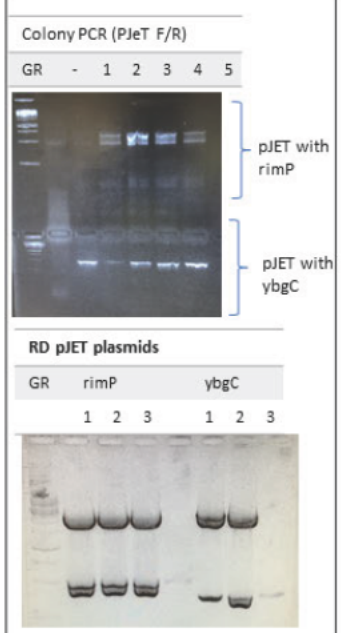
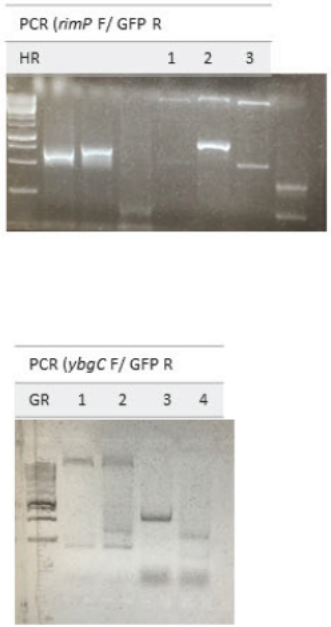
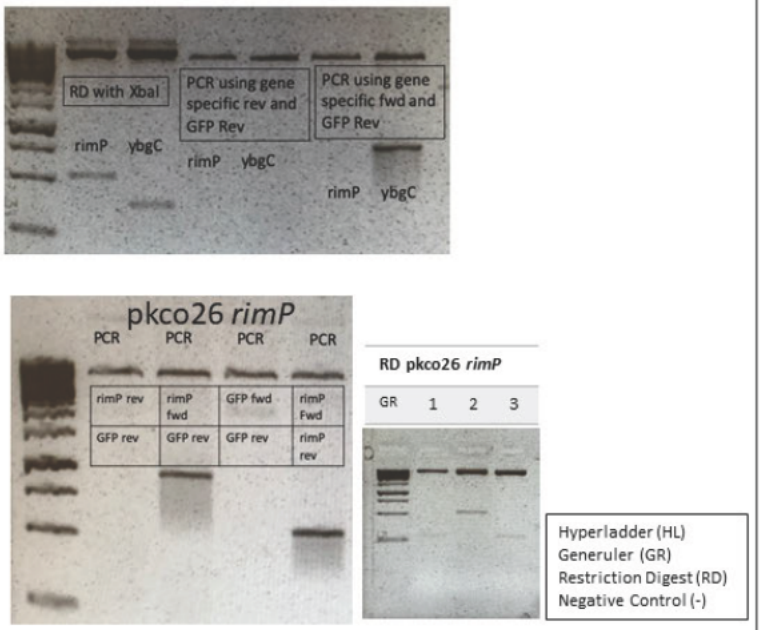
Gene	Fragment	pJET Cloning	pkCO26 Cloning	pKCO26 confirmations
ybgC	364bp	DH10β pJET *ybgC* 3, 4, 5 frozen	DH10β pkCO26 *ybgC 3* (frozen)	pkCO26 *ybgC 3*
rimP	512bp	DH10β pJET *rimP* 3, 4, 5 frozen	DH10β pkCO26 *rimP2* (frozen)	pkCO26 *rimP2*
				

Figure 13: Figure summarising the cloning process for making GFP expressing vectors for reporter gene fusion assay. The first column shows gel image corresponding to the two fragments that were cloned into the GFP expression plasmid, i.e. *ybgC (364bp))* and *rimP (512bp).* The second column shows the colony PCR result after cloning these fragments into the pJET1.2 vector. A confirmatory restriction digest (RD) wit XbaI is done to see observe the release of the right fragments. Next these plasmids were extracted and XbaI digested so that they can be cloned into a digested pkCO26. This was followed by a confirmatory restrictiom digest. The positive clones that gave a correct sized PCR product for *rimP* and *ybgC* GFP clones with corresponding forward primer and GFP reverse primer were selected and were transformed int Ecl8 and Ecl8 Δ*ramR.*

6.2.2 GFP assay

Emission intensity of bacterial culture was measured at 509 nm. To calculate the fold change, the data was first normalized to the vector only control. The fold change was then calculated by dividing the relative fluorescence observed in the Ecl8 ΔramR background by the relative fluorescence seen in Ecl8. Since a fold change value of 1 would imply that there has been no change in promoter activation under RamA overexpression, a fold change below 1 would mean downregulation was observed. Both *ybgC* and *rimP* showed a fold change of less than 1. However, since 10µl of overnight culture was used to inoculate a 200µl culture, we can see that the culture was already over late-exponential phase at OD600 of 0.5 (Figure 17) hence we are unable to decipher the activation of promoter regions of these genes during the lag phase and the beginning of the exponential phase. Another concern is the high standard deviation observed specially at higher OD600 values.

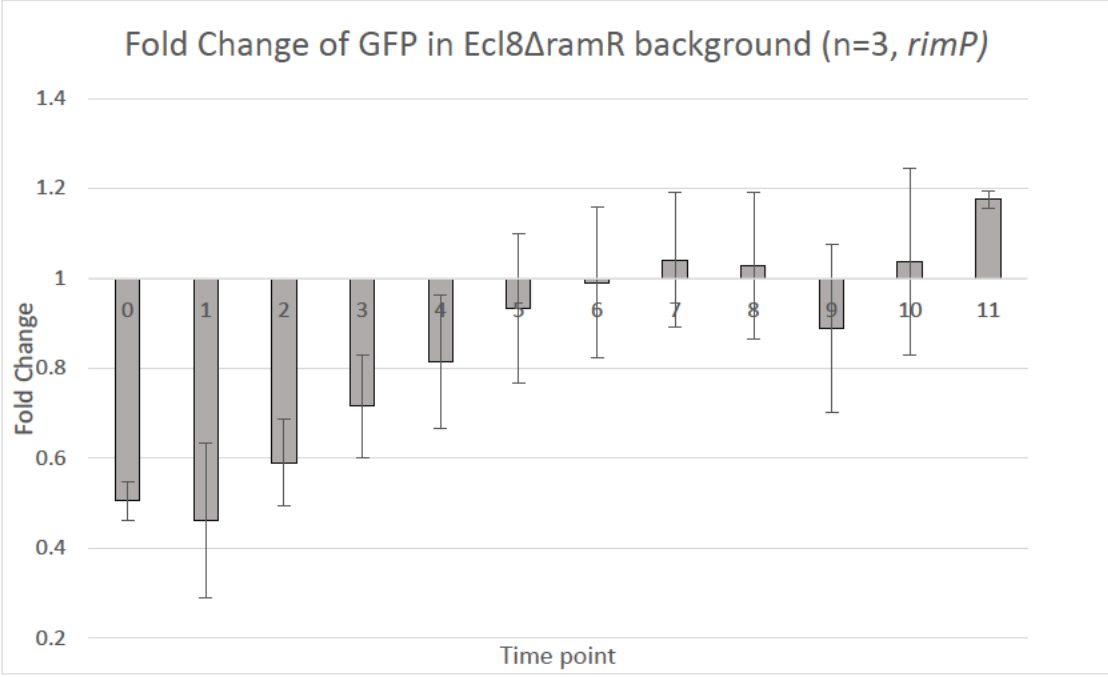


Figure 14: Bar chart showing fold change of GFP expression in *K pneumonaie* Ecl8 *ΔramR* containing GFP vector with promoter region of *rimP* relative to *K pneumonaie* Ecl8 cloned

with the same vector. The fluorescence and OD600 was measured at an interval of every half an hour. Error bar indicate standard deviation from three repeats.

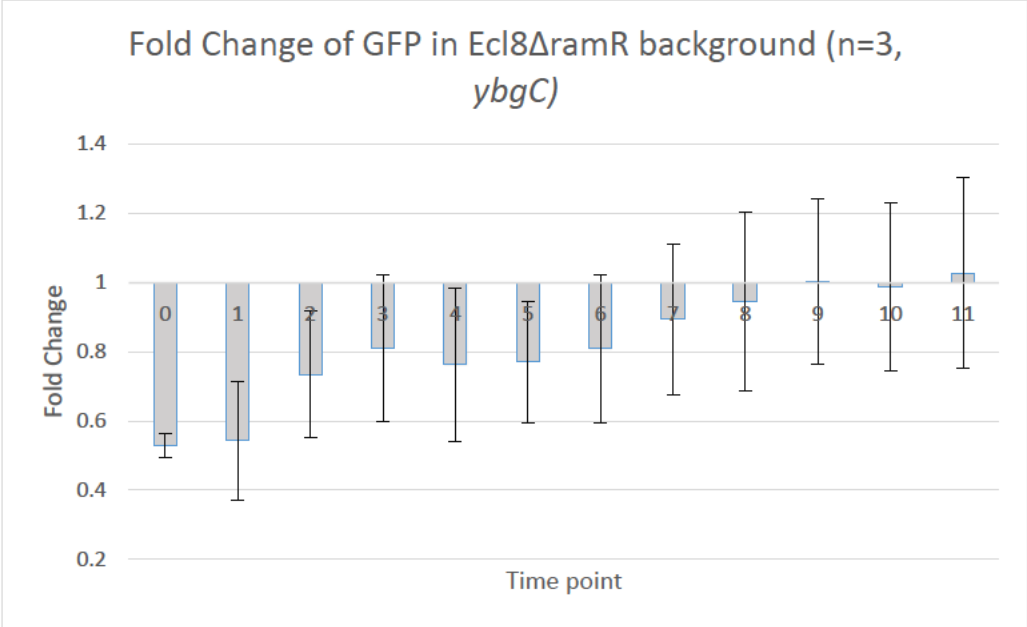


Figure 15: Bar chart showing fold change of GFP expression in *K pneumonaie* Ecl8 *ΔramR* containing GFP vector with promoter region of *ybgC* relative to *K pneumonaie Ecl8* cloned with the same vector. The fluorescence and OD600 was measured at an interval of every half an hour. Error bar indicate standard deviation from three repeats.

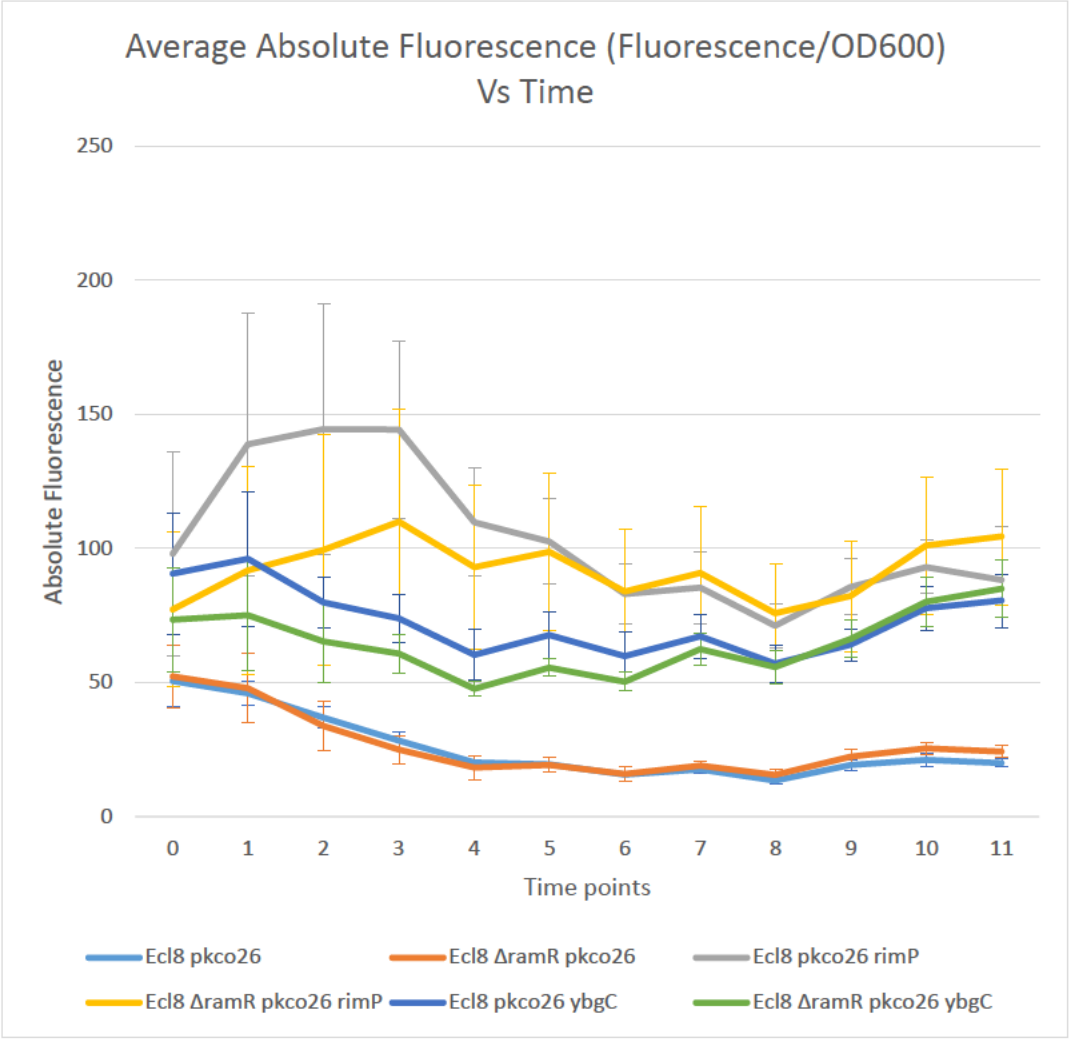


Figure 16: Average of three Absolute Fluorescence against time. The emission fluorescence intensity was divided by the observed OD600 at each time point for all the GFP strains and plotted against the time point. Error bar indicate standard deviation from three repeats.

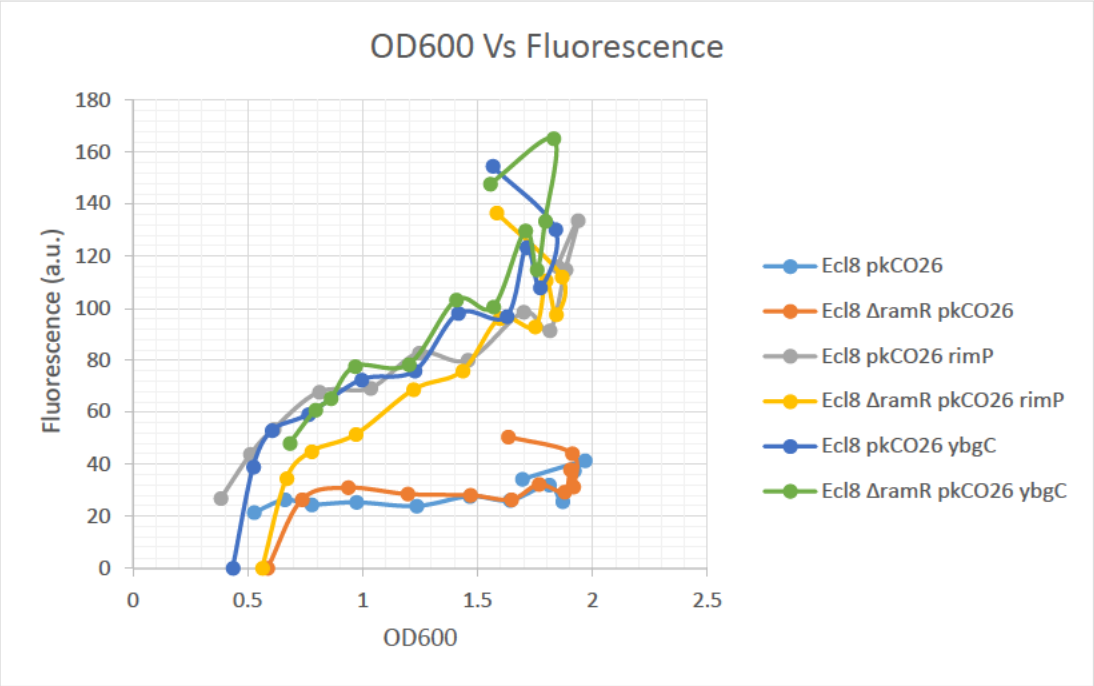


Figure 17: Scatter plot of average measure fluorescence against the average OD600 observed for all the GFP strains used in the GFP assay. Each time point is an average of three replicates.

6.3 Constructing the new strains for ChIPseq

For the second phase of ChIPseq, expression vectors containing tags (FLAG/MYC) suitable for both N-terminal and C-terminal tagging were used to clone the *ramA* gene. The section below summarises the cloning strategy employed for cloning each of the four tagged plasmids. This is followed by validation of these strains using susceptibility and immunoblotting assays.

6.3.1 Construction of tagged plasmids

Figure 18 A shows the amplification of *ramA* from *K pneumoniae* Ecl8 using gene specific primers with required restriction sites which followed cloning it into pJET commercialized cloning vector. Figure 18 B, further depicts the gel images of colony PCR performed to select the pJET clones with the correct insert size. Next, these *ramA* containing vector were digested with KpnI and HindIII which

showed two bands owing to a HindIII restriction site present in the pJET1.2 vector itself (Appendix 3)

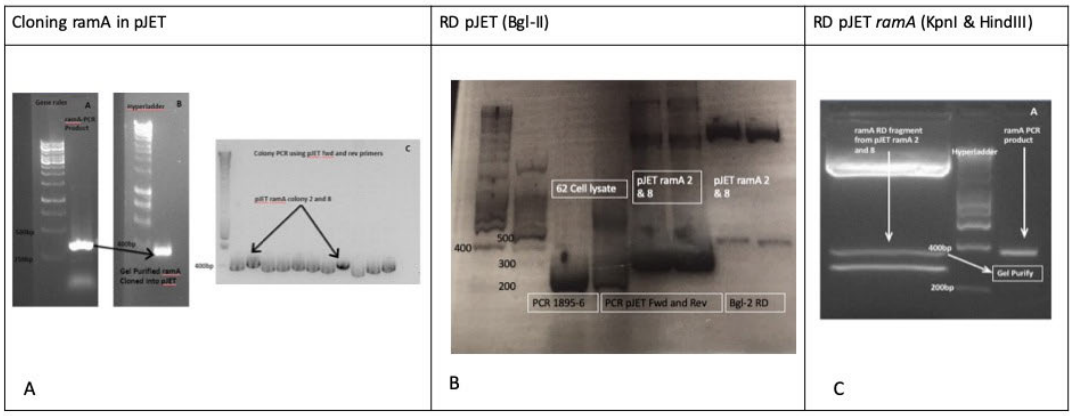


Figure 18: Figure depicting cloning of *ramA* from *K pneumoniae* into pJET1.2 (A). The selection of the right clones using pJET Forward and Reverse primer to do colony PCR (A). Digesting the selected plasmids with Bgl-II to observe the release of the right fragment (B). Followed by running on agarose gel products from the digestion of the pJET *ramA* clones with KpnI and HindIII along with the PCR product (to verify the correct size of interest). This gel was used to perform gel purification of the band corresponding to *ramA* which was used in the next steps of cloning.

The fragment excised from the restriction digest of pJET *ramA* clones using KpnI and HindIII restriction digest enzymes were cloned into NTD FLAG, NTD MYC, CTD FLAG and CTD MYC plasmids. Colony PCR was used to determine positive clones which were retained and stored as bacterial stocks. For a molecular biology confirmation, plasmid extraction was performed on these potential clones and restriction digest was then performed using KpnI and HindIII to observe the release of the right size of fragment. All cloning steps were performed in *E coli* Dh10β. See Appendix 4 for all the new plasmid maps.

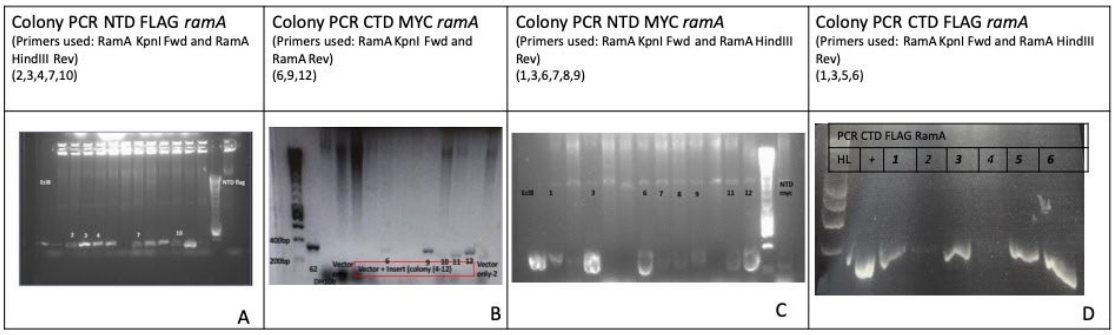


Figure 19: Figure showing gel images of the confirmatory colony PCR performed after the ligation of *ramA* into each of the tagged plasmids. In brackets numbers refer to the clone with positive results that was taken to the next stage of confirmation by restriction digest have been specified. (HL: Hyperladder, Ecl8/62: *K pneumoniae Ecl8*)

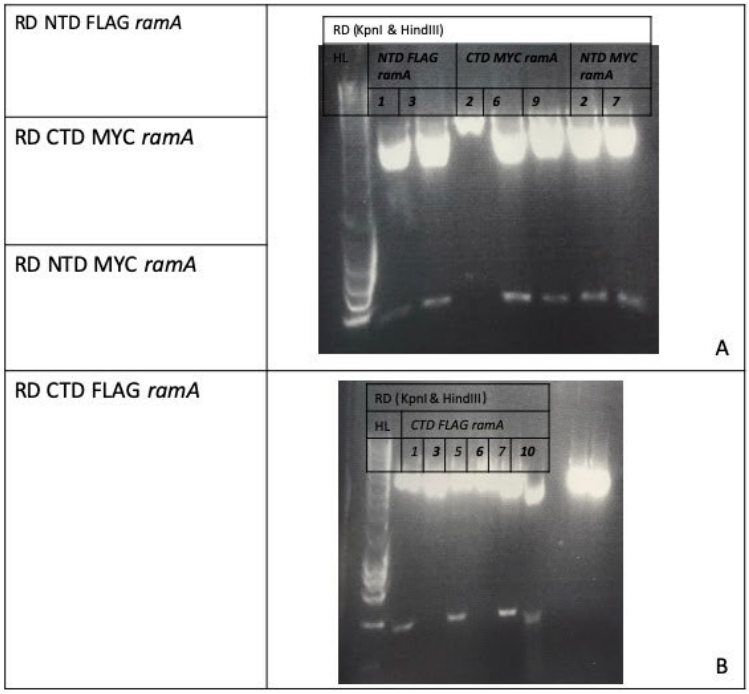


Figure 20: Gel images showing results of restriction digest performed on potential clones of NTD FLAG *ramA*, CTD MYC *ramA*, NTD myc *ramA* (A) and CTD FLAG *ramA* using KpnI and HindIII restriction digest enzymes. Clones with the correct size was then used for further

6.3.2 Validating the strains by MIC

In order to assess the functionality of RamA cloned into these tagged plasmids, the Minimum Inhibitory Concentration of *K pneumoniae* Ecl8 electroporated with these plasmids were checked under tigecycline.

Table 14: Table below is the result from a Agar MIC of Tigecycline performed on the specified strains. The results are from one repeat.

Strain	MIC tigecycline	
	Neat	Dilute (10^4)
K pneumoniae Ecl8	0.5 μg/ml	1μg/ml
K pneumoniae Ecl8 Δ*ramR*	4μg/ml	4μg/ml
K pn. Ecl8 NTD Flag 1.1	4μg/ml	1μg/ml
K pn. Ecl8 NTD Flag 1.2	4μg/ml	1μg/ml
K pn. Ecl8 NTC Myc 1.1	4μg/ml	1μg/ml
K pn. Ecl8 NTC Myc 1.2	4μg/ml	1μg/ml
K pn. Ecl8 CTD Flag 1.1	1μg/ml	1μg/ml
K pn. Ecl8 CTD Flag 1.2	1μg/ml	1μg/ml
K pn. Ecl8 CTD Myc 1.1	2μg/ml	0.5μg/ml
K pn. Ecl8 CTD Myc 1.2	4μg/ml	1μg/ml
K pn. Ecl8 NTD Flag *ramA* 1.1	>4μg/ml	>4μg/ml
K pn. Ecl8 NTD Flag *ramA* 1.2	>4μg/ml	>4μg/ml
K pn. Ecl8 NTD Flag *ramA* 3.1	>4μg/ml	>4μg/ml
K pn. Ecl8 NTD Flag *ramA* 3.2	>4μg/ml	>4μg/ml
K pn. Ecl8 NTC Myc *ramA* 2.1	>4μg/ml	>4μg/ml
K pn. Ecl8 NTC Myc *ramA* 2.2	>4μg/ml	>4μg/ml
K pn. Ecl8 NTC Myc *ramA* 7.1	>4μg/ml	>4μg/ml
K pn. Ecl8 NTC Myc *ramA* 7.2	>4μg/ml	>4μg/ml
K pn. Ecl8 CTD Myc *ramA* 6.1	>4μg/ml	>4μg/ml
K pn. Ecl8 CTD Myc *ramA* 6.2	>4μg/ml	>4μg/ml
K pn. Ecl8 CTD Myc *ramA* 9.1	>4μg/ml	>4μg/ml
K pn. Ecl8 CTD Myc *ramA* 9.2	>4μg/ml	>4μg/ml

6.3.2 Validating by Western Blotting

To check whether RamA was tagged with the appropriate MYC and FLAG tags, SDS Page gel electrophoresis was performed on protein extracts of *E coli* DH10β containing the plasmids, NTD FLAG *ramA*, CTD MYC *ramA* and NTD MYC *ramA*. The gels were then transferred on to a PVDF membrane which was taken through immunoblotting by either anti MYC or anti FLAG antibody. Our results show successful tagging of the RamA protein as the resulting RamA-MYC should and RamA-FLAG.

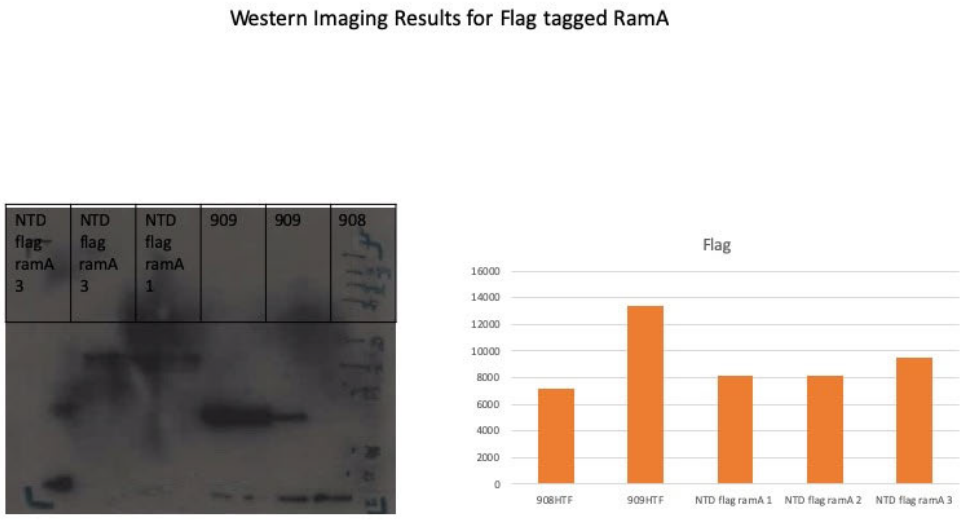


Figure 21: Western blot images of Flag antibody along with the ImageJ analysis. NTD Flag *ramA* clones 1, 2, and 3 were assessed along with 908 and 909 which are Ecl8 *ramA HTF* and Ecl8 *ΔramR ramA HTF* respectively.

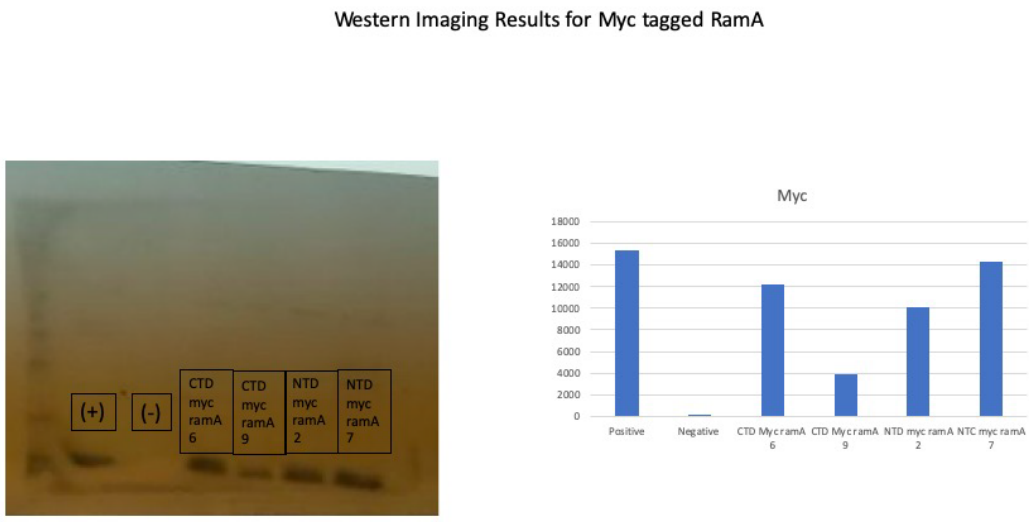


Figure 22: Western blot images of Myc antibody along with the ImageJ analysis. NTD myc *ramA* clones 2, and 7 & CTD myc *ramA* 6 and 9 were assessed along with an appropriate positive and negative control.

Section 7: Discussion

ChIPseq is an important technique that has been used to determine protein DNA binding association in numerous eukaryotic studies in the past. Recently the use of ChIPseq technique in determining association of protein and DNA interactions has also been done in prokaryotic models. This technique is efficient in performing a whole genome screen of all the possible recognition sites of a protein. This is of great importance when studying a transcriptional factor such as RamA which has implication on development of antibiotic resistance in a clinically relevant pathogen, *K pneumoniae.* It will not only allow us to study RamA as a regulator of resistance but also elucidate to us new specific pathways that can be targeted for combating antibiotics.

The first phase of RamA ChIPseq utilised chromosomally tagged Histidine-Tetracycline-FLAG (HTF) RamA in two backgrounds, i.e. the wild type Ecl8 and the RamA overexpressor (Ecl8 *⊿ramR*) (Bharathwaj, M.A., 2018). This study identified some key targets such as OmpK36/OmpC.

In this project efforts were aimed at validating other targets such as *tolQ*, *ybgC* and *rimP*. *tolQ* and *ybgC* are components of the TolPal operon and *rimP* encodes for an accessory ribosomal protein (See Appendix 5).

TolPal system is involved in directly or indirectly regulating the outer membrane lipid asymmetry in *E coli* and *S enterica* serovar Typhimurium (Chong, Woo, and Chng, 2015; Masilamani,Cian and Dalebroux,2018) . Additionally, this system helps in promoting OM constriction that assists in cell division by fission (Gerding, et al. 2007). It is comprised of seven proteins encoded by seven genes organised in two operons (Figure 7). The *ybgC* gene which expresses an acyl-CoA

thioesterase that forms a complex with an acyl carrier protein (ACP) and interacts with enzymes involved in GPL biosynthesis (Angelini, A. *et al.* 2008) and hence has a potential role in OM biogenesis. The second operon encodes for TolQRA that constitute the proton motif force in the inner membrane (IM) (Figure 5). TolQR transduces energy leading to conformational changes in TolA that releases Pal from TolB and promotes OM invagination. The biological function of *cpoB,* is not yet established but it has been suggested to bind with TolA and assist in septal constrictions.

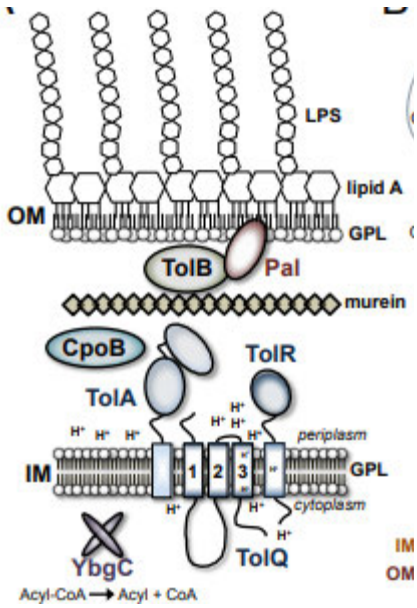


Figure 23: Tol-Pal assembly in *S. enterica.* Constituents of Tol-Pal system are present in the cytoplasm, periplasm, outer and inner membrane. The inner membrane (IM) is made up of phospholipids (PLs) and the outer membrane (OM) consists of an inner leaflet of PLs and an outer leaflet containing lipopolysaccharides (LPS). Gram-negative bacteria produce peptidoglycan which is attached to the OM by lipoproteins. B. TolQRA makes up the inner membrane system that transduces energy using proton motif force which allows TolA to span across the murein and displaces the TolB and Pal association. Image taken from Masilamani, Cian and Dalebroux, 2018.

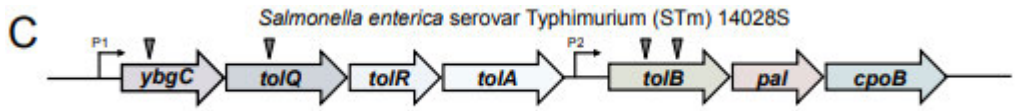


Figure 24: Genetic organization of Tol-Pal system in *S. enterica serovar Typhimurium*. The enterobacterial Tol -Pal system is transcribed from at least two promoters as two operons, *ybgC-tolQRA* and *tolB-pal-cpoB*. Image taken from Masilamani,Cian and Dalebroux, 2018

Salmonella TolPal mutants result in decrease rifampicin resistance and attenuated survival in macrophages and mice (Masilamani, Cian and Dalebroux,2018). Additionally, in *E coli* these mutants are associated with hypersensitivity to detergents and altered cell morphology (Chong, Woo, and Chng, 2015). These processes are attenuated more so by *tolQRA* and *cpoB* mutants than by *ybgC*, *tolB* and *Pal*. Hence there was a keen interest in understanding the possible role of TolPal in regulating barrier function and OM biogenesis and its regulation by RamA in *K. pneumoniae*. As promoter regions of *tolQ* and *ybgC* were a hit for RamA in the ChIPseq analysis, qPCR was performed to understand the nature of this *ramA* dependent regulation on their expression.

The regulation of *tolQ* has been briefly validated as part of the previous project, but the possible regulation of *ybgC* was yet to be explored. As part of this study, the relative abundance of *ybgC* and *tolQ* transcripts in *K pneumoniae* Ecl8 Δ*ramR*, where RamA is overexpressed using qPCR analysis was analysed. Even though there was an average upregulation of 3-4 folds observed for both the genes, the exceeding value of standard deviation observed in this analysis makes the data inconclusive to report any changes in the level of expression of these under RamA upregulation. There was a considerable challenge faced in RNA extraction without DNA contamination which can be attributed to technical errors and introduction of DNA contamination. As a confirmation of these results, a repeat of qPCR with RNA extracted at different phases of bacterial cell

growth, i.e. in lag, exponential and stationary phase is recommended. This would aid in concluding that the levels of *ybgC* and *tolQ* are unchanged under the overexpression of RamA. Alternatively, it would be helpful to use a strain with a Δ*ramA* mutation as an appropriate control. The validation of *ybgC* and *tolQ* transcriptional regulation by RamA would confirm its over-riding role in OM biogenesis and maintenance.

The regulation of *ybgC* and *rimP* was also assessed by cloning it's promoter region in a GFP expressing vector to observe its activation under RamA. Of the two promoter regions in the TolPal operon, i.e. upstream of *ybgC* and *tolQ* was cloned in to an expression plasmid of GFP. As part of this study the protocol for the GFP gene fusion assay was optimised. Black and clear 96 well plate was used to grow the strains. All the strains were grown in M9 media where the OD600 of the cultures was measured using the clear plate and the black bottom plate was used to measure the fluorescence every half an hour from the time of inoculation until 5 hours. The use of no washing steps made the assay less labour intensive (lower operator errors), allows shorter gaps between each time point and can reduce loss of cells from the washing of the pellet, hence resulting in less variability from one biological repeat to another. However, two different plates were used to assess the fluorescence (black plate) and optical density at OD600, we can reduce the variability even more by using 96 well black plates with clear bottoms.

The expression vectors with promoter regions from *ybgC* and *rimP* were cloned in Ecl8. The relative expression of GFP protein under excess of RamA by the release of its repression in the Δ*ramR* strain was done with respect to its expression in the wild type strain. Promoter regions of both the genes didn't show any increase in GFP expression under RamA. There was an observed trend of lower absolute fluorescence in the Ecl8 Δ*ramR* version of both pkCO26 *ybgC*

and pkCO26 *rimP*(Figure 14 & 15*)*. This observation is clearly different from the results of qPCR of *ybgC* and *tolQ* which indicate a small increase in their expression*.* But it should also be noted that since the culture was inoculated with 10μl of subculture in 200μl (1 in 10 dilution), the bacterial cultures was already in late exponential phase (0.5 OD600). Hence the difference in growth phase between the two assays doesn't allow us to make any conclusions about the promoter activation of the TolPal operon under RamA from lag to mid exponential phase. A lower dilution of the sub-culture (1 in 100 dilution) would be a recommended change in the protocol that can be used along with longer period of assessment of fluorescence and optical density.

The GFP results from the other putative target, *rimP,* suggested its down regulation under RamA in late exponential phase. RimP is an accessory ribosomal protein that stabilizes the 16s rRNA. In *E coli, ΔrimP* leads to accumulation of immature 16s rRNA, associated with 50S and 30S ribosomal unit(Nord *et al.*, 2009). *rimP* mutant models in *E coli* and Salmonella *enterica* serovar Enteritidis result in increased sensitivity to temperature and reactive oxygen intermediates. Furthermore, infection models using *S. enterica ΔrimP* cleared much faster from spleen and liver (Chang *et al.*, 2008). Similarly, a homologue of the *rimP* gene in *Streptomyces coelicolor*, *rimP-SC* has been involved in the increased synthesis of secondary metabolites like antibiotics(Pan *et al.*, 2013). It does so by increasing the translation speed but decreasing its fidelity via stabilisation of the translation machinery with near-cognate aminoacyl tRNA(Pan *et al.*, 2013). Hence, *rimP* facilitates faster growth by stabilisation of the translation machinery and allows for more translation errors. Its downregulation under RamA is rather unexpected, because of two reasons. Firstly, due to the known role of RamA as an activator of transcription rather than repressor. Secondly, due to the understanding that it is RimP's

overexpression that will help the bacteria accumulate more mutations and hence result in higher chances of resistance development.

To further assess the role of RimP attempts were made to develop Tigecycline mutants of Ecl8. It will be interesting to see the relation between tigecycline resistance which targets the 16s rRNA unit and RimP, involved in stabilizing the 30S ribosomal unit. Role of RamA in conferring tigecycline resistance by AcrA upregulation is well established (Ruzin *et al.*, 2005; Keeney, Ruzin and Bradford, 2007; R. Rosenblum *et al.*, 2011) and it would be interested to see whether RamA overexpressing can confer tigecycline resistance via modulating expression of other genetic determinants, like *rimP.*

Other than the changes specified in performing qPCR and GFP fusion assay it might be helpful to look at the other assays such as β galactosidase assay and Electrophoretic Mobility Shift Assay to confirm the targets identified by ChIPseq.

Other than validation of the previous targets, expression vectors expressing RamA tagged with MYC or FLAG were constructed and validated in this project. A first challenge faced in the cloning procedure was the release of two bands (Figure 18A and 18C)from the pJET1.2 vector due to the presence of a HindIII site in this commercial vector. This issue was resolved by running the original RamA PCR product so that the band corresponding to it can be excised from the gel. Of the four strains constructed, three of them (CTD/NTC MYC *ramA* and NTD FLAG *ramA*) were further assessed using antibiotic susceptibility assay and immunoblotting. The MIC of these strains were assessed under tigecycline as upregulation of RamA is known to confer tigecycline resistance. As observed in our results there was an increase in MIC (>4μg/ml) of *K pneumoniae* Ecl8 containing these tagged RamA expression vectors, like *K pneumoniae* Ecl8 Δ*ramR.* Further to this the results from Western blotting using anti-MYC and anti-FLAG antibody in the background of *E coli* DH10β confirms the expression

of the tagged RamA in all three assessed strains. The expression of this tagged RamA is an improved version from the last due to the smaller size of the tag, the absence of the tetracycline cassette and the ability to overexpress the protein when expressing from a plasmid.

Further to the use of tagged RamA to perform ChIPseq analysis, this phase of ChIPseq can also utilise the anti-sigma antibody to determine the binding sites of the RNA polymerase subunit across the genome of *K pneumoniae* Ecl8. This is an approach that has been previously used in the ChIPseq analysis of MarA and it would help identify RamA binding sites closer to the promoter regions identified(Sharma *et al.*, 2017). This will enrich the data and help screen for binding artefacts that can arise during the experiment.

Section 8: Further Expectations

This study will help define the role of RamA in the regulation of these target genes and give information that will aid to outlining the parameters (*rambox*) required for RamA mediated regulation which is yet largely unknown in *K. pneumoniae*. The information of the binding motif will open doors to look for targets in other strains of *K. pneumoniae* that are more pathogenic and virulent to understand the employment of cellular mechanisms in cell survival.

Section 9: References

Abouzeed, Y. M., Baucheron, S. and Cloeckaert, a. (2008) 'ramR Mutations Involved in Efflux-Mediated Multidrug Resistance in Salmonella enterica Serovar Typhimurium', *Antimicrobial Agents and Chemotherapy*, 52(7), pp. 2428–2434. doi: 10.1128/AAC.00084-08.

Alekshun, M. N. and Levy, S. B. (1997) 'Regulation of chromosomally mediated multiple antibiotic resistance: the mar regulon.', *Antimicrobial agents and chemotherapy*, 41(10), pp. 2067–75. Available at: https://www-ncbi-nlm-nih-gov.docadis.ups-tlse.fr/pmc/articles/PMC164072/ (Accessed: 13 March 2015).

Amábile-Cuevas, C. F. and Arredondo-García, J. L. (2013) 'Nitrofurantoin, phenazopyridine, and the superoxide-response regulon soxRS of Escherichia coli', Journal of Infection and Chemotherapy. Springer Japan, 19(6), pp. 1135–1140. doi: 10.1007/s10156-013-0635-4.

Amábile-Cuevas, C. F. and Demple, B. (1991) 'Molecular characterization of the soxRS genes of Escherichia coli: two genes control a superoxide stress regulon.', *Nucleic acids research*, 19(16), pp. 4479–84. doi: 10.1093/nar/19.16.4479.

Andersen, C. L., Matthey-Dupraz, A., Missiakas, D., & Raina, S. (1997). A new Escherichia coli gene, dsbG, encodes a periplasmic protein involved in disulphide bond formation, required for recycling DsbA/DsbB and DsbC redox proteins. Molecular Microbiology, 26(1), 121–132.

Angelini, A., Cendron, L., Goncalves, S., Zanotti, G., Terradot, L. (2008) 'Structural and enzymatic characterization of HP0496, a YbgC thioesterase from Helicobacter pylori', Proteins: Structure, Function, and Bioinformatics. Wiley-Blackwell, 72(4), pp. 1212–1221. doi: 10.1002/prot.22014.

Bailey, A. M., Ivens, A., Kingsley, R., Cottell, J. L., Wain, J., Piddock, L. J. V. (2010) 'RamA, a Member of the AraC/XylS Family, Influences Both Virulence and Efflux in Salmonella enterica Serovar Typhimurium', *Journal of Bacteriology*, 192(6), pp. 1607–1616. doi: 10.1128/JB.01517-09.

Barbosa, T. M. and Levy, S. B. (2002) 'Activation of the Escherichia coli *nfnB* gene by MarA through a highly divergent marbox in a class II promoter', Molecular Microbiology. Wiley/Blackwell (10.1111), 45(1), pp. 191–202. doi: 10.1046/j.1365-2958.2002.03006.x.

Bharathwaj, M.A., 2018. Defining the RamA regulon in Klebsiella pneumoniae using ChIP sequencing.

Bialek-Davenet, S., Marcon, E., Leflon-Guibout, V., Lavigne, J.P., Bert, F., Moreau, R. and Nicolas-Chanoine, M.H., 2011. In vitro selection of ramR and soxR mutants overexpressing efflux systems by fluoroquinolones as well as cefoxitin in Klebsiella pneumoniae. Antimicrobial agents and chemotherapy.

Blair, J.M., Webber, M.A., Baylay, A.J., Ogbolu, D.O. and Piddock, L.J., 2015. Molecular mechanisms of antibiotic resistance. Nature Reviews Microbiology, 13(1), p.42.

Boucher, H.W., Talbot, G.H., Bradley, J.S., Edwards, J.E., Gilbert, D., Rice, L.B., Scheld, M., Spellberg, B. and Bartlett, J., 2009. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clinical infectious diseases, 48(1), pp.1-12.

CDDEP, The Centre for Disease Dynamics Economics & Policy. ResistanceMap:[First reported cases of bacterial resistance against key antibiotics]. 2018.[https://cddep.org/tool/first_reported_cases_bacterial_resistance_against_key_antibiotics/]. Date accessed: [July 31st, 2019].

CDDEP (1), The Centre for Disease Dynamics Economics & Policy. ResistanceMap:[Antibiotic Resistance]. 2018.[https://resistancemap.cddep.org/AntibioticResistance.php]. Date accessed: [July 31st, 2019].

Chollet, R., Chevalier, J., Bollet, C., Pages, J.M. and Davin-Regli, A., 2004. RamA is an alternate activator of the multidrug resistance cascade in Enterobacter aerogenes. Antimicrobial agents and chemotherapy, 48(7), pp.2518-2523.

Chong, Z.-S., Woo, W.-F. and Chng, S.-S. (2015) 'Osmoporin OmpC forms a complex with MlaA to maintain outer membrane lipid asymmetry in *Escherichia coli*', *Molecular Microbiology*, 98(6), pp. 1133–1146. doi: 10.1111/mmi.13202.

Cohen, S. P., McMurry, L. M. and Levy, S. B. (1988) 'marA locus causes decreased expression of OmpF porin in multiple-antibiotic-resistant (Mar) mutants of Escherichia coli.', *Journal of bacteriology*, 170(12), pp. 5416–22. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=211632&tool=pmcentrez&rendertype=abstract (Accessed: 5 August 2015).

Cohen, S.P., McMurry, L.M., Hooper, D.C., Wolfson, J.S. and Levy, S.B., 1989. Cross-resistance to fluoroquinolones in multiple-antibiotic-resistant (Mar) Escherichia coli selected by tetracycline or chloramphenicol: decreased drug accumulation associated with membrane changes in addition to OmpF reduction. Antimicrobial agents and chemotherapy, 33(8), pp.1318-1325.

Cohen, S. P., Levy, S. B., Foulds, J., & Rosner, J. L. (1993). Salicylate induction of antibiotic resistance in Escherichia coli: activation of the mar operon and a mar-independent pathway. J. Bacteriol., 175(24), 7856–7862. Retrieved from http://jb.asm.org/content/175/24/7856.short

Cohen, S. P., Yan, W., & Levy, S. B. (1993). A Multidrug Resistance Regulatory Chromosomal Locus Is Widespread among Enteric Bacteria. Journal of Infectious Diseases, 168(2), 484–488. https://doi.org/10.1093/infdis/168.2.484

Dangi, B., Pelupessey, P., Martin, R.G., Rosner, J.L., Louis, J.M. and Gronenborn, A.M., 2001. Structure and dynamics of MarA-DNA complexes: an NMR investigation1. Journal of molecular biology, 314(1), pp.113-127.

De Majumdar, S., Veleba, M., Finn, S., Fanning, S. and Schneiders, T., 2013. Elucidating the Regulon of the Multi Drug Resistance Regulator RarA in K. pneumoniae. Antimicrobial agents and chemotherapy, pp.AAC-01998.

De Majumdar, S., Yu, J., Fookes, M., McAteer, S.P., Llobet, E., Finn, S., Spence, S., Monaghan, A., Kissenpfennig, A., Ingram, R.J. and Bengoechea, J., 2015. Elucidation of the RamA regulon in Klebsiella pneumoniae reveals a role in LPS regulation. PLoS pathogens, 11(1), p.e1004627.

Du, D. et al. (2014) 'Structure of the AcrAB–TolC multidrug efflux pump', Nature. Nature Publishing Group, 509(7501), pp. 512–515. doi: 10.1038/nature13205.

European Centre for Disease Prevention and Control. (2014) *Antimicrobial resistance (EARS-Net) - Annual Epidemiological Report for 2014*. Available at: https://ecdc.europa.eu/en/publications-data/antimicrobial-resistance-ears-net-annual-epidemiological-report-2014#copy-to-clipboard (Accessed: 13 August 2018).

Fernández, L. and Hancock, R. E. W. (2012) 'Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance.', *Clinical microbiology reviews*. American Society for Microbiology Journals, 25(4), pp. 661–81. doi: 10.1128/CMR.00043-12.

Fookes, M. et al. (2013) 'Genome Sequence of Klebsiella pneumoniae Ecl8, a Reference Strain for Targeted Genetic Manipulation.', Genome announcements. American Society for Microbiology (ASM), 1(1). doi: 10.1128/genomeA.00027-12.

Forage, R. G. and Lin, E. C. (1982) 'DHA system mediating aerobic and anaerobic dissimilation of glycerol in Klebsiella pneumoniae NCIB 418.', Journal of bacteriology. American Society for Microbiology Journals, 151(2), pp. 591–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6284704 (Accessed: 16 July 2019).

Gallegos, M.T., Schleif, R., Bairoch, A., Hofmann, K. and Ramos, J.L., 1997. Arac/XylS family of transcriptional regulators. Microbiology and Molecular Biology Reviews, 61(4), pp.393-410.

Gambino, L., Gracheck, S. J. and Miller, P. F. (1993) 'Overexpression of the MarA positive regulator is sufficient to confer multiple antibiotic resistance in Escherichia coli.', Journal of bacteriology, 175(10), pp. 2888–94. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=204606&tool=pmcentrez&rendertype=abstract (Accessed: 5 August 2015).

Geertz, M. and Maerkl, S. J. (2010) 'Experimental strategies for studying transcription factor-DNA binding specificities.', Briefings in functional genomics. Oxford University Press, 9(5–6), pp. 362–73. doi: 10.1093/bfgp/elq023.

George, A. M. and Levy, S. B. (1983) 'Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in Escherichia coli: involvement of a non-plasmid-determined efflux of tetracycline.', *Journal of bacteriology*, 155(2), pp. 531–40. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=217720&tool=pmcentrez&rendertype=abstract (Accessed: 7 April 2015).

George, A. M., Hall, R. M. and Stokes, H. W. (1995) 'Multidrug resistance in Klebsiella pneumoniae: a novel gene, ramA, confers a multidrug resistance phenotype in Escherichia coli', *Microbiology*. Microbiology Society, 141(8), pp. 1909–1920. doi: 10.1099/13500872-141-8-1909.

Geneva: World Health Organization (2017) *Global Antimicrobial Resistance Surveillance System (GLASS) Report: Early Implementation 2016-17*. Available at: http://apps.who.int/iris/bitstream/handle/10665/259744/9789241513449-eng.pdf?sequence=1 (Accessed: 13 August 2018).

Gerding, M.A., Ogata, Y., Pecora, N.D., Niki, H. and De Boer, P.A., 2007. The trans-envelope Tol–Pal complex is part of the cell division machinery and required for proper outer-membrane invagination during cell constriction in E. coli. Molecular microbiology, 63(4), pp.1008-1025.

Gervais, F. G. and Drapeau, G. R. (1992) 'Identification, cloning, and characterization of rcsF, a new regulator gene for exopolysaccharide synthesis that suppresses the division mutation ftsZ84 in Escherichia coli K-12.', Journal of bacteriology. American Society for Microbiology Journals, 174(24), pp. 8016–22. doi: 10.1128/JB.174.24.8016-8022.1992.

Golubeva, Y. A. et al. (2016) 'Intestinal Long-Chain Fatty Acids Act as a Direct Signal To Modulate Expression of the Salmonella Pathogenicity Island 1 Type III Secretion System.', mBio. American Society for Microbiology (ASM), 7(1), pp. e02170-15. doi: 10.1128/mBio.02170-15.

Gordân, R., Hartemink, A. J. and Bulyk, M. L. (2009) 'Distinguishing direct versus indirect transcription factor-DNA interactions.', Genome research. Cold Spring Harbor Laboratory Press, 19(11), pp. 2090–100. doi: 10.1101/gr.094144.109.

Gossett, A. J. and Lieb, J. D. (2008) 'DNA Immunoprecipitation (DIP) for the Determination of DNA-Binding Specificity', Cold Spring Harbor Protocols, 2008(4), p. pdb.prot4972-pdb.prot4972. doi: 10.1101/pdb.prot4972.

Griffith, K.L., Shah, I.M., Myers, T.E., O'Neill, M.C. and Wolf Jr, R.E., 2002. Evidence for “pre-recruitment” as a new mechanism of transcription activation in Escherichia coli: the large excess of SoxS binding sites per cell relative to the number of SoxS molecules per cell. Biochemical and biophysical research communications, 291(4), pp.979-986.

Griffith, K.L., Fitzpatrick, M.M., Keen III, E.F. and Wolf Jr, R.E., 2009. Two functions of the C-terminal domain of Escherichia coli Rob: mediating “sequestration–dispersal” as a novel off–on switch for regulating Rob’s activity as a transcription activator and preventing degradation of Rob by Lon protease. Journal of molecular biology, 388(3), pp.415-430.

Hächler, H., Cohen, S. P., & Levy, S. B. (1991). marA, a regulated locus which controls expression of chromosomal multiple antibiotic resistance in Escherichia coli. Journal of Bacteriology, 173(17), 5532–5538. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=208267&tool=pmcentrez&rendertype=abstract

Hentschke, M., Wolters, M., Sobottka, I., Rohde, H. and Aepfelbacher, M., 2010. ramR mutations in clinical isolates of Klebsiella pneumoniae with reduced susceptibility to tigecycline. Antimicrobial agents and chemotherapy, 54(6), pp.2720-2723.

Hobbs, E.C., Yin, X., Paul, B.J., Astarita, J.L. and Storz, G., 2012. Conserved small protein associates with the multidrug efflux pump AcrB and differentially affects

antibiotic resistance. Proceedings of the National Academy of Sciences, 109(41), pp.16696-16701.

Jair, K. W. et al. (1995) 'Purification and regulatory properties of MarA protein, a transcriptional activator of Escherichia coli multiple antibiotic and superoxide resistance promoters.', Journal of bacteriology. American Society for Microbiology (ASM), 177(24), pp. 7100–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8522515 (Accessed: 22 January 2018).

Jair, K.-W. et al. (1996) 'Ambidextrous transcriptional activation by SoxS: requirement for the C-terminal domain of the RNA polymerase alpha subunit in a subset of Escherichia coli superoxide-inducible genes', Molecular Microbiology, 19(2), pp. 307–317. doi: 10.1046/j.1365-2958.1996.368893.x.

Keeney, D., Ruzin, A. and Bradford, P. A. (2007) 'RamA, a Transcriptional Regulator, and AcrAB, an RND-Type Efflux Pump, are Associated with Decreased Susceptibility to Tigecycline in Enterobacter cloacae', Microbial Drug Resistance, 13(1), pp. 1–6. doi: 10.1089/mdr.2006.9990.

Kukurba, K. R. and Montgomery, S. B. (2015) 'RNA Sequencing and Analysis.', *Cold Spring Harbor protocols*. NIH Public Access, 2015(11), pp. 951–69. doi: 10.1101/pdb.top084970.

Malinverni, J. C. and Silhavy, T. J. (2009) 'An ABC transport system that maintains lipid asymmetry in the gram-negative outer membrane.', *Proceedings of the National Academy of Sciences of the United States of America*, 106(19), pp. 8009–14. doi: 10.1073/pnas.0903229106.

Mariscotti, J. F. and García-Del Portillo, F. (2008) 'Instability of the Salmonella RcsCDB signalling system in the absence of the attenuator IgaA'. doi: 10.1099/mic.0.2007/015891-0.

Martin, R. G., Gillette, W. K., Martin, N. I. & Rosner, J. L. Complex formation between activator and RNA polymerase as the basis for transcriptional activation by MarA and SoxS in Escherichia coli. Mol. Microbiol. 43, 355–370 (2002).

Martin, R. M. and Bachman, M. A. (2018) 'Colonization, Infection, and the Accessory Genome of Klebsiella pneumoniae', *Frontiers in Cellular and Infection Microbiology*, 8, p. 4. doi: 10.3389/fcimb.2018.00004Magill, S. S. *et al.* (2014) 'Multistate Point-Prevalence Survey of Health Care–Associated Infections', *New England Journal of Medicine*. Massachusetts Medical Society , 370(13), pp. 1198–1208. doi: 10.1056/NEJMoa1306801.

Masilamani, R., Cian, M. B. and Dalebroux, Z. D. (2018) 'Salmonella Tol-Pal Reduces Outer Membrane Glycerophospholipid Levels for Envelope Homeostasis and Survival during Bacteremia.', *Infection and immunity*. American Society for Microbiology Journals, 86(7), p. IAI.00173-18. doi: 10.1128/IAI.00173-18.

Merlin, C., McAteer, S. and Masters, M. (2002) 'Tools for characterization of Escherichia coli genes of unknown function.', Journal of bacteriology, 184(16), pp. 4573–81. doi: 10.1128/jb.184.16.4573-4581.2002.

Myers, K.S., Park, D.M., Beauchene, N.A. and Kiley, P.J., 2015. Defining bacterial regulons using ChIP-seq. Methods, 86, pp.80-88.

Nikaido, E., Yamaguchi, A. and Nishino, K. (2008) 'AcrAB multidrug efflux pump regulation in Salmonella enterica serovar Typhimurium by RamA in response to environmental signals.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 283(35), pp. 24245–53. doi: 10.1074/jbc.M804544200.

Nord, S., Bylund, G.O., Lövgren, J.M. and Wikström, P.M., 2009. The RimP protein is important for maturation of the 30S ribosomal subunit. Journal of molecular biology, 386(3), pp.742-753.

O'Neill, J. (2016) *Tackling drug-resistant infections globally: final report and recommendations*, *the Review on Antimicrobial Resistance*. doi: 10.1016/j.jpha.2015.11.005.

Oz, T., Guvenek, A., Yildiz, S., Karaboga, E., Tamer, Y.T., Mumcuyan, N., Ozan, V.B., Senturk, G.H., Cokol, M., Yeh, P. and Toprak, E., 2014. Strength of selection pressure is an important parameter contributing to the complexity of antibiotic resistance evolution. Molecular biology and evolution, 31(9), pp.2387-2401.

Padilla, E., Llobet, E., Doménech-Sánchez, A., Martínez-Martínez, L., Bengoechea, J.A. and Albertí, S., 2010. Klebsiella pneumoniae AcrAB efflux pump contributes to antimicrobial resistance and virulence. Antimicrobial agents and chemotherapy, 54(1), pp.177-183.

Pan, Y., Lu, C., Dong, H., Yu, L., Liu, G. and Tan, H., 2013. Disruption of rimP-SC, encoding a ribosome assembly cofactor, markedly enhances the production of several antibiotics in Streptomyces coelicolor. Microbial cell factories, 12(1), p.65.

Pendleton, J. N., Gorman, S. P. and Gilmore, B. F. (2013) 'Clinical relevance of the ESKAPE pathogens.', *Expert review of anti-infective therapy*. Informa HealthcareLondon, 11(3), pp. 297–308. doi: 10.1586/eri.13.12.

Pomposiello, P. J. and Demple, B. (2000) 'Identification of SoxS-regulated genes in Salmonella enterica serovar typhimurium.', *Journal of bacteriology*. American Society for Microbiology, 182(1), pp. 23–9. doi: 10.1128/JB.182.1.23-29.2000.

Rosenblum, R., Khan, E., Gonzalez, G., Hasan, R. and Schneiders, T., 2011. Genetic regulation of the ramA locus and its expression in clinical isolates of Klebsiella pneumoniae. International journal of antimicrobial agents, 38(1), pp.39-45.

Ruzin, A., Visalli, M.A., Keeney, D. and Bradford, P.A., 2005. Influence of transcriptional activator RamA on expression of multidrug efflux pump AcrAB and tigecycline susceptibility in Klebsiella pneumoniae. Antimicrobial agents and chemotherapy, 49(3), pp.1017-1022.

Rice, L. B. (2008) 'Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE.', *The Journal of infectious diseases*, 197(8), pp. 1079–81. doi: 10.1086/533452.

Seo, S.W., Kim, D., Szubin, R. and Palsson, B.O., 2015. Genome-wide reconstruction of OxyR and SoxRS transcriptional regulatory networks under oxidative stress in Escherichia coli K-12 MG1655. Cell Reports, 12(8), pp.1289-1299.

Sharma, P., Haycocks, J.R., Middlemiss, A.D., Kettles, R.A., Sellars, L.E., Ricci, V., Piddock, L.J. and Grainger, D.C., 2017. The multiple antibiotic resistance operon of enteric bacteria controls DNA repair and outer membrane integrity. Nature Communications, 8(1), p.1444.

Schenley Laboratories (1944) 'Thanks to Penicillin...He will come home', *Life Magazine*, August.

Schneiders, T., Amyes, S. G. B. and Levy, S. B. (2003) 'Role of AcrR and ramA in fluoroquinolone resistance in clinical Klebsiella pneumoniae isolates from Singapore.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology, 47(9), pp. 2831–7. doi: 10.1128/AAC.47.9.2831-2837.2003.

van Straaten, M., Missiakas, D., Raina, S., & Darby, N. J. (1998). The functional properties of DsbG, a thiol-disulfide oxidoreductase from the periplasm of Escherichia coli. FEBS Letters, 428(3), 255–258.\

Bessette et al., 1999)

Veleba, M., De Majumdar, S., Hornsey, M., Woodford, N. and Schneiders, T., 2013. Genetic characterization of tigecycline resistance in clinical isolates of

Enterobacter cloacae and Enterobacter aerogenes. Journal of Antimicrobial Chemotherapy, 68(5), pp.1011-1018.

Viveiros, M., Dupont, M., Rodrigues, L., Couto, I., Davin-Regli, A., Martins, M., Pagès, J.M. and Amaral, L., 2007. Antibiotic stress, genetic response and altered permeability of E. coli. PloS one, 2(4), p.e365.

Wall, E., Majdalani, N. and Gottesman, S. (2018) 'The Complex Rcs Regulatory Cascade', Annual Review of Microbiology. Annual Reviews 4139 El Camino Way, PO Box 10139, Palo Alto, California 94303-0139, USA , 72(1), p. annurev-micro-090817-062640. doi: 10.1146/annurev-micro-090817-062640.

Wang, Z., Gerstein, M. and Snyder, M. (2009) 'RNA-Seq: a revolutionary tool for transcriptomics', *Nature Reviews Genetics*. Nature Publishing Group, 10(1), pp. 57–63. doi: 10.1038/nrg2484.

Webber, M. A. and Piddock, L. J. (2001) 'Absence of mutations in marRAB or soxRS in acrB-overexpressing fluoroquinolone-resistant clinical and veterinary isolates of Escherichia coli.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology (ASM), 45(5), pp. 1550–2. doi: 10.1128/AAC.45.5.1550-1552.2001.

Webber, M. A. and Piddock, L. J. V. (2003) 'The importance of efflux pumps in bacterial antibiotic resistance', *Journal of Antimicrobial Chemotherapy*. Oxford University Press, 51(1), pp. 9–11. doi: 10.1093/jac/dkg050.

WHO News Release (2018) 'WHO | High levels of antibiotic resistance found worldwide, new data shows', *WHO*. Available at: http://www.who.int/mediacentre/news/releases/2018/antibiotic-resistance-found/en/#.W3FPGbi2yFM.mendeley (Accessed: 13 August 2018).

World Health Organization (2018) *Antimicrobial resistance*. Available at: http://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance (Accessed: 13 August 2018).

Yamasaki, S., Nikaido, E., Nakashima, R., Sakurai, K., Fujiwara, D., Fujii, I. and Nishino, K., 2013. The crystal structure of multidrug-resistance regulator RamR with multiple drugs. Nature communications, 4, p.2078.

Yassien, M.A., Ewis, H.E., Lu, C.D. and Abdelal, A.T., 2002. Molecular cloning and characterization of the Salmonella enterica serovar Paratyphi B rma gene, which confers multiple drug resistance in Escherichia coli. Antimicrobial agents and chemotherapy, 46(2), pp.360-366.

Yelin, I. and Kishony, R., 2018. Antibiotic resistance. Cell, 172(5), pp.1136-1136.

Zheng, J., Cui, S. and Meng, J. (2009) 'Effect of transcriptional activators RamA and SoxS on expression of multidrug efflux pumps AcrAB and AcrEF in

fluoroquinolone-resistant Salmonella Typhimurium', *Journal of Antimicrobial Chemotherapy*. Oxford University Press, 63(1), pp. 95–102. doi: 10.1093/jac/dkn448

Section 10: Appendix

Appendix 1: MIC of strains used in the first phase of MIC

	FOX	CTX	IMP
Ecl8	2	<0.125	0.25
Ecl8acrAB<Km>	<0.5	<0.125	0.125
Ecl8 ΔompC	4	0.25	0.5
Ecl8ramAHTF	32	0.25	0.25
Ecl8ramAHTFompC<Km>	>32	1	0.5
Ecl8ΔramR	32	0.25	0.125
Ecl8ΔramRacrAB<Km>	1	<0.125	<0.0625
Ecl8ΔramRΔompC	>32	1	0.5
Ecl8ΔramRramAHTF	32	0.25	0.25
Ecl8ΔramRramAHTFompC<Km>	>32	1	0.5
Ecl8ΔramA	1	<0.125	0.125
Ecl8ΔramApACramA	2	<0.125	0.125
Ecl8ΔramApACYC177	1	<0.125	0.125

Figure 22. MICs for cefoxitin, cefotaxime and imipenem (n=3; all units are in µg/ml)

Figure 3: MIC Results from the first phase of ChIPseq performed by the previous MScR student. The table highlights the difference in the MIC of the wild type, Ecl8 and Ecl8 *ramA-HTF,* for all the three tested antibiotics, i.e. cefoxitin (FOX), cefotaxime (CTX), imipenem (IMP). The observed MIC for Ecl8*ΔramR ramA-HTF* and is similar to Ecl8 *ramA-HTF and* Ecl8*ΔramR* (Bharathwaj, M.A., 2018).

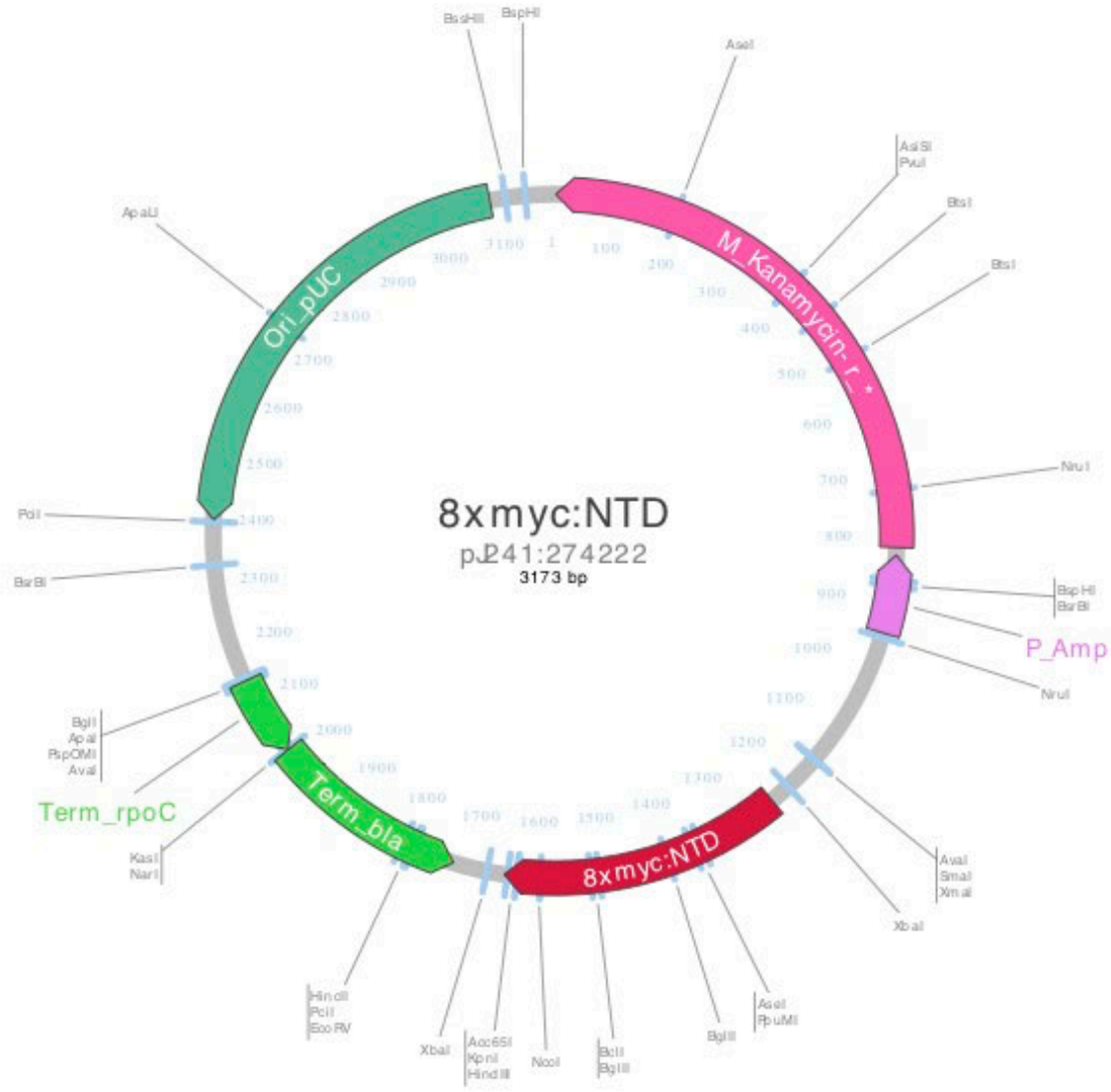
Appendix 2: Plasmid Maps of the tag containing expression vectors

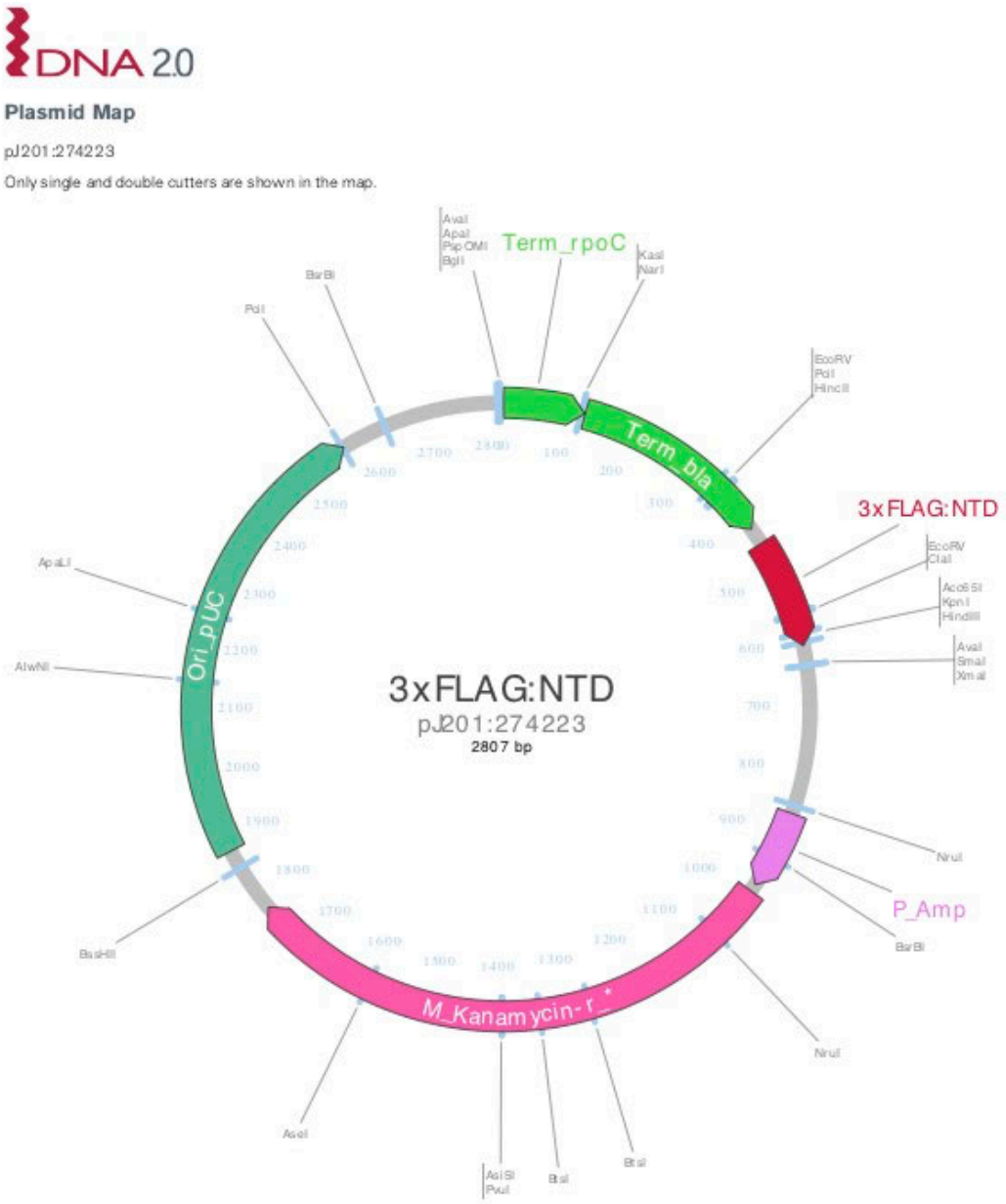
DNA 2.0

Plasmid Map

pJ241:274222

Only single and double cutters are shown in the map.



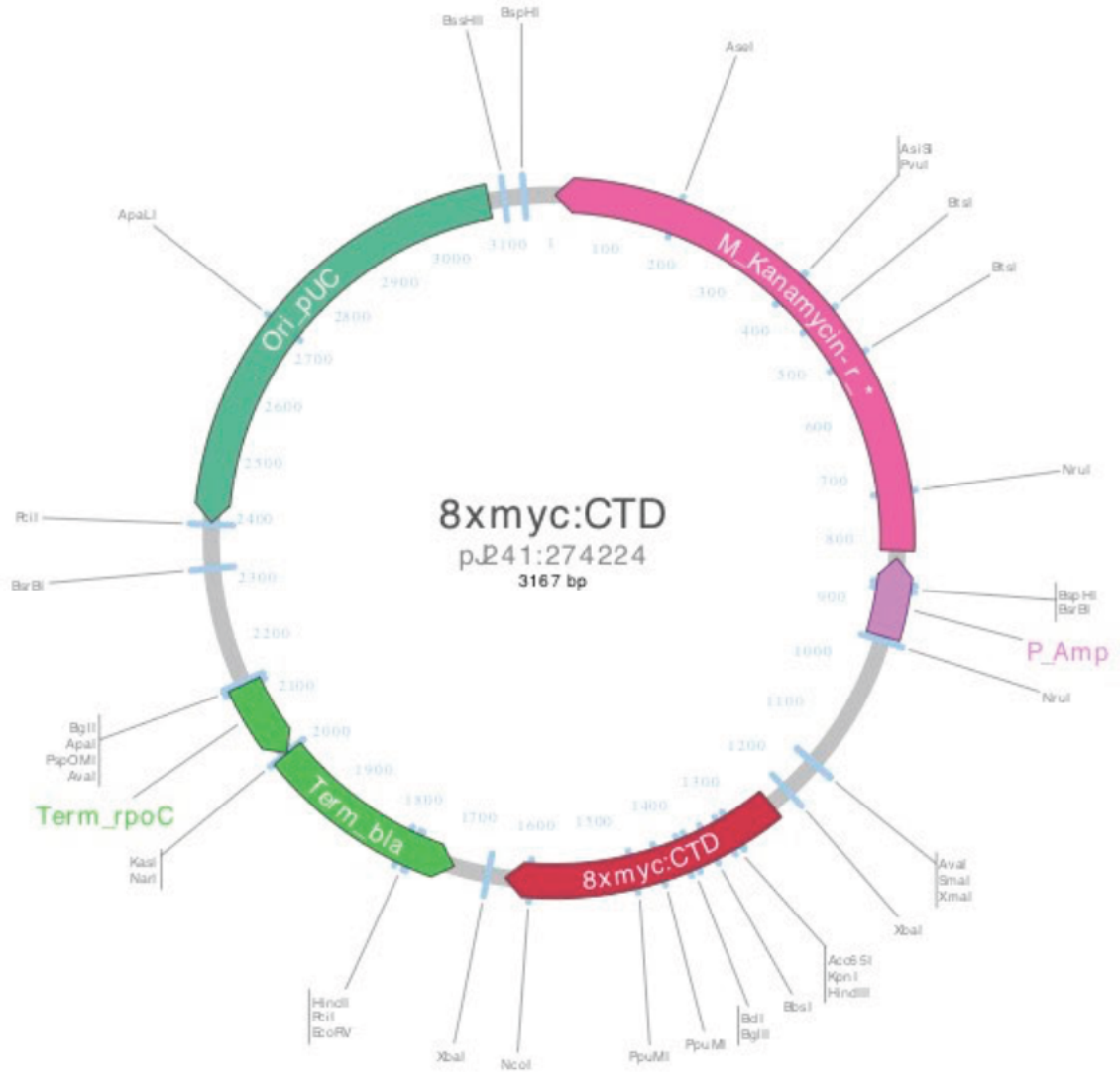

DNA 2.0
Plasmid Map
pJ201:274223
Only single and double cutters are shown in the map.
3xFLAG:NTD
pJ201:274223
2807 bp
Term_rpoC
Term_bla
3xFLAG:NTD
P_Amp
M_Kanamycin-r_*
Ori_pUC
AvaI
ApaI
PspOMI
BglI
KasI
NarI
BsrBI
PstI
EcoRV
PciI
HincII
EcoRV
ClaI
Acc65I
KpnI
HindIII
AvaI
SmaI
XmaI
NruI
BsrBI
NruI
BtsI
BtsI
AsiSI
PvuI
AseI
BssHII
AlwNI
ApaLI

DNA 2.0

Plasmid Map

pJ241:274224

Only single and double cutters are shown in the map.

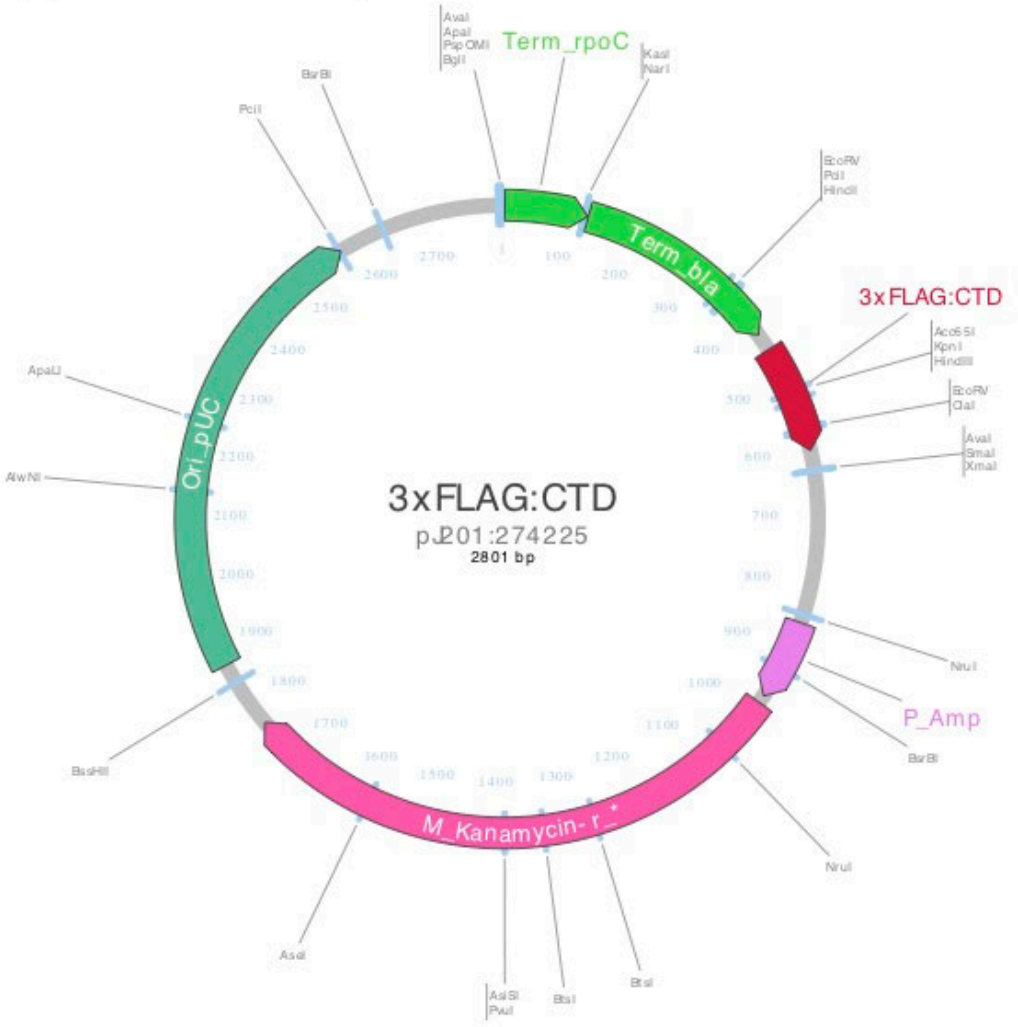


DNA 2.0

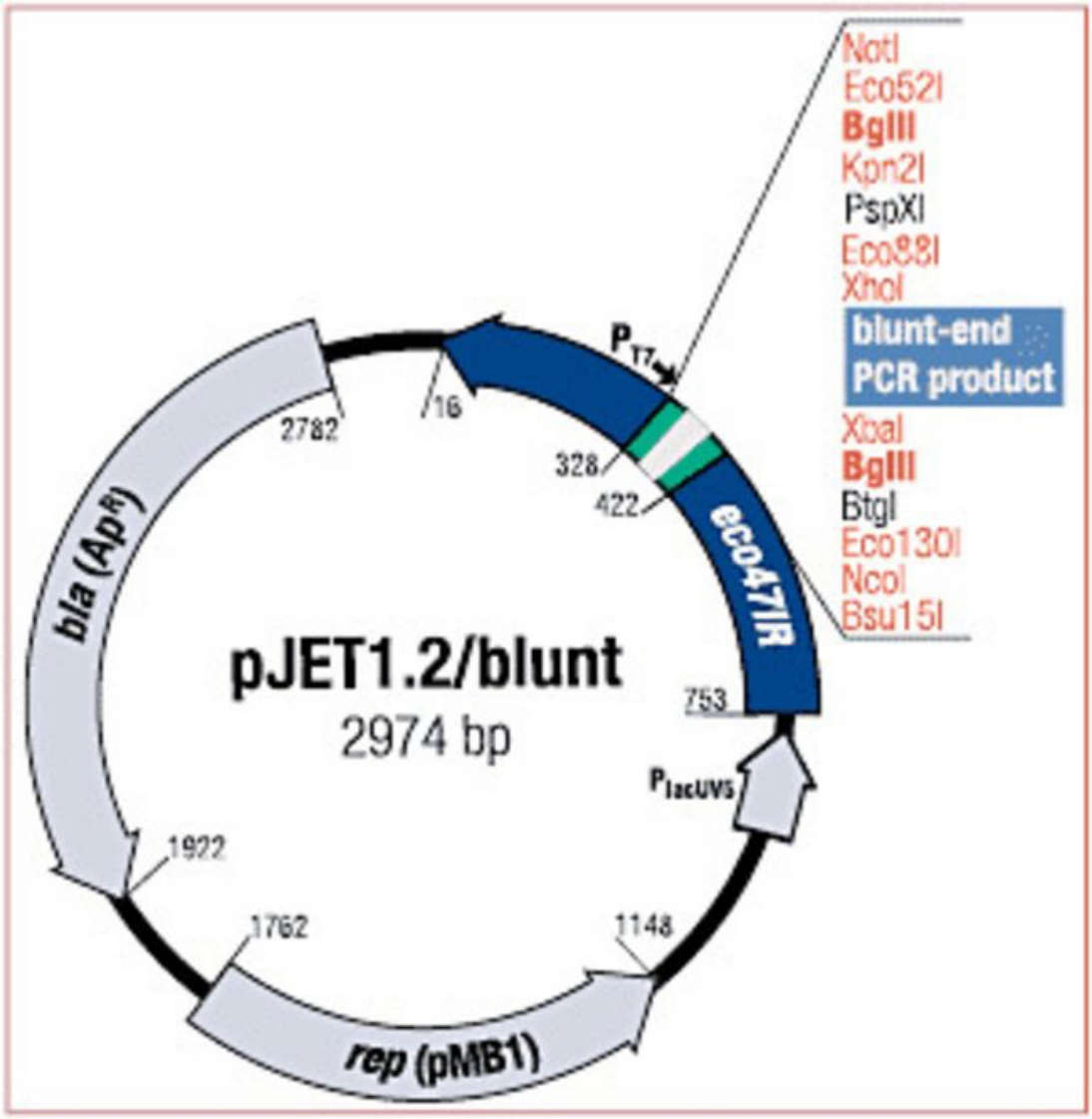
Plasmid Map

pJ201:274225

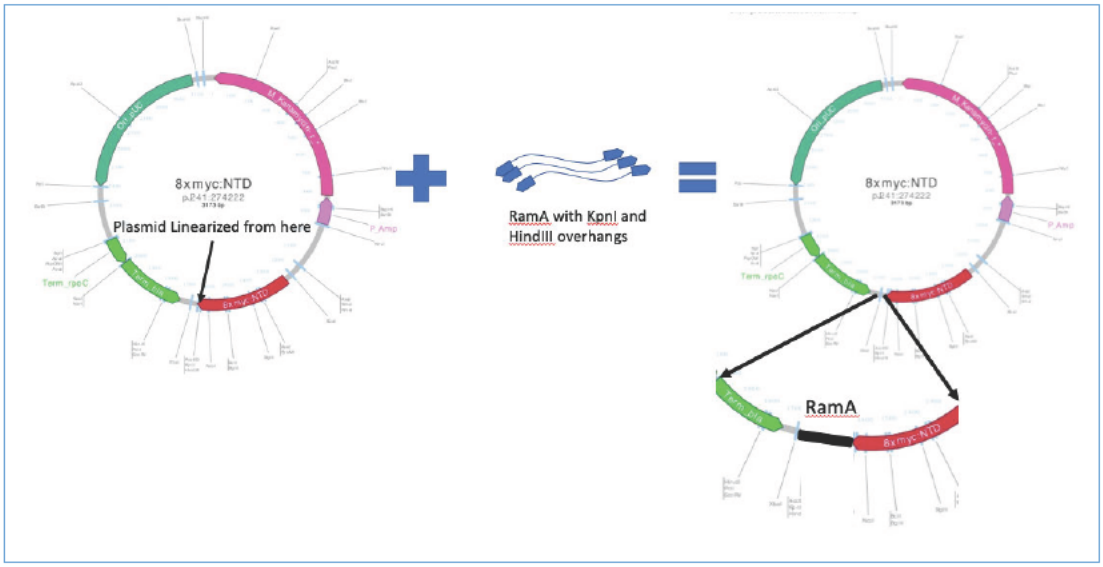
Only single and double cutters are shown in the map.

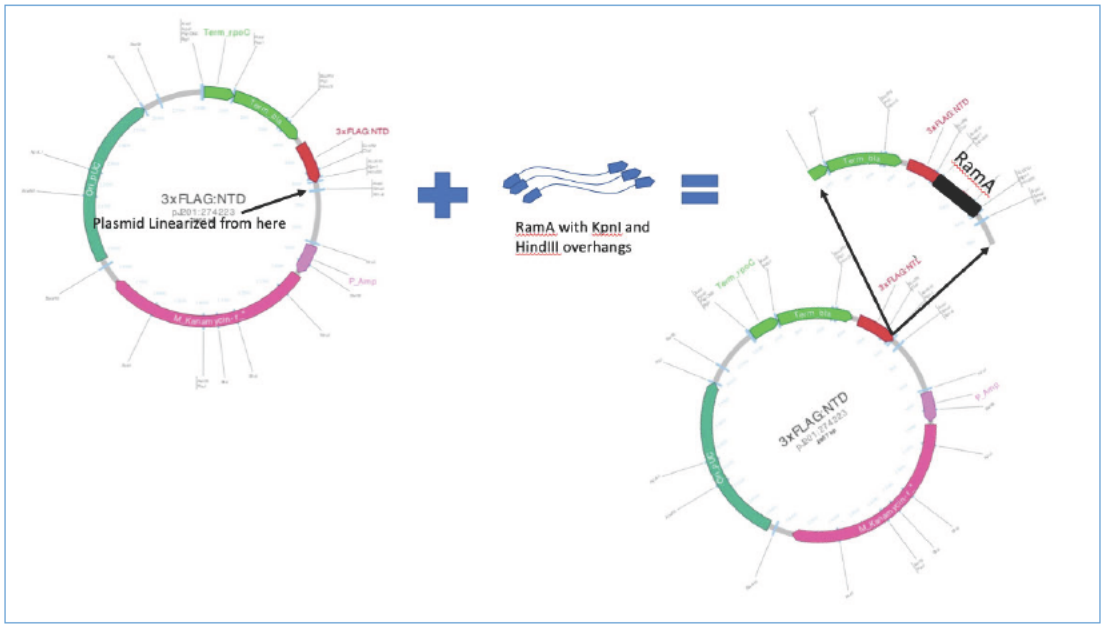


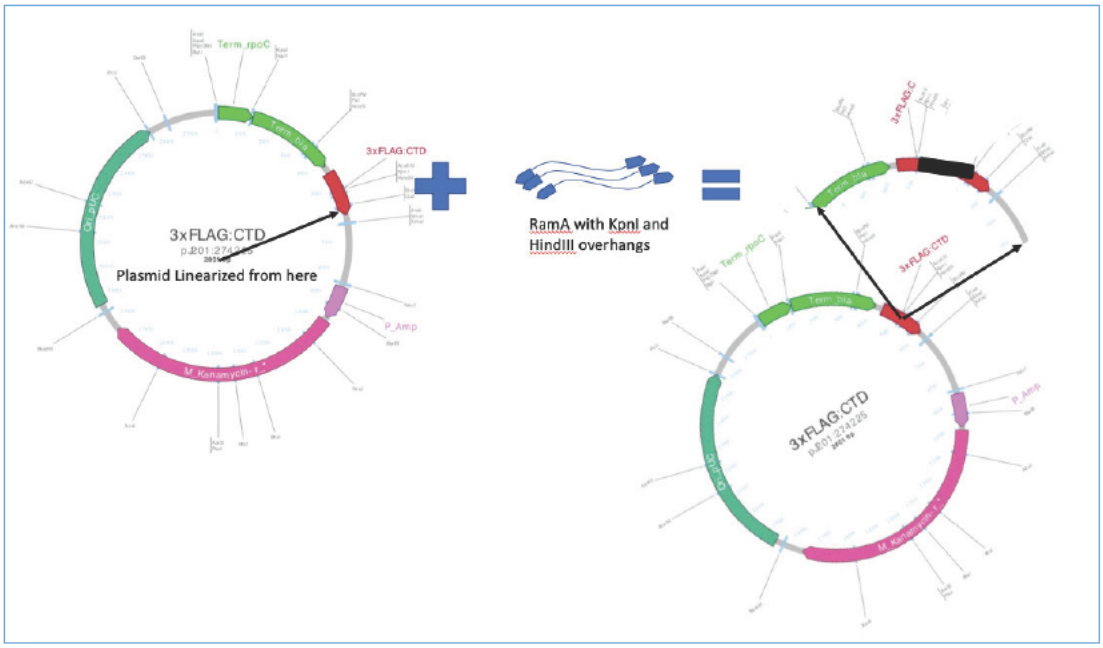
Appendix 3: Plasmid map of pJET1.2

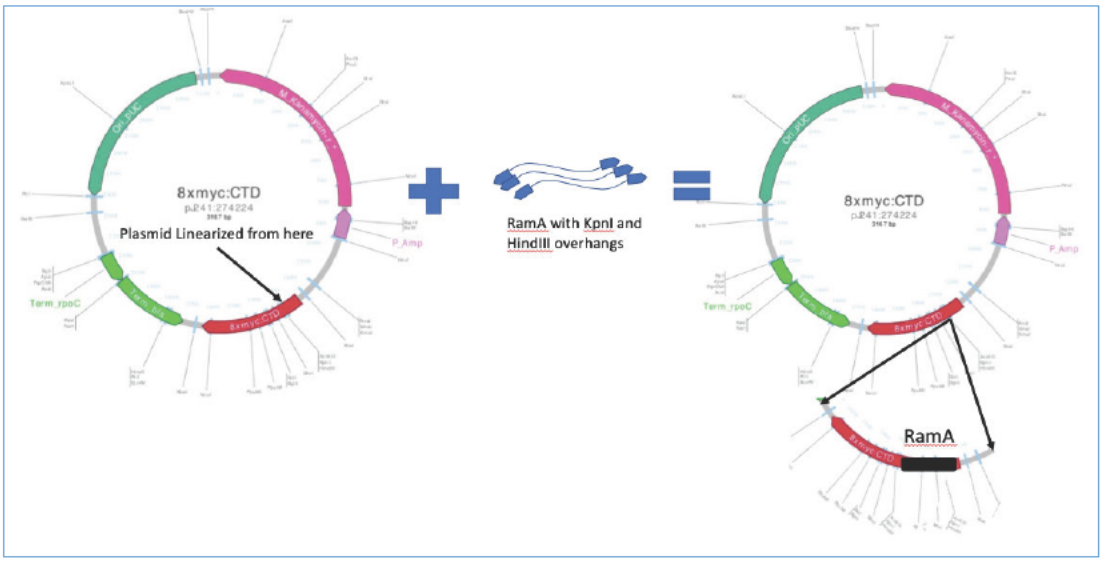


Appendix 4: Plasmid map for the tagged plasmids






Term_rpoC
3xFLAG:CTD
3xFLAG:CTD
Plasmid Linearized from here
P_Amp
M_Kanamycin-Y
RamA with KpnI and
HindIII overhangs
3xFLAG:CTD
3xFLAG:CTD
P_Amp


8xmyc:CTD
Plasmid Linearized from here
P_Amp
Term_rpoC
8xmyc:CTD
RamA with KpnI and
HindIII overhangs
8xmyc:CTD
P_Amp
Term_rpoC
RamA

Appendix 5: Results of the first phase of ChIPseq