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# CrrAB-dependent capsule formation in *K.pneumoniae*

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**Contents**

**Anti-microbial resistance**

The pace at which multidrug-resistant bacteria (MDR) are increasing and spreading around the world is worrying and it is now recognised by the World Health Organization as a public health threat (Shrivastava, Shrivastava and Ramasamy, 2018). To give a scale on the pressing issue, Jim O' Neill in his report on anti-microbial resistance (AMR) predicted a drastic increase in deaths caused by MDR bacteria, potentially reaching 50.000.000 deaths per year by 2050 (Review on Antimicrobial Resistance, 2016). Bacteria can acquire resistance through transmission of antimicrobial resistance genes (carried either on bacterial chromosome, plasmids, or transposons), to multiple antibiotics. Thus, creating MDR bacteria (Paterson, 2004).

In order to raise awareness, the World Health Organization (WHO) has recently identified the ESKAPES (*E. faecium, S. aureus, K.pneumoniae, A. baumannii, P.aeruginosa, Enterobacter spp. , S. maltophila*) group of pathogens which pose an imminent threat to human and animal health as antibiotic options against these bacteria are limited (Antibiotic-resistant priority pathogens list, 2017).

To be precise, carbapenem resistant *A. baumannii* and *P. aeruginosa*, extended spectrum β-lactamase (ESBL) or **carbapenem** resistant *K. pneumoniae* and *Enterobacter spp*. are listed in the critical priority list of pathogens. Vancomycin resistant *E. faecium* (VRE) and methicillin and vancomycin resistant *S. aureus* (MRSA and VRSA) are in the high priority group (Antibiotic-resistant priority pathogens list, 2017).

### *Klebsiella pneumoniae* in the clinical setting

The critical priority list consists of three pathogens which amount for most nosocomial infections. *Klebsiella pneumoniae* and *Acinetobacter spp.* interchange between the highest rate of infection (Agaba, Tumukunde, Tindimwebwa and Kwizera, 2017). The high infection rate is attributed to their ability to exhibiting multiple pathogenesis routes leading to a plethora of symptoms. These can range from pneumoniae to liver abscesses and septic shock, ultimately proving lethal if left untreated. Furthermore, their virulence can greatly increase through the acquisition of mobile elements (such as pathogenicity islands) or through chromosomal mutations leading to increased avoidance of the host immune system (Podschun and Ullmann,

1998). Overall, these capabilities make them successful in colonization of the host and in producing disease. But they do not render treatment regimens ineffective. Thus, the major threat lies when these capabilities are coupled to extensive resistance to antibiotics. One of the most studied examples is the increased mortality rate associated with MDR *K.pneumoniae* arising from the recent surge in AMR.

### Emergence of β-lactam resistance in *K.pneumoniae*

β-lactams retain the central role in treatment of gram-negative bacteria, such as *K.pneumoniae*. These include penicillins, cephalosporins, carbapenems and monobactams. The central component of all is the β-lactam ring which prevents cell wall synthesis in gram-negative bacteria. Resistance to β-lactams, arises from β-lactamase enzymes that hydrolyze the amide bond of the four-membered β-lactam ring (Tooke et al., 2019). Multiple enzymes disseminate on mobile elements across *Enterobacteriaceae* thus proliferating and spreading. The most prevalent genes, guiding β-lactam resistance, are KPC, NDM and OXA-48 which are frequently encountered in *K.pneumoniae* (Tooke et al., 2019).

The threat imposed by the acquisition of genes is portrayed in a meta-analysis of 10000 clinical isolates of *K.pneumoniae* collected in Asia. The findings reveal the increased resistance to numerous β-lactams. To be precise: aztreonam (monobactam)(73.3%) ceftazidime(3$^{rd}$ generation cephalosporin) (75.7%), cefotaxime (3$^{rd}$ generation cephalosporin) (79.2%), cefepime (4$^{th}$ generation cephalosporin) (72.6%) and imipenem (carbapenem)(65.6%) (Effah, Sun, Liu and Wu, 2020).

As it can be seen in the data above more than half of the *K.pneumoniae* clinical isolates exhibit resistance to their respective treatment regime thus decreasing the effectiveness of β-lactam antibiotics as the standard treatment regime. The perpetual increase in resistance to β-lactams and specifically carbapenems, required the introduction of novel antibiotics and prolongation of old antibiotic use. Due to the lack of novel antibiotics and the necessity of immediate treatments the re-introduction of old antibiotics as the predominant coping mechanism. One of the most effective in the treatment of carbapenem resistant *K.pneumoniae* is colistin.

**Colistin**

Also known as polymyxin E, colistin is a peptide antibiotic that is a natural product isolated from *Bacillus polymyxin*. The polymyxin antibiotics were first discovered in 1947, with the first clinical use of colistin recorded in the US in 1959(Govil et al., 2009). Colistin has significant toxicity, primarily renal and neurologic. The renal side effects include kidney injury and, in some cases, complete failure. Since colistin is eliminated by renal excretion, where toxicity occurs, it is imperative to assess renal function closely during therapy, decreasing the dosage if any degree of renal insufficiency is noted. Due to the complications associated with the treatment regime, colistin is only administered in cases where no other antibiotic proves effective (Govil et al., 2009).

Due to the nature of colistin cross-resistance with β-lactams is rarely seen. Subsequently, colistin has been used as a viable option in the treatment of the critical priority carbapenem resistant *K.pneumoniae* . Its effectivity lies on the peptide structure which consists of 10 linked amino acids, 6 of which form a peptide ring structure attached to a fatty acid side chain (Figure 1).

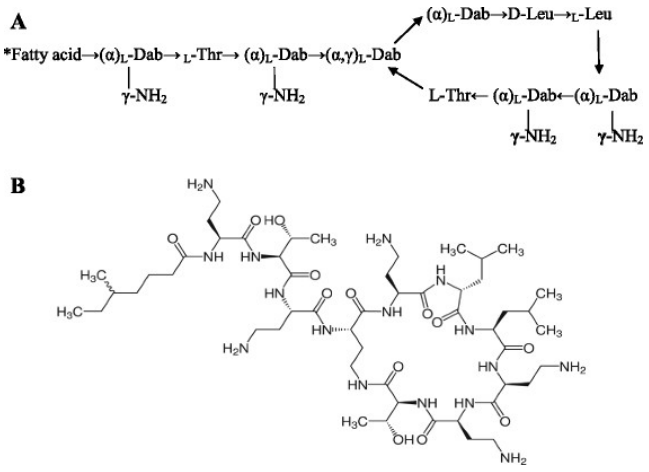


Figure 1:. Molecular (A) and Structural (B) formula of colistin. The formulas are a result of Mass Spectrometry conducted by (Gurjar, 2015).

## Requirements for colistin susceptibility:

There are two requirements for susceptibility to colistin. Firstly, the pathogen needs to be a gram-negative bacterium. Secondly, the mechanism of action of colistin requires *de novo* synthesis of lipopolysaccharides (LPS) (Sabnis et al., 2018). Situated on the outer leaflet of the outer membrane, LPS contributes to the stability of the membrane.

Moreover, it enhances the pathogenicity of the bacteria, and is a key factor in septic shock.

Single molecules of LPS can be divided in three main structural compartments based on their spatial orientation with respect to the center of the outer membrane (OM) bilayer and their chemical structure (Figure 2). Firstly, the base of the molecule is called lipid A, abundant in long chains of polysaccharides attached to the phosphate groups of the bilayer and fixating the molecule on the OM. Secondly, is the core of LPS, consisting of the inner and outer core, acting as a bridge between lipid A and the O-antigen. Finally, the outer compartment of LPS, namely O-antigen defines the antigenic character of the molecule, it is highly variable and crucial in immune evasion and toxicity (Bertani and Ruiz, 2018).

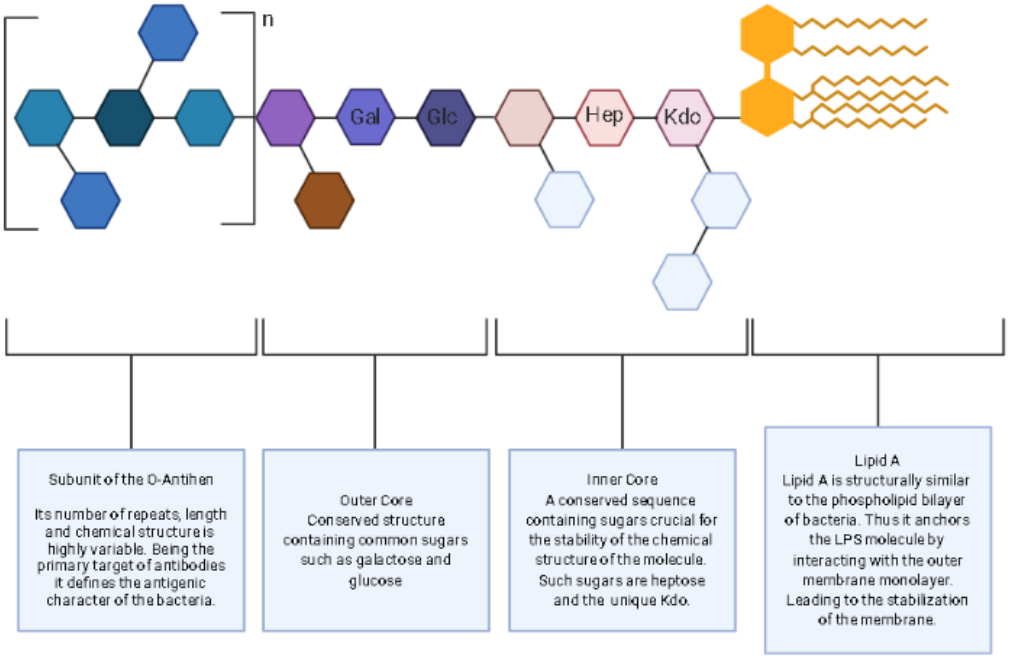


Figure 2: Molecular structure of LPS. Carbohydrate molecules are represented by colored hexagons to represent different types. Each major subunit is represented by closed brackets and their characteristics are given in the respective boxes(Bertani and Ruiz, 2018).

## Interaction of colistin with LPS:

The stabilizing effect that LPS has on the outer membrane is believed to be due to strong intermolecular interactions between molecules bridged by divalent ions. Broadly, the current model for colistin bactericidal effect suggests a primary disruption of OM followed by an equivalent effect on the inner or cytoplasmic

membrane (Khadka, Aryal and Pan, 2018) (Figure 3).

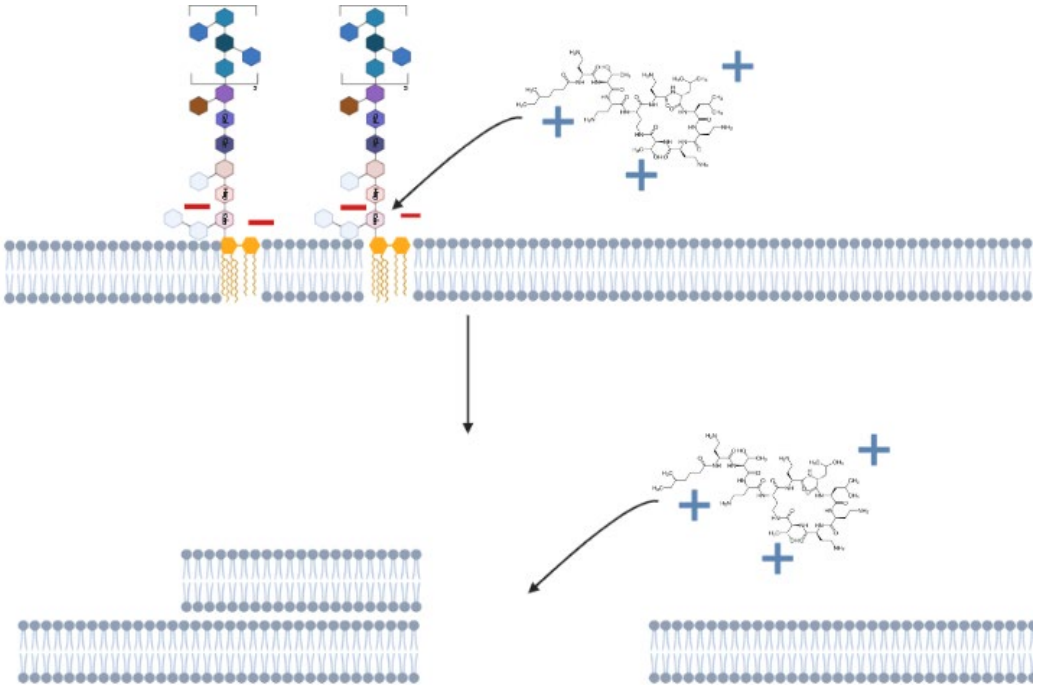

Figure 3: Electrostatic interaction of colistin with LPS on the outer membrane of gram-negative cells. (+), signs represent a positive net charge in the molecule of colistin. Minus signs (-) represent a negative net charge in the molecule of LPS. The electrostatic interaction of colistin-Lipid A is represented by the arrow in the first part of the figure. The second part of the figure represents the clustering of the phospholipid bilayer and entry of colistin in the inner-membrane space. Conceptualized from (Khadka, Aryal and Pan, 2018)

The precise model suggests that cationic polypeptides of colistin interact with anionic LPS molecules on the OM of gram-negative bacteria, leading to displacement of calcium ($Ca^{2+}$) and magnesium ($Mg^{2+}$) (normally meant to stabilise the LPS membrane) thus causing lipid clustering (Khadka, Aryal and Pan, 2018) (Figure 3). This results in a destabilisation of the inner membrane (IM), drastically increasing cell membrane permeability, leakage of contents, inhibition of nucleic acid production and ultimately cell death.

### Resistance to colistin in K.pneumoniae :

Since its re-introduction to the medical field, colistin has been used with success in the treatment of previously lethal, carbapenem-resistant MDR bacterial infections such as *K.pneumoniae* . Therefore, and in addition to misuse as a growth factor in agriculture, resistance has emerged. (Gharaibeh and Shatnawi, 2019)

Since its reintroduction in clinical use, extensive research has been conducted to understand the underlaying mechanism of resistance. Resistance to colistin can either be acquired (from mobile elements such as plasmid) or intrinsic (chromosomal mutations).

### Acquired Colistin Resistance :

Acquired colistin resistance has become a significant threat, following the isolation of plasmids from *Escherichia coli* carrying the *mcr-1* gene in late 2015 at China. *Mcr* genes code for the enzyme phosphoethanolamine (PEA)-lipid A transferase that adds a PEA group to lipid A of the LPS. This alters the net negative charge of LPS and consequently reduces the binding affinity of colistin (Anandan et al., 2017). The mobile element can be transferred across different species of gram-negative bacteria of which the most important are *Enterobacteriaceae*, *K.pneumoniae* , *P.aeruginosa* and *A.baumanii* (Gharaibeh and Shatnawi, 2019). The opportunistic bacterium *E.coli* acts as the vector for spreading of the plasmid to a variety of other species within the host and across to other hosts. As a result, in the short span of 5 years *mcr-1* is known to exist in clinical samples of 40 countries (Wang et al., 2018). Additionally, nine different *Mcr* genes have been described.

### Intrinsic Colistin Resistance:

Intrinsic colistin resistance is increasingly studied since the re-introduction of the antibiotic as treatment method for carbapenem resistant *K.pneumoniae* . The first colistin resistance *K.pneumoniae* strain was isolated in Athens 2004. Ever since, there has been a steady increase of reported colistin resistance caused by intrinsic elements. In Europe, the rate doubled in the span of only 6 years from 1.1% in 2003 to 2.2% 2009 (Li et al., 2019).

According to the European meta-analysis (EARS-Net) 29% of MDR *K.pneumoniae* strains exhibit resistance to colistin, mainly stemming from previous use of the antibiotic (European Antimicrobial Resistance Surveillance Network (EARS-Net), 2014). In comparison to acquired resistance which can be introduced to a colistin naïve population, intrinsic resistance arises due to alterations, caused by exposure to colistin, on pre-existing adaptation mechanisms. (Macesic et al., 2019). These mechanisms are regulated by two-component regulatory systems (TCS). Apart, from

their role in the adaptation of bacteria in variable environments, TCS have been shown to confer resistance to multiple antibiotics. Indeed, twenty such systems have been related to antibiotic resistance in *K.pneumoniae* , but only three guide colistin resistance (Bhagirath et al., 2019).These three are PhoPQ, PmrAB and CrrAB.

**Canonical function and structure of two-component systems mediating colistin resistance in *K.pneumoniae* :**

The PhoPQ system:

The PhoPQ regulatory system is a well-studied global regulator governing the transcriptional activation of at least 40 genes in *Salmonella* and about 600 genes in *E.coli* (Alteri et al., 2011). This disparity in regulon size may be a consequence of species-specific regulation (Groisman, 2001).

One of the most important functions of PhoPQ is the sensing and acquisition of $Mg^{2+}$ ions in limiting conditions. This function allows survival during early host infection and intra-macrophage survival. Some of the most studied examples are the PhoP regulated, and highly conserved amongst *Enterobacteriaceae*, genes *mgtA* and *mgtB*. The proteins encoded are $Mg^{2+}$ transporters and are increasingly expressed in $Mg^{2+}$ limiting conditions on liquid culture. In fact, the MgtA and MgtB protein are P-type adenosine triphosphate(ATP)ases functioning as cotransporters of $Mg^{2+}$ and ligands down an electrochemical gradient. MgtA and MgtB null mutants exhibit a growth deficit under low [$Mg^{+2}$] conditions. (Soncini et al., 1996).

As described previously, divalent ions such as $Mg^{+2}$ act to stabilize LPS and the outer membrane. In $Mg^{+2}$ limited conditions, LPS can therefore lose its cross-linking properties, leading to an increase in membrane permeability. PhoP can independently activate the *arn* operon leading to decoration of LPS with 4-aminoarabinose (L-Ara4N) to stabilize the membrane while increase the acquisition of Mg2+ ions through MgtA and MgtB protein expression. This structural change results in a change in net charge of LPS resulting in the decrease of required divalent ions for membrane stability (Kim, Choi and Ko, 2014).

### The PmrAB system:

The PmrAB TCS is shown to be involved in the LPS modification in *K.pneumoniae* and *P.aeruginosa*. The genes *pmrB* and *pmrA* encode for the histidine kinase and its cognate response regulator (RR), respectively. The *pmrCAB* operon has a fundamental role in the survival of bacteria under a variety of environmental stressors. This stress-response capability is based on the variability of the LPS molecule. The modification of LPS according to stress can occur in its whole structure, but it mainly occurs in the subunit lipid A (Anandan et al., 2017). The response is guided by PmrAB. Specifically, phosphorylation of PmrA (from PmrB) causes the transcription of the *pmrCAB* and *arn* operon resulting in the decoration of lipid A with phosphoethanolamine (pEtN) and (L-Ara4N) respectively. This structural change causes a shift on the net negative charge of the membrane resulting in a more positively charged membrane (Kim, Choi and Ko, 2014).

### The CrrAB system:

The CrrAB operon was first described in *K. pneumoniae* no more than a decade ago. It was then attributed the name colistin resistance regulon due to the highly resistant phenotype arising from substitutions within the *crrA* and *crrB* genes. Furthermore, this type of substitutions has been found in clinical isolates of *K. pneumoniae* such as ST258, ST65, ST23, ST14 and ST43 (Macesic et al., 2019)

Whilst the canonical activation of CrrAB is not known it is likely that CrrB responds to environmental stimuli. It is shown that mutations in CrrB cause an upregulation of the *arn* operon and *pmrC*, both of which are under the control of PmrAB, thus suggesting that CrrAB is an activator of PmrAB. However, the mechanistic details surrounding the interaction between the two systems have not been established yet. (Cheng, Lin, Lin and Wang, 2018).

### Domains of TCS and their functionality:

In the past ten years, the structure to function relation of the three TCS has been thoroughly investigated through visualization techniques such as X-ray crystallography. As a result, the relation between activation of the HK and in turn the activation of the RR has been established.

HKs can respond to a plethora of signals, both internal and external due to their unique quaternary structure as proteins. The best studied example out of the three TCS contributing to colistin resistance is PhoPQ. The general structure of PhoQ can be used to elucidate the conserved composition of HK's in *K.pneumoniae* (Groisman, 2001) . Although there are differences on the amino acid level the overall structure of these kinases has been broadly divided to three functionally distinct parts; The transmembrane domain (TM)(a), the HAMP domain(b), and the Histidine Kinase A domain (HKA)(c) (Bhate et al., 2015).

a) TM domain: It was observed that most signaling TM domains have in common a helix at the plasmid interface directly next to the ligand-binding domain. This helix was thus named periplasmic-helix and suggested that the binding of ligand causes a conformational change ultimately leading to a twist in the Tm domain (Bhate et al., 2015).
b) HAMP domain: The HAMP domain is found as an immediate C-terminal extension of the TM helix. Thus, the twist on the TM helical part directly arrives at the N-terminus of the HAMP domain. The signal is transduced due to the unique four-helical structure of the domain and transferred to the HK catalytic core (Bhate et al., 2015).
c) HKA domain: The HK's catalytic core or HKA comprises two domains: the dimerization histidine-phosphotransferase (DHP) domain and the catalytic and ATP-binding domain. The signal transmitted from the HAMP domain causes a conformational change in DHP leading to its autophosphorylation, aided by the catalytic and ATP-binding domain (Zschiedrich, Keidel and Szurmant, 2016). Ultimately, this leads to the transfer of a high energy phosphate in the response regulator.

RRs act as the diffusible part of the TCS. Upon phosphorylation, thus activation, by the HK they act as transcription factor to upregulate expression of genes. The

binding sites of the different regulons can be unique to a single RR or recognized by more than one RR (Jung, Fried, Behr and Heermann, 2012).

Combinatory function of PhoPQ, PmrAB and CrrAB giving rise to colistin resistance*:* In the context of chromosomal colistin resistance in *K.pneumoniae* PhoPQ, PmrAB and CrrAB are closely interlinked. Firstly, the most studied end points of regulatory pathways concerning these three TCS, are the lipid A associated operons*: pmrCAB* and *arn* operon (Aghapour et al., 2019). Under normal conditions the expression of these operons ultimately results in the modification of LPS the effect of which is limited to acquisition of ions.

Specific alterations, mostly chromosomal mutations, in these systems result in excessive modification of LPS with cationic moieties (PetN and L-Ara4N) to prevent the binding of colistin (Olaitan, Morand and Rolain, 2014). Precisely, they decorate Lipid A (and Kdo less frequently) with variable numbers of these moieties thus causing a shift in the net charge of the LPS molecule (towards a cationic profile) and in its spatial conformation (Li et al., 2019). As a result, the affinity of colistin is decreased thus the permeability of the OM remains intact giving rise to colistin resistance. A model based on protein expression profiles, for the regulation of these operons by TCS is given in Figure 4.

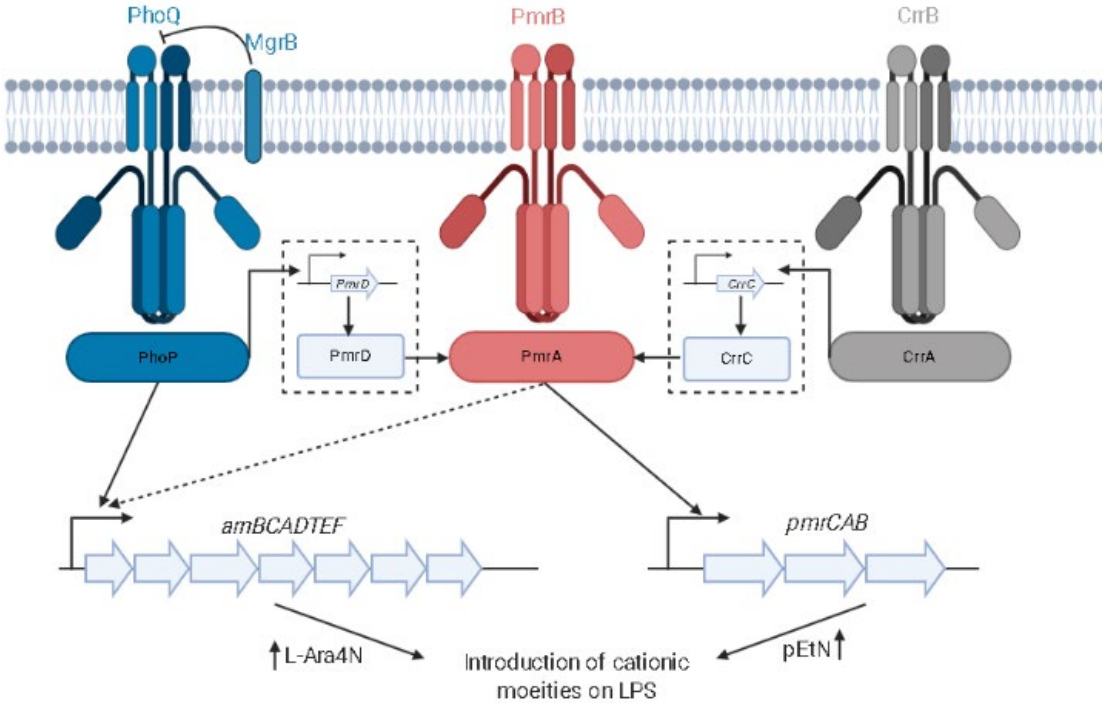


Figure 4: Molecular model for the interaction of the three TCS; PhoPQ, PmrAB and CrrAB conceptualized from data of mutational studies on the systems. Genes are given in wide arrows and their 5'-3' transcription is indicated by orthogonal arrows. Conceptualized from (Cheung, Dulyayangkul, Heesom and Avison, 2020)

## Prevalence of mutations in TCS associated with colistin resistance:

As mentioned above, specific alteration in these systems result in the constitutive transcription of the operons. A study meant to elucidate the frequency of these alternations in a total of 164 colistin resistant isolates was conducted by Macesic et al. (2019). Firstly, the most frequently occurring is the inactivation of, the PhoPQ inhibitor, MgrB (41% of 164) which leads to the constitutive activation of PhoQ. PhoQ in turn increasing the activity of PhoP and PmrD leading to the activation of PmrAB and ultimately to the excessive transcription of the operons guiding LPS decoration.

Secondly, substitutions in colistin resistance canonical genes (PhoQ, PhoP) and CrrB, these two are grouped together because they have comparable frequencies of occurrence (24% and 17% of 164, respectively). While PhoQ substitutions follow a similar pathway to that of inactivation of MgrB, CrrB substitutions have a completely different impact. Substitutions in CrrB are believed to cause resistance in a PmrAB-dependent manner. Thus, they activate PmrB which in turn causes an increase in PmrA activity leading to the excessive transcription of both operons.

The frequency of occurrence does not necessarily depict the "clinical" importance of the mutations. Most studies like those carried out by Macesic et al. (2019) analyze the chromosomal mutations at a single point in time through WGS rather than an exposure-based analysis over time. From these studies it can be concluded that MgrB inactivation is in fact the most stable of all mutations. As a result, other mutations appear to have less clinical significance at first glance, such as the ones occurring in the newly discovered and understudied CrrAB TCS.

**Phenotypic observations in CrrAB mutations and their clinical importance:**

### CrrAB mediated Phenotypic alterations:

The main function of resistance is attributed to substitutions in CrrB which lead to the overexpression of the *CrrB* gene and are thought to increase the activity of the protein. Most publication are limited to a qPCR analysis portraying its increased expression as mean of colistin resistance. There are two main groups which further described this system (Cheng, Lin, Lin and Wang, 2016) (Cain et al., 2018).

As described by (Cheng, Lin, Lin and Wang, 2016), CrrAB causes colistin resistance in a PmrAB dependent manner. The main substitution they investigated was CrrBN141I, previously identified in a ST258 clinical isolate, replicated in a *K.pneumoniae* A4528 colistin susceptible strain. As previously stated, CrrBN141I substitution caused a significant increase in expression and activity of the protein. In their study was identified the connector between these two systems, named CrrC, guiding the cross activation between CrrAB and PmrAB which is in the same operon as CrrAB. Additionally, another gene belonging to the same operon and coding for a putative RND-type efflux pump (H239_3064) was also described as contributing to resistance(Cheng, Lin, Lin and Wang, 2018). Furthermore, there was no explicit evidence that PhoPQ does not play a role in this system, but it was concluded that due to its low expression profile it probably does not contribute to the phenotype. This study shed light on the pathway at which CrrAB activates the *arn* and *pmrC* operon. However, secondary phenotypic effects arising from CrrAB overexpression, were not investigated.

Cain et al., (2018) undertook *in vitro* exposure of *K.pneumoniae* Ecl8 (susceptible non-capsule producing strain) to increasing concentrations of colistin in liquid broth.

Colistin exposure selected for  mutations in the CrrB protein e.g.  CrrBS93N and CrrBG175A. Extensive phenotypic effects identified that the CrrB variants had altered fimbrial and phage production, and increased capsule production relative to the wild type strain Ecl8(Figure 5). To be precise there was no significant increase in the cps operon regulatory proteins thus the mechanistic basis of capsule formation remained unknown.

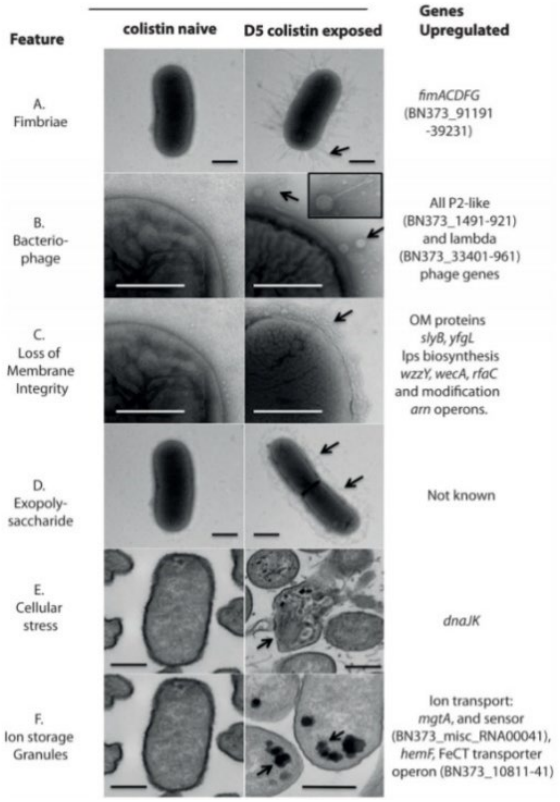


Figure 5: Phenotypic effects observed on TEM on a heterogenous population of CrrB single mutants. Features indicate the observed phenotype, while each panel represents colistin naïve or colistin exposed populations of *K.pneumoniae  Ecl8*. The genes upregulated are speculated to be the cause for the apparent phenotype (Cain et al., 2018).

### *Capsule regulation in K. pneumoniae:*

As demonstrated by Dorman et al. (2018) there are multiple genes affecting the regulation and capsule formation. In this study a transposon mutant library was created and coupled with an analysis of capsule levels. Genes relating to capsule formation were given a high to low contribution towards formation of capsule. Thus 78 genes were identified as producing of low or high concentrations of capsule

(Dorman et al., 2018). An overview of identified genes to be central in the production of capsule in *K.pneumoniae* is given in Figure 6.

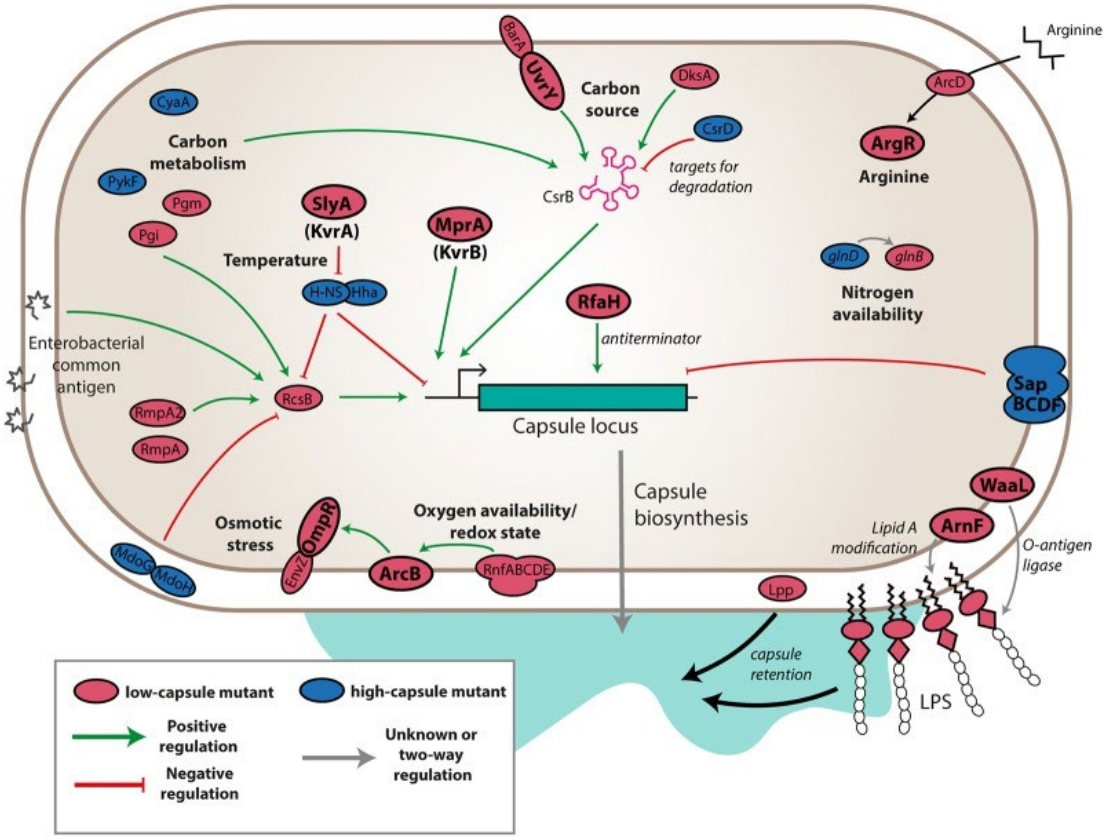


Figure 6: An overview of the capsule regulatory network found from the library of mutants reported by (Dorman et al., 2018)

## Clinical significance of capsule production in K.pneumoniae :

Recent data by Ernest et al., (2020) clearly demonstrates the importance of mutations resulting to increased capsule production and its impact on infection routes as well as its effect on pathogenicity.

They reported mutations in the core capsule biosynthesis genes resulting in differential capsule production in *K.pneumoniae* . Two gain of function mutations were identified in *wzc*, a core capsule gene, in clinical strains of *K.pneumoniae* . The *wzc* gene is part of the *cps* operon thus it plays a fundamental role in production of capsule.

The gain of function mutations in *wzc* resulted in hyper-capsulated bacteria (>300 nmol of uronic acid per 10^9 cells). On the other hand, low capsule producing mutants (<50 nmol of uronic acid per 10^9 cells) arise from loss of function mutations. It was then established that high capsule production results in systemic

infections and higher dissemination of *K.pneumoniae* on the blood stream as identified in murine hosts. Thus, highlighting the significance of capsule as a determining factor in systemic infections which result in the higher mortality rate.

To identify the clinical relatability of the gain of function mutations, 966 genomes from clinical isolates of *K.pneumoniae* were analyzed. 20 of the 966 had the two gain of function mutations characterized in this study result in a hyper-capsule producing phenotype. Suggesting that the capsule is a key element in establishing severe systemic infections in animal models (Ernst et al., 2020).

Taken together, we hypothesize that CrrAB is a regulator of capsule production which can contribute to both pathogen-host and drug resistance phenotypes. Thus, the aim of this thesis is to mechanistically elucidate how CrrAB substitutions affects capsule production. Our specific aims are to characterize the role of CrrAB alterations in capsule production in *K. pneumoniae*. Establish the regulatory pathways which facilitates capsule production. Relate capsule production to colistin resistance.

## Materials and Methods:

### Analysis of literature

Search terms “CrrB” AND “Klebsiella” AND “Pneumoniae” were used to identify publications concerning alteration in the histidine kinase CrrB. The predominant search engine used was provided by the University of Edinburgh, Library Search. Furthermore, it was coupled, and cross referenced to the search engines NCBI and Google Scholar. The publications were then categorized according to relevance to and novelty of CrrB alterations. Post-analysis publication, where alterations in CrrB were referenced to previous publication did not contribute to the “Count” number of reported CrrB alterations. Moreover, all “Counts” of mutations in CrrB stem from clinical isolates (*in vivo*) rather than artificially (*in vitro*) created mutations.

### Structural modelling of the CrrB histidine kinase

Structural modelling was conducted using analytic software (SWISS-model) to estimate the global equality of CrrB compartments to other histidine kinases, mainly PhoQ. To validate the functionality of compartments within CrrB, the software PredictProtein was used. The output was filtered to reveal known structures, such as dimeric helices and motifs, which relate to function.

### Bacterial strains and plasmids used.

*K.pneumoniae* Ecl8 (Fookes et al., 2013),*K.pneumoniae* Ecl8 ΔPhoPQ (Unpublished provided in the lab) and *K.pneumoniae* 52145 (Provided in the lab) were the final vectors harboring the pBR-322::crrB variants. *E.coli* DH10β chemically competent cells were used as vectors for plasmid amplification and retrieval. The overexpression vector used was pBR-322 thus LB+ Tetracycline (30 mg/L) at 37**°C** conditions were used upon its presence. The cloning vector used was pJET2.1 with selection in LB+ Ampicillin (50 mg/L) at 37**°C**.

### Construction of the altered CrrB overexpression strains.

The *crrB* gene was amplified by PCR reaction to introduce restriction enzyme cutting sites PstI (Primer: 5’- CTGCAG CACGCCAGTATAACCAC-3’) and ScaI (Primer: 5’- AAA AGTACT TTAAACAGGCCATGAGAG-3’). The chromosomal material of *K.pneumoniae* Ecl8 was extracted using Wizard Genomic Purification Kit (Promega), and used as a template in the PCR reaction, set up with Q5® High-Fidelity DNA Polymerase according to manufacturer’s instructions (New England Biolab).

The *crrB* amplicon was inserted into the pJET2.1 blunt cloning vector according to manufacturer's instructions (ThermoFisher, CloneJET PCR Cloning Kit). The resulting plasmid was inserted in *E.coli* DH10β (100μl of CC cells/ 10μl of the reaction) and was grown overnight at appropriate selection.

Following, extraction of the plasmid using the GeneJET Plasmid Miniprep kit (Thermo Scientofoc), the cloning plasmid was digested with PstI and ScaI according to manufacturer's instructions to retrieve the *crrB* gene (New England Biolab). In parallel the overexpression vector pBR-322 was digested with PstI and ScaI (New England Biolab). The two products were ligated using T4 DNA ligase according to the manufacturer's instructions (Thermo Scientific). The product of ligation (pBR-322::*crrB* construct) was inserted in *E.coli* DH10β (100μl of CC cells/ 10μl of the reaction) and grown overnight at appropriate selection.

Five CrrB protein substitutions (I27V, F84S, S93N, N141I and S195N) were created by site directed mutagenesis (QuikChange Lightning Site-Directed Mutagenesis Kit). Primers were designed according to the provided online software by the manufacturer. (https://www.agilent.com/store/primerDesignProgram.jsp).

Subsequently, PCR reactions were carried out as instructed by the manufacturer (Agilent Technologies). The final PCR product was inserted in *E.Coli* DH10β (100μl of CC cells/ 10μl of the reaction) and grown on appropriate selection for successful pBR-322:*crrB* constructs. Resistant colonies were further validated by PCR reactions for the presence of the *crrB* gene, using the respective plasmid extractions as templates, with *crrB* primers (PstI and ScaI). Successful plasmids were then sequenced (Eurofins Genomics).

The pBR-322:*crrB* variants were then extracted (GeneJET Plasmid Miniprep kit, Thermo Scientific) and inserted in *K.pneumoniae* Ecl8, *K.pneumoniae* Ecl8 ΔPhoPQ and *K.pneumoniae* 52145 electrocompetent cells with appropriate selection.

### Capsule quantification; Percol and Uronic acid assays.

The percol separation protocol was adapted from (Dorman et al, 2018). Briefly, single colonies of *crrB* variants were inoculated overnight in separate batch of 5 ml LB broth with Tetracycline (30 mg/L) at 37**°C** and 180rpm shaking. 500μl of overnight culture

was then inoculated in 50ml Lb broth with Tetracycline (30 mg/L) at 37**°C** and 180rpm shaking to an initial Optical Density at 600 nm ($OD_{600}$) of ≈0.05-0.08.

To retrieve the bacteria, cultures were centrifuged at 6000 rpm, 10 min, 4oC. They were then resuspended in 2ml of phosphate buffer solution (1XPBS) of which 600µl was applied to the top of the discontinuous percol column (Consisting of three phases: 10%, 30% and 50% v/v).

The capsule quantification protocol was adapted from (Blumenkrantz and Asboe-Hansen, 1973). Briefly, 600µl of the bacteria were retrieved from the percol column and washed twice in 1xPBS total volume of 2ml (Centrifugion at 10000rpm, 10 min, 4**°C**). The pellet was then resuspended in 1.5ml of 1xPBS. 500µl of the resuspended sample were added to 100µl of capsule extraction buffer (500 mM citric acid pH 2.0, 1% Zwittergent 3-10) and incubated for 20 minutes at 50**°C** static. The samples were then centrifuged (10000rpm, 5 min, 4**°C**) and 300ul of the supernatants were transferred in clean Eppendorf tubes. 1.2ml of absolute ethanol (at -20**°C**) was added and the resuspension was incubated for 30 minutes at 4**°C** static. Subsequently, the samples were centrifuged (10000rpm, 5 min, 4**°C**), and air dried. The supernatant were then resuspended in 200µl of MQ-Water of which 100µl was transferred in 600µl of capsule quantification solution (0.0125M $Na_2[B_4O_5(OH)_4]\cdot 8H_2O$ in $H_2SO_4$). The solutions were incubated for 10 minutes in ice followed by 5 minutes at 95**°C**. Absorbance was measured at 520nm.

### Transcriptome analysis

The *K.pneumoniae* Ecl8 variants (vector only control, pCrrBWT and pCrrBF84S) were prepared for transcriptome analysis as follows. They were inoculated into separate stoppered flasks in 20mL LB broth (Lenox) with 30 mg/L Tetracycline at 37**°C** shaking (180 rpm) until an OD 600 of 0.6-0.8 was reached. To retrieve the bacteria, cultures were centrifuged at 6000rpm, 10 min, 4**°C** and washed twice with 1xPBS. Following, the pellet was disrupted with 100µl of Zirkonia Beads (RPI, Research Products International)) and the proteins denatured with 100µl of GTC phenol mix (Sigma) and incubated for 4 minutes at 4**°C**. Followed by addition of 600µl of GTC phenol mix (Sigma). RNA was purified using the phenol-chloroform/isoamyl alcohol (IAA) method. The purified RNA was dissolved in 35µl of DEPC-treated water. RNA integrity and concentration measurement were identified using the Agilent 4200 TapeStation System (Agilent). All samples with

RIN value ≥ 7.0 were sent for sequencing. All library preparation and sequencing were undertaken at Novogene where the RNAseq data was generated using Illumina PE150 reads at 2GB raw data/ per sample. Differential gene expression analyses were undertaken using DEseq (Agilent).

### Proteome analysis

The *K.pneumoniae* Ecl8 variants (vector only control, pCrrBWT and pCrrBF84S) were prepared for proteome analysis as follows. They were inoculated into separate stoppered flasks with 20mL LB broth (Lenox) with 30 mg/L Tetracycline at 37**°C** shaking (180 rpm) until an OD 600 of 0.6-0.8 was reached. Cells were retrieved by centrifugation at 6000rpm, 10 min and 4**°C**. Followed by resuspension in 20mL of 30mM Tris-HCL (pH= 8) and sonication (ON-OFF cycle of 1 second and 0.5 seconds for 3 minutes) at amplitude of 63% using Sonics Vibracell VC-505TM (Sonics and Materials Inc., Newton, Connecticut, USA). The sonicated samples were centrifuged 8000 rpm, at 15 min, 4**°C** (Sorval RC5B PLUS using an SS-34 rotor). The supernatant was isolated and centrifuged at 20000rpm, 60 min, 4**°C** to pellet the envelope (Sorval RC5B PLUS using an SS-34 rotor). To isolate envelope proteins, the pellet was resuspended in 200µl of 30 mM Tris-HCl pH 8 containing 0.5% (w/v) SDS. Protein concentration was identified according to manufacturer's instructions (Biorad Protein Assay Dye Reagent Concentrate). 5ug of proteins were loaded on SDS-page (11% acrylamide, 0.5% bis acrylamide) into a Biorad Min-Protein Tetracell chamber model 3000X1. A 200V current was used to fixate the protein on the SDS-page. Proteins were then stained for 20 minutes using Instant Blue (Expedeon).

The gel lane containing envelope proteins was cut out and subjected to in-gel tryptic digestion using an automated ProGest digestion unit (Digilab UK*).* The resulting peptides were fractionated using an Ultimate 3000 nanoHPLC system in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific).

Raw data files were processed using Proteome discoverer v1.2 (ThermoScientific). Annotation of proteins was performed using SEQUEST (Ver. 28 Rev. 13) by searching against *K.pneumoniae* ATCC700721/ MGH78578 protein database (5126 protein entries, ACC:272620). Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.8 Da. Search criteria included

carbamidomethylating of cysteine (+57.0214) as a fixed modification and oxidation of methionine (+15.9949) as a variable modification. Searches were performed with full tryptic digestion and a maximum of 1 missed cleavage was allowed. The reverse database search option was enabled, and all peptide data was filtered to satisfy false discovery rate (FDR) of 5 %.

Three pairwise comparisons were carried out using the raw data set. To identify significantly different values a paired t-test was used in excel. Significant values were those with a p-value of less than 0.05. Relative fold-change within each pair of data was defined as the fold-change of average absolute abundance of protein.

### Identification of Minimum Inhibitory Concentration (MIC) of colistin.

Minimum Inhibitory Concentrations (MICs) were determined by broth micro-dilution according to CLSI methodology and interpreted using CLSI performance standard breakpoints (Cockerill, 2015). Briefly, all samples were grown in 5ml of LB broth+ Tetracycline selection in 37**°C**, 180rpm until an $OD_{600}$ of 0.1 was reached. Doubling concentrations of colistin where placed in a 96-well plate with a starting point of 0.25 (ng/µl) to an ending point of 256 (ng/µl). A total volume of 200µl was used in each well of which 100µl was 2X the concentration of colistin in LB broth and the rest 100µl was *K.pneumoniae* Ecl8 pBR-322:*crrB* variants or *K.pneumoniae* 52145 pBR-322:*crrB* variants (including the vector only control and Wild-Type) . The samples were incubated at 37**°C** overnight.

### Real-time PCR (qPCR) for the quantification of gene expression

*K.pneumoniae* Ecl8 pBR-322::*crrB* variants were inoculated into separate batches of 20 mL LB broth (30mg/L tetracycline) Cultures were incubated at 37 **°C** with shaking (180 rpm) until the $OD_{600}$ had reached 0.6-0.8.

RNA of samples was stabilized using RNAprotect Bacteria Reagent (Qiagen, Crawley, UK) according to the manufacturer's instructions. 200 µL of TE buffer containing lysozyme (20 mg/mL) was mixed into the samples to disrupt the pellet. The mixture was incubated for 10 min at 25**°C**, with 20 seconds of vortexing every 2 min. Following the incubation cycle, 700 µL of RLT buffer (Qiagen) containing 150 mM β-mercaptoethanol were added to the samples. RNA was purified using a Qiagen

RNeasy RNA purification kit (Qiagen), according to the manufacturer's instructions. RNA from bacterial culture was dissolved in 35µl RNase free water (provided by the manufacturer). The samples were then purified, by digesting unwanted nucleic acids, using TURBO DNA-free™ Kit (Ambion, Foster City, CA, USA) and following the manufacturer's instructions. The concentration of RNA in each sample was measured using a NanoDrop ND-100 spectrophotometer (Labtech, UK). One microgram of total RNA was converted into cDNA using LunaScript reverse transcriptase supermix (New England Biolabs) in a 20 µl reaction ,which included random hexamer primers, and incubated according to the manufacturer's instructions. cDNA samples were preserved at -20**°C** sterile freezers until used as templates. The reference gene in each case was *rrsE* (Berghoff et al., 2017). Each real-time qPCR (QPCR) reaction was prepared using the Luna Universal qPCR Master MIX kit according to the manufacturer's instruction (New England Biolabs). Samples were run as 3 technical repeats nested in each of the 4 biological replicates. The PCR amplification cycles were adapted according to the cDNA of interest. The ratio of target to reference cDNA in a sample of comparator B (*K.pneumoniae* Ecl8 pCrrB variants) was calculated relative to that in a sample of comparator A (vector only control) according to the ΔΔCT method (Livak and Schmittgen, 2001).

### Identification of growth rates

Each *K. pneumoniae* pBR-322::*crrB* variant was grown in 5ml of LB Lenox (30mg/L Tetracycline) until an $OD_{600}$ of ≈0.01 was reached. Bacterial growth was estimated. Shortly, M9 media was prepared (10X M9 salts, 1M MgSO4, 1M CaCl2, 50X essential amino acids, 100X non-essential amino acid and supplemented with 20mM glucose) and 450µl were placed in a flat-bottomed 96-well plate (Greiner Bio-one, Stonehouse, UK). 50µl of each *K. pneumoniae* pBR-322::*crrB* were added to the respective well. Measurements were taken in a Fluostar Optima (Aylesbury, UK) plate reader. The conditions were as follows: 37**°C** and shaking at 180 rpm, absorbance ($OD_{600}$) measurements were taken every 30 minutes in a grand total of 50 measurements. The readout was bacterial growth over time of 3 biological repeats with 3 technical repeats per point.

# Chapter 1: Review of known CrrB mutations and their effect on colistin resistance

## 1.1. Review of CrrB changes reported in literature.

Multiple mutations in the CrrAB locus have been reported in the literature. Thus far, an exhaustive Web of Science and Pubmed search demonstrates that a total of 52 papers report the role of CrrAB in colistin resistance in *K. pneumoniae*. However, closer reading of these papers shows that only a subset of these studies proceeds to attribute colistin resistance to these mutations (Table 1). A total of 54 mutations were reported in the selected publications (Table1).

| ST[A] | Amino acid substitutions within the CrrB histidine kinase | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | No. of counts[C] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 10 | 13 | 27 | 31 | 57 | 63 | 66 | 68 | 84 | 87 | 93 | 94 | 99 | 133 | 140 | 141 | 151 | 175 | 183 | 191 | 195 | 224 | 239 | 276 | 281 | 287 | 296 | 303 | 322 | 325 | |
| ECL8 WT | Q | T | I | Y | D | M | I | C | F | L | S | L | G | L | W | N | P | G | G | L | S | P | Q | T | F | Q | L | F | S | K | |
| ST258 | L,L,L | K | | | V | | | S,S | | V,V | | M, M | | | | Y | S,L,T | | V | F | N | | | | Y | | | | W | | 20 |
| ST11 | L | | | H | | | | | | | | | | | | I,I | S | | | | N | | | | | | | | | | 6 |
| ST20 | | | | | | | V | | | | | | | | | | | | | | | | H | A | | | | | | | 3 |
| ST392 | | | | | | | | | | | | | | | | | L,S | | | | | | | | | | Q | | | | 3 |
| 20.70 | | | | | | | | | S | | | | | | R | | | | | | | | | | | | | | | | 2 |
| B0608-134 | | | | | | | V | | | | G | | | | | | | | | | | | | | | | | | | | 2 |
| ECL8 | | | | | | | | | | | N | | | | | | | A | | | | | | | | | | | | | 2 |
| ST3375 | | | | | | | | | | | | | R | | | | | | | | | | H | | | | | | | | 2 |
| ST3380 | | | V | | | | | | | | | | | | | | | | | | N | | | | | | | | | | 2 |
| ST37 | | | V | | | | | | | | | | | | | | | | | | | | | | | | | S | | | 2 |
| ST629 | | | | | | | | | | | | | | | | | | | | | | | | | | K | Q | | | | 2 |
| Af44b | | | | | | | | | | | | | | | | | L | | | | | | | | | | | | | | 1 |
| B0701-068 | | | | | | | | | | | | | | DEL | | | | | | | | | | | | | | | | | 1 |
| C7 | | | | | | | | | | | | | | | | | | | V | | | | | | | | | | | | 1 |
| G104 | | | | | | | | | | | | | | | | Y | | | | | | | | | | | | | | | 1 |
| ST22 | | | | | | I | | | | | | | | | | | | | | | | | | | | | | | | | 1 |
| ST29 | L | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 1 |
| ST34 | | | | | | | | | | | | | | | | | | | | | | L | | | | | | | | | 1 |
| ST485 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | R | 1 |
| No. of counts[B] | 5 | 1 | 2 | 1 | 1 | 1 | 2 | 2 | 1 | 2 | 2 | 2 | 1 | 1 | 1 | 4 | 7 | 1 | 2 | 1 | 3 | 1 | 2 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 54[D] |

Table 1: Published and characterized substitutions found in the CrrB histidine kinase of *K.pneumoniae* strains. (A) The strain of *K.pneumoniae* clinical isolates as reported in the publications. (B) Number of novel amino acid changes within CrrB reported (C) Number of amino acid changes reported in the specific sequence type (D) Grand total of CrrB alterations. In this analysis, the clinical significance of the alterations is measured by the MIC of colistin. In the clinical setting, it is used as an ON-OFF characterization for resistance: if the MIC is >2, then the strain is colistin resistant (ON); if the MIC is <2 then the strain is colistin sensitive (OFF). Thus, neutral (OFF) mutations are denoted by red characters. (Cain et al., 2018)(Cheng et al., 2016)(Esposito et al., 2018)(Hernández et al., 2018)(Jayol et al., 2017)(Kim and Ko, 2018)(Longo et al., 2019)(Macesic et al., 2019)(McConville et al., 2018)(Pishnian, Haeili and Feizi, 2020)(Pitt et al., 2018)(Wright et al., 2014)

All changes reported within CrrB do not lead to a change in colistin susceptibility. Thus, these are labelled “neutral” changes. These “neutral” changes are crucial in providing functional insight into the CrrB protein. Amino acid substitutions in “neutral” changes result in an unaltered amino acid character in most cases (58%). On the other hand, “active” substitutions (which alter the susceptibility to colistin) show a high frequency (83%) of change in amino acid character. Thus, the electrostatic interactions between amino acids happening within the protein have a crucial role in determining the extend of protein activity and in turn the resulting phenotype. To identify the location and the effect of the amino acid character change of the “active” mutations in the CrrB protein a model was build.

### 1.2. Functionality-based modelling of the CrrB protein:

The functionality-based model of CrrB is based on theoretical homology profiles to TCS. The theoretical structure can reveal the location of mutations within the CrrB protein, which in turn can aid in finding the frequency of mutations in each compartment.

The CrrB histidine kinase consists of 353 amino acids. Its overall structure is comparable to the well-studied PhoQ but has a low, (less than 30%), model similarity. The approach of protein modelling requires a break down to three functionality-based compartments, as mentioned in the introduction. The three compartments being the TM, the HAMP and HKA domain.

### 1.2.1 The transmembrane sensory domain (TM)

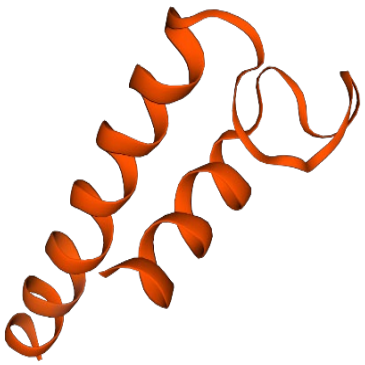

Figure 7: Transmembrane domain of CrrB

Homology modelling for the sensor domain is unreliable, the highest global model equality estimation detected using SWISS-model was approximately 30%. Albeit 2 transmembrane helices with aa 42-50 forming an exposed loop between them were detected (Figure 7). This structure resembles sensory domains responding to external stimuli. Furthermore, at least one GxxxG motif was detected, this motif is known to bind divalent cations thus activating the sensory domain (Teese and Langosch, 2015). Overall, the functionality of aa 1-80 is that of sensing divalent ions and relaying to the rest of the histidine kinase.

### 1.2.2 The transductor HAMP domain (HAMP)

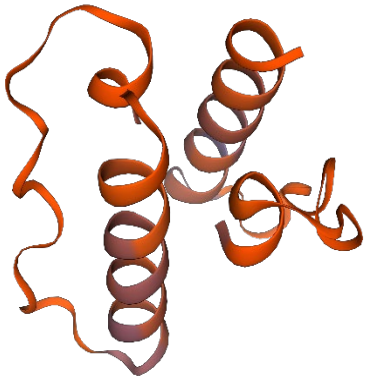

Figure 8: HAMP domain of CrrB.

The HAMP domain was detected from aa 81-153. The character, folding and sequence of the region strongly resembled those of dimeric HAMP domains. Furthermore, a global model equality estimation of 57%, when juxtaposed to the HAMP domain of the structurally similar histidine kinase EnvZ(Yoshida, Phadtare and Inouye, 2007)Thus, it can be concluded that the functionality of the domain ranging from aa 81 to 153 is that of signal transduction and amplification.

### 1.2.3 The histidine kinase A domain (HKA)

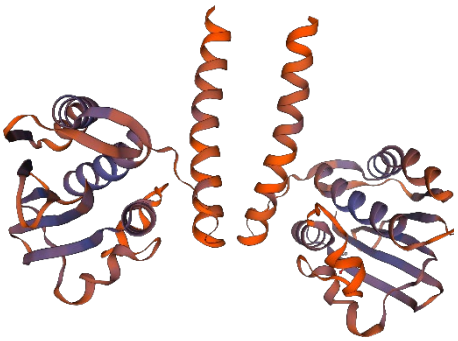

Figure 9: The HKA domain of CrrB.

The rest of the structure 153-353 was found to have a classical protein-L-histidine N-phosphotransferase structure, indicative of a histidine kinase domain. Furthermore, a global model equality estimation of 64% was found when compared to other histidine kinase type 1 groups (Figure 9). This domain is central in the histidine kinase facilitating the phosphorylation of the cognate response regulator of CrrB, CrrA.

### 1.3 CrrB compartment-based frequency of changes:

The changes in CrrB, resulting to colistin resistance, found in literature were associated to their respective CrrB compartment according to their amino-acid position (Figure 10). As seen in Figure 10, 50% of the described mutations are found in the HKA compartment followed by 31% in the TM and 19% in the HAMP domain.

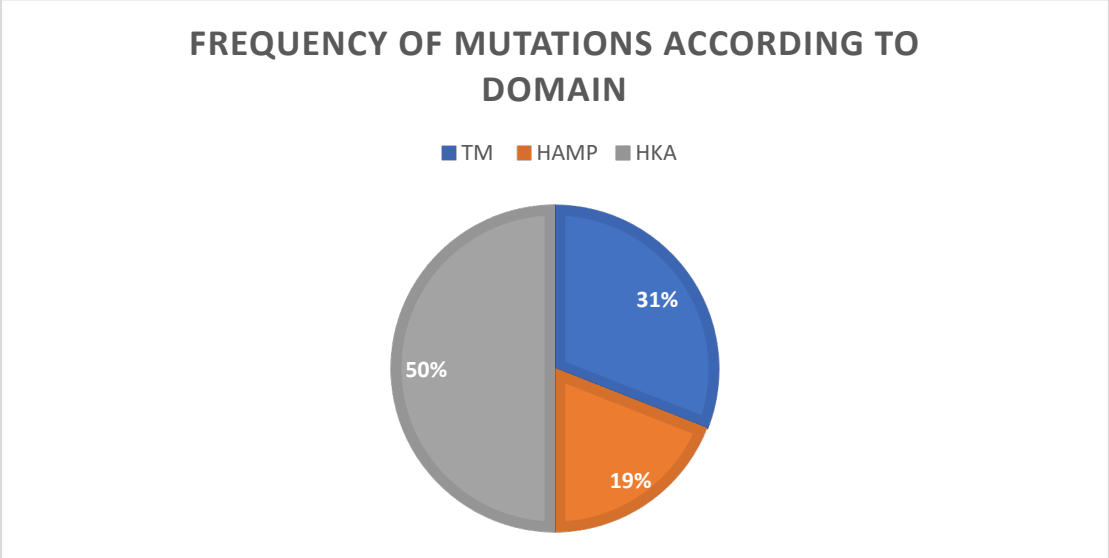


Figure 10: Frequency of substitutions in CrrB according to functional domain in the protein. “neutral” mutations were excluded in the analysis of the data. Data are adapted from Table 1 and categorized according to CrrB protein domains; Tm Domain ( 1-80 aa), HAMP Domain (81-153aa) and HKA Domain (153-353).

The high frequency of mutations within HKA coupled with the change in amino acid character reveals a preference for the activation of the domain. Subsequently, the observation suggests a potential benefit of HKA mutations in comparison to other compartments. Potential benefits in the context of antibiotic resistance revolve around increased survival. Therefore, upon exposure to colistin mutations in HKA might be the first to spontaneously occur. Moreover, due to the change in amino acid character in the specific compartment a higher protein activity is to be expected which in turn could result in the expression of a plethora of phenotypes. Due to factors such as sequence type, previous exposure and changes irrelevant to CrrB conclusions cannot be drawn from the set of data provided. To study the relationship between compartment and phenotype, specific mutations were selected and reproduced in the background of *K.pneumoniae* Ecl8.

### 1.4 Recreation of CrrB changes in *K.pneumoniae* Ecl8:

Firstly, the well characterized mutations N141I and S93N were recreated (Cheng, Lin, Lin and Wang, 2016) (Cain et al., 2018). As mentioned in the introduction the N141I, belonging to the HKA domain of CrrB, portrays the highest level of resistance indicative of increased protein activity and has been extensively used to study CrrB-dependent colistin resistance (Cheng, Lin, Lin and Wang, 2016). The S93N was recreated as it is known to portray phenotypic alteration such as the formation of capsule (Cain et al., 2018).

In addition, three more mutations were recreated: I27V, F84S and S195N. The S195N mutation was recreated because it belongs to the HKA domain which has the highest frequency of occurrence for alterations. Secondly, the I27V mutation was recreated to represent mutations within the TM domain. Finally, the F84S mutation was recreated due to its high proximity to the S93N mutation (known to produce capsule) to further investigate the unique phenotypic changes reported.

The selected mutations were carried forward. Furthermore, they were all recreated in the same genetic background to eliminate variables such as Sequence type, exposure to antibiotics, growth conditions and presence of plasmids/mobile elements. The overexpression vector pBR-322, containing the CrrB constructs, was introduced in *K.pneumoniae* Ecl8. Thus, an expression system for CrrB mutations, found in clinical isolates, was created. The genome of *K.pneumoniae* Ecl8 can be found in (Fookes et al., 2013).

**Chapter 2: Identifying the CrrB dependent capsule formation in K.pneumoniae.**

Following the analysis of the current literature, and the recreation of the *K.pneumoniae* Ecl8 pCrrBWT variants (pCrrBI27V, pCrrBS93N, pCrrBF84S and pCrrBN141I ) selected in Chapter 1 we investigated phenotypic effect arising from the changes. As mentioned in the introduction the most relevant observation is capsule formation. Its impact on virulence could determine the disease outcome especially in colistin resistance strains.

Intriguingly, the mechanistic basis of this phenotype was not apparent in the transcriptomic analyses of the mutants performed by Cain et al, (2018). Thus, the exact CrrB-dependent mechanism which results in increased capsule formation is unknown. As such, the focus of this chapter is to elucidate the CrrB-dependent mechanism in conferring increased capsule formation in *K.pneumoniae*. Firstly, the capsule production was determined in a two-step process.

## 2.1 Identification of extracellular density (ECD) by percol column:

In the first part of the process to identify capsule production the percoll gradient was conducted. This semi-quantitative measurement provides insights into the extracellular density (ECD) of the cells based on differences in sedimentation rates. The distance from the bottom of the column is directly proportional to the ECD i.e., less dense closer to the bottom of the density gradient. Using our recombinant constructs overexpressing the different CrrB variants, we undertook percoll density gradient analyses. A representation of 1 biological repeat is shown in Figure 11.

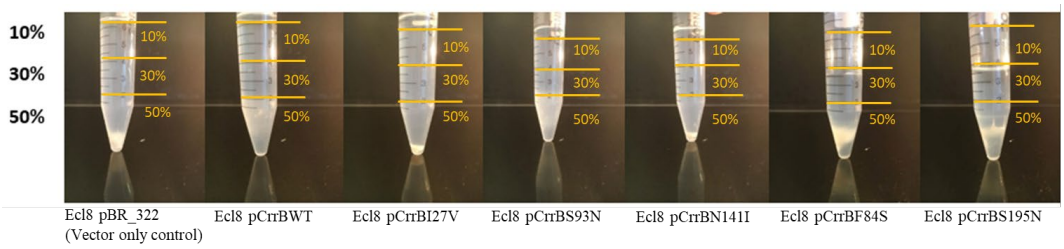


Figure 11: Percoll assay of the selected mutants. Percentages indicate the different phases of the column, and the isogenic mutants are given on the bottom of each image. Because of the qualitative nature of the assay image represents n=1.

Three biological repeats were conducted, each nested with 3 technical repeats. The strains pCrrBWT, pCrrBS93N and pCrrBF84S migrated to the bottom of the 30% fraction while vector only control, pCrrBI27V and pCrrBN141I migrated to the bottom of the 50% fraction. Migration patterns in percol columns indicate a difference

in the density of exopolysaccharides thus in this case they are indicative of capsule production but do not act as proof for its increased production based on CrrB alterations. To quantify capsule production, the samples are isolated from the column and uronic acid production is quantified.

### 2.2 Quantification of Uronic Acid to establish capsule production:

In the second part of the process, the denser part of each percol column was isolated to retrieve the samples. The samples are then treated to extract 4-O-(l-1-Carboxyethyl)-d-glucuronic acid, a major component of capsule (Clements et al., 2008). Subsequently, the glucuronic acid is quantified to reveal the density of the capsule. The results of the assay are shown in Figure 12 and are relative to capsule production in the vector only control.

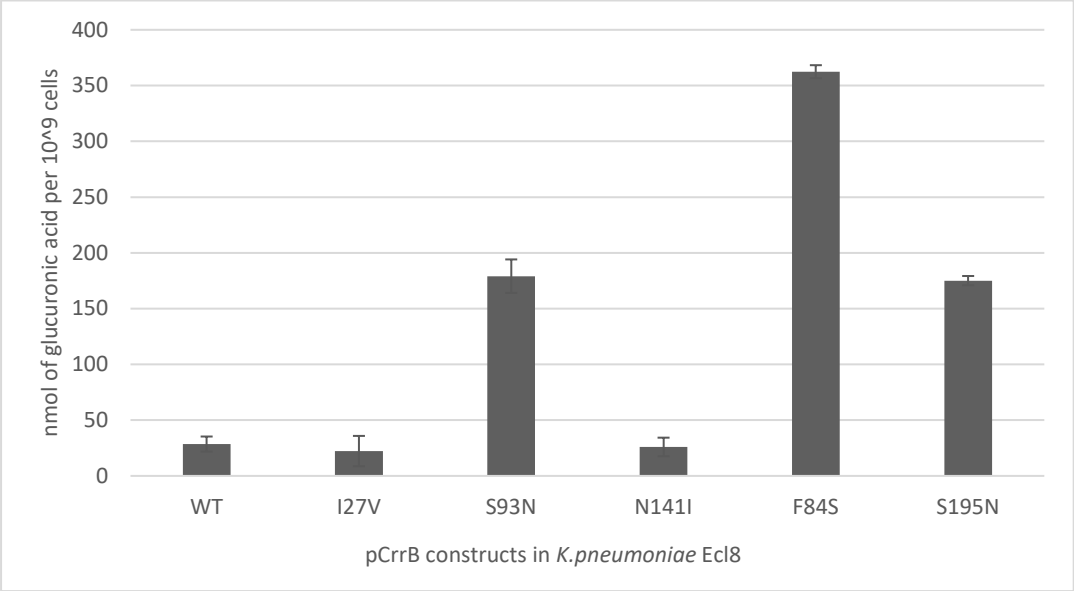


Figure 12 : Quantification of capsule production in selected CrrB mutants (pCrrBI27V, pCrrBS93N,pCrrBN141I, pCrrBS195N and pCrrBF84S) expressed in *K.pneumoniae* Ecl8 fixed for vector only control. Glucuronic Acid is one of capsule main subunits thus its amount relative to a fixed number of cells (10^9 cells) gives an accurate measurement for capsule production within CrrB specific mutations. Error bars indicate ±SEM. Statistical testing was conducted using ANOVA-Friedman (n=3)

Our results show that pCrrBF84S, confers the highest capsule production (>300 nmol/ 10^9 cells of uronic acid/ 13-Fold) followed by pCrrBS93N (>150 nmol/ 10^9 cells of uronic acid/ 6-Fold). The remaining variants pCrrBI27V and pCrrBN141I are comparable to the WT pCrrB gene (< 50nmol/ 10^9 cells of uronic acid). Thus, capsule production is dependent on the CrrB variant and suggests that these alterations are mutation specific.

## 2.3 Strain independent capsule formation in CrrB mutants.

However, in order to confirm the mutation dependency and rule out any strain specific effects we expressed four of the CrrB variants in *K.pneumoniae* 52145 (Figure 13).

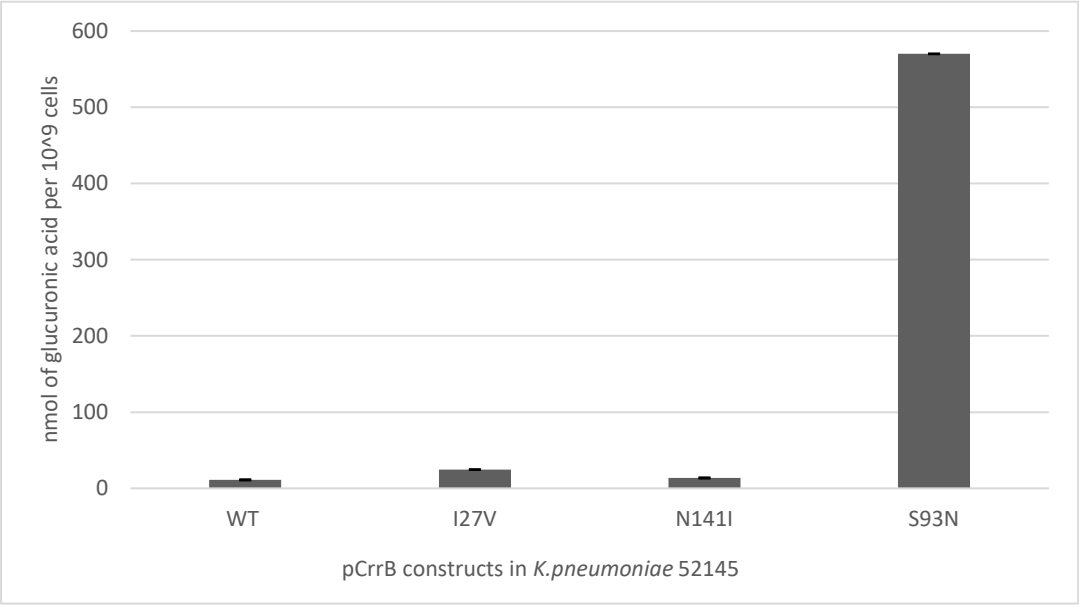


Figure 13: Quantification of capsule production in selected mutants (I27V, S93N andN141I) expressed in *K.pneumoniae* A4345 to validate the strain-independent effect of capsule production. Bars indicate the mean measurement of 3 biological repeats containing three technical repeats each. Error bars indicate ±SEM. Statistical testing was conducted using ANOVA-Friedman.

Our results as shown in Figure 12 demonstrate the increase in capsule production is observed in a non-strain dependent manner. PCrrBS93N has a 50-fold increase in capsule production when compared to WT pCrrB WT. While the other two variants pCrrBI27V and pCrrBN141I maintaining the same level of production as seen in the pCrrBWT. This trend is observed in the Ecl8 strain thus supporting the strain independent role of CrrB mutations in producing capsule. Although the trend is similar, there is a difference in the fold increase of capsule production between *K.pneumoniae* Ecl8 pCrrBS93N (6-Fold) and *K.pneumoniae* 52145 pCrrBS93N (50-Fold).

Overall, in this chapter we established the production of capsule according to CrrB alteration. Interestingly, we found that capsule formation in *K.pneumoniae* Ecl8 is not present in all alterations within CrrB but it occurs in specific alterations such as F84S, S93N and S195N and not in I27V and N141I (Figure 12). To identify if this effect is carried across different strains we analysed three mutations each belonging to a different CrrB protein domain: the two non-capsule producing I27V (TM domain) and

N141I (HKA domain) alteration and the capsule producing S93N (HAMP domain) alteration in the genetic background of *K.pneumoniae* 52145. As expected, the N141I and I27V did not result in an upregulation of capsule production. On the other hand, the S93N alteration resulted in a significant upregulation in capsule production, of >500nmol/10^9 cells. This value is 4-fold higher than that observed in the expression of S93N on the genetic background of *K.pneumoniae* Ecl8. *K.pneumoniae* 52145 as opposed to K.pneumoniae Ecl8 is a more virulent strain with higher mucoviscosity and an activated K-2 locus (Gomez-Simmonds and Uhlemann, 2017).Thus, the data supports that specific alterations such as S93N can increase capsule formation in a non-strain dependent manner. But the extent to which they do so, depends on the genetic background of the strain.

Furthermore, the quantity of capsule produced is not dependent of the protein domain, as S93N and F84S (both of which belong to the HAMP domain) portray different quantities of capsule, >150 and >300 nmol of uronic acid /10^9 cells respectively. The same applies to the alterations S195N and N141I (both belonging in the HKA domain) of which the first results in >150 nmol of uronic acid /10^9 cells while the latter does not alter capsule production. Thus, the definitive factor for capsule production are the alterations irrespective of the domain to which they belong. This unique observation suggests that specific alterations, increase the effectivity of CrrB as a signaling protein regulating capsule production. The highest increase of which is observed in F84S. To establish the mechanistic basis of the pCrrBF84S dependent regulation of capsule production, we undertook both transcriptomic and proteomic analyses using the pCrrBF84S variant.

## Chapter 3: Characterizing the mechanistic basis for CrrB-dependent capsule regulation.

### 3.1 Transcriptomic investigation of capsule regulation in *K.pneumoniae* Ecl8 pCrrBF84S:

The transcriptomic analysis consisted of three comparative analysis of gene expression. Those three are pCrrBF84S against vector only, pCrrBWT against vector and the derived comparison of pCrrBF84S against pCrrBWT. The analysis was performed to identify differences in gene regulation between the pCrrBF84S and pCrrBWT thus the set of data presented for the transcriptomic analysis is the comparison between the two. This set of data was p-value adjusted and filtered for values of $p<0.05$. Additionally, all values with a less than 2-Fold expression were excluded. The resulting data set is presented in Figure 14.

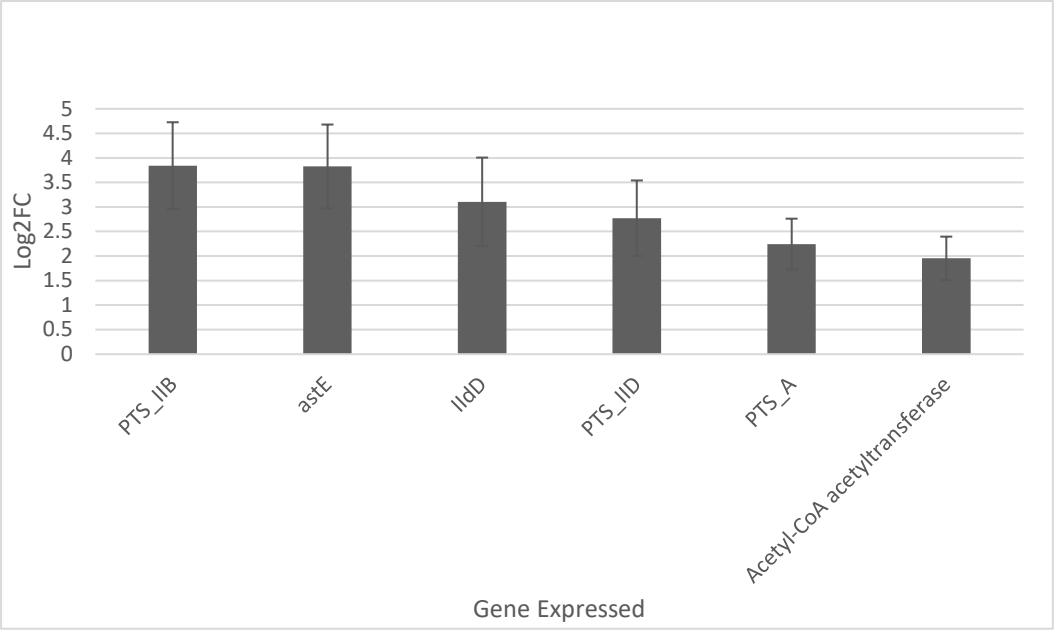


Figure 14: Presenting gene expression profile comparison of K.pneumoniae Ecl8 pCrrBF84S against K.pneumoniae Ecl8pCrrBWT. Fold-change indicates the increased expression of genes as a ratio of pCrrBF84S:pCrrBWT. All the values are significant as identified by t-test ($p<0.05$). Bars indicate ±SEM.

Our results as shown in Figure 14 demonstrate an increase in expression of genes related to metabolic processes, when overexpressing the CrrB F84S substitution compared to overexpressing CrrB WT. The genes are *PTS_IIB* and *astE* both of which have a 4-Fold increase in expression followed by *IIdD* with a 3-fold increase. Then *PTS_IID*, *PTS* and *acetyl-CoA acetyltransferase* have a >2-fold and <3-fold increase in expression.

To iterate, most of the significantly upregulated genes (*PTS_IIB, lldD, PTS_IID, PTS_*) belong to the phosphotransferase system (PTS). The PTS facilitates the entrance of carbon sources (mannose, fructose, N-acetylgalactosamine) in the cell. The carbon sources mentioned can be biosynthesized to produce capsule (Zeidan et al., 2017). With the strongest case being that of mannose which is exclusively brought inside the cell by a channel formed from the dimerization of PTS_IIB and PTS_IID. Mannose is fermented to GDP-D-mannose, a main capsular component (Pan et al., 2015).

The current data suggests that expressing the pCrrBF84S variant results in the overexpression of genes associated with the introduction of new carbon sources into the cell. Similar to our findings Dorman et al. (2018) identified the protein PtsN as an important regulator of capsule production. PtsN is a compartment of PTS_IIA belonging to the same system (PTS) as all the genes we identified as being important for capsule production. Thus, further supporting the role of the PTS cycle and the identified genes in capsule regulation. The transcriptome results reveal an overexpression on the genetic level, but to further support the current data and to formulate a clearer image of the mechanistic basis behind capsule production, the overexpression on the protein level is investigated.

### 3.2 Proteomic investigation of capsule regulation in *K.pneumoniae* Ecl8 pCrrBF84S:

To elucidate the effect of a single CrrB substitution on the protein expression profile, three strains of Ecl8 were analyzed. Namely, Ecl8 pBR-322, Ecl8 pCrrBWT and Ecl8 pCrrBF84S. In the same manner to the transcriptome processing, protein expression differences between pCrrBWT and pCrrBF84S were identified. Firstly, the p-value is adjusted to identify protein hits with (n=3). Secondly, the proteins are filtered based on two criteria. Those being a p-value of less than 0.05 ($p<0.05$) and a Fold increase of more than 2 ($Log2FC>2$).

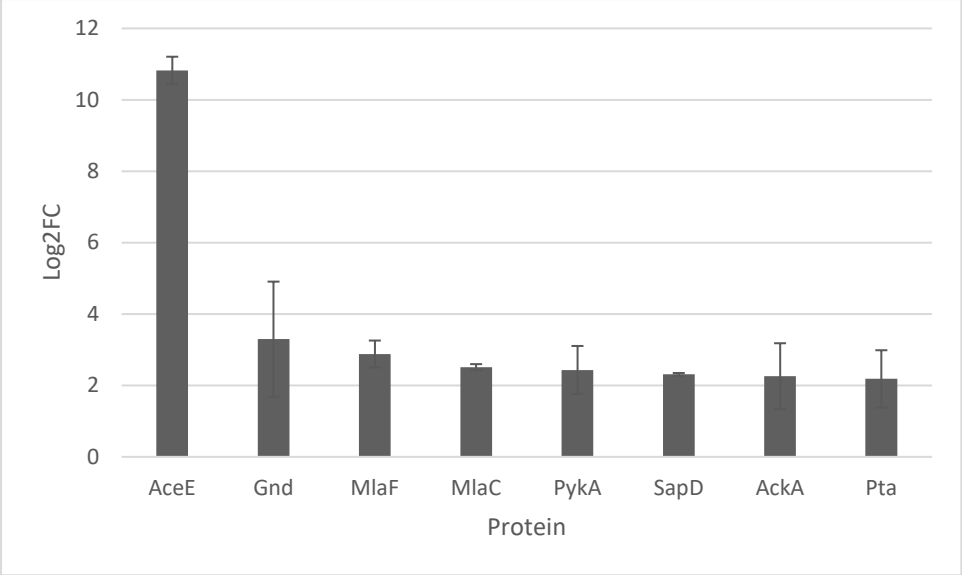


Figure 15: Presenting protein expression profile comparison of K.pneumoniae Ecl8 pCrrBF84S against K.pneumoniae Ecl8pCrrBWT. Fold-change indicates the increased expression of genes as a ratio of pCrrBF84S:pCrrBWT. All the values are significant as identified by t-test ($p<0.05$).

As shown in Figure 15, 8 proteins were identified which are associated with capsule regulation and significantly upregulated in the pCrrBF84S strain. The greatest difference in protein expression is found in AceE. In pCrrBF84S AceE has a 11-fold increased expression compared to pCrrBWT. Moreover, the seven proteins (Gnd, MlaF, MlaC, PykA, SapD, AckA, Pta) range from 3-fold to 2-fold increased expression compared to pCrrBWT.

Most of these proteins (AceE, Gnd, PykA, AckA, Pta) directly facilitate the fermentation of pyruvate and acetyl-CoA to carbon compounds, nicotinamide adenine dinucleotide phosphate (NADPH) and ATP (Enjalbert et al., 2017). Thus, their upregulation is indicative of increased metabolic processes inside the cell potentially leading to an increase capsular density.

To further support their role in capsule regulation, the upregulated proteins were cross-checked with the data base for known capsule regulatory proteins (Dorman et al., 2018).

| Protein[a] | Fold-Increase (pCrrBF84S: pCrrBWT)[b] | Capsular Density[c] |
|---|---|---|
| AceE | 10.82173121 | High |
| Gnd | 3.295829797 | Low |
| MlaF | 2.881259841 | Low |
| MlaC | 2.512690505 | Low |
| PykA | 2.433533903 | - |
| SapD | 2.310778096 | - |
| AckA | 2.258256208 | High |
| Pta | 2.183939399 | High |

Table 2: Upregulated proteins in *K.pneumoniae* Ecl8 pCrrBF84S and their previously published effect on capsule regulation (a) Three letter identification for proteins (b) Fold-change of protein in *K.penumoniae* Ecl8 pCrrBF84S in comparison to K.pneumoniae Ecl8 pCrrBWT (c) Impact of the proteins on capsular density in *K.pneumoniae* NTUH-K2044 as reported by Dorma et al. (2018).

According to the library of mutants most of the proteins upregulated in pCrrBF84S are required for capsule production in the strain *K.pneumoniae* NTUH-K2044 (Table 2c). Thus, their upregulation in our strains *K.pneumoniae* Ecl8 pCrrBF84S is indicative of the underlying mechanism for capsule production. To summarize, the data supports a unique mechanism in the production of capsule, in which the pCrrBF84S variant has an effect in metabolic processes.

### 3.3Merging transcriptome and proteome analysis to reveal a pathway for CrrB-dependent capsule production:

Overall, there is no overlap between the transcriptomic and proteomic data which is indicative of a more complex regulatory pathway than in canonical capsule production. Our data suggests that alteration both on the genetic expression and protein expression level alter the metabolic processes responsible for carbon processing ultimately leading to capsule production. To further elucidate this the significantly upregulated genes and proteins are compiled and presented in Figure 16.

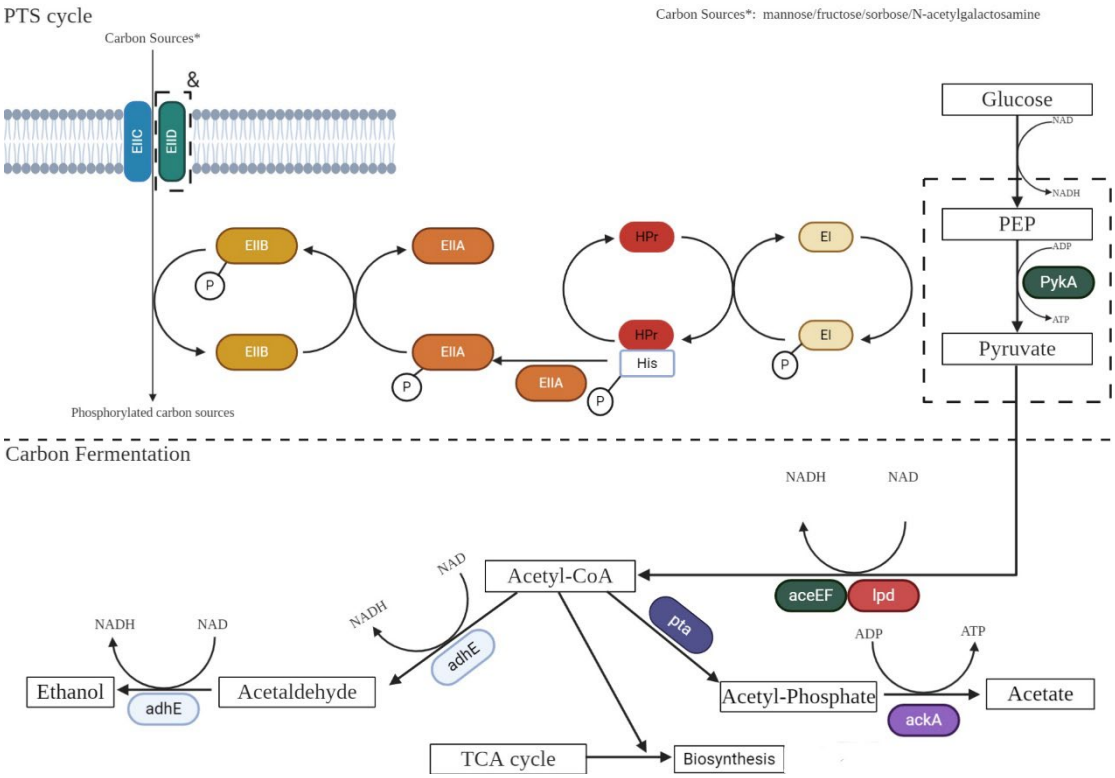


Figure 16: Metabolic cycles identified to be upregulated in the pairwise comparison of transcriptomic and proteomic datasets between *K.pneumoniae* Ecl8 pCrrBWT and *K.pneumoniae* Ecl8 pCrrBF84S. The top panel consists of the phosphotransferase system (PTS), solely upregulated in the transcriptomic data. The bottom panel presents the Acetyl-CoA cycle, solely upregulated in the proteomic data. Arrows indicate direction of the reactions. Square boxes indicate carbohydrates. Oval shapes represent proteins, as identified by their acronyms. (Zeidan et al., 2017) (Wolfe, 2005)

As it can be seen in Figure 16 both metabolic processes that we identified as being upregulated are closely interlinked. Specifically, the PTS cycle utilizes the energy (in the form of ATP) produced by the catabolism of PEP to pyruvate, a process facilitated by the upregulated protein PykA (Deutscher et al., 2014). Pyruvate is then directly processed by AceEF to produce acetyl-CoA. Which in turn is modified by pta and AckA to produce acetate (Enjalbert et al., 2017). Overall, through the PTS cycle and Acetyl-CoA cycle the cell increases its influx in carbon sources and energy production, respectively. Capsule formation is a biosynthetic process requiring the modification of carbohydrates to capsular components and their fixation on the outer membrane. Both processes require carbon sources and energy sources. These resources are provided in the form of mannose, fructose, and N-acetylgalactosamine by the PTS cycle and in the form of ATP, NADPH, and acetate by the Acetyl-CoA cycle. Thus, suggesting that the regulation of metabolic processes in a CrrB-dependent manner results in the formation of capsule.

*K.pneumoniae* Ecl8 is also susceptible to colistin. To identify if the changes in CrrB have a dual effect in guiding both colistin resistance and capsule formation, the strains were moved forward.

## Chapter 4: Characterizing the CrrB-dependent colistin resistance in *K.pneumoniae.*

Following the identification of differential capsule production, according to CrrB changes we established that pCrrB variants have different impact on the phenotype. Thus, we proceeded on analyzing their clinically significant phenotype of susceptibility to colistin.

### 4.1MIC of colistin of the CrrB variants in the genetic background of *K.pneumoniae* Ecl8:

A plate microdilution assay was set up according to CSLI. Subsequently, a standard measurement for MIC of colistin was conducted in all strain. Results are shown in Table 3.

| **Construct** [a] | **MIC**[b] (ng/µl) | **Fold-Change**[c] (x) |
|---|---|---|
| *K.pneumoniae* Ecl8 WT | 1 | 1 |
| *K.pneumoniae* Ecl8 pBR-322 (Vector only) | 1 | 1 |
| *K.pneumoniae* Ecl8 pCrrB | 4 | 4 |
| *K.pneumoniae* Ecl8 pCrrBI27V | 8 | 8 |
| *K.pneumoniae* Ecl8 pCrrBS93N | 128 | 128 |
| *K.pneumoniae* Ecl8 pCrrBN141I | ≥256 | 256 |
| *K.pneumoniae* Ecl8 pCrrBF84S | 128 | 128 |
| *K.pneumoniae* Ecl8 pCrrBS195N | 16 | 16 |

Table 3: MIC of colistin for *K.pneumoniae* Ecl8 strains containing the CrrB overexpression system. (a): Klebsiella pneumonia Ecl8 strains with the pBR-322 plasmid vector overexpressing different CrrB alterations. (b) Identified MIC of colistin in ng/ µl. (c) Fold-Change of colistin MIC with respect to *K.pneumoniae* Ecl8 WT, value presented is the most frequently occurring in a series of 3 biological repeats with 3 technical repeats in each.

Our results, Table 3 show that changes in CrrB result in a decrease to susceptibility of colistin. Furthermore, each CrrB alteration has a different impact on the resistance phenotype. The highest impact being that of CrrBN141I with a 64-Fold increase in MIC compared to overexpressing CrrBWT. Followed by CrrBS93N and CrrBF84S

with a 32-Fold increase in MIC and CrrBS195N, CrrBI27V with a 4-Fold and 2-Fold respectively. Thus, each CrrB alteration can affect colistin susceptibility in a unique manner in *K.pneumoniae* Ecl8.

### 4.2 Strain independent impact of CrrB variants on MIC of colistin:

To identify if this effect is strain dependent, the same methodology was used for analysis in the genetic background of *K.pneumoniae* 52145. The results are shown in Table 4.

| **Construct**[a] | **MIC of *K.pneumoniae* 52145**[b](ng/µl) | **Fold-Change in *K.pneumoniae* 52145**[c] (x) | **Fold-Change in *K.pneumoniae* Ecl8**[d] (x) |
|---|---|---|---|
| WT | 4 | 1 | 1 |
| pBR-322 (Vector only) | 4 | 1 | 1 |
| pCrrB | 16 | 4 | 4 |
| pCrrBI27V | 16 | 4 | 8 |
| pCrrBS93N | 128 | 32 | 128 |
| pCrrBN141I | ≥256 | 64 | 256 |
| pCrrBF84S | 128 | 32 | 128 |
| pCrrBS195N | 16 | 4 | 16 |

Table 4: MIC of colistin for *K.pneumoniae* 440 and K.pneumoniae Ecl8, both containing the different overexpression mutants. MIC presented is the most frequently occurring in a series of 3 biological repeats with 3 technical repeats in each. (a): Wild-type, vector only and plasmid vector overexpressing different CrrB alterations. (b) Identified *K.pneumoniae* 52145 MIC of colistin in ng/ µl. (c) Fold-Change of colistin MIC with respect to *K.pneumoniae* 52145 WT, value presented is the most frequently occurring in a series of 3 biological repeats with 3 technical repeats in each. (d) Fold-Change in *K.pneumoniae* Ecl8 strains with respect to WT.

The trend of colistin resistance in *K.pneumoniae* 52145 is similar to that of K.pneumoniae Ecl8. Specifically, in the genetic background of both *K.pneumoniae* Ecl8 and *K.pneumoniae* 52145, pCrrBN141I has the highest impact on MIC. Followed by the in the intermediate pCrrBS93N and pCrrBF84S and the low

pCrrBS195N and pCrrBI27V. Thus, the effect on colistin susceptibility of overexpressing CrrB alterations is not strain-dependent.

### 4.3 Expression profiles of genes regulating colistin resistance in *K.pneumoniae*:

To identify the underlying mechanism of colistin resistance we filtered our transcriptomic data for upregulation of known mediators of resistance (Cheng, Lin, Lin and Wang, 2016). Specifically, the expression of *crrA* , *crrB* and *crrC* which are part of the c*rrCAB* operon were found for an initial description of the CrrAB TCS. Following, the expression profiles for genes associated with colistin resistance and hypothesized to be under the direct control of the CrrAB were found (Cheng, Lin, Lin and Wang, 2018). The two response regulators (RR) *phoP* and *pmrA* the expression of which is linked to colistin resistance. *pmrl (ArnA)* a component of the *arn* operon, its expression used as a standard measurement for the activation of the *arn* operon, the central component in LPS modification (Cheung, Dulyayangkul, Heesom and Avison, 2020). And the theoretical connector, between the TCSs CrrAB and PmrAB, namely CrrC (Cheng, Lin, Lin and Wang, 2018). In the transcriptomic dataset of the pairwise comparison between overexpression of CrrBWT (colistin susceptible) and CrrBF84S (colistin resistant), none of the listed genes were significantly upregulated. To be precise the p-value adjustment resulted in p-values of more than 0.05 ($p>0.05$) for the genes *crrA, crrB, phoP, pmrA, pmrl and crrC*. To substantiate the result of the transcriptome data we performed a qPCR test for the same genetic elements (Figure 17).

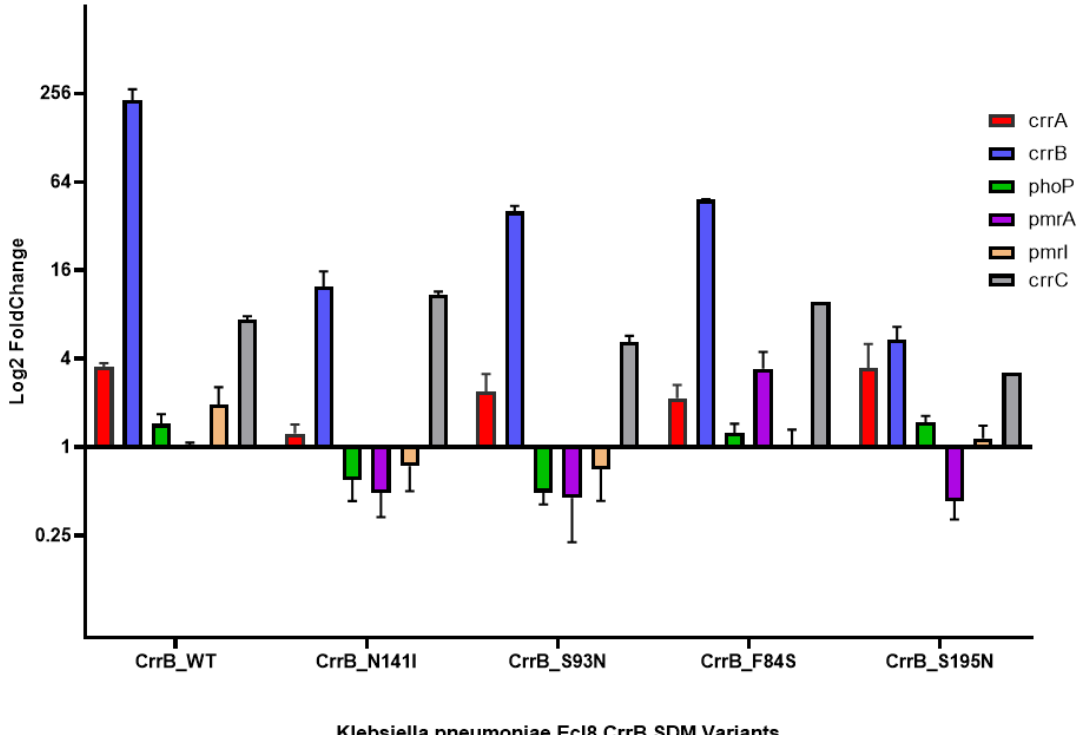


Figure 17: Transcriptomic analysis of key elements, associated with colistin resistance, in the *K.pneumoniae Ecl8::pBR* overexpression vector with different substitutions in CrrB protein and WT. Bars represent the average identified by 3 biological repeats each containing 3 technical repeats. Error bars represent SEM.

Firstly, the expression of *CrrC* ranges between the constructs with the highest being that of CrrBN141I, CrrBF84S, CrrBWT at 10-fold, 9-fold and 8-fold respectively. Followed by CrrBS93N and CrrBS195N at 6-fold and 3-fold respectively. In previous publication it was shown that the chromosomal CrrBS93N mutation has a >256-fold in CrrC gene expression, which acted as supporting evidence for the profound MIC of 128ng/ul (Cheng, Lin, Lin and Wang, 2016). In the current data the highest MIC (>256ng/ul) producing mutation pCrrBN141I, has a 10-fold increase in CrrC expression and it is not significantly different from the expression of CrrC found in the colistin susceptible pCrrBWT. Suggesting that CrrC expression is not responsible for the high resistance phenotype observed in pCrrBN141I.

Similarly, the expression of *pmrI* and *pmrA*, key genes in identifying colistin resistance are downregulated in pCrrBN141I compared to pCrrBWT. Finally, the expression of CrrB in pCrrBN141I is 16-Fold less than that of the CrrBWT. Subsequently, we hypothesized that there is a growth defect associated with overexpressing the CrrB variants. To identify a potential growth defect, standard growth analysis was conducted in minimal media (M9 media).

## 4.4 Growth impact of the overexpression of mutations:

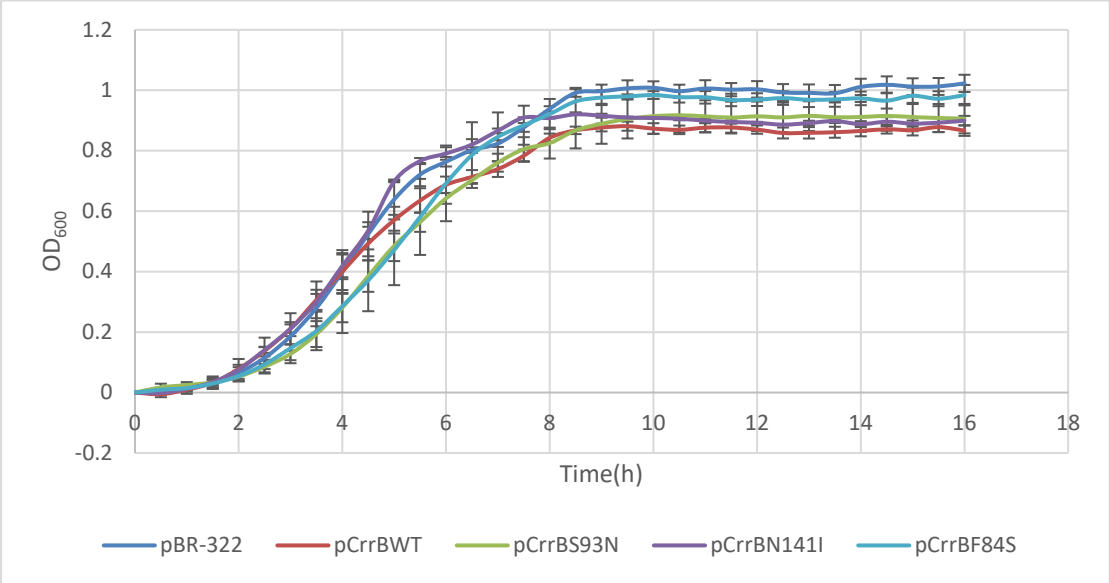


Figure 78: Growth curves for the different mutants of CrrB in *K.pneumoniae* Ecl8. M9 minimal media was used to see growth deficits arising from mutations within CrrB. No significant differences were found between vector only control (known to have no impact on growth) and any of the mutants or the WT gene. Lines indicate the average of three biological repeats with three technical repeats. Error bars indicate ±SEM. Statistical analysis was conducted using.

Our results shown in Figure 18 reveal no significant growth defects at the exponential phase of growth. Thus, the expression of the six genes previously associated with colistin resistance are unique and vary according to the impact of the substitution. The set of data produced by the qPCR analysis supports the finding found in the whole transcriptome analysis. But it does not provide a clear pathway by which high levels of colistin resistance arise upon the overexpression of CrrB alterations. Thus, we proceeded on taking a different route to establish the later. It is known that the most important regulator of colistin resistance is the TCS PhoPQ (Bhagirath et al., 2019). thus, we created an isogenic *K.pneumoniae* Ecl8 mutant lacking the system in which we expressed the CrrB variants.

## Chapter 5: Characterizing the dependability of CrrB-dependent phenotypic changes on PhoPQ.

In Chapters 2 and 3 we have established phenotypic alterations, capsule production and decreased susceptibility to colistin, arising from the expression of CrrB changes. The mechanistic basis of capsule production has been identified using proteome and transcriptome analysis. PhoPQ is arguably the most important of the three TCS. As mentioned in the introduction it is known to have variable regulon sizes and can alter the expression of up to 600 genes in *E.coli* (Alteri et al., 2011). Previous publications have shown that PhoPQ is closely interlinked with CrrAB (McConville et al., 2020). Naturally, the next step into describing phenotypic alteration guided by the CrrB system is to identify its dependency on PhoPQ.

Subsequently, we created a *K.pneumoniae* Ecl8 isogenic mutant lacking the PhoPQ TCS. Following, we expressed the pCrrB variants in the isogenic mutant in which we identified the formation of capsule.

### 5.1Capsule production of CrrB variants in *K.pneumoniae* Ecl8 ΔPhoPQ:

The capsule production observed in the WT genetic background is unique characteristic of CrrB substitutions. It has not yet been described if this phenotype is dependent on the PhoPQ system. Thus, three CrrB alteration resulting in low (N141I), intermediate (S93N) and high (F84S) capsule production were analyzed in the genetic background of a PhoPQ null mutant of *K.pneumoniae Ecl8* (Figure 19).

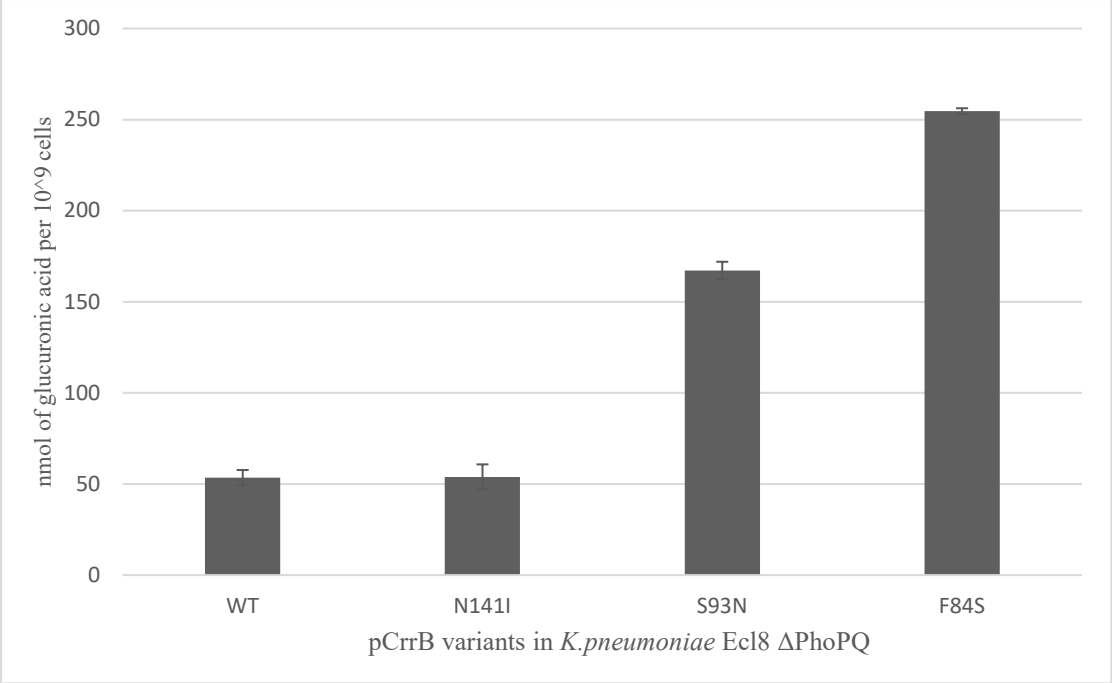


Figure 89: Quantification of capsule production in selected mutants (I27V, S93N, N141I and F84S) expressed in the genetic background of K.pneumoniae Ecl8 PhoP -/PhoQ-. Glucuronic Acid is one of capsule main subunits thus its amount relative to a fixed number of cells (10^9 cells) gives an accurate measurement for capsule production within CrrB Specific mutations. Bars indicate the mean measurement of 3 biological repeats containing three technical repeats each. Error bars indicate ±SEM. Statistical testing was conducted using ANOVA-Friedman (*

The trend for production of capsule in the PhoPQ null background are comparable to those in the WT strain. To be precise, pCrrBF84S, confers the highest capsule production (>250 nmol/ 10^9 cells of uronic acid/ 5-Fold) followed by pCrrBS93N (>150 nmol/ 10^9 cells of uronic acid/ 3-Fold). The remaining variant pCrrBN141I is comparable to the WT pCrrB gene (50nmol/ 10^9 cells of uronic acid). Thus, we concluded that capsule production is PhoPQ independent (Figure 19).

**Discussion**

Our analyses show that the colistin resistance regulatory locus, CrrAB, can confer pleiotropic phenotypes including capsule formation in a mutation specific and non-strain dependent manner.

Firstly, we described the existing knowledge on alteration in the system. We associated changes in CrrB following a review of 14 publications. This work revealed that mutations were more likely to occur in the HKA domain of CrrB. Suggesting that kinase activity is likely elevated resulting in increased rates of phospho-transfer from the sensor kinase (CrrB) to the transcriptional regulator (CrrA). Accordingly, these alterations in the HKA domain led to increased CrrB levels, potentially via constitutive induction, and the subsequent colistin resistance. In addition, alterations in TM and HAMP domains which have a lower frequency of occurrence result in the increased signal transduction within CrrB thus decreasing the activation energy threshold required for phosphorylation of the protein. With regards to MIC of colistin alterations in each domain have been shown to result in colistin resistance.

Previous work has demonstrated that CrrB alterations results in multiple phenotypes unconnected to colistin resistance which include capsule formation (Cain et al., 2018). Intriguingly, previous studies on the CrrAB mutations had not reported such phenotypes. Alterations in the HAMP domain of CrrB with changes at S93N contribute to the capsule production (Cain et al., 2018). Given the preferential accumulation of mutations within the HKA domain, we wondered which of the changes within the CrrB protein would confer increased capsule formation. Thus, we quantified capsule formation in multiple CrrB changes (I27V, F84S, S93N, N141I, S195N). Firstly, the variants S93N and N141I were chosen as they were reported to cause capsule formation and the highest reported MIC of colistin, respectively (Cain et al., 2018) (Cheng, Lin, Lin and Wang, 2016). Two further CrrB variants (F84S and S195N) were chosen to support the reported phenotypes. F84S was chosen as it is proximal to the capsule producing S93N change thus we hypothesized that it would produce a similar phenotype. In contrast, S195N was chosen as it is located within the HKA domain of CrrB which is over-represented in literature. Finally, I27V was chosen to represent changes within the TM domain of the CrrB protein. Overall, the chosen CrrB alterations located across the different domains of the protein provide a small but relevant group of changes in characterizing CrrB-dependent regulation.

Our results show that different mutations within CrrB result in variable effects on capsule formation in a strain independent manner. We identified differential expression of capsule according to CrrB alteration. The values of capsule quantity ranged from high (>300 nmol/ 10^9 cells of uronic acid) in F84S, to intermediate (>150 nmol/ 10^9 cells of uronic acid) in S93N and S195N, to low capsule (<50 nmol/ 10^9 cells of uronic acid) in I27V and N141I. Our results indicate a correlation between alteration within the HAMP domain of CrrB (F84S and S93N) and capsule production. On the other hand, alterations within the TM domain did not result in capsule production. Interestingly, only one of the alterations, S195N, within the HKA domain resulted in capsule production. Our data suggests a preference of mutations within the HAMP domain of CrrB for capsule production.

The differential effects of CrrB alteration on capsule formation are novel. Thus, to understand the mechanistic basis of the capsule formation phenotype we undertook proteomic and transcriptomic analyses. This specific alteration, F84S, was chosen as it is located within the HAMP domain of CrrB,

Pairwise comparisons between CrrB-F84S, CrrB-WT and the vector-only control of both the proteomic and transcriptomic data identified closely interlinked pathways. Our results show 6 genes were significantly upregulated in the transcriptomic data and of 8 proteins significantly upregulated in the proteomic data. Specifically, the genes *PTS_IIB*, *astE*, *lldD* were upregulated more than 3-fold and the genes *PTS_IID*, *PTS_*, *Acetyl-CoA* acetyltransferase more than 2-fold in the transcriptomic data. In contrast, the proteomic data revealed 8 significantly upregulated proteins and consisted of a more than 10-fold increase in AceE while the rest of the proteins (Gnd, MlaF, MlaC, PykA, SapD, AckA, Pta) had a 2-fold to 3-fold upregulation. Most of the upregulated genes and proteins have been shown to be important for capsule production in a series of deletion experiments (Dorman et al., 2018). Albeit their importance in maintaining capsule production the exact mechanism by which they do so was not described.

Capsule formation in *K.pneumoniae* in most cases a result of the increased expression of the regulatory *cps* operon. The *cps* operon regulates two processes; The transport of capsular monomers to the outer membrane of the cell and the processing of substrates to yield these monomers. Transport occurs through the Wzx/Wzy pathway which is essentially a concoction of channels and kinases responsible for the transfer of polysaccharides to the outer membrane of the bacterium facilitated by *cps* genes

(*wcaI*, *wcaG*, *gmd*, *atf*, *wzy*, *wzx*, *wzc*, *wzb*, and *wza*) (Ho et al., 2011). These polysaccharides are synthesized from pentose sugars, with the primary example being that of mannose-6-phosphate (M-6-P). To be precise M-6-P undergoes a series of modifications facilitated by *cps* genes
(*manB*, *manC*, *wcaH*, *gnd*, *wcaG*, *gmd*, *galF*, *ugd* and *uge*) to produce one of the repeating units of capsule, namely mannose-α-2/3-mannose (Ho et al., 2011). Thus, to produce sufficient capsule, the cell requires an excess of the primary substrate, mannose, and other similar substrates.

The phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) catalyzes the transport and phosphorylation of these substrates. The catalytic function of the PTS cycle utilizes PEP as an energy source and phosphoryl donor. The conversion of PEP to pyruvate (facilitated by AckA) releases a phosphoryl group which is transported via four PTS domains (EI, HPr, EIIA, and EIIB) to the substrate bound to the membrane components (EIIC and EIID) of PTS (Deutscher, Francke and Postma, 2006).

As previously mentioned upon expression of the F84S alteration, the only the PTS what was identified to be significantly what. The membrane components EIID and EIIB and the phosphoryl transfer component EIIA were significantly upregulated. None of the *cps* operon genes were differentially expressed at both transcript levels. The overexpression of these components suggests that increased capsule production because of CrrB regulation is linked to increased capsule biosynthesis. A direct link between capsule production and upregulation of the PTS has not been established before. On the other hand, the influx of exogenous carbon inside bacterial cells has been shown to be a promoter of virulence (Wei et al., 2019). Our data suggests an upregulation in the acquiree of primary substrates leading to the biosynthesis of capsule. Specifically, the acquisition and processing of mannose and other substrates important for capsule production. The upregulation of the PTS cycle responsible for the influx of mannose and other sugar sources is important for the metabolic drive of capsule production. It provides an excess of the capsular substrates. It is well known that substrate concentration can affect enzyme activity i.e., a high concentration of substrates leads to a high enzyme activity. Since the metabolic drive of capsule is present (excess of substrate) we surmised that there will be an impact on enzyme activity. The increased activity decreases the turn-over of enzymes potentially leading

to a build-up (Sousa, Ramos, Lim and Fernandes, 2015). Thus, the actual structural loci which contribute to capsule on the surface may remain unchanged in gene expression but altered at protein levels. Accordingly, we measured the protein levels in the F84S alterations.

Our proteomic data did not reveal an upregulation in proteins of the *cps* operon. Instead, we identified an upregulation in proteins ultimately leading to acetate dissimilation (Figure 15). We identified an upregulation in PykA which facilitates the conversion of PEP to pyruvate with the production of ATP. The upregulated protein PykA feeds both cycles identified in our analyses. The ATP is utilized as an energy source for the PTS cycle, while pyruvate is converted to acetate through a series of reactions. This common first step in both cycles unifies them on the metabolic level. Pyruvate is initially converted to Acetyl-CoA and NADPH, facilitated by the vastly upregulated AceEF. These processes directly feed Acetyl-CoA for acetate dissimilation. Acetate dissimilation is catalyzed by the upregulated enzymes pta and Acka. Pta reversibly converts acetyl-CoA and inorganic phosphate to acetyl-Phosphate. AckA reversibly converts Acetyl-phosphate and ADP to acetate and ATP (Wolfe, 2005). Thus, the pta-AckA pathway couples energy metabolism with those of carbon and phosphorus. The combination of acetate dissimilation (identified in proteomics) and the PTS cycle (found in transcriptomics) provides the necessary groundwork for capsule formation. Both cycles produce the necessary substrates for capsule production, those being M-6-P and acetate. Both can be utilized by the cell to build carbohydrate polymers known to constitute capsule. Furthermore, the excess sources of energy in the form of ATP and NADPH produced by acetate dissimilation can be utilized for the active transport of these polymers in the outer membrane of *K.pneumoniae*.

The role of these pathways in virulence of Gram-negative organisms is not completely clear, but it has been shown that similar metabolic pathways are activated upon alterations within CrrB. Such a process is the (pentose phosphate pathway) PPP responsible for production of NADPH and carbon intermediates, mostly glucose products, was upregulated upon expression of the S151N CrrB mutant (McConville et al., 2020). Through a series of deletion experiments it was deducted that the S151N CrrB alteration resulted in shunting of sugars towards the PPP pathway and away from glycolysis. This process increased the in vivo virulence of *K.pneumoniae* as it

was determined in a *Galleria mellonella* infection model (McConville et al., 2020). Similarly, the upregulated protein pathway identified in our results, shunts pyruvate towards the production of acetate. This process produces ATP and NADPH both of which are necessary for capsule production. Furthermore, acetate has been shown to be a structural component of capsule. Thus, its excess presence suggests its utilization as a capsular component in CrrB-dependent capsule formation.

The second phenotype we investigated was colistin susceptibility. In the selected alteration we observed high (N141I), intermediate (S93N, F84S) and low (I27V, S195N) strain-independent decrease in colistin susceptibility.

Interestingly, the highest colistin MIC was identified in the alteration N141I (found in HKA) which has minimal to no capsule production while both intermediate colistin resistant alterations F84S and S93N (Found in HAMP) have significantly more capsule production. This high MIC identified in N141I is speculated to be a result of increased kinase activity which increases the phosphorylation of CrrA and in turn the expression of the downstream genes leading to colistin resistance. Similar to this finding a group identified two alteration in CrrB (94, 87) as producing the same MIC (>512 ng/µl) but with significantly different effect on virulence *in vivo* (McConville et al., 2020). Thus, our data supported by previous work suggest a CrrA phosphorylation dependent regulation of genes guiding colistin resistance and/or pathways associated with virulence. Expression of these virulence pathways is solely determined by the alteration occurring within CrrB and the respective activation of CrrA. A clear motif, for alterations regulating virulence pathways, has not been determined yet but our data suggests a preference for the HAMP domain. In contrast, the pathway by which CrrB-dependent colistin resistance occurs has been previously described.

To identify how different CrrB alteration affect the pathway of CrrB-dependent colistin resistance, we quantified the expression of genes known to confer it through qPCR analysis. According to previous publication CrrB-dependent resistance to colistin results in a MIC which is proportional to the expression of the *arn* operon (Cheung, Dulyayangkul, Heesom and Avison, 2020). Furthermore, PmrAB and PhoPQ expression is also increased as it is believed to be the main pathways by which CrrAB acts to increase the expression of *arn*. Strikingly, we did not identify a relation between MIC levels and induction of the *arn* operon in any of the alterations. There was no correlation between the expression of the *arn* operon (quantified through

expression of pmrl) and MIC of colistin. On the other hand, the levels of both *crrB* and *crrC* were significantly upregulated in all constructs including the CrrB WT. Since our constructs are overexpressed the upregulation in *crrB* expression is to be expected. Interestingly, the levels of *crrC* were upregulated in a similar manner. The overexpression of the CrrB WT (colistin susceptible) resulted in a 10-fold increase in *crrC* which was comparable to that of CrrBN141I (colistin resistance). Thus, there was no correlation between *crrC* expression and MIC of colistin. The limited capsule in N141I was not attributed to any genetic elements. The qPCR did not reveal any evidence on differential regulation of the selected genes due to the expression levels of all genes (*crrA, crrB, phoP, pmrA, pmrl, crrC*) being comparable to those of the capsule producing S93N.

In the context of global regulators, PhoPQ is the most important of the three TCS. As mentioned in the introduction it is known to have variable regulon sizes and can alter the expression of up to 600 genes in *E.coli* (Alteri et al., 2011). Previous publications have shown that PhoPQ is closely interlinked with CrrAB. Subsequently, we wanted to investigate the potentiality of a PhoPQ-CrrAB interaction guiding the CrrB-dependent capsule synthesis.

Thus, we quantified capsule production in the CrrB alteration expressed on the genetic background of *K.pneumoniae* Ecl8 ΔPhoPQ. Interestingly, capsule production was unaffected and comparable to that of CrrB alterations expressed in the genetic background of *K.pneumoniae* Ecl8 WT. CrrB regulates capsule production in a PhoPQ-independent manner. Based on these results we surmised that the TCS CrrAB can independently activate a regulon which confers capsule production.

Overall, we have described the pleiotropic character of the CrrB histidine kinase. We identified and described the regulation of capsule production and resistance to colistin by single amino acid substitutions. The impact of capsule as a virulence factor is well established. It can lead to persistence of infection through avoidance of the host immune system. In addition to the capsular phenotype the substitutions also resort to resistance to the last-line antibiotic colistin. Thus, in the case of MDR *K.pneumoniae* infections, the presence of substitutions in CrrB can facilitate pathogen viability in the host- and against the drug. This raises important antibiotic usage concerns and treatment concerns for MDR *K.pneumoniae*. Unnecessary use of colistin could lead to selection of CrrB alterations which result in the highest MIC and the highest capsule

production. Virtually creating a pan-drug resistant and virulent strain of *K.pneumoniae*.

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