A Novel Two-Component System Involved in the Growth and Antimicrobial Resistance of *Burkholderia cenocepacia*

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Abstract

The *Burkholderia cepacia* complex (Bcc) are multi-drug resistant, opportunistic pathogens that cause infections in immunocompromised patients and those with cystic fibrosis. A chemogenomic screen of a transposon mutant library of essential genes revealed a novel two-component system, named *esaSR* that affects the antimicrobial resistance of *Burkholderia cenocepacia.* To characterize *esaSR*, an *esaS* knockout mutant and *esaR* knockdown mutant were created. To characterize the phenotype of resultant mutants, viability, minimum inhibitory concentrations, RND efflux expression, membrane protein profiles were assessed. Results demonstrate that *esaR* is essential for growth and viability in rich media. Mutants also exhibit hypersusceptibility to antimicrobials, altered cell size and morphology, altered RND efflux expression, in addition to having compromised cell envelopes. In summary, results indicate that EsaSR plays a major role in the viability and antimicrobial resistance of *B. cenocepacia.* EsaSR represents an attractive system to study for its potential as a drug target to lower resistance and render resistant pathogens susceptible to antibiotics currently in use.

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List of Abbreviations

atb	antibiotic
bp	base pair
Bcc	*Burkholderia cepacia* complex
CF	cystic fibrosis
CG	conditional growth
DNA	deoxyribonucleic acid
HK	histidine kinase
hrs	hours
kDa	kilodaltons
LB	lysogeny broth
MIC	minimum inhibitory concentration
mM	millimolar
min	minutes
OD_{600nm}	optical density at 600nm light
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
psi	pounds per square inch
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
rha	rhamnose
RNA	ribonucleic acid

RND	resistance-nodulation-division
RR	response regulator
SDS	sodium dodecyl sulfate
sec	seconds
TCS	two-component system
Tn-seq	transposon sequencing
wt	wild type

Chapter 1 – Introduction

1.1 The Genus *Burkholderia*

The genus *Burkholderia* is comprised of rod-shaped, motile Gram-negative β-Proteobacteria found ubiquitously in the soil environment (Mahenthiralingam et al. 2005). In 1950 Walter Burkholder described a plant pathogen causing sour skin rot in onions and named the organism *Pseudomonas cepacia* (Burkholder 1950). The genus *Burkholderia* was established in 1992, when seven species of *Pseudomonas* (including *Pseudomonas cepacia*) was transferred to a new genus on the basis of 16S rRNA sequences, DNA–DNA homology values, cellular lipid and fatty acid composition analysis, and phenotypic characteristics (Yabuuchi et al. 1992).

Bacteria of the genus *Burkholderia* can be found in the soil environment, closely associated with plants as some species have beneficial effects on plants such as promoting root system growth, plant growth, phytopathogen protection (producing antimicrobials and antifungals), nitrogen fixation, and degradation of chemical contaminants in the water and soil environment (Mahenthiralingam et al. 2005). Additionally, strains have been shown to provide protection to abiotic stresses such as low temperature tolerance, drought tolerance, and salt tolerance (Theocharis et al. 2012, Naveed et al. 2014, Pinedo et al. 2015, Su et al. 2015). Some species have been shown to promote growth in maize and protect against phytopathogens, improve rice crop yields, suppress cucumber and soybean diseases caused by fungal pathogens in the soil environment as well as antagonizing parasitic plant nematodes (Van et al. 2000, Li et al. 2002, Bevivino et al. 2005).

Additionally, *Burkholderia* spp. are able to tolerate and degrade many toxic compounds, such as phenol, polychlorinated biphenyl compounds, as well as some of the most common organic pollutants in groundwater including toluene and trichloroethylene among others (Mahenthiralingam et al. 2005, Chiarini et al. 2006, Hamid et al. 2014, Chen et al. 2017).

With the above list of advantageous properties, *Burkholderia* spp. have been proposed to be used in agriculture and bioremediation efforts, to promote plant growth and reduce levels of toxic compounds in the environment (Coenye and Vandamme 2003, Chiarini et al. 2006, Eberl and Vandamme 2016). However, species of *Burkholderia* can occupy different niches, including that of a pathogen (Mahenthiralingam et al. 2005). Different species of *Burkholderia* can have symbiotic and pathogenic interactions with plants, some being symbiotic with plants while others are pathogens of the same plant (Coenye and Vandamme 2003). Additionally, some species of *Burkholderia* are animal and human pathogens, causing illness such as melioidosis, food poisoning, lung infections, septic shock, etc. (Dance 2000, Mahenthiralingam et al. 2005). Pathogenic species include *Burkholderia pseudomallei* and *Burkholderia mallei* which are primary human pathogens capable of causing melioidosis and glanders, respectively, as well as the closely related *Burkholderia* species known was the *Burkholderia cepacia* complex (Bcc), a group of emerging pathogens capable of causing severe infections in cystic fibrosis (CF) and immunocompromised patients (Mahenthiralingam et al. 2005, Eberl and Vandamme 2016).

1.2 The *Burkholderia cepacia* complex (Bcc)

The *Burkholderia cepacia* complex is a group of at least 20 closely related species within the genus *Burkholderia* found ubiquitously in the soil environment (Mahenthiralingam et al. 2005, Eberl and Vandamme 2016). The Bcc are extremely versatile owing to their large genomes (~7 Mbp to 9.7 Mbp) encoding over 7000 genes, some of the largest observed in Gram negatives (Mahenthiralingam et al. 2005, Ussery et al. 2009, Sousa et al. 2017). These large genomes provide Bcc members with an extraordinary degree of flexibility in ecological niches within which to occupy, with extensive metabolic pathways to allow adaptation to different environments ranging from soil, to plant rhizospheres, to the CF lung (Coenye and Vandamme 2003, Mahenthiralingam et al. 2005, Eberl and Vandamme 2016).

CF is a genetic condition wherein there is a defect in the CF transmembrane conductance regulator (CFTR), which leads to the characteristic symptoms of chronically inflamed airways and altered mucous clearance leading to mucous build-up (Luciani et al. 2011). Because of this, the CF lung is an environment where pathogens can establish chronic infections due to the accumulation of mucous (Folescu et al. 2015). Chronic pulmonary infections are the leading cause of death in CF patients, with some main causative organisms being members of the Bcc, *Pseudomonas aeruginosa*, *Staphylococcus aureus, Haemophilus influenza*, and *Stenotrophomonas maltophilia* (Ratjen and Döring 2003). The most common organism isolated from infections in CF patients is *P. aeruginosa*, and Bcc infections only make up a small percentage of total isolates. Infection with Bcc, however, is feared because of the difficulty in treatment as well as the clinical outcomes which range from asymptomatic infection to cepacia syndrome, which involves a rapid decline of lung function characterized by

necrotizing pneumonia and septicemia (Sousa et al. 2011). In an example from a clinic in Manchester, the five-year survival rate for *P. aeruginosa* infections in CF patients was 85.3%, whereas the five-year survival rate for infection with *B. cenocepacia* was significantly lower at 66.6% (Jones et al. 2004). Bcc infection also is a major setback in the selection of the patient for a lung transplant, as patients colonized with Bcc are at high risk of post-transplant sepsis and death, eliminating one of the possible treatments of the CF disease itself (Chiarini et al. 2006).

The first reports of significant Bcc infection in CF patients appeared in 1984 and 1985, in which the virulent nature of infection, a rapid and uncontrollable decline in health, and fatality in ~10% of patients was described (Isles et al. 1984, Mahenthiralingam et al. 2005). In the United States, the majority of Bcc infections in CF patients are caused by *B. cenocepacia* and *B. multivorans*, at 45.6% and 38.7% respectively (Reik et al. 2005), with the percentages varying by region. In recent years, *B. multivorans* has taken over *B. cenocepacia* as the predominant Bcc species infecting CF patients in North America, as well as in the United Kingdom (Pope et al. 2010, Zlosnik et al. 2014). The particular reason for the dominance of *B. cenocepacia* and *B. multivorans* is unknown, with incidences of the two species accounting for 85-97% of all Bcc infections in CF patients. (Drevinek and Mahenthiralingam 2010). Infection control methods are implemented to decrease the incidences of newly acquired Bcc infections. However, chronic Bcc infections are also hard to deal with, as members of the Bcc are able to survive most conventional antimicrobial therapies because of their remarkable survival/persistence capabilities (Mahenthiralingam et al. 2005). Genomic flexibility provides members of the Bcc various intrinsic methods of resistance to antimicrobials and disinfectants, allowing them to

survive many common cleaning regimens as well as in an infection setting within a host (Eberl and Vandamme 2016).

1.3 Intrinsic Resistances of the Bcc

Members of the Bcc have been shown to be quite persistent in diverse environments including their natural soil environment, as well as in hospital/clinical settings, in addition to occasionally contaminating commonly used commercial and pharmaceutical products (Marigliano et al. 2010, Martin et al. 2011, Torbeck et al. 2011). Among these commonly used items in hospital settings are gels used for echocardiographic and electrocardiographic tests (composed of water, EDTA, sodium hydroxide, and preservatives which have a broad spectrum of action for the control of bacteria, yeast and moulds), as well as distilled water, tap water, and dialysis machines, highlighting the ability of these bacteria to persist in substances seemingly devoid of nutrients required for growth of microorganisms (Marigliano et al. 2010, Torbeck et al. 2011). In addition, Bcc members have been found to survive and persist in materials used for cleaning and disinfecting hospital surfaces as well as in direct patient care. Examples of these products include povidone-iodine solutions (a commonly used and commercially available general-use antiseptic), chlorhexidine solutions (used as an antiseptic as well as in cosmetics such as creams, deodorants, toothpaste, eye drops, and antiseptic mouthwash), moisturizers, intravenous flush solutions, and many other substances used in the hospital setting as well as in general household use (Held et al. 2006, Holden et al. 2009, Loutet and Valvano 2010, Marigliano et al. 2010, Martin et al. 2011, Torbeck et al. 2011). In 2008, there was an outbreak of *Burkholderia contaminans* (a member of the Bcc) in a German

university hospital, in which the source of the outbreak was reported to be prefabricated moist washcloths (Martin et al. 2011). These washcloths were introduced in the early 2000's as a part of the hygiene system for immobile patients, and patients with special needs such as those in the intensive care unit (ICU), to make daily care faster and easier (Martin et al. 2011). In addition to the ability to survive in distilled water and other water-based pharmaceuticals, members of the Bcc display high levels of intrinsic resistance to several of the antibiotics used in clinics. This can be attributed to the extensive genomes of the Bcc providing a large array of genes with a variety of functions to afford them different mechanisms of resistance to antimicrobial molecules (Mahenthiralingam et al. 2005, Holden et al. 2009, Bazzini et al. 2011).

Resistance of the Bcc, as well as most other bacteria, can be grouped into four main mechanisms, which include membrane impermeability, target modification, antimicrobial modification, and efflux activity. The primary method of resistance is the impermeability of the Gram-negative outer cell membrane (Burns et al. 1996), which blocks entry of antimicrobial drugs, as well as causing the inability of some drugs to bind (Burns et al. 1996; Mahenthiralingam et al. 2005). The modification of LPS composition is implicated in the intrinsic resistance of Bcc members to aminoglycosides (inability of aminoglycosides to bind to the LPS), as well as modifications to the lipid A and core components of LPS providing resistance to cationic antimicrobial peptides and polymyxin. Bcc strains have penicillin-binding proteins that are less susceptible to the binding and action of β-lactam drugs, in addition to readily inducible β-lactamases, which are enzymes that degrade β-lactam antimicrobials (Mahenthiralingam et al. 2005; Sherrard et al. 2014). Another example of target modification is

the enzyme dihydrofolate reductase (DHFR), which is responsible for resistance to trimethoprim (Mahenthiralingam et al. 2005).

The greatest range of resistance is brought about because of efflux systems, which are present in multiple types and copies in *Burkholderia spp.* (Burns et al. 1996, Coenye and Vandamme 2003, Mahenthiralingam et al. 2005, Holden et al. 2009, Loutet and Valvano 2010, Sherrard et al. 2014). With some *B. cenocepacia* strains having pumps from six different families including: major facilitator superfamily (MFS), ATP binding cassette (ABC) family, resistance nodulation division (RND) family, multidrug and toxic compound extrusion (MATE) family, small multidrug resistance (SMR) family, and fusaric acid resistance family proteins (Holden et al. 2009). Efflux pumps generally work by transporting antimicrobial compounds that manage to get into the cell outside, through consumption of energy generated by proton motive force, with the exception of the ABC family which uses ATP hydrolysis as its energy source (Mahenthiralingam et al. 2005, Guglierame et al. 2006, Holden et al. 2009, Sherrard et al. 2014, Podnecky et al. 2015). Efflux pumps can confer resistance to specific antimicrobials such as metals, or a range of molecules such as one pump that confers resistance to chloramphenicol, trimethoprim, and ciprofloxacin (Burns et al. 1996, Holden et al. 2009), and another that is associated with resistance to fluoroquinolones, streptomycin, and ethidium bromide (Holden et al. 2009). Efflux pumps play a large part in the resistance of Bcc members to antimicrobials used in the treatment of human infections, as they can have a broad range of substrate specificity. The RND family of efflux pumps is possibly the main contributor to resistance, as genomic analysis has identified at least 16 open reading frames (ORFs) encoding putative RND family efflux pumps alone (Guglierame et al. 2006, Holden et al. 2009).

The examples of resistance mechanisms mentioned above highlight the remarkable ability of the Bcc to survive in various environments/niches including plants, the rhizosphere, soil, animals and humans, as well as seemingly nutrient-free environments such as distilled water or pharmaceutical cleaning products. This incredible range of adaptability highlights the mechanisms of regulatory systems in Bcc species, allowing them to adapt to and survive in different environments and to various stresses (Loutet and Valvano 2010, Sousa et al. 2017). Bacteria use many different methods to regulate gene expression, whether it be down-regulation or up-regulation, including quorum sensing systems, toxin-antitoxin systems, sRNAs, and transcriptional regulators (Sousa et al. 2017). However, the dominant type of regulation relies on the use of two-component system (TCS) signal transduction pathways (Stock et al. 2000)

1.4 Two-Component System Signal Transduction

TCS are used to sense and respond to various environmental stimuli and conditions. TCS consist of a histidine kinase (HK) and a response regulator (RR), which are usually situated together in the genome (Stock et al. 2000). TCS are activated by many different environmental stimuli including changes in pH, nutrient level, redox state, osmotic pressure, quorum signaling, and the presence of antimicrobial molecules, although the specific molecular basis of signal recognition is unknown for most TCS (Worthington et al. 2013). The ability of TCS to be activated by so many different signals allows for the regulation of multiple genes, leading to changes in motility, cell growth, virulence factor expression, biofilm formation and maintenance, quorum sensing, and antimicrobial resistance (Gotoh et al. 2010).

The HKs and RRs both have conserved domains that allow phosphorylation and transfer of the phosphate group to facilitate a response to extracellular/environmental stimuli (Stock et al. 2000). The conserved nature of these domains and the advent of next-generation sequencing makes it possible to analyze the entire genome of a bacterial organism, and then predict and identify the complete selection of TCS present in the genome (Borland et al. 2015). HK's have a diverse sensing domain used to perceive a variety of environmental signals/stimuli and have a highly conserved histidine core with a unique fold, with larger HK's having up to six structurally and functionally unique domains (Stock et al. 2000). The HK is capable of ATP-dependent autophosphorylation in response to an environmental stimulus/signal, and the RR then interacts with the phosphorylated HK (Stock et al. 2000). Unlike typical protein kinase signal cascades, a phosphotransfer event happens where the phosphate group is transferred from the HK stoichiometrically to a conserved amino acid residue on the RR (Stock et al. 2000, Gotoh et al. 2010). In this phosphorylated state, the RR can interact with regulatory regions of genes and control expression (Worthington et al. 2013), Figure 1 shows an illustration of the phosphotransfer pathway between a HK and its cognate RR. TCS can work as a two-member chain or involve a signaling pathway involving other proteins (Skerker and Laub 2004). TCS are found predominantly in prokaryotic organisms, with a small number being identified in eukaryotic microorganisms such as fungi and slime molds, as well as plants (Mavrianos et al. 2013, Worthington et al. 2013); TCS have yet to be identified in animals including humans (Mavrianos et al. 2013). There are a limited number of TCS found in eukaryotes, with some organisms such as *Saccharomyces cerevisiae* containing one TCS within its genome that controls osmoregulation, and *Candida albicans* which has two TCS regulating osmoregulation and hyphal

development (Stock et al. 2000). In contrast to eukaryotic organisms, prokaryotes encode many more TCSs within their genomes, with numbers ranging from 11 (in *Helicobacter pylori*) to 80 (in *Synechocystis* sp.) (Stock et al. 2000)

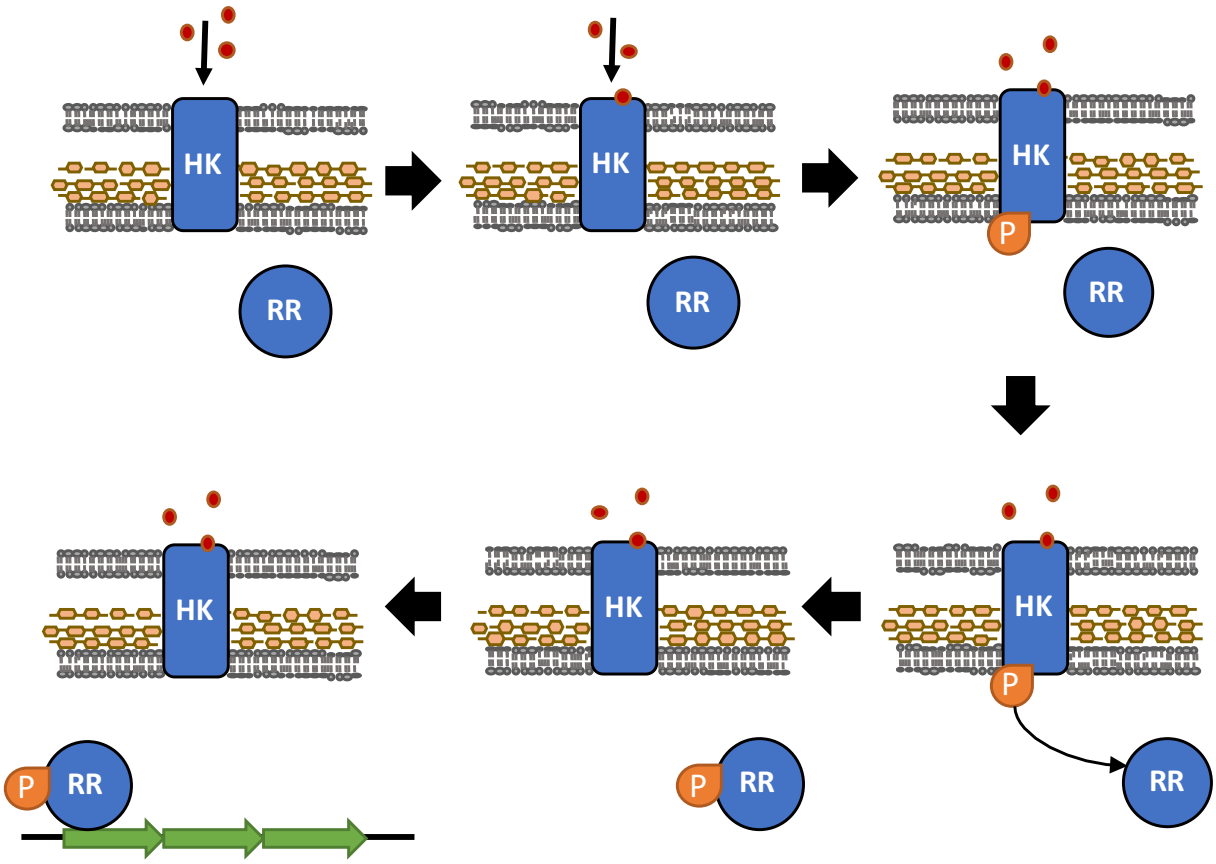


Figure 1. Representation of the typical TCS phosphorylation/signaling pathway between a HK and its cognate RR. Upon sensing of a signal (red dots), the histidine kinase (HK) is able to undergo autophosphorylation (orange "P"). Following this, the HK is able to stoichiometrically transfer the phosphate group to its cognate response regulator (RR), which can then elicit an appropriate response to the signal received (Stock et al. 2000).

1.5 TCS Regulation of Virulence

TCS have been shown to contribute to bacterial virulence, regulating genes required for survival in different environments (such as the host environment) to biofilm formation and maintenance, an important behavioral modification that increases survival and persistence in harsh environments. An example of a TCS having a broad range of activity, including virulence, is the PhoQ/PhoP TCS pair in *Salmonella* spp. As the major regulator of virulence, PhoP is estimated to regulate up to 3% of the *Salmonella* genome, including genes responsible for survival in macrophages and resistance to antimicrobial peptides (Gotoh et al. 2010). PhoQP is also present in some Gram-negative pathogens such as *P. aeruginosa, Escherichia coli, Salmonella enterica, Klebsiella pneumoniae* and *Yersinia pestis*, and it also regulates genes responsible for resistance to cationic antimicrobials such as polymyxins and antimicrobial peptides in addition to biofilm formation (Gooderham and Hancock 2009, Worthington et al. 2013). In *P. aeruginosa* the GacS/GacA HK/RR pair regulates genes related to production of acyl-homoserine lactones (AHLs) involved in quorum sensing, pyocyanin, lipase, elastase, biofilm formation and maintenance, and motility; all of which are non-essential for survival, but are implicated in virulence of the pathogen (Gotoh et al. 2010). The three TCS pairs BfiRS, BfmRS and MifRS from *P. aeruginosa* regulate biofilm formation and maturation at different stages, and if inhibited, stop biofilm maturation at the step for which they are responsible (Worthington et al. 2013). TCS regulate extracellular membrane-derived vesicle (EMV) production in another human pathogen*, Streptococcus pyogenes* or Group A streptococcus (Resch et al. 2016). These EMVs have been implicated in playing a part in bacterial pathogenesis including delivery of toxins, triggering host immune responses, as well as providing protection

from phages and antimicrobial peptides (Resch et al. 2016). Another *P. aeruginosa* TCS important in the regulation of virulence is AlgZ/AlgR HK/RR pair, which is essential for twitching motility and alginate production (Pritchett et al. 2015). Alginate is an exopolysaccharide that leads to the formation of mucoid colony phenotype, which is associated with increased resistance to phagocytosis and antimicrobial treatment (Pritchett et al. 2015).

1.6 TCS Regulation of Antimicrobial Resistance

TCS can also regulate genes that affect antimicrobial resistance, turning on genes encoding β-lactamases, those involved in membrane modifications, and most notably efflux pumps (Stock et al. 2000, Hirakawa et al. 2003, Marchand et al. 2004, Worthington et al. 2013). *Acinetobacter baumannii* is an important nosocomial pathogen able to colonize and infect patients in hospitals, causing illnesses such as pneumonia, urinary tract infections, septicemia, and meningitis (Marchand et al. 2004). The TCS AdeRS HK/RR pair regulates the tripartite resistance-nodulation-division (RND) efflux pump AdeABC, which has been shown to be responsible for resistance to aminoglycosides, β-lactams, chloramphenicol, erythromycin, tetracyclines, and ethidium bromide (Marchand et al. 2004). AdeABC has also been implicated in resistance to tigecycline, a drug of last resort in the treatment of *A. baumannii* infections (Worthington et al. 2013). Like *A. baumannii*, many other pathogens possess TCS to regulate genes related to antimicrobial resistance such as inducing or upregulating efflux pumps, inducing enzymes to inactivate or modify the antimicrobials such as β-lactams or acetylases, downregulation of porins or modification of the cell envelope structure to reduce permeability, among many of their widespread functions (Gotoh et al. 2010, Worthington et al. 2013, Harris

et al. 2014, Brown and Wright 2016, Wu et al. 2016). This makes the study of TCS, and potentially targeting TCS, a valuable effort in the continued race for the discovery of new treatment methods to overcome antibiotic resistance. Antibiotic resistance is one of the most pressing issues facing human health, as we are encountering more and more human pathogens that are resistant to most conventional treatments, with some resistant to all clinically available drugs (Silver 2011, Brown and Wright 2016, Chen 2017).

1.7 TCS Regulation of Cell Envelope Processes

The bacterial cell envelope is the barrier between the contents of the cell and the harsh exterior environment. Generally, in Gram-positive bacteria the primary structure of the cell envelope includes a cell membrane, a thick peptidoglycan layer, as well as teichoic and lipoteichoic acids; whereas the Gram-negative cell envelope generally consists of a cell membrane, a thin peptidoglycan layer, and an outer membrane containing lipopolysaccharides (Silhavy et al. 2010). There are many TCS involved in the regulation of cell envelope functions such as biogenesis, maintenance, and homeostasis (Raivio and Silhavy 1997, Skerker et al. 2005, Kolar et al. 2011, Svensson et al. 2015, Guest and Raivio 2016). One of the most studied of these TCS is WalKR, which is conserved among low GC content Gram-positive bacteria (such as *Bacillus subtilis, Staphylococcus aureus, Enterococcus faecalis, Listeria monocytogenes, Streptococcus pneumoniae, and Streptococcus mutans*)(Dubrac et al. 2007). WalKR, also known as YycG/YycF, VicK/VicR, or MicA/MicB, plays an important role in cell membrane synthesis and fluidity, cell division, exopolysaccharide synthesis, and oxidative stress (Dubrac et al. 2008). WalKR is involved in the regulation of autolysins and the peptidoglycan

metabolism/remodelling activities of the cell (Yamaguchi et al. 2004, Bisicchia et al. 2007, Shah and Dworkin 2010). Additionally, this TCS has been shown to be possibly linked to vancomycin resistance, as WalKR is drastically upregulated in some strains with intermediate resistance to vancomycin (Jansen et al. 2007). Table 1 lists additional TCS that regulate various aspects of the cell envelope, including modification of lipid components, permeability, peptidoglycan synthesis and remodelling, in addition to activities relating to cell envelope stress response which can results in increased resistance to antimicrobials (Bisicchia et al. 2007, Dubrac et al. 2007, Plocinska et al. 2012, Henst et al. 2012, Bretl et al. 2014, Svensson et al. 2015, Guest and Raivio 2016, Dorr et al. 2016).

Table 1. Two-Component Systems Involved in Regulation of Cell Envelope Functions/Processes. Adapted from Cardona and Choy, 2017, Journal of Membrane Biology; submitted.

Organism	**TCS**	**Essential?**	**Regulation of cell envelope functions**	**Regulation of other functions**	**Reference**
Bacillus subtilis	YycFG	Yes (Fabret and Hoch 1998b)	Cell wall metabolism (Bisicchia et al. 2007)	Modulation of FtsAZ operon (Fukuchi et al. 2000)	(Fabret and Hoch 1998b, Fukuchi et al. 2000, Bisicchia et al. 2007)
Brucella abortus	PdhS	Yes	Cell cycle/division progression	Not described	(Hallez et al. 2007, Henst et al. 2012)
Burkholderia cenocepacia	EsaSR	Yes (Gislason et al. 2017)	Cell envelope integrity (Gislason et al. 2017)	Drug Efflux (Gislason et al. 2017)	(Gislason et al. 2017)
Campylobacter jejuni	CprRS	Yes (Svensson et al. 2009)	Cell envelope biogenesis and maintenance (Svensson et al. 2009)	Not described	(Svensson et al. 2009)
Caulobacter crescentus	CckA-CtrA	Yes (Quon et al. 1996b, Jacobs et al. 1999)	Cell wall metabolism (Laub et al. 2002)	Cell cycle (Quon et al. 1996b)	(Quon et al. 1996b, Jacobs et al. 1999, Laub et al. 2002)
Escherichia coli	CpxAR	no	Cell envelope stress (Pogliano et al. 1997, Raivio and Silhavy 1997)	Not described	((Pogliano et al. 1997, Raivio and Silhavy 1997)

Organism	**TCS**	**Essential?**	**Regulation of cell envelope functions**	**Regulation of other functions**	**Reference**
Lactococcus lactis	CesSR	no	Cell envelope stress (Martínez et al. 2007)	Not described	(Martínez et al. 2007)
Listeria monocitogenes	CesRK	no	Cell wall stress in response to antibiotics (Gottschalk et al. 2008)	Not described	(Gottschalk et al. 2008)
Listeria monocytogenes	LiaFSR(Lm)	no	Cell envelope biogenesis and remodeling (Fritsch et al. 2011)	Response to cell wall active antibiotics (Fritsch et al. 2011)	(Fritsch et al. 2011)
Mycobacterium tuberculosis	MprB	no	Cell envelope stress (Bretl et al. 2014)	Not described	(Bretl et al. 2014)
Mycobacterium tuberculosis	MtrAB	Yes (Zahrt and Deretic 2000)	Control of peptidoglycan hydrolase RipA (Plocinska et al. 2012)	Chromosome replication (Purushotham et al. 2015)	(Zahrt and Deretic 2000, Plocinska et al. 2012, Purushotham et al. 2015)

Organism	**TCS**	**Essential?**	**Regulation of cell envelope functions**	**Regulation of other functions**	**Reference**
Salmonella Typhimrium	PmrAB	no	Incorporation of aminoarabinose to lipid A and regulation of O-antigen length (Farizano et al. 2012)	Not described	(Farizano et al. 2012)
Staphylococcus aureus	AirSR (YhcS)	Yes	Cell wall synthesis (Sun et al. 2013)	Nitrate respiration (Yan et al. 2011)	(Yan et al. 2011, Sun et al. 2013)
	VraSR	No (Kuroda et al. 2003)	Peptidoglycan synthesis (Chen et al. 2016)	Adaptation to cell wall stress (Muthaiyan et al. 2008)	(Kuroda et al. 2003, Muthaiyan et al. 2008, Chen et al. 2016)
Staphylococcus aureus	WalKR (YycFG)	Yes (Dubrac and Msadek 2004b)	Cell permeability, cell wall metabolism (Dubrac et al. 2007, Martin et al. 1999)	Cell division, through cross talk with PknB (Hardt et al. 2017) Virulence (Delaune et al. 2011)	(Martin et al. 1999, Dubrac and Msadek 2004b, Dubrac et al. 2007, Delaune et al. 2011, Hardt et al. 2017)

Organism	TCS	Essential?	Regulation of cell envelope functions	Regulation of other functions	Reference
Streptococcus mutans	VicRKX (YycFG)	Yes (Ng et al. 2003)	Cell envelope biosynthesis (Ng et al. 2004)	Interaction with cell division proteins through cell wall hydrolase PcsB (Sham et al. 2011) Biofilm formation (Duque et al. 2011) Virulence (Alves et al. 2017)	(Ng et al. 2003, 2004, Duque et al. 2011, Sham et al. 2011, Alves et al. 2017)
***Streptomyces coelicolor* and others**	VanSR	No (Hutchings et al. 2006)	Not described	Response to vancomycin, inducing resistance by remodeling cell wall precursors (Hong et al. 2008)	(Hutchings et al. 2006, Hong et al. 2008)
Vibrio cholerae	WigKR	No (Cheng et al. 2015)	Monitors cell wall synthesis and increases cell wall production upon damage (Dorr et al. 2016)	Regulation of type VI secretion system (Cheng et al. 2015)	(Cheng et al. 2015, Dorr et al. 2016)

Organism	**TCS**	**Essential?**	**Regulation of cell envelope functions**	**Regulation of other functions**	**Reference**
Vibrio parahaemolyticus	VbrKR	No (Li et al. 2016)	Not described	Response to β-lactam antibiotic, inducing β -lactamase production (Li et al. 2016)	(Li et al. 2016)

1.8 Antimicrobials and Essential Genomes

Almost all antimicrobials work by targeting essential cellular processes in an effort to halt the growth of, or kill the bacterial pathogen (Silver 2011). In this era of data and computers, the push in research has been towards high-throughput screens with the aid of computer analysis, such as docking simulations of vast libraries of molecules and protein structures or screens of whole bacterial genomes (Worthington et al. 2013, Brown and Wright 2016). Many putatively essential TCS have been identified using these genomic screens (Table 2), underscoring their capability to identify novel antimicrobial targets. It is interesting to consider the essentiality of TCS, as it is most likely not the encoding genes themselves, but the signaling pathways and processes they regulate that make them essential. Figure 2 illustrates how signaling pathways regulated by TCS can be essential, explaining why a mutation of genes encoding the TCS can lead to cell death.

Table 2. Essential Two-Component Systems Identified Using Genomic Screens of Transposon Mutants. Adapted from Cardona and Choy, 2017, Journal of Membrane Biology; submitted.

Organism(s)	Gene Name	Notes	Method	Number of Genes	Number of TCS genes	Reference
***Acinetobacter baumannii* ATCC 17978**	A1S_3229	OmpR homolog. Response regulator	INseq	3803	17 HK 17 RR 3 hybrid HK/RR	(Wang et al. 2014, Weber et al. 2015)
	A1S_3230	Sensory histidine kinase				
***Burkholderia pseudomallei* K96243**	BPSL0128	Putative response regulator	TraDIS	5729	33 HK 49 RR 6 hybrid HK/RR	(Holden et al. 2004, Moule et al. 2014)
	BPSL2065	Putative response regulator				
	BPSS0813	Putative sensor histidine kinase				
	BPSS1358	Putative sensor histidine kinase				
	BPSS1646	Probable two-component sensor kinase				

Organism(s)	Gene Name	Notes	Method	Number of Genes	Number of TCS genes	Reference
***Burkholderia thailandensis* E264**	BTH_I0135	Response regulator	TN-seq	5712	40 HK 60 RR 6 hybrid HK/RR	(Baugh et al. 2013)
***Campylobacter jejuni* 81-176**	CJJ81176_0379	Response regulator.	TN-seq	1754	6 HK 9 RR	(Gao et al. 2014)
	CJJ81176_1241	DNA-binding response regulator				
	CJJ81176_1277	*racR* response regulator				
	CJJ81176_1595	Putative DNA-binding response regulator				
Caulobacter crescentus	*tacA*	Response regulator	TN-seq	3767	62 HK 44 RR	(Skerker et al. 2005, Christen et al. 2011)
	divK	Response regulator. Cell division				

ctrA	Response regulator. Cell division, stalk synthesis, cell cycle
cenR	Response regulator. Cell envelope regulator
petR	Response regulator
cenK	Sensory signal transduction protein kinase
cckA	Histidine kinase/receiver protein
divL	Sensor histidine kinase
shkA	Hybrid HK/RR. High fitness cost

Organism(s)	Gene Name	Notes	Method	Number of Genes	Number of TCS genes	Reference
***Helicobacter pylori* G27**	*arsR*	Response regulator	MATT (Microarray tracking of transposon mutants)	1515	4 HK 6 RR	(Salama et al. 2004, Baltrus et al. 2009)
***Herbaspirillum seropedicae* SMR1**	HSERO_RS19780	Response regulator	Tn-Seq	4804	41 HK 53 RR	(Pedrosa et al. 2011, Rosconi et al. 2016)
	HSERO_RS00340	Response regulator				
***Mycobacterium tuberculosis* H37Rv strain**	*mtrA*	Response Regulator.	TN-seq	3924	14 HK 13 RR	(Cole et al. 1998, Zhang et al. 2012)
Neisseria gonorrhoeae strain MS11	*ntrX*	Response regulator	Tn-Seq	2185	3 HK 4 RR	(Remmele et al. 2014)
Porphyromonas gingivalis ATCC 33277	PGN_1019	Response regulator	Tn-Seq	1909	2 HK 4 RR	(Klein et al. 2012)
Rhizobium leguminosarum	RL0030	Putative histidine kinase	INSeq	7263	51 HK 63 RR 6 Hybrids	(Young et al. 2006, Perry et al. 2016)
	RL2281	NtrY				
	RL2282	NtrX				
	RL3758	Putative two-component sensor/regulator				

Organism(s)	Gene Name	Notes	Method	Number of Genes	Number of TCS genes	Reference
Rhodopseudomonas palustris	RPA0283	Two-component transcriptional regulator	Tn-seq	4836	63 HK 79 RR	(Larimer et al. 2004, Pechter et al. 2016)
	RPA0357	Sensor signal transduction histidine kinase				
	RPA0358	Two-component transcriptional regulator ChvI				
	RPA1632	Two-component cell cycle transcriptional regulator CtrA				
	RPA2862	Exopolysaccharide regulatory protein exoR. Regulation of TCS				
	RPA3882	Histidine kinase				
	RPA4226	Histidine kinase				
	RPA4368	Two-component transcriptional regulator				

Organism(s)	Gene Name	Notes	Method	Number of Genes	Number of TCS genes	Reference
***Salmonella enterica* serotype Typhimurium**	*ompR*	Response regulator	Tn-Seq	5372	17 HK 17 RR	(Khatiwara et al. 2012)
***Snodgrassella alvi* strain wkB2**	SALWKB2_1717	Two component system histidine kinase	Tn-seq	2226	7 HK 6 RR	(Powell et al. 2016)
	SALWKB2_1718	Copper-sensing two-component system response regulator CpxR				
***Staphylococcus aureus* ST398**	*yycG (walK)*	Histidine Kinase	TN-seq	2779	15 HK 16 RR	(Golding et al. 2012, Christiansen et al. 2014)
Streptococcus agalactiae A909	SAK_0392	Response regulator	Tn-seq	2034	19 HK 21 RR	(Tettelin et al. 2005, Hooven et al. 2016)
	SAK_0845	Response regulator				
***Streptococcus pyogenes* SF370**	*vicR*	Response regulator.	TN-seq	1752	13 HK 11 RR	(Ferretti et al. 2001, Breton et al. 2015)

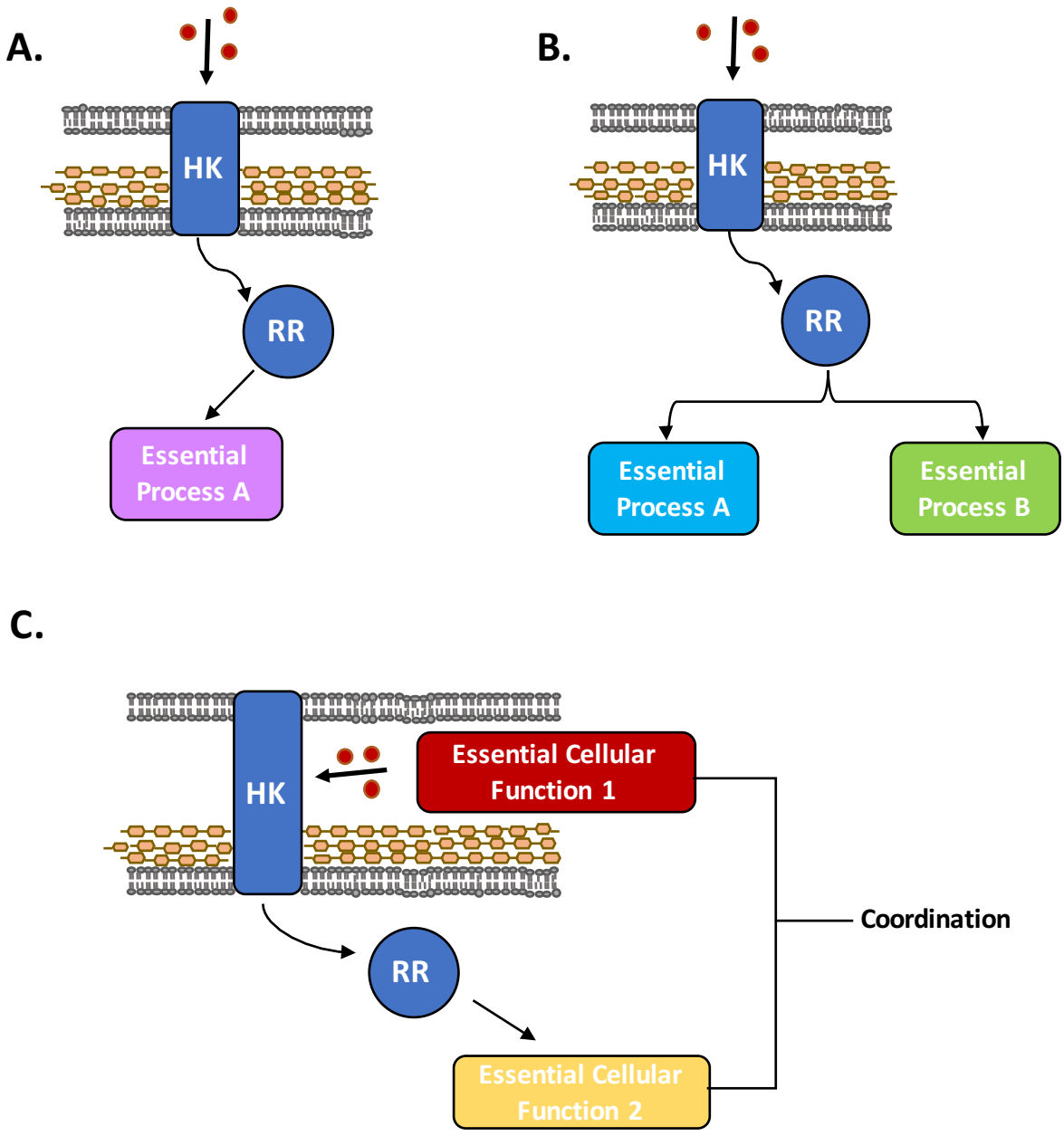


Figure 2. Proposed pathways for TCS regulation of essential gene processes. A) A HK interacts with its cognate RR to regulate an essential gene function/process. B) A TCS could be responsible for the timing of two essential processes that are both regulated by the RR. C) A TCS could be responsible for regulating and coordination of two essential functions/processes that creates a feedback system. This could be when the products/effects of one essential function is the signal for activation of the TCS to regulate another essential function/process. Adapted from Cardona and Choy, 2017, Journal of Membrane Biology; submitted.

Screening for essential genes together with drug-target matches can be achieved through the use of a transposon mutant library (Bloodworth et al. 2013, Cardona et al. 2015). Such libraries allow for the determination of the essential genome, and are a useful tool in high-throughput screens to determine a drug-target match, and elucidating the mechanism of action (MOA) of novel compounds. If a compound with antimicrobial effects exerts its action by binding an essential protein to inhibit bacterial growth, then underexpression of the target protein should lead to hypersusceptibility to the antimicrobial (DeVito et al. 2002). We refer to this specific hypersusceptibility to a growth inhibitor as enhanced susceptibility (Bloodworth et al. 2013).

1.9 Identification and Characterization of a TCS Involved in Growth and Antimicrobial Resistance

Gislason et al. demonstrated that it is possible to match an antibiotic to its target using an assay based on Illumina next-generation sequencing, with novobiocin and GyrB as a known drug-target control (Gislason et al. 2017). The assay uses a conditional growth mutant library and the concept of enhanced susceptibility to identify antimicrobial-target matches. As previously mentioned, a mutant underexpressing the gene target of a compound should be hypersusceptible to its inhibitory action, and thus be rendered less fit compared to other strains that are not underexpressing the gene target.

To demonstrate this, Gislason et al. showed that at subinhibitory concentrations of novobiocin, a CG mutant of *gyrB* exhibited more than a 10-fold reduction compared to the wt (Gislason et al. 2017). The *gyrB* CG mutant did not exhibit enhanced susceptibility to other antimicrobials tested, and with one exception, other CG mutants tested were not hypersensitive to novobiocin (Gislason et al. 2017). This CG mutant exhibited enhanced susceptibility to various antimicrobials tested such as chloramphenicol, tetracycline, kanamycin, and carbonyl cyanide m-chlorophenylhydrazone, in addition to novobiocin (Gislason et al. 2017). However, this CG mutant did not show enhanced susceptibility to hydrogen peroxide, colistin, or dimethyl sulfoxide (Gislason et al. 2017). This CG mutant was called 73-14C5 and had a rhamnose-inducible promoter introduced in a previously uncharacterized TCS. This rhamnose-inducible promoter system allows for regulation of gene expression when introduced into the genome of *B. cenocepacia*, allowing the study of the effect of underexpression of genes (Cardona and Valvano 2005). The transposon insertion was 58bp upstream of the 3' end of BURCENK56V_RS04770, a gene annotated as a HK. Immediately downstream from this HK lies BURCENK56V_RS04765, an XRE family transcriptional regulator, suggested to form a TCS HK/RR pair (Gislason et al. 2017). Due to the insertion site of the transposon, it has been suggested that the RR is regulated by the rhamnose inducible promoter and it is the underexpression of the RR that is the underlying cause of the growth defect and antibiotic susceptibility. The results of this screen formed the basis of the current project, focused on characterizing the effects of downregulation of the TCS, named EsaSR for **e**nhanced **s**ensitivity to **a**ntimicrobials **s**ensor and response regulator.

My hypothesis is that the EsaSR TCS is essential for growth in laboratory conditions and is involved in antimicrobial resistance by regulating efflux and cell envelope integrity of *Burkholderia* cenocepacia K56-2. The objectives of this study are to confirm the essentiality of *esaSR*, in addition to the phenotype arising from downregulation of this TCS.

Chapter 2 – Materials and Methods

2.1 Bacterial Strains and Growth Conditions

Bacterial strains and plasmids used in this project are listed in Table 3. All *B. cenocepacia* strains used were mutants genetically engineered from *B. cenocepacia* K56-2 (also designated LMG 18863). Strains were cultured in Luria-Bertani (LB) media (Difco, Becton, Dickenson and Company) with shaking at 37°C in a New Brunswick Scientific Excella E24 shaking incubator (New Brunswick Scientific, USA). Media was supplemented with 100 μg/ml trimethoprim and 0.2% rhamnose (rha) for *B. cenocepacia* conditional expression mutants when required.

Standardized glycerol stocks of *B. cenocepacia* K56-2 (wild type) or mutants used in experiments were prepared as described by Bloodworth *et al.* 2013. To prepare stocks, overnight cultures of strains grown in LB were washed twice with sterile LB, adjusted to an OD_{600nm} of 0.2 in LB supplemented with 20% glycerol, and aliquoted into polymerase chain reaction (PCR) tubes and stored at -80°C until use. In subsequent experiments, standardized glycerol stocks were diluted to give an OD_{600nm} of 0.001.

Table 3. Strains and Plasmids Used in this Study

Strain or Plasmid	Features	Source
Strains		
Burkholderia cenocepacia K56-2	Cystic fibrosis clinical isolate	(Mahenthiralingam et al. 2000)
Burkholderia cenocepacia MKC2	Site-directed CG mutant; P*rhaB* promoter upstream of esaR	This study
Burkholderia cenocepacia MKC4	Δ*esaS*	This study
Burkholderia cenocepacia 84-37D12	Transposon inserted P*rhaB* in BURCENK562V_RS02270, phosphoribosylaminoimidazole-succinocarboxamide synthase, CG mutant; unrelated CG mutant control	(Bloodworth et al. 2013)
Escherichia coli SY327	*araD; Δ(lac pro); argE(*Am*); recA56;* Rif^r; *nalA; λpir*	(Miller and Mekalanos 1988)
Plasmids		
pRK2013	ori$_{colE1}$; RK2 derivative; Kan^r; mob$^+$; tra$^+$	(Figurski and Helinski 1979)
pSC201	ori$_{R6K}$; *rhaR; rhaS;* P*rhaB;* tmp^r	(Ortega et al. 2007)
pMC2	pSC201 derivative; containing 5' *esaR* fragment	This study
pMC4	pGPI-SceI derivative; containing upstream and downstream regions of *esaS*	This study
pMC5	pGPI-SceI derivative; containing upstream and downstream regions of *esaSR*	This study
pGPI-SceI	ori$_{R6K}$; tmp^r; mob$^+$; I-SceI recognition/cut sequence	(Flannagan et al. 2008)
pDAI-SceI	ori$_{pBBR1}$; tet^r; mob$^+$; P$_{dhfr}$; constitutive expression of I-SceI endonuclease	(Flannagan et al. 2008)

2.2 Construction of Unmarked Deletion *esaS* Mutant, MKC4

An unmarked deletion of HK *esaS* (BURCENK56V_RS04770) in *B. cenocepacia* K56-2 was carried out as described by Flannagan et al. (2008). Primers 666 and 665 were used to amplify a 450bp region immediately upstream of *esaS*; and primers 664 and 663 were used to amplify a 450bp region immediately downstream of *esaS* (Primer sequences in Table 4). The amplicons were digested with BamHI (New England BioLabs Inc., USA) and ligated with T4 DNA ligase (New England BioLabs Inc., USA). The resulting DNA fragment was digested with XbaI and XmaI (New England BioLabs Inc., USA) and ligated into the XbaI- and XmaI-digested pGPI-SceI to create plasmid pMC4. pMC4 was conjugated into *B. cenocepacia* K56-2, and merodiploids were selected on LB agar plates supplemented with 100μg/ml trimethoprim and 50μg/ml gentamicin. To initiate the second recombination event, pDAI-SceI, which constitutively expresses the yeast homing endonuclease I-SceI gene, was introduced by triparental mating into trimethoprim-resistant clones. Tetracycline-resistant clones were screened for loss of trimethoprim resistance, and the recovered trimethoprim-susceptible clones were screened by colony PCR to identify deletion mutants.

Table 4. Primers Used in this Study

PRIMER NAME	BINDING SITE	GENE NAME	SEQUENCE
591	BCAL2821	RND-4 Transporter	CCGCTGGCTCATCATCTATCTGGTC
593	BCAL2821		TCAACAGGTAGTCGGAAATGTTCGC
594	BCAL1675	RND-3 Transporter	GACTATATCGGCGACTTCATGCACG
595	BCAL1675		GGATAACCGTTGTAGCGCGTGAG
596	BCAM2550	RND-10 Transporter	GAAGTACGGCGCGATGAAGGATTC
597	BCAM2550		GTTGATCTGGTAGCTCGTGAACAGC
615	BCAL0471	*esaS*	AATTAACATATGGTGATCGTCTCGACCGTCG
616	BCAL0471		ATATAATCTAGAGATGTAGATGATCCCGCCCG
633	BCAL0472	*esaR*	AATTAACATATGATGGCAACCATCCTGGTG
634	BCAL0472		ATATAATCTAGACATTCCTTGAGCAGCGTGAC
645	pSC201	plasmid	GCCCATTTTCCTGTCAGTAACGAGA
646	BCAL0472	*esaR*	TGCTTGAGCTTGCGATACAGG
663	BCAL0472		AATTATCCCGGGGCTTGAGCTTGCGATACAG
664	BCAL0472		ATATGGATCCCAAAGGCAGCGTAAATGGCA
665	BCAL0470	hypothetical protein	ATATGGATCCGTTCGGTCACCGTGAAG
666	BCAL0470		AGATAATCTAGAGACTTCGAGCTGAATCCGA
750	BCAM0918	*sigE*	TACGCCGAAATCAACGACCA
751	BCAM0918		ACTTCGGCTTCCTCTTCGAC
754	BCAM1946	RND-9 transporter	GTTGCAGGTGTATCTCGGCT
755	BCAM1946		ATCGGTACCATCTCGCCCTT
884	BCAM1931	porin	GACAGGTCGCCGTTGTAGAT
885	BCAM1931		CTGGGTGCTGCTTACACGTA

Note: Restriction enzyme sites in primers are underlined.

2.3 Construction of Conditional Expression Mutant of *esaR*, MKC2

A rhamnose inducible promoter (P_{rhaB}) was introduced using homologous recombination to insert plasmid pMC2 into the genome of *B. cenocepacia* K56-2, immediately upstream of response regulator *esaR* (BURCENK562V_RS04765). Primers 615 and 616 were used to amplify 170bp of the 5' end of *esaR* using PCR with HotStar HiFidelity polymerase (Qiagen, Germany). The PCR fragment was digested with NdeI and XbaI (New England BioLabs Inc., USA), and cloned into the same sites of pSC201 immediately downstream of the rhamnose-inducible promoter. The resulting mutagenesis plasmid, pMC2, was introduced into *B. cenocepacia* K56-2 by triparental mating. Exconjugants were selected on LB agar plates supplemented with 0.2% rhamnose, 100μg/ml trimethoprim, and 50μg/ml gentamicin. Insertional mutants were confirmed by PCR using primers 645 and 652, which anneal with pSC201 and a region downstream of the 170bp 5'-end fragment of *esaR*, respectively.

2.4 Triparental Mating

Mutagenic plasmids were introduced into *B. cenocepacia* K56-2 using a triparental mating conjugation process with the help of helper plasmid pRK2013. Antibiotic markers of each bacterial strain and plasmid used can be found in Table 4. The bacterial strains were grown overnight with their respective antibiotics, with the *E. coli* strains being plated on LB agar in petri plates, and a subculture of *B. cenocepacia* K56-2 overnight culture grown in liquid LB broth. The *E. coli* strains were collected with a plastic inoculation loop and suspended in 5ml sterile LB broth and vortexed to mix thoroughly. The *E. coli* cell suspensions were diluted 1/20 and the OD_{600nm} was measured. *B. cenocepacia* OD_{600nm} was measured without dilution. The

triparental mating mixture was made to a ratio of the different strains, with volumes added from the calculations of: *E. coli* SY327 0.45/OD_{600nm}, *E. coli* pRK2013 0.45/OD_{600nm}, and *B. cenocepacia* K56-2 0.5/OD_{600nm}. The three strains were mixed together and then spun at 6000 rpm in an Eppendorf Centrifuge 5424 (Eppendorf AG, Germany), and the supernatant was removed. The pellet was resuspended in 100µL fresh LB broth and then plated on a LB + 0.2% rhamnose plate, making the spot as small as possible to help facilitate the mating process. The plate was incubated right side up at 37°C overnight. The cells were collected with a plastic loop and resuspended in 2mL sterile LB broth, and vortexed to create a suspension. 200µL of the cell suspension was then spread onto LB+ 0.2% rhamnose +100µg/mL trimethoprim + 50µg/mL gentamicin agar, and incubated at 37°C overnight.

Colonies were selected and picked onto a grid to be grown overnight on the same media. Exconjugants were streaked onto agar and glycerol stocks prepared in LB supplemented with 20% glycerol for storage at -80°C.

2.5 Growth Kinetics

To assess the effect of each mutation on growth, kinetics were run in 96-well plates using a BioTek Synergy 2 plate reader (BioTek Instruments, Inc., USA). Strains were cultured in LB broth (Difco, USA) supplemented with 0.2% rhamnose when needed. For growth kinetics, standardized glycerol stocks were thawed and diluted to a final OD_{600nm} of 0.001 in a final well volume of 200µl and incubated in a BioTek Synergy 2 plate reader at 37°C with constant shaking for at least 20hrs. 96-well plates were sealed with parafilm prior to incubation to prevent

excess evaporation of the media. The OD_{600nm} was read every hour and plotted against time to give a representation of growth.

2.6 Viability Assay

Viability of strains was assessed by taking samples every two hours and spread plating to count cfu/ml at each time point. Strains were inoculated to a final OD_{600nm} of 0.001 in 5ml LB broth or 5ml LB broth supplemented with 0.2% rha and incubated at 37°C with shaking at 220rpm. 200µL of each sample was taken and spread onto LB agar supplemented with 0.2% rha as the time zero sample to assess the number starting cells in each culture. Subsequent sampling was done at 12hrs, 14hrs, 16hrs, 18hrs and 20hrs. At each time point, 200µL of sample was taken and added to a well in column 1 of a 96-well plate. This 96-well plate had 180µL of sterile LB broth added to all the other wells for 10-fold dilutions of each strain. Serial dilutions were carried out with a multi-channel micropipettor, taking 20µL between each well for a 10-fold dilution. 5µL of a strain was spotted onto a LB+0.2%rha agar plate using a multichannel micropipettor with eight tips, allowing the spotting of eight dilutions. Each dilution was spotted in duplicate. For time zero, dilutions from 10^{0}-10^{-7} were spotted; for time 12, 14, 16, 18, and 20hrs, dilutions from 10^{-4}-10^{-11} were analyzed. Plates were incubated for at least 24hrs and colonies were counted using a Nikon SMZ745 stereomicroscope (Nikon Instruments, USA). Colony counts for each time point were averaged and compared.

2.7 MIC Ratios

Assays were performed to compare the MIC of *B. cenocepacia* K56-2 (wild type; wt) to *B. cenocepacia* MKC2, *B. cenocepacia* MKC4, and *B. cenocepacia* 84-37D12 (CG transposon mutant with similar growth phenotype to MKC2). Standardized glycerol stocks were diluted to a final OD_{600nm} of 0.001 in LB broth and added to 96-well plates containing 2-fold serial dilutions of the antibiotic to be tested. The plates were incubated for 22 h at 37°C without shaking. The MIC ratio for each strain was calculated as the MIC of the wt divided by the MIC of the mutant, grown without rhamnose.

2.8 Microscopic Analysis

To prepare for microscopic observation, bacterial strains were inoculated into 5ml of LB plus 0.2% rhamnose to a final OD_{600nm} of 0.001 and incubated at 37°C with shaking at 220 rpm for 16 to 17hr. 15µL of each strain was then subcultured into 5 ml of LB or LB+ 0.2% rhamnose and incubated at 37°C with shaking at 220 rpm. After 24hrs of incubation, the cultures were diluted 1/20 with phosphate-buffered saline (PBS) to prepare for staining using SYTO9 dye and propidium iodide from the BacLight LIVE/DEAD Bacterial Viability Kit (Molecular Probes, USA). Controls were set up using wt type *B. cenocepacia* K56-2 added at a final 1/10 dilution to 4ml PBS or 4ml 70% isopropanol as the live and dead controls, respectively. Control tubes were incubated at room temperature for 1hr, with quick vortexing every 15min. Diluted bacterial samples were stained with 3µL SYTO9 dye and PI staining solution for 15min; a 10µL aliquot of each sample and control was spotted onto 1% agarose-coated microscope slides and topped with a cover slip. The slides were imaged using an AxioCamMR attached to an Axio Imager Z1

(Carl Zeiss) at 1,000x magnification using DIC, rhodamine, and green fluorescent protein (GFP) fluorescence filters. To determine the proportion of cells with a compromised cell envelope, 100 fluorescent cells from each biological replicate were counted.

2.9 Cell Fractionation

Cellular fractions were collected and isolated by using a modified version of Method 4 as described by (Thein et al. 2010). A 1L volume of bacteria was grown in LB or LB+ 0.2% rhamnose at 37°C to an OD_{600nm} of approximately 1.5 and cells were harvested by centrifugation at 4 000rpm for 20min. The cell pellets were resuspended in 10ml of Buffer 1 (0.2M Tris-HCl pH 8, 1M Sucrose, 1mM EDTA) containing 1mg/ml of lysozyme, mixed thoroughly and allowed to incubate at room temperature for 10min to digest the cell wall and create spheroplasts. The spheroplast suspension was transferred to ultracentrifuge tubes and spun at 44 500 rpm (203 459x g) for 45 minutes at 4°C using a Beckman Coulter Type 70 Ti rotor in Beckman Coulter Optima LE-80K Ultracentrifuge (Beckman Coulter, USA). The supernatant was removed and 10ml of ice-cold Buffer 2 (10mM Tris-HCl pH 7.5, 5mM EDTA, 0.2 mM DTT) was used to resuspend the pellets. The ultracentrifuged pellets were quite compacted, so a serological pipette was used to dislodge and break up the pellet. Resuspended spheroplasts were then passed through a French pressure cell at 20 000psi. Samples were centrifuged at 4 000rpm for 10min at 4°C to remove unbroken cells. Samples were then transferred to ultracentrifuge tubes and centrifuged at 54 000rpm (299 602x g) for 4 hours at 4°C. After ultracentrifugation, the resulting supernatant contains the cytoplasmic fraction and the pellet contains the inner and outer membranes. The cytoplasmic fraction was removed and stored at -20°C in 50ml conical

centrifuge tubes. The membrane pellet was resuspended in 1ml Buffer 3 (10mM Tris-HCl pH 7.5, 15% sucrose (w/v), 5mM EDTA, 0.2mM DTT), homogenized using a Potter-Elvehjem tissue grinder (Kimble Chase, USA) and stored at -20°C until use.

2.10 Molecular Biology Techniques

PCR was performed using an Eppendorf Mastercycler ep gradient S thermal cycler (Eppendorf AG, Germany) and HotStar HiFidelity polymerase (Qiagen, Germany). PCR programs were typically: 95°C for 5min, 27 cycles 95°C for 30sec, XX°C (where "XX" was the optimal annealing temperature for the primer pair being used) for 30sec, and 72°C for 30sec, followed by a final extension at 72°C for 10min.

Plasmids were prepared using a QIAprep Spin Miniprep Kit (Qiagen, Germany), restriction enzymes were acquired from New England Biolabs (USA), gel extraction of PCR products was done using a QIAquick Gel Extraction Kit (Qiagen, Germany), and PCR purification was done using a QIAquick PCR Purification Kit (Qiagen, Germany).

For SDS-PAGE analysis, proteins were run on 12% acrylamide gels using a Bio-Rad Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories, USA).

2.11 RNA Extraction and Preparation

Cellular RNA was extracted from strains grown in different conditions for qRT-PCR experiments using an Ambion Bacteria RiboPure RNA Purification Kit (Invitrogen, USA) according to manufacturer's protocol with some minor modifications. All tubes utilized in the preparation steps were provided in the kit unless noted. All pipette tips used were filter tips.

Micropipettors, work area, and equipment were cleaned with RNase Away (Thermo Scientific, USA). Bacterial strains were grown overnight in LB broth or LB+ 0.2% rhamnose, incubated at 37°C with shaking at 220rpm for 10-12hrs. After incubation, each strain was adjusted to an OD_{600nm} in microcentrifuge tubes and cells were pelleted at 13 000rpm for 3min. The supernatant was removed and cells were immediately frozen at -80°C until RNA extraction. ~250µL of ice cold zirconia beads from the kit were dispensed into screw cap tubes (up to the fill line marked on the tubes). 350µL of RNAwiz was added to each sample and then vortexed at maximum speed horizontally for 10min to disrupt cells. After centrifugation at 13 000rpm at 4°C for 5min., samples were transferred to a fresh 1.5ml tube, excluding the pelleted beads. A 0.2 volume of chloroform was then added, samples were shaken vigorously for 30sec and allowed to incubate at 10min at room temperature before centrifuging at 15 000rpm at 4°C for 5min. The aqueous phase (clear top layer) was transferred to a fresh 1.5ml tube. 0.5 volumes of 100% ethanol was added to each sample and mixed thoroughly. A filter cartridge was placed in a 2ml collection tube. Samples were transferred to the filter cartridge and centrifuged at 13 000rpm for 1min at room temperature. The flow-through was removed and filters were placed back into the same collection tube before addition of 700µL Wash Solution 1. Tubes were centrifuged at 13 000rpm for 1min, and the flow-through was discarded. The same process was repeated twice with 500µL Wash Solution 2/3. The filter cartridges were then placed into a fresh 2ml collection tubes. 30µl of Elution Solution, previously heated to 97°C in RNase-free PCR tubes (not provided in the kit), was added to the center of each filter and centrifuged at 13 000rpm for 1min at room temperature. To increase RNA yield, another 30µl of Elution Solution was added to the filter and centrifuged again. The manufacturer's protocol recommends DNase

treatment for 30 minutes; however, these samples were DNase treated for 1.5hrs to ensure minimal genomic DNA contamination in samples. DNase buffer (1/9th of sample volume; 6.67µl) and DNase I (4µl) was added and mixed gently but thoroughly. After incubation, 0.2 volumes (12µl) of DNase Inactivation Reagent was added to each RNA sample and incubated at room temperature for 2min, with some agitation to keep the reagent suspended. Samples were centrifuged at 13 000rpm for 1min to pellet the DNase Inactivation Reagent, and the purified RNA samples were transferred to RNase-free microcentrifuge tubes. The quality of RNA was assessed by running samples on a 2% agarose gel to check for degradation and samples were quantified using a NanoDrop Lite Spectrophotometer (ThermoFisher Scientific, USA). Samples were standardized to 500ng total RNA input into each tube for cDNA synthesis. cDNA was synthesized from RNA samples using an iScript Reverse Transcriptase Supermix for RT-qPCR (Bio-Rad Laboratories, USA) according to manufacturer's instructions, with a modification of the extension time (doubling of time from 20min to 40min) in the thermocycler program. cDNA synthesis was performed using the following parameters for one cycle: 25°C for 5min, 46°C for 40min, and 95°C for 1min. cDNA samples were diluted ½ using pH8.5 water.

2.12 qRT-PCR

The qPCR primer efficiency was assessed before running qRT-PCR using *B. cenocepacia* K56-2 genomic DNA. DNA was diluted 10-fold, five times using sterile pH8.5 water to yield samples containing 10^{-1} to 10^{-5} of original DNA concentration. IQ SYBR Supermix (7µl; Bio-Rad Laboratories, USA), the appropriate primers (300nM final concentration) and water were aliquoted into wells of a 96-well skirted PCR plate (Eppendorf AG, Germany). 3µl of template

DNA was added to each well and samples were run in triplicate. PCR plates were centrifuged at 1400rpm for 3min. Primer efficiency analysis was performed using a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, USA) with the following program: 95°C for 3min, 40 cycles of 95°C for 15sec and 59°C for 30sec, followed by a melt curve from 55°C to 95°C, 5sec for each 0.5°C temperature increase. Primer efficiencies are calculated by plotting the log10 of the DNA input (the dilutions of the genomic DNA) against the Ct values of each gene. The slope of the trendline is then used in the equation Efficiency = $(10^{-1/\text{slope}} - 1)*100$. Primers with efficiencies between 95-110% were deemed acceptable for use in subsequent qRT-PCR experiments.

A mastermix of IQ SYBR Supermix (Bio-Rad Laboratories, USA) was prepared with appropriate primers (300nM). Each well of the PCR plate contained 7µl of mastermix and 3µl of template cDNA (giving a final concentration of 150 ng/µl of cDNA). The plate was sealed using a clear PCR plate seal and centrifuged at 1400rpm for 3min. before qRT-PCR was performed according to the aforementioned conditions. *sigE* was chosen as a reference gene as it is expressed stably under antibiotic conditions. All data was compared using $\Delta\Delta$CT method and expressed as relative fold expression (Livak and Schmittgen 2001, Tseng et al. 2014). Two biological replicates were performed for each strain and condition, with three technical replicates per strain per condition.

Chapter 3 – Results

3.1 The *esaSR* Locus is Essential for Growth in Rich Media

Figure 3 depicts the genomic area around *esaSR* in the *B. cenocepacia* K56-2 genome. BLAST results showing percent amino acid identity of *esaS* and *esaR* homologs, as well as homologs that have been identified as being essential are outlined in Table 5. These findings give an idea of the conserved nature of *esaSR* among *Burkholderia* species; the high percent identity of this TCS in other *Burkholderia* species suggests that it may be essential in these bacteria.

To evaluate the essentiality of the *esaSR* locus, the genetic manipulation system described by Flannagan et al., was employed in order to delete *esaS* and *esaSR* (Flannagan et al. 2008). Mutagenic plasmids were created using pGPI-SceI as a backbone, and cloning the flanking regions immediately upstream and downstream of the gene of interest. These mutagenic plasmids, carrying a trimethoprim resistance cassette and I-SceI homing endonuclease recognition site, were introduced into the genome via a homologous recombination event. The second recombination event occurs after introduction of the second plasmid, pDAI-SceI, which constitutively expresses the I-SceI endonuclease. This homing endonuclease creates double-stranded DNA breaks at the recognition site, which are lethal if not corrected. If a gene is essential, then it can be reasoned that all colonies from the second recombination event will have the wt genotype, as loss of the gene results in death. Analysis of clones after the second event of *esaS* recombination showed that over 10% of colonies exhibited the deletion genotype (Figure 4). However, attempts to delete *esaSR* were unsuccessful, as all the colonies screened exhibited the wt configuration.



Figure 3. Visualization of the local genomic region around the *esaSR* TCS in *B. cenocepacia* K56-2.

Table 5. BLAST search of selected *Burkholderia* species comparing *esaS* (HK) and *esaR* (RR) homologs. The selected strains have minimum 95% coverage of HK and RR relative to *B. cenocepacia* K56-2 *esaSR*. All E values were 0.0.

Strain Name	HK Amino Acid Identity	RR Amino Acid Identity	Essential? (Reference)
***Burkholderia cepacia* strain DDS 7H-2**	99.88%	100%	
***Burkholderia cenocepacia* J2315**	99.87%	99.87%	Yes (Wong et al. 2016)
***Burkholderia* sp. 383 (Burkholderia lata)**	97.76%	99.14%	
***Burkholderia contaminans* strain MS14**	98.01%	99.14%	
***Burkholderia pyrrocinia* strain DSM 10685**	98.13%	98.28%	
***Burkholderia multivorans* ATCC 17616**	93.88%	98.28%	
***Burkholderia ambifaria* AMMD**	96.77%	97.84%	
***Burkholderia dolosa* AU0158**	96.17%	97.84%	
***Burkholderia vietnamiensis* LMG 10929**	95.90%	96.98%	
***Burkholderia gladioli* strain ATCC 10248**	86.44%	88.41%	
***Burkholderia thailandensis* E264**	88.97%	88.41%	Yes (Baugh et al. 2013)
***Burkholderia glumae* LMG 2196**	86.69%	86.70%	
***Burkholderia pseudomallei* K96243**	89.04%	88.41%	Yes (Moule et al. 2014)

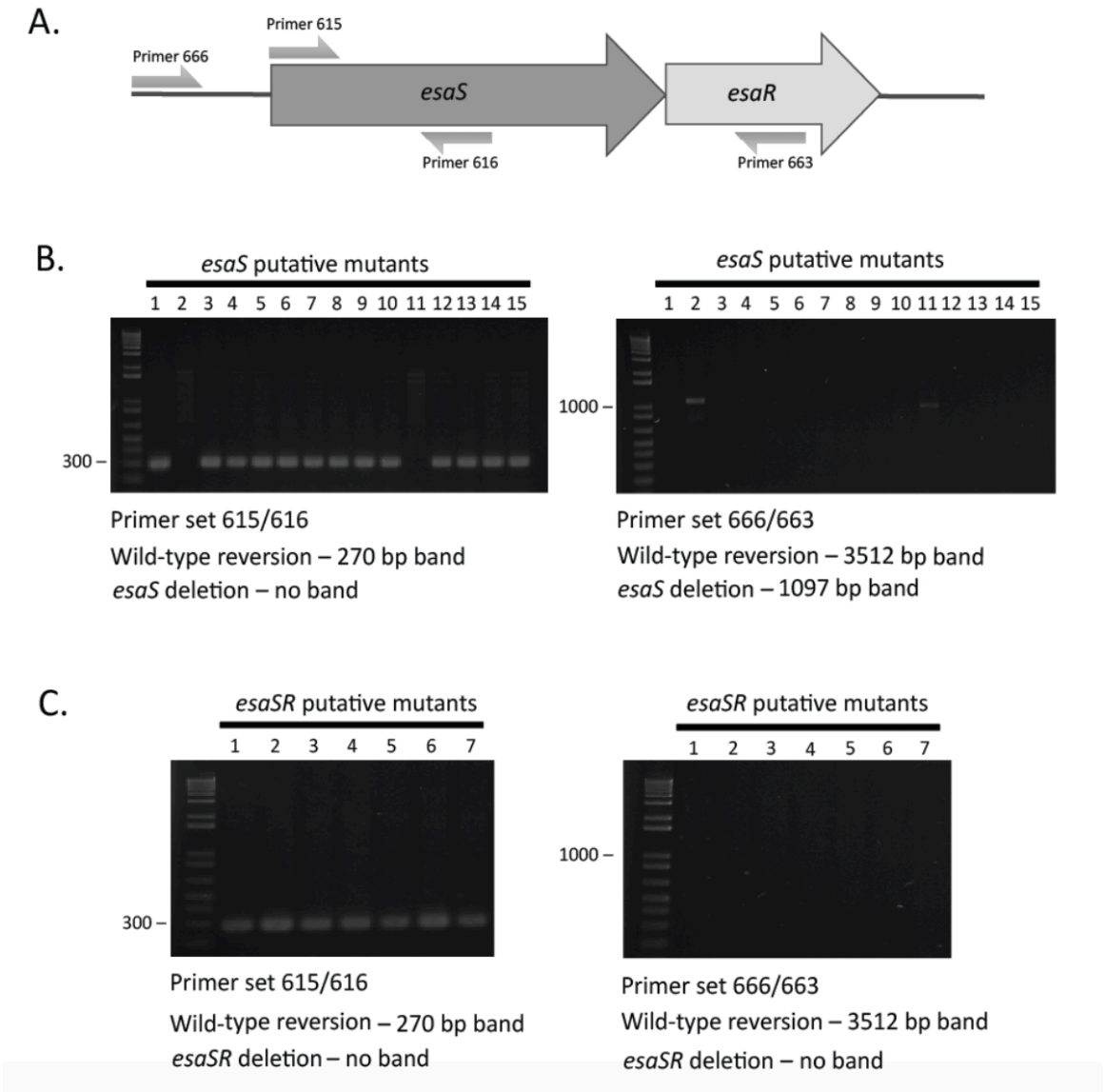


Figure 4. Genetic manipulation of *esaSR* in *Burkholderia cenocepacia* K56-2. A) Diagrammatic representation of the *esaSR* TCS and flanking regions with primer binding sites used for PCR screening. B) Agarose gel electrophoresis of PCR amplicons from putative colonies with *esaS* deletion. Left gel shows primer set 615/616. Colonies 2 and 11 show an absence of the expected 270bp band, suggesting successful deletion of *esaS*. All other colonies display a 270bp band, indicating the presence of *esaS* in the genome. The right gel shows primer set 666/663, which amplifies regions upstream and downstream of *esaS*. Colonies 2 and 11 show a 1100bp band, suggesting that *esaS* has successfully been deleted. The expected 3512pb amplicon expected from primer set 666/663 was not observed, suggesting PCR conditions need to be optimized. C) Agarose gel electrophoresis of attempted deletions of *esaSR*. The left gel shows primer set 615/616 amplifying the 5' end of *esaS*, indicating the presence of the gene. The right gel shows no amplification of the 3512bp band, again, probably due to the length as well as the high GC content of the amplicon region. Adapted from Gislason et al. 2017, Antimicrobial Agents and Chemotherapy.

The deletion was attempted multiple times, with over four hundred colonies screened; however, no colonies exhibiting the *esaSR* deletion genotypes were recovered. The *esaS* deletion mutant (ΔesaS) was named MKC4 and stocked at -80°C for further characterization.

To characterize the depletion of EsaR*,* a knockdown mutant was created by introducing a rhamnose-inducible promoter directly upstream of *esaR* to control expression. A severe growth defect was exhibited by this strain (when measured by OD_{600nm}) when grown in media without the supplementation of rhamnose. This conditional growth (CG) mutant was named MKC2 and stocked at -80°C for further characterization.

The mutation of *esaS* and *esaR* both lead to a defect in growth measured by OD_{600nm} when compared to the wt *B. cenocepacia* K56-2 (Figure 5). The growth defect due to the *esaS* deletion is moderate, but the growth defect caused by mutagenesis of *esaR* is more severe. In the absence of rhamnose, the OD_{600nm} of MKC2 reaches just above 1 before decreasing with further incubation. With rhamnose supplementation, the growth phenotype of MKC2 is almost identical to that of the wt. The growth of MKC4 is unaffected by the presence or absence of rhamnose, and the growth is the same in both conditions. The growth of the wt is also unaffected by rhamnose as *B. cenocepacia* cannot utilize rhamnose as a carbon source. The CG mutant 84-37D12 was included as a functionally-unrelated control. 84-37D12 has a low growth phenotype in the absence of rhamnose and harbors BURCENK562V_RS02270, phosphoribosylaminoimidazole-succinocarboxamide synthase, a gene involved in purine metabolism under control of PrhaB. This transposon-mutant strain was used in subsequent experiments to demonstrate that the hypersusceptibility to antibiotics exhibited by MKC2 is not a general effect due to poor growth without the supplementation of rhamnose.

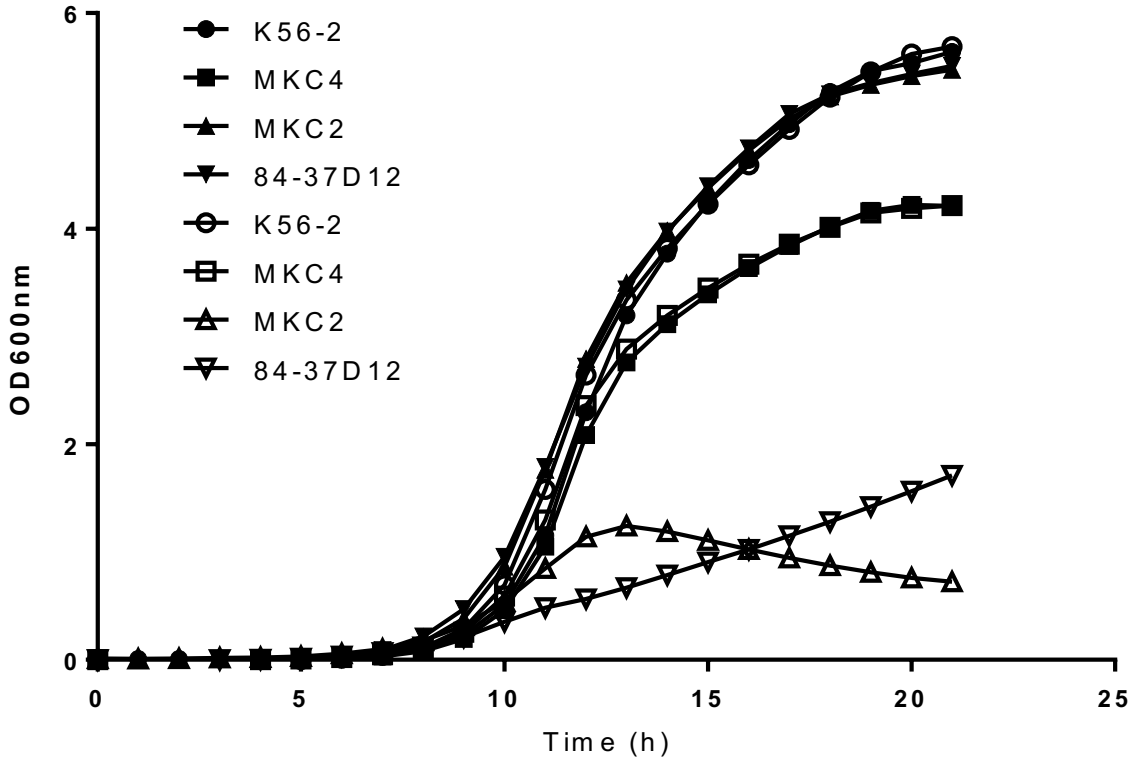


Figure 5. Growth kinetics of *Burkholderia cenocepacia* K56-2, *B. cenocepacia* MKC2 (*esaR* knockdown mutant), *B. cenocepacia* MKC4 (*esaS* deletion mutant), and *B. cenocepacia* CG mutant 84-37D12 (unrelated CG mutant control). Strains were grown in LB and LB broth supplemented with 0.2% rhamnose, at 37°C shaking at 220rpm with a starting inoculum of OD_{600nm} 0.001. Filled markers represent growth with rhamnose supplementation and empty markers represent growth in no rhamnose conditions. Adapted from Gislason et al. 2017, Antimicrobial Agents and Chemotherapy.

3.2 EsaR is Required for Viability and Membrane Integrity

Looking at the growth curve of MKC2 without added rhamnose, the OD_{600nm} is observed to increase up to around 12hrs after which the OD_{600nm} starts to decrease (Figure 6). This lead to the hypothesis that EsaR underexpression causes a reduction in viability. The viability of the wt and MKC2 was monitored by taking samples every two hours and plating on LB agar supplemented with 0.2% rha and then plotting the percent viability relative to the first sample taken (at time=12hrs post-inoculation). Figure 6 shows that at eight hours, the viability of MKC2 declines to 2% compared to viability at the beginning of the experiment. This experiment suggests that the depletion of EsaR influences the viability of *B. cenocepacia*, as without expression of EsaR, the viability of the cells markedly drops over time.

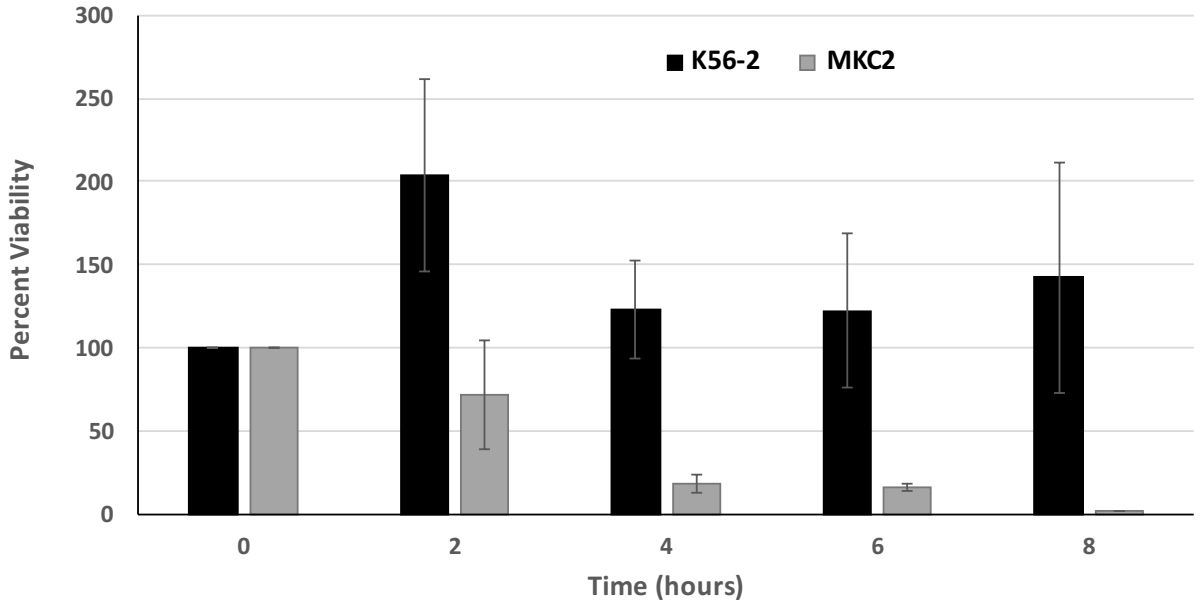


Figure 6. Viability of *B. cenocepacia* K56-2 and *B. cenocepacia* MKC2 (*esaR* knockdown mutant) in LB broth tracked over a period of eight hours, twelve hours after initial inoculation at an OD_{600nm} of 0.001. Percent viability is relative to the twelve-hour time point which is set to 100%.

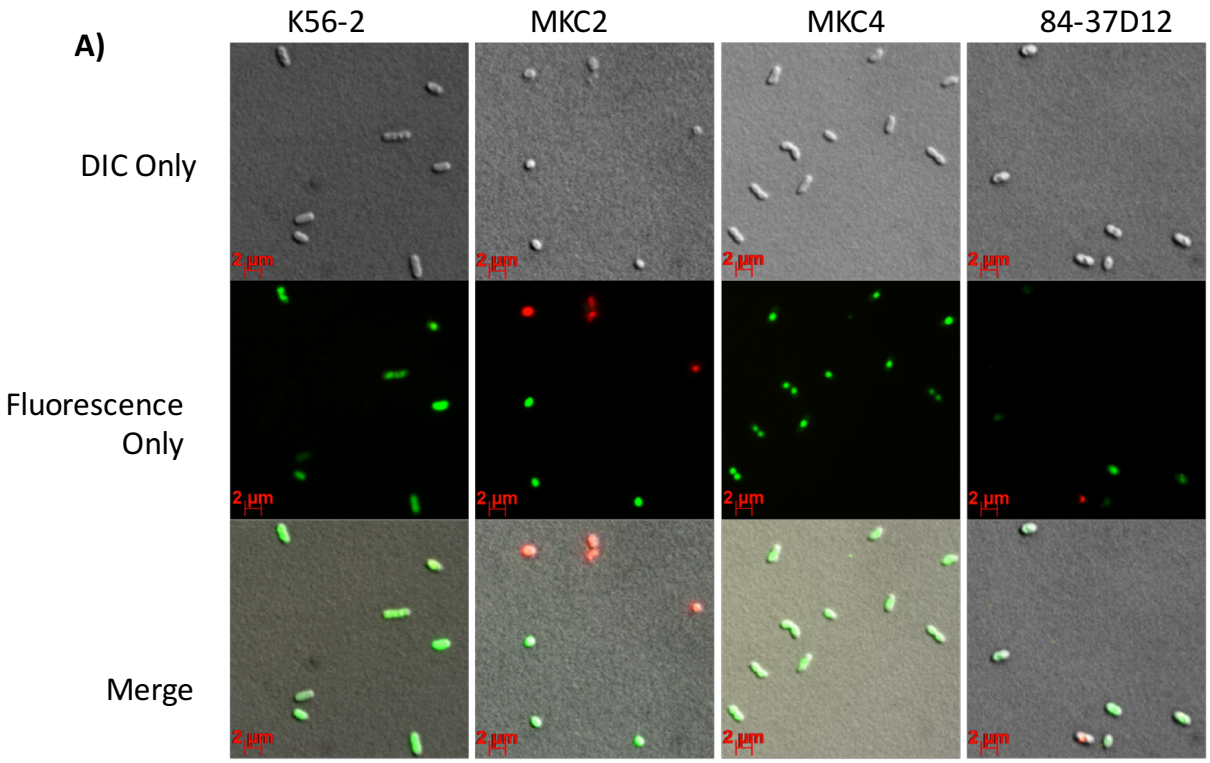
To investigate if *esaSR* plays a role in maintaining cell envelope integrity, the BacLight LIVE/DEAD Bacterial Viability Kit was employed to stain each strain for observation under a microscope. This assay uses the differential envelope penetrating ability of two DNA staining dyes, SYTO9 and propidium iodide. SYTO9 is able to penetrate healthy, intact cell envelopes and stain DNA, whereas propidium iodide is only able to enter the cell if the cell envelope has been compromised in some way. However, if propidium iodide is able to enter the cell, it is able to displace SYTO9 due to its higher DNA binding affinity, and thus the cells fluoresce red. If the cell envelope is intact then propidium iodide is excluded, and the cells fluoresce green due to the binding of SYTO9 to DNA.

Cells were incubated for 24hrs, and after treatment, observed using a microscope with differential interference contrast (DIC), green fluorescent protein (GFP), and rhodamine filters. The typical cell size and morphology of *B. cenocepacia* K56-2 is that of a rod-shaped bacterium approximately 3µm in length. The vast majority of wt cells were shown to fluoresce green, and all exhibited a rod-shaped morphology. MKC4 and 84-37D12 (without rhamnose supplementation) also exhibited rod-shaped morphology, albeit 84-37D12 cells were slightly smaller when compared to the wt. Without added rhamnose, MKC2 cells displayed a spherical morphology and appeared to be extremely small, approximately 1µm. Additionally, about half of the MKC2 cells fluoresced red, suggesting that a compromised cell envelope and propidium iodide was able to enter the cell (Figure 7).

The membrane fraction of wt and MKC2 cells was collected after growth in LB with and without rha supplementation. Protein profiles in both the cytoplasmic and membrane fractions looked very similar for wt rha, wt no rha, and MKC2 rha samples. Some minor differences were

observed in the cytoplasmic fraction of MKC2 no rha. However, there were some very evident differences in the membrane protein fraction of MKC2 no rha compared the other three samples. Proteins were noticeably missing/downregulated, while some bands became more evident, suggesting upregulation. Figure 8 shows the polyacrylamide gel, with arrows added to emphasize noticeable differences in the membrane protein profile of MKC2 grown without rha supplementation.

A)



B)

	Wild type	MKC2	MKC4	84-37D12
Rhamnose	2.5%	1%	5%	1%
No rhamnose	1.5%	50%	3.5%	1.5%

Figure 7. Fluorescence microscope images of the Live/Dead assay using SYTO9 and propidium iodide. Cells were grown for 24hrs in LB broth at 37°C shaking at 220rpm, before staining and imaging. A) For each column, there are three panels; top panel: differential interference contrast (DIC) filter only; middle panel: fluorescence filter only; bottom panel: merged DIC and fluorescence filter images. B) The total number of cells and number of cells fluorescing red were counted and reported. Percentages shown are an average of two biological replicates. Adapted from Gislason et al. 2017, Antimicrobial Agents and Chemotherapy.

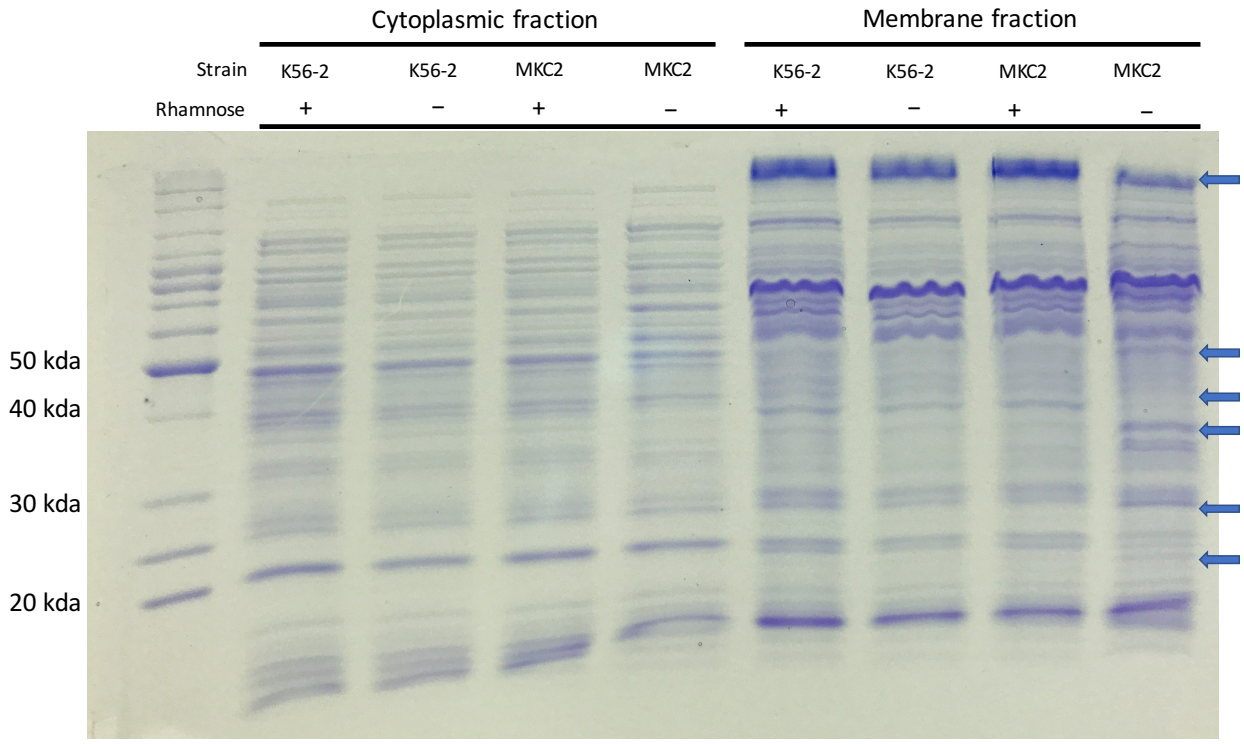


Figure 8. 12% SDS-PAGE gel of cell fractions of *B. cenocepacia* K56-2 and MKC2 grown in LB broth, or LB broth supplemented with no rhamnose. Cells were fractionated using an ultracentrifugation method. Lane 1: protein ladder; Lanes 2-5: cytoplasmic fraction samples; Lanes 6-9: membrane fraction samples. The arrows on the right side of the image point to areas of noticeable differences between MKC2 no rhamnose compared to MKC2 with rhamnose supplementation, as well as the wild type with and without rhamnose supplementation.

3.3 Underexpression of EsaSR Increases the Susceptibility of *B. cenocepacia* to Antimicrobials

To assess the effect of *esaSR* mutation on the antimicrobial susceptibility of *B. cenocepacia*, MIC ratio experiments were performed using wt, MKC2, MKC4, and CG mutant 84-37D12. MIC ratios are a representation of the difference in MIC between the wt and mutant strains, and are calculated by dividing the MIC of the wt divided by the MIC of the mutant grown without rhamnose. Higher MIC ratios indicate a higher susceptibility to the antimicrobial tested, as the MIC of the mutant is lower than that of the wt. Compounds of different mechanisms of action (MOA) were chosen for activity against the strains, including those that inhibit cell wall synthesis, DNA replication and synthesis, RNA polymerase, and protein synthesis. H_2O_2 and DMSO were included as general antimicrobial compounds, and show that mutation of *esaSR* does not cause increased susceptibility to all compounds with an antimicrobial effect. The CG mutant 84-37D12 was included as a control to show that the increased susceptibility to antimicrobials was not due to the introduction of the rhamnose-inducible promoter into the genome as well as the low growth phenotype exhibited by MKC2 and 84-37D12.

Figure 9 shows that the deletion of *esaS* in MKC4 increased the susceptibility to some antimicrobial molecules, but not to the same degree as underexpression of EsaR in MKC2. MKC2 exhibits a higher MIC ratio for ceftazidime (MIC ratio = 16), as well as some protein synthesis inhibitors, with the largest being tetracycline (MIC ratio = 8). MKC2 exhibits greater susceptibility to more antimicrobial compounds than MKC4 and 84-37D12, and to have higher MIC ratios for those antimicrobials. The MIC ratios of 84-37D12 were almost all at 1, indicating that that MIC of 84-37D12 is the same as the wt strain. The general trend was that the highest

MIC ratios resulted from cell wall synthesis inhibitors and DNA synthesis inhibitors, followed by the protein synthesis inhibitors and rifampicin.

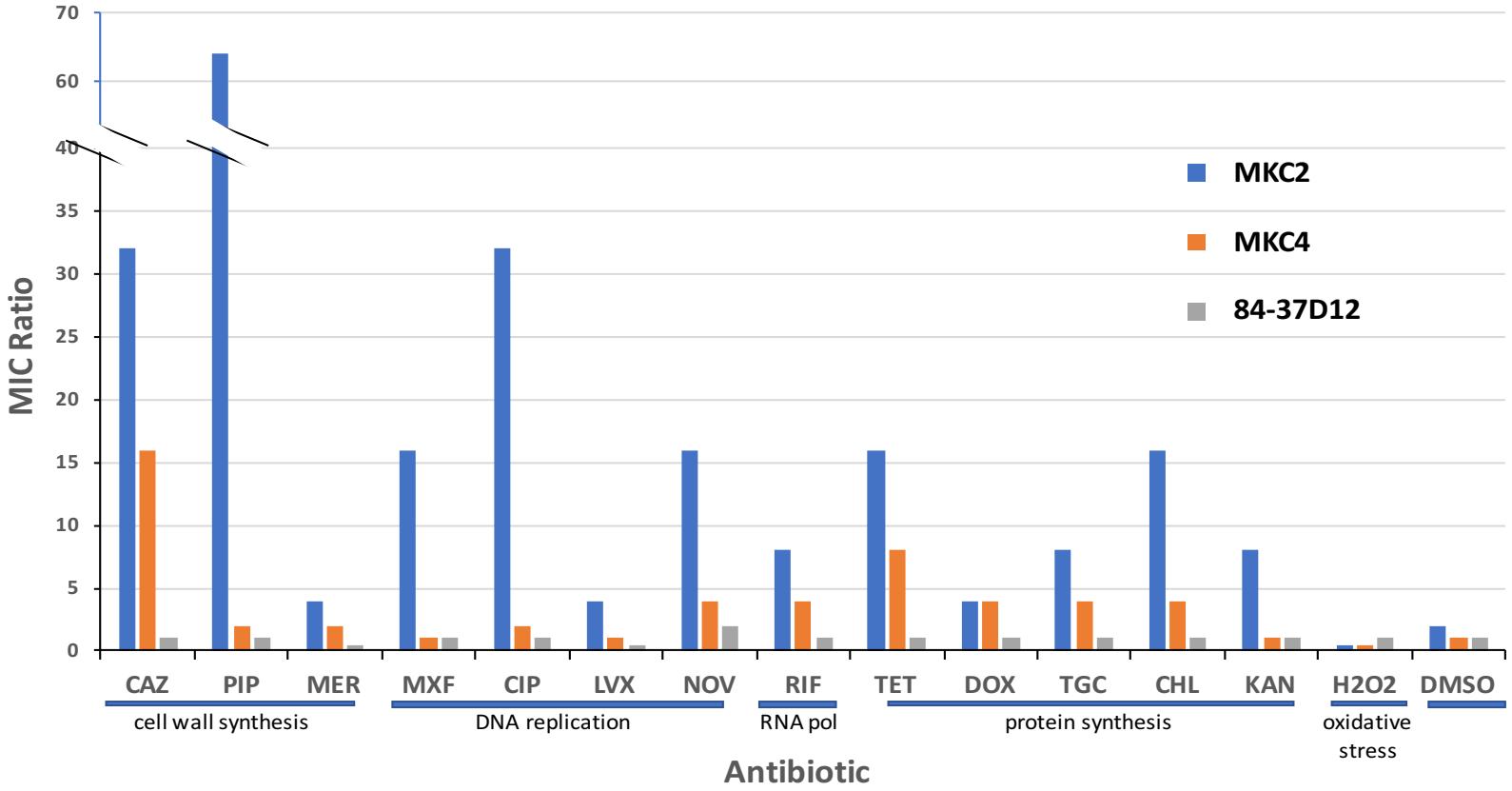


Figure 9. MIC ratios of antimicrobials of varying mechanisms of action of *esaSR* mutants and unrelated conditional growth mutant 84-37D12. MIC ratio is a comparison of the MIC of the mutant compared to the wild type and is calculated by $[MIC_{wt}]/[MIC_{mutant}]$. This is a representation of the shift in the MICs of the mutants, with higher MIC ratio values indicating increased susceptibility to the antimicrobial, and MIC ratios of 1 indicating the same level of susceptibility to the wild type. Partial data adapted from Gislason et al. 2017, Antimicrobial Agents and Chemotherapy.

Note: CAZ, ceftazidime; PIP, piperacillin; MER, meropenem; MXF, moxifloxacin; CIP, ciprofloxacin; LVX, levofloxacin; NOV, novobiocin; RIF, rifampicin; TET, tetracycline; DOX, doxycycline; TGC, tigecycline; CHL, chloramphenicol; KAN, kanamycin; H_2O_2; hydrogen peroxide; DMSO, dimethyl sulfoxide.

3.4 EsaSR Plays a Role in the Efflux Activity of *B. cenocepacia*

To investigate the role of EsaSR in regulating efflux, expression of four efflux pumps previously demonstrated to be involved in antimicrobial efflux was analyzed using qPCR. Cells were grown for 10hrs and cells were frozen at -80°C for later RNA extraction. This time was chosen because at 10hrs MKC2 grown without rhamnose had a similar OD_{600nm} to the other samples. As shown in Figure 6, 10hrs the optical density starts to decrease and viability is compromised. To demonstrate that rhamnose has minimal effect on altering expression of the selected RND-efflux genes, the wt was grown with and without 0.2% rhamnose supplementation and relative gene expression was compared. Substantial changes in relative gene expression was observed in both the presence and the absence of rhamnose (Figure 10A), indicating that rhamnose itself did not have an impact on altering gene expression of the four RND-efflux transporters. Without rhamnose supplementation, MKC2 exhibited some altered expression of efflux genes, with RND-3 being underexpressed and RND-9 being slightly overexpressed (Figure 10B). This suggests that RND-9 is negatively regulated by EsaR, while RND-3 is positively regulated by EsaR. Additionally, the outer membrane porin (J2315 locus BCAM1931) was shown to be underexpressed relative to the wt (Figure 11), providing more support towards the hypothesis that EsaR plays a role in the regulation of cell envelope proteins illustrated by the acrylamide protein gel (Figure 8). BCAM1931 is a major porin protein in *Burkholderia* species, being one of the most abundant membrane proteins expressed under all conditions (Schell et al. 2011). Additionally, in transposon mutant library screens for essential genes, this porin was noted to be essential in other *Burkholderia* species (Baugh et al. 2013, Moule et al. 2014, Wong et al. 2016).

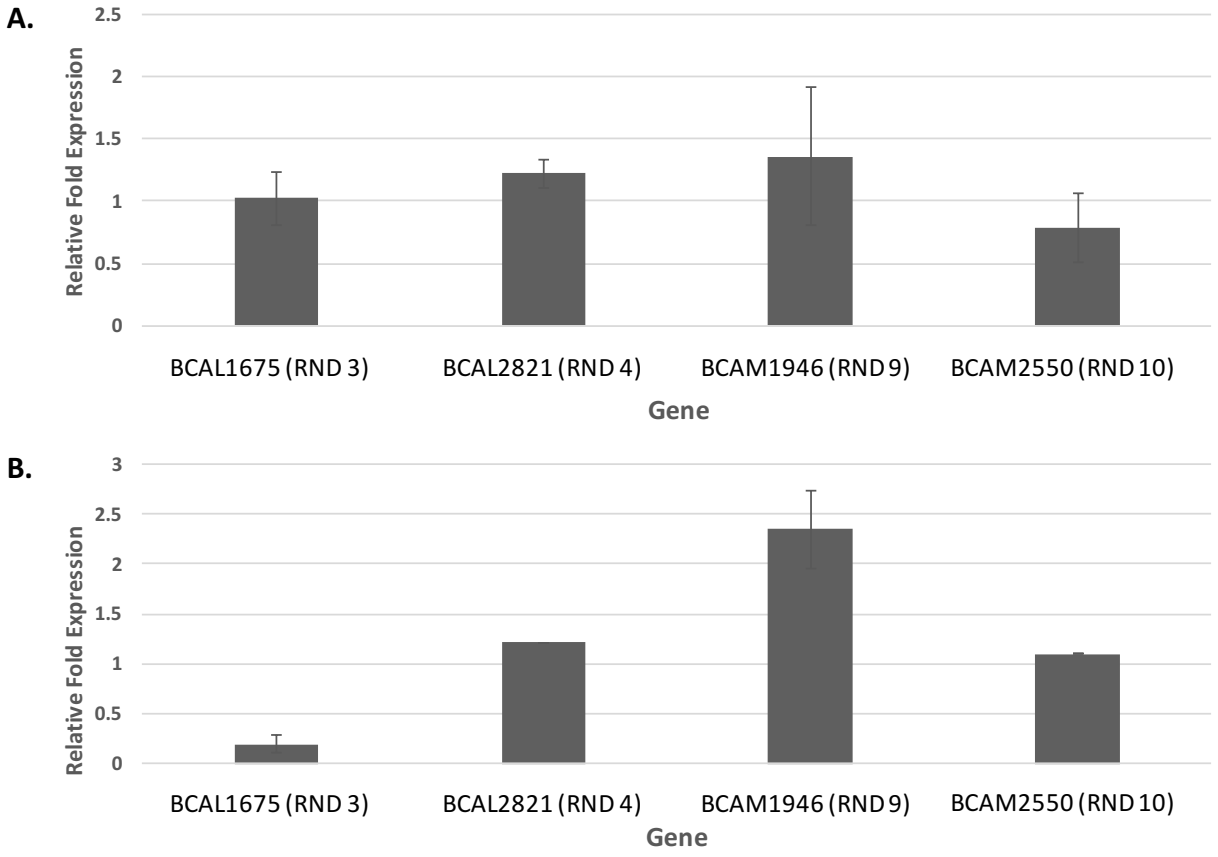


Figure 10. Changes in expression of four RND efflux transporters from *Burkholderia cenocepacia* grown in LB for 10hrs at 37°C shaking at 220rpm. A) Comparison of the effect of the growth of *B. cenocepacia* K56-2 wild type in LB + 0.2% rhamnose compared to LB broth on the expression of RND efflux transporters. B) Comparison of the effect of the growth of *B. cenocepacia* MKC2 in LB broth compared to LB + 0.2% rhamnose broth.

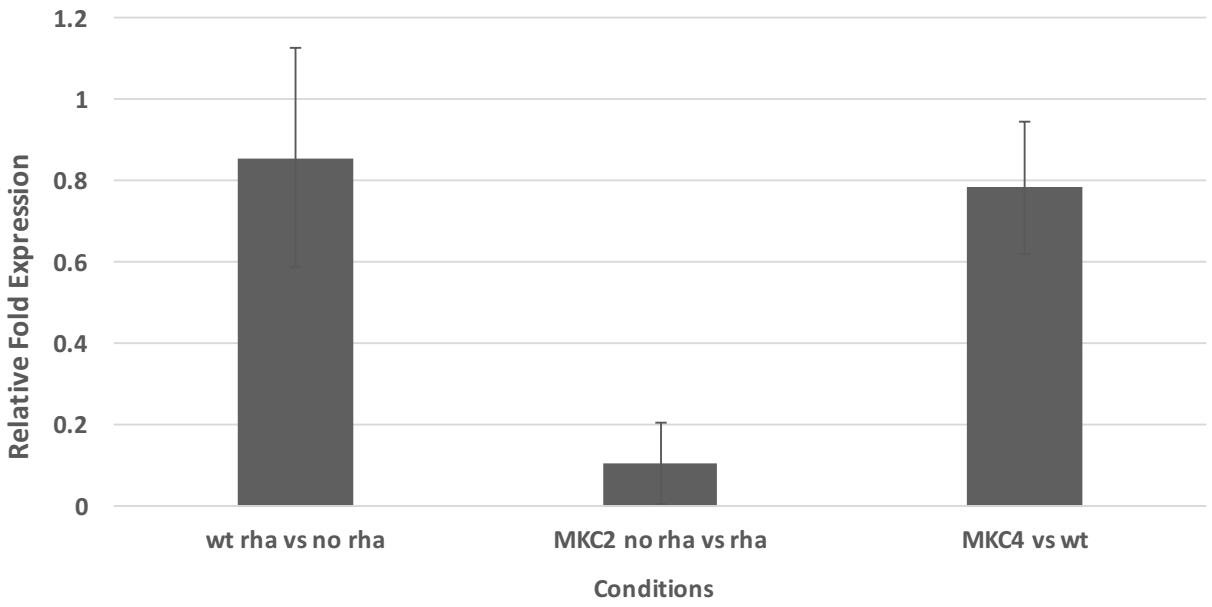


Figure 11. qRT-PCR results showing changes in expression of putative membrane porin BURCENK562V_RS12925, homolog of BCAM1931 from *B. cenocepacia* J2315 and BPSS0879 from *B. pseudomallei*) from *Burkholderia cenocepacia* grown in LB for 10hrs at 37°C shaking at 220rpm.

MKC4 also shows some altered expression of efflux pumps after 10hrs of growth; however, expression profiles differ from those of MKC2 (Figure 12A). MKC4 is shown to slightly underexpress the RND-4 and RND-9 transporters relative to the wt. Interestingly, when MKC4 is grown for 13hrs (Figure 12B), the expression of the efflux pumps change. RND 3 and RND 10 show large amounts of upregulation, whereas RND 4 and RND 9 show slight upregulation (2.7 and 2.2 fold, respectively). At the 13hr time point, the growth of the *esaSR* mutants starts to deviate from the wt with the growth rate of MKC4 slowing down; whereas the OD_{600nm} of MKC2 without rhamnose hits its peak and begins to decline (Figure 6).

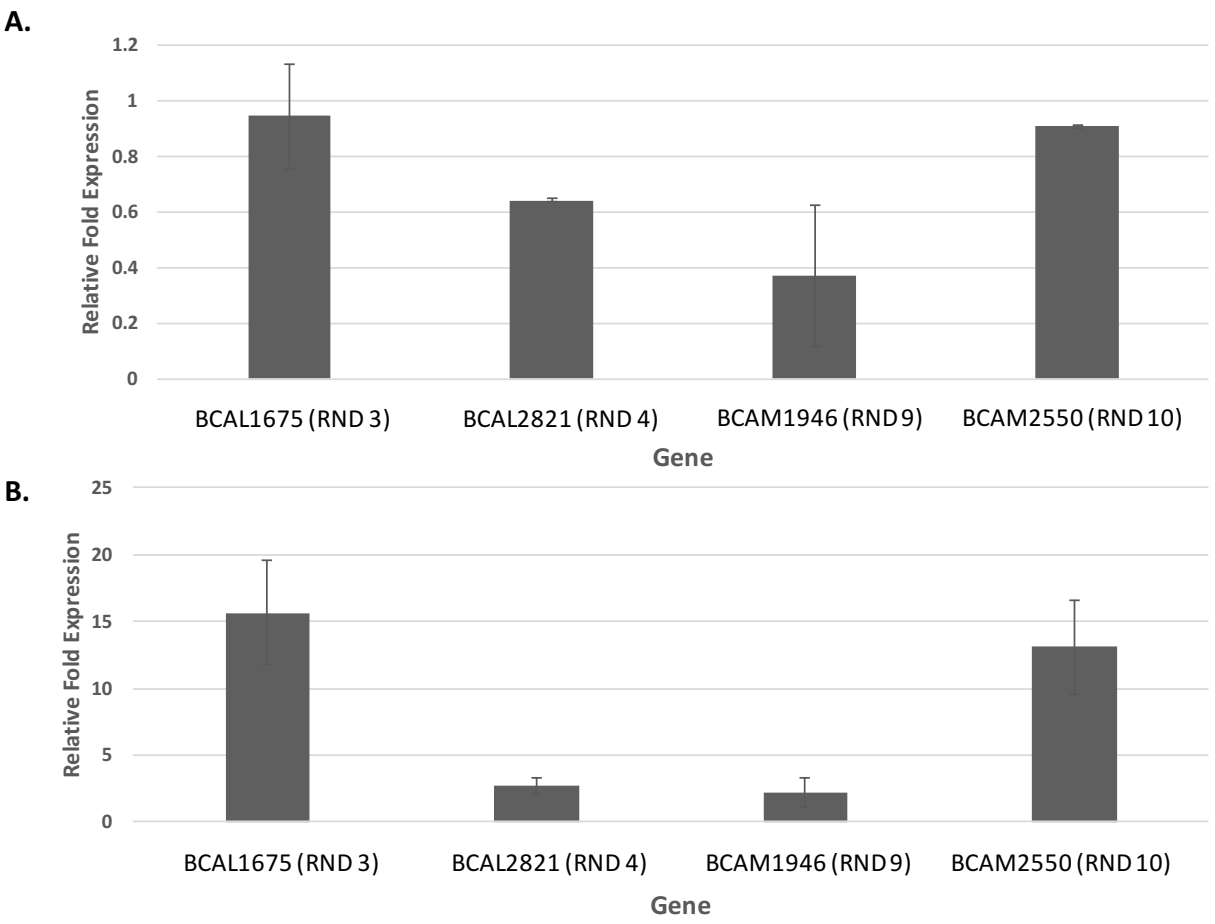


Figure 12. qRT-PCR results looking at the change in expression of four RND efflux transporters from *Burkholderia cenocepacia* MKC4 relative to wild type K56-2 after growing at different times at 37°C shaking at 220rpm. A) Comparison of the RND efflux transporters in MKC4 compared to wildtype K56-2 after 10hrs of growth. B) Comparison of RND efflux transporters in MKC4 compared to wild type K56-2 after 13hrs of growth.

Chapter 4 – Discussion

A screen of a transposon mutants lead to the identification of a CG mutant that exhibited a growth defect and hypersusceptibility to multiple antimicrobials. The transposon insertion in CG mutant 73-14C5 introduced a rhamnose-inducible promoter at the 3' end of *esaS,* regulating the expression of *esaR*; *esaS* and *esaR* encode the *B. cenocepacia* EsaSR TCS. To better characterize this TCS, attempts to delete the HK, RR, and both genes were carried out. The deletion attempts suggest that the HK *esaS* is dispensable, while *esaR* is essential as attempts to delete *esaSR* were unsuccessful, while deletion of *esaS* was achieved. Gene essentiality can be confirmed when the deletion of a gene from the genome can only be achieved when it is provided in trans on a plasmid (Gislason et al. 2017). However, genetic manipulation tools in *B. cenocepacia* are fairly limited partly because of its high intrinsic resistances to antimicrobials as well as the limited availability of genetic tools, restricting the selection methods in genetic engineering (Flannagan et al. 2008). Deletion of *esaS* showed about 14% of colonies recovered after the second recombination event possessed the deletion (Figure 4). Multiple attempts to delete *esaSR* resulted in the screening of over four hundred colonies, with 100% of colonies screened reverting to the wt genotype. A transposon mutant library was constructed by Wong et al. using *B. cenocepacia* J2315, a clonal isolate from the same lineage as *B. cenocepacia* K56-2, and utilized this library of mutants for TraDIS (Transposon Directed Insertion-site Sequencing) to analyze the *B. cenocepacia* genome and identify essential genes (Wong et al. 2016). In this study, BCAL0472 (the J2315 homolog of K56-2 *esaR*, with 99.86% nucleotide match) was noted as being an essential gene, providing further evidence that *esaR* is indeed an essential gene in *B. cenocepacia*. Transposon-mutant library

aided genomic screens of other *Burkholderia* species have also demonstrated that the *esaR* homologs in *B. pseudomallei* (BPSL0128) and *B. thailandensis* (BTH_I0135)are essential (Baugh et al. 2013, Moule et al. 2014). Additionally, Figure 13 shows *esaR* being identified and demonstrated as being essential in a TN-seq experiment to determine essential genes of *B. cenocepacia* K56-2, as the empty area represents a region where all insertions were lethal (Gislason et al., 2017, submitted). A site-directed insertional mutant of the *esaR* homolog, Bmul_3117, in *B. multivorans* was created and the strain exhibits hypersusceptibility to antimicrobials of different MOA (Figure 14). The aforementioned findings strongly suggest that the response regulator encoded by BURCENK562V_RS04765 (*esaR*) is essential in *B. cenocepacia*, and probably many other Bcc members as well.

When supplemented with rhamnose in the medium the growth of the *esaR* knockdown mutant MKC2 is almost identical to the wt; however, without added rhamnose, MKC2 exhibits a severe growth defect, growing to about 20% the OD_{600nm} of the wt strain (Figure 5). Because of the decreasing OD_{600nm} of MKC2 without rhamnose seen in the kinetics, the viability of these cells came into question and thus viability was investigated. By taking samples over a period of eight hours and calculating cfu/ml for each time point, the number of viable cells decreases relative to the starting time. This is in contrast to the wt strain, which more or less maintains viability (Figure 6). These findings provide further evidence that EsaR is an essential gene, as the underexpression of this RR leads to a gradual decline in cell viability.

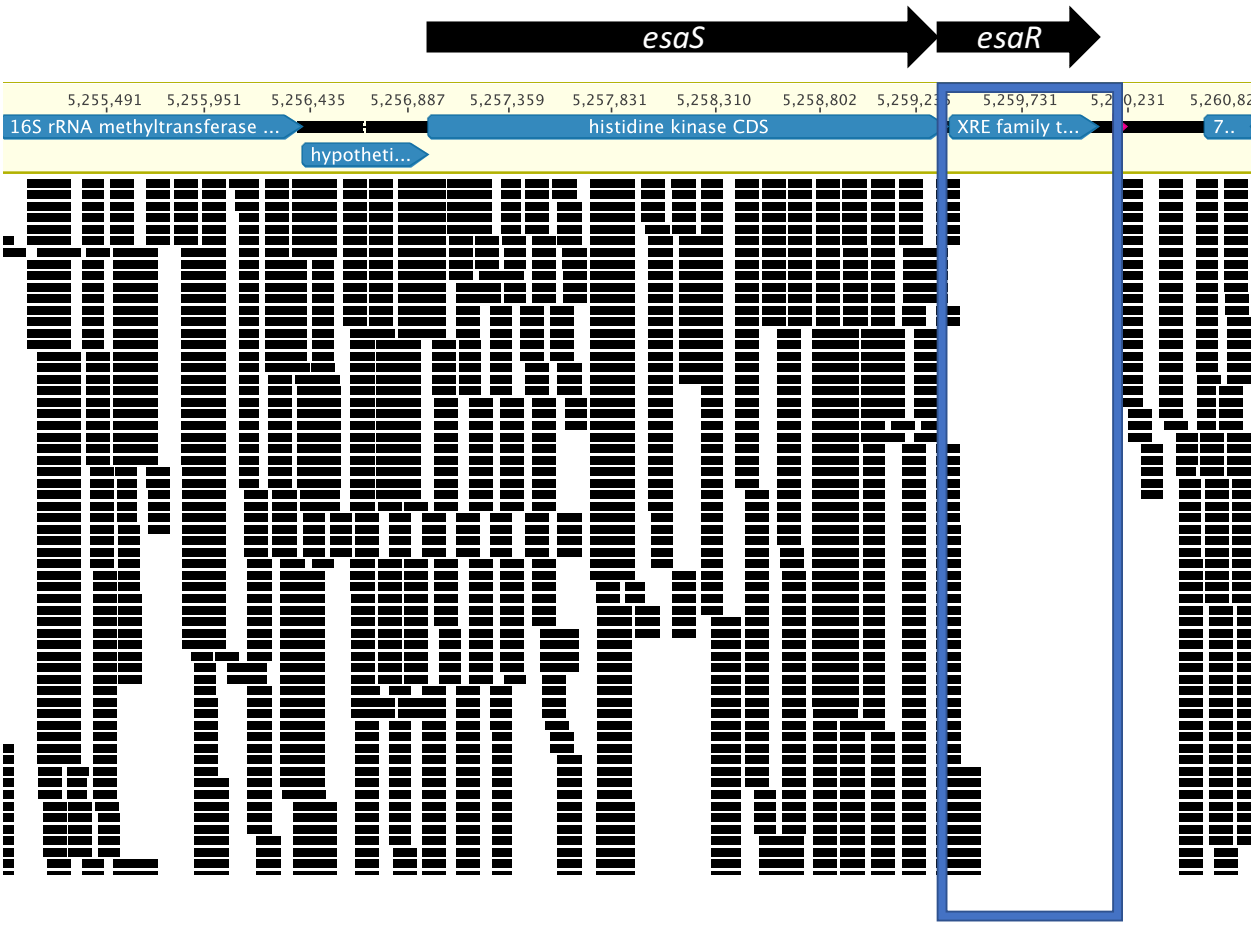


Figure 13. Area shown is a representation of the genomic area around *esaSR* from TN-seq data of a transposon mutant library of *B. cenocepacia* K56-2 mutants. Each black bar representing one read from Illumina next-generation sequencing data. Areas with no insertions indicate that all insertions in the region were lethal, and resulted in no mutants being recovered to be sequenced. Data from Gislason et al 2017, submitted, "Comparative analysis of the *Burkholderia cenocepacia* K56-2 essential genome reveals cell envelope functions that are uniquely required for survival in *Burkholderia* species".

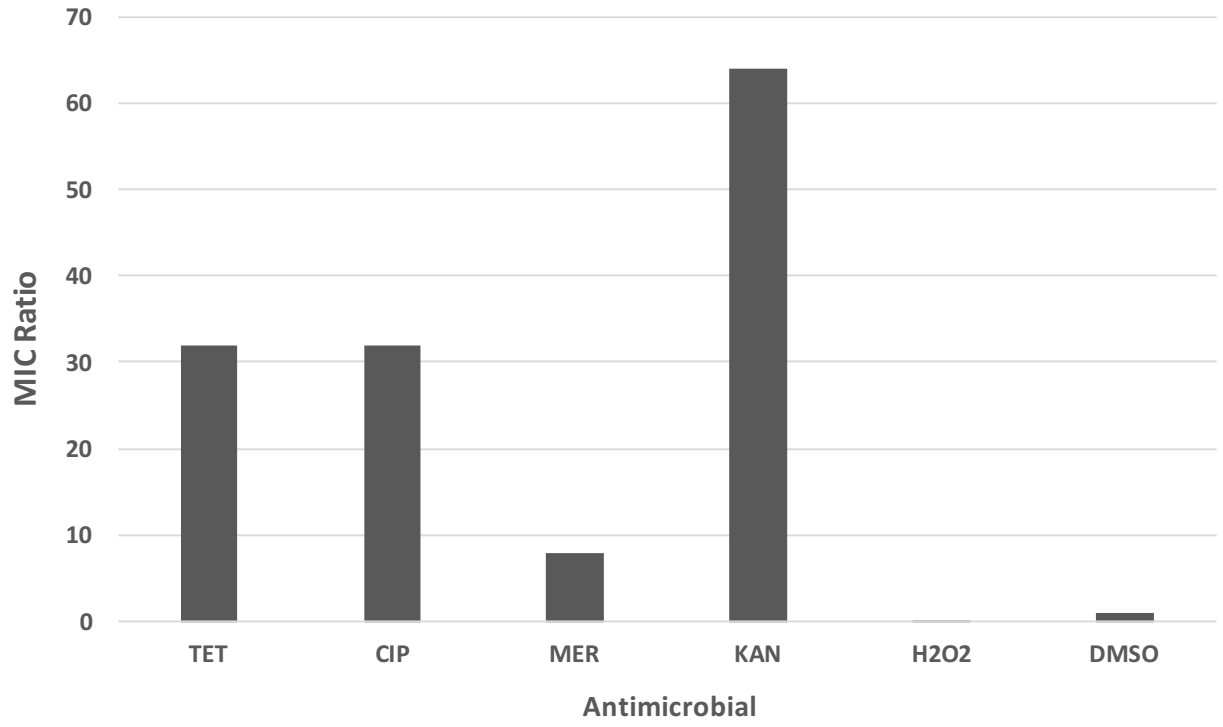


Figure 14. MIC ratio comparison of *B. multivorans* MKC3 (CG mutant of *esaR* homolog Bmul_3117) relative to the wild type strain *B. multivorans* strain C5393. MIC ratio is a comparison of the MIC of the mutant compared to the wild type and is calculated by $[MIC_{wt}]/[MIC_{mutant}]$.
Note: TET, tetracycline; CIP, ciprofloxacin; MER, meropenem; KAN, kanamycin; H_2O_2; hydrogen peroxide; DMSO, dimethyl sulfoxide.

Skerker et al. (2005) also provide evidence of a pair of essential HK and RR genes, *cenK* and *cenR,* that play an important role in the growth and cell envelope integrity of *Caulobacter cresentus.* In their study of TCS related to growth and cell cycle progression, the authors systematically deleted TCS and identified 39 TCS genes related to growth and/or cell cycle progression. Nine of these TCS genes were shown to be essential in *C. cresentus* (Skerker et al. 2005). *cenK* and *cenR*, two of the nine essential TCS genes with functions in growth and cell envelope progression, were successfully deleted from the genome with the addition of a copy of the gene on a low copy plasmid under the control of a xylose inducible, glucose-repressible promoter. Deletion of these genes was not possible without complementation, as well as with the empty vector control (Skerker et al. 2005). Without induction, the mutants exhibited a marked growth defect when compared to growth with xylose supplementation, similar to the large difference in growth of MKC2 in the presence and absence of rhamnose. In addition to affecting growth, depletion of *cenKR* leads to a compromised cell envelope, with the experiments suggesting that *cenR* plays an important role in maintaining proper cell envelope structure and cell wall synthesis/maintenance (Skerker et al. 2005).

Another TCS demonstrated to have a role in the regulation of cell wall metabolism is the MtrAB TCS in members of the suborder *Corynebacterineae,* which includes human pathogens such as *Corynebacterium diphtheriae, Mycobacterium tuberculosis* and *Mycobacterium leprae*. This TCS is essential in *M. tuberculosis*, however Möker et al. demonstrated that the MtrAB homolog in *C. glutamicum* could be deleted, although at some fitness cost to the bacteria (Möker et al. 2004). In this study, deletion of MtrAB affected the growth of *C. glutamicum* and caused changes in cellular morphology and susceptibility to cell wall active compounds (Möker

et al. 2004), similar to what was demonstrated with the *esaR* knockdown mutant MKC2. When grown in the absence of rhamnose, MKC2 cells exhibit a small, spherical morphology, differing from the rod-shape that is typical of bacteria from the genus *Burkholderia*. In addition to the change in morphology, MKC2 cells were shown to have compromised cell membranes in the live/dead staining assay. The underexpression of EsaR leads to a dramatic change in in the permeability of propidium iodide (an increase from 1% of cells fluorescing red with rhamnose supplementation to 50% of cells fluorescing red in the absence of rhamnose) (Figure 7). Collectively these findings suggest that EsaR plays an important role in cell envelope metabolism and homeostasis.

To further demonstrate EsaR's role in the regulation of cell envelope function, differences in total membrane proteins were examined by SDS-PAGE of proteins isolated by cell fractionation methods. The cytoplasmic and membrane proteins were separated to obtain well-isolated fractions using ultracentrifugation (Thein et al. 2010). This process first uses lysozyme to create spheroplasts. An interesting observation was made in that MKC2 cells grown with no rhamnose seem to be more sensitive to the action of lysozyme, as the MKC2 no rha pellet had an appearance that differed from the other three samples (wt rha, wt no rha, and MKC2 rha) which looked identical (Appendix Figure 1). When comparing the membrane protein profiles of the four samples, three samples (wt rha, wt no rha, and MKC2 rha) looked almost identical, whereas there were major differences in the protein bands in the MKC2 no rha sample (Figure 8). The membrane profiles reveal differences in the proteins present as well as protein abundance. One protein of note, approximately 37 kDa in size, almost entirely disappears in the MKC2 no rha sample. Based on data from studies investigating the membrane proteins of other

Burkholderia species, this 37kDa protein correlates with a major membrane porin. Schell et al. demonstrate that a 37kDa membrane protein is a porin encoded by the gene *BPSS0879*, one of the most abundant membrane proteins in *B. pseudomallei* and *B. mallei* (Schell et al. 2011). Based on the size and abundance of this protein band at approx. 37kDa, it is possible that this membrane protein is the K56-2 homolog of *BPSS0879*. The homologs of *BPSS0879* in *B. cenocepacia* K56-2 and *B. cenocepacia* J2315 are called *BURCENK562V_RS12925* and *BCAM1931*, respectively. The homologs of this 37kDa major porin are noted to be essential in *B. cenocepacia*, *B. thailandensis,* and *B*. *pseudomallei* from genomic screens for the essential genes of these bacteria (Baugh et al. 2013, Moule et al. 2014, Wong et al. 2016). qRT-PCR results also supports the evidence of downregulation of this major porin, as the porin is downregulated in MKC2 without rhamnose supplementation. Confirmation of the protein's identity using mass spectrometry is needed to help confirm the identity of this 37kDA protein, as well as other membrane proteins differentially expressed for characterization of the phenotype due to EsaR underexpression.

Essential TCS have been described in other bacteria that have regulatory functions related to cell envelope metabolism and homeostasis (Quon et al. 1996a, Fabret and Hoch 1998a, Zahrt and Deretic 2000, Dubrac and Msadek 2004a, Sun et al. 2013, Svensson et al. 2015). One of the most studied essential TCS, the WalKR system of *Staphylococcus aureus,* is highly conserved among low GC Gram-positive bacteria (such as *Bacillus subtilis, S. aureus, Enterococcus faecalis, Listeria monocytogenes, Streptococcus pneumoniae, and Streptococcus mutans*). The WalKR TCS and its homologs play a role in regulating metabolism, stress responses, virulence, host-pathogen interactions, transport, and other regulatory pathways

(Bem et al. 2015). The interest in this TCS is its essentiality and potential as an antimicrobial target to combat infection, as well as its putative role in antibiotic resistance in some species, such as *Staphylococcus aureus*. Other TCS have been associated with antimicrobial resistance such as PhoPQ in *Pseudomonas aeruginosa* and *Enterobacteriaceae* which plays a role in aminoglycoside, polymyxin and cationic antimicrobial peptide resistance (Macfarlane et al. 2000, Shi et al. 2004). Similarly PmrAB in *Acinetobacter baumannii, P. aeruginosa, K. pneumoniae,* and *Enterobacteriaceae* regulates changes in cell envelope to confers resistance to polymyxins and cationic antimicrobial peptides (Moskowitz et al. 2004, Beceiro et al. 2011, Harris et al. 2014). And finally VraRS in *Staphylococcus aureus* facilitates resistance to methicillin, vancomycin, and daptomycin (Gardete et al. 2006, Boyle-Vavra et al. 2013).

MIC testing of MKC2 and MKC4 suggest that the EsaSR TCS plays an important role in facilitating antimicrobial susceptibility of *B. cenocepacia*. Mutation of either *esaS* or *esaR* leads to an increase in antimicrobial susceptibility, with the *esaR* knockdown mutant MKC2 exhibiting a large increase in susceptibility to many different antimicrobial compounds of varying MOA (Figure 9). Hypersusceptibility to many different antimicrobials with different MOA helps reinforce the hypothesis that EsaR plays an important role in cell envelope maintenance and/or homeostasis. This hypersusceptibility could be caused by increased penetration of the antimicrobial molecules due to the compromised cell envelope nature resulting from EsaR depletion. MKC4 cells did not show a large difference from the wt in the live/dead assay; however, MKC4 exhibits increased susceptibility to some antimicrobials, suggesting EsaS could be involved in other pathways related to antimicrobial resistance aside from cell envelope integrity.

Another major mechanism of resistance to antimicrobials is associated with efflux pumps, which are energy-dependent systems that pump antimicrobials out of the cell to prevent interaction with their intended targets (Bazzini et al. 2011, Podnecky et al. 2015). Efflux pumps are able to pump out a wide variety of substrates including the antibiotics used to treat infections and are categorized into several different families. The resistance nodulation cell division (RND) efflux pumps are the most relevant to this study, as they have the ability to pump out many clinically relevant antibiotics (Bazzini et al. 2011, Tseng et al. 2014, Podnecky et al. 2015, Venter et al. 2015). *B. cenocepacia* has 16 annotated RND-efflux pumps in its genome (Bazzini et al. 2011). In comparison, ESKCAPE pathogens *Acinetobacter baumannii* and *Pseudomonas aeruginosa* are two prevalent, multi-drug resistant human pathogens which encode 7 and 10 RND-efflux pumps in their genomes, respectively (Stover et al. 2000, Coyne et al. 2011). Gislason et al. demonstrate that deletion of *esaS* leads to altered efflux activity in *B. cenocepacia* using a fluorescent assay (Gislason et al. 2017). qRT-PCR results of four select efflux pumps showed altered expression due to underexpression of EsaS or EsaR (Figures 10 and 12). The four efflux pumps (RND-3, RND-4, RND-9, and RND-10) were selected because of previous research describing efflux pump activity in *B. cenocepacia* (Buroni et al. 2009, 2014, Bazzini et al. 2011, Tseng et al. 2014). Interestingly the RND-efflux gene expression levels of MKC4 differ when cells are collected at 10hrs of incubation versus 13hrs of incubation (Figure 12). When looking at the growth kinetics in Figure 5, the 13hr time point is when MKC2 without rhamnose supplementation hits its peak OD_{600nm} and then declines. These time points offer an interesting subject of study, as investigating the differences in gene expression in this phase could help provide information as to what role EsaSR plays in growth and cell envelope

maintenance, as well as efflux pump expression and antimicrobial resistance. Many TCS have been demonstrated to regulate efflux pumps, such as the AdeRS TCS in *A. baumannii,* SmeRS in *Stenotrophomonas maltophilia*, CpxAR in *Klebsiella pneumoniae,* among others (Srinivasan et al. 2012, Yoon et al. 2013, Wu et al. 2016). The altered expression of the RND-efflux transporter genes differs between MKC4 and MKC2, suggesting that they each play a slightly different role when regulating RND-efflux expression. It is likely that the other signalling or regulation pathways are working cooperatively to regulate efflux pump expression in *B. cenocepacia*.

The qRT-PCR results seem contradictory as they suggest overexpression of RND transporters, which logically leads one to assume that there should be an accompanying increase in efflux activity and subsequent increase in MICs. However, the MIC ratio data does not show decreases in the susceptibility to antimicrobials, which is expected if there is overexpression of RND efflux systems. On the contrary, the MIC ratio data shows no change, or an increase in susceptibility to antimicrobial compounds of MKC4 and MKC2 in comparison to the wt. One possible explanation is the concept that modifications in levels of transcription do not necessarily lead to altered protein levels (Maier et al. 2011, Vogel and Marcotte 2012, Taylor et al. 2013). In general, the abundance of proteins can be correlated to the amounts of the respective mRNA; however, Vogel and Marcotte argue that it is not a strong correlation (Vogel and Marcotte 2012). Post-transcriptional processes such as differences in translational efficiency and regulation of protein degradation have been shown to have a larger net effect on the abundance of some proteins (Vogel and Marcotte 2012, Taylor et al. 2013). If there is indeed a higher abundance of some RND efflux systems, as suggested by the qRT-PCR results, it is possible that the uncontrolled overexpression of these systems is detrimental and leads to

changes in bacterial physiology (Sánchez et al. 2002, Alonso et al. 2004, Alvarez-Ortega et al. 2013). Either case represents an interesting possibility that warrants further research into the regulation of RND efflux systems by EsaSR. An "-omics" (such as transcriptomics and proteomics) approach would be helpful to further study the regulatory functions of this TCS, as evidence so far suggests that EsaSR plays a role in regulating a wide array of cellular functions. Further characterization of the EsaSR TCS would lead to a better understanding of the many resistance mechanisms intrinsic to *B. cenocepacia*, and provide a lucrative target for antimicrobial therapies.

In an age of "rising antibiotic resistance" or even what some are calling the "post-antibiotic era", pathogens are acquiring more and more mechanisms of resistance to the drugs being used to treat infection, and the problem is compounded by the decreasing number of new antibiotics being discovered and released onto the market for treatment (Gotoh et al. 2010, Silver 2011, Worthington et al. 2013, Brown and Wright 2016). The issue with drug discovery is the challenge of finding a novel mechanism to inhibit bacterial growth while at the same time avoiding existing resistance mechanisms. The targeting of TCS has been proposed as a method to overcoming resistance mechanisms such as efflux or enzymatic inactivation/modification of drugs (Gotoh et al. 2010, Worthington et al. 2013, Bem et al. 2015, Velikova and Wells 2016). Adjuvants are used to potentiate the action of currently available drugs and provide effective treatments for bacterial infection while at the same time limiting resistance (Worthington et al. 2013). Traditional adjuvants, such as β-lactamase inhibitors and efflux pump inhibitors, attempt to directly address the mechanism of resistance to allow effective treatment with available drugs (Worthington et al. 2013). However, there is immense

selection pressure on the pathogen when exposed to antimicrobials. Evolution has led to increasing prevalence of even more effective resistance mechanisms that are no longer impacted by the adjuvants, such as β-lactams that are not affected by the presence of β-lactamase inhibitory compounds. Thus, alternative approaches are needed, such as targeting the regulatory systems of bacteria to prevent or delay response to the presence of antibiotics. TCS have been studied as either antibacterial targets themselves, or as targets to reduce resistance and virulence of the pathogen (Watanabe et al. 2008, Okada et al. 2010, Gotoh et al. 2010, Harris et al. 2014). One of the most studied TCS as a potential target for small molecule treatment is the essential conserved HK WalK of the low GC Gram-positive bacteria, which includes pathogens such as *S. aureus* and *Bacillus subtilis* (Velikova et al. 2013). Several compounds have been shown to target WalK and inhibit its function, such as the family of compounds called walkmycins (Worthington et al. 2013, Bem et al. 2015). However, research into TCS inhibitors has been on-going for many years and there has yet to be a TCS inhibitor available for commercial use as an adjuvant in antibacterial therapies. This likely results because of the many challenges involved in identifying potential new drugs, as the compound must be proven efficacious against its intended target organism while minimizing the damage to the patient (Stephenson and Hoch 2004, Silver 2011, Brown and Wright 2016).

Chapter 5 – Concluding Remarks

With the ability of TCS to affect so many different cellular functions as well as the potential for inter-TCS communication/regulation, evidence suggests that EsaSR plays a central role in cellular growth, cell envelope biogenesis and/or homeostasis, as well as efflux. This makes EsaSR an interesting signalling system to study as underexpression of this TCS, especially the RR EsaR, leads to such dramatic changes in growth, morphology, as well as susceptibility to antimicrobial molecules. Further study of EsaSR would be valuable if not in its potential as an antimicrobial target, but also to explore the mechanisms of resistance and growth of *B. cenocepacia*, as not much is currently known about its intrinsic resistances. Using –omics approaches to study the effect of EsaSR depletion would be ideal as evidence so far suggests that this TCS has an extensive regulon, playing a role in growth, cell envelope integrity, efflux activity, as well as the viability of *B. cenocepacia* K56-2. Transcriptomics would allow a comprehensive look at the global gene expression changes associated with EsaSR depletion. Proteomics would allow a comprehensive look at the alterations in protein levels, particularly the cell envelope protein. And lastly, a lipidomic approach would reveal variations in the lipid profile providing a more comprehensive overview of how the cell envelope is impacted by EsaSR depletion. EsaSR represents an interesting and lucrative potential target to reduce the antibiotic resistance of members of the multi-drug resistant Bcc and render strains susceptible to currently available antibiotics, circumventing the problem of having to develop new drugs. Ideally, future research would work towards better understanding the regulon of EsaSR as well as screening for potential inhibitors to reduce the antibiotic resistance of *B. cenocepacia*, in

addition to other members of the Bcc. Such findings may lead to better therapeutic options for those stricken with Bcc infection.

References

Alonso, A., Morales, G., Escalante, R., Campanario, E., Sastre, L., and Martinez, J.L. 2004. Overexpression of the multidrug efflux pump SmeDEF impairs Stenotrophomonas maltophilia physiology. J. Antimicrob. Chemother. **53**(3): 432–434. doi:10.1093/jac/dkh074.

Alvarez-Ortega, C., Olivares, J., and Martínez, J.L. 2013. RND multidrug efflux pumps: what are they good for? Front. Microbiol. **4**. doi:10.3389/fmicb.2013.00007.

Alves, L.A., Harth-Chu, E.N., Palma, T.H., Stipp, R.N., Mariano, F.S., Höfling, J.F., Abranches, J., and Mattos-Graner, R.O. 2017. The two-component system VicRK regulates functions associated with Streptococcus mutans resistance to complement immunity. Mol. Oral Microbiol. doi:10.1111/omi.12183.

Baltrus, D.A., Amieva, M.R., Covacci, A., Lowe, T.M., Merrell, D.S., Ottemann, K.M., Stein, M., Salama, N.R., and Guillemin, K. 2009. The Complete Genome Sequence of Helicobacter pylori Strain G27. J. Bacteriol. **191**(1): 447–448. doi:10.1128/JB.01416-08.

Baugh, L., Gallagher, L.A., Patrapuvich, R., Clifton, M.C., Gardberg, A.S., Edwards, T.E., Armour, B., Begley, D.W., Dieterich, S.H., Dranow, D.M., Abendroth, J., Fairman, J.W., Iii, D.F., Staker, B.L., Phan, I., Gillespie, A., Choi, R., Nakazawa-Hewitt, S., Nguyen, M.T., Napuli, A., Barrett, L., Buchko, G.W., Stacy, R., Myler, P.J., Stewart, L.J., Manoil, C., and Voorhis, W.C.V. 2013. Combining Functional and Structural Genomics to Sample the Essential Burkholderia Structome. PLOS ONE **8**(1): e53851. doi:10.1371/journal.pone.0053851.

Bazzini, S., Udine, C., Sass, A., Pasca, M.R., Longo, F., Emiliani, G., Fondi, M., Perrin, E., Decorosi, F., Viti, C., Giovannetti, L., Leoni, L., Fani, R., Riccardi, G., Mahenthiralingam, E., and Buroni, S. 2011. Deciphering the Role of RND Efflux Transporters in Burkholderia cenocepacia. PLOS ONE **6**(4): e18902. doi:10.1371/journal.pone.0018902.

Beceiro, A., Llobet, E., Aranda, J., Bengoechea, J.A., Doumith, M., Hornsey, M., Dhanji, H., Chart, H., Bou, G., Livermore, D.M., and Woodford, N. 2011. Phosphoethanolamine Modification of Lipid A in Colistin-Resistant Variants of Acinetobacter baumannii Mediated by the pmrAB Two-Component Regulatory System▽. Antimicrob. Agents Chemother. **55**(7): 3370–3379. doi:10.1128/AAC.00079-11.

Bem, A.E., Velikova, N., Pellicer, M.T., Baarlen, P. van, Marina, A., and Wells, J.M. 2015. Bacterial Histidine Kinases as Novel Antibacterial Drug Targets. ACS Chem. Biol. **10**(1): 213–224. doi:10.1021/cb5007135.

Bevivino, A., Peggion, V., Chiarini, L., Tabacchioni, S., Cantale, C., and Dalmastri, C. 2005. Effect of Fusarium verticillioides on maize-root-associated Burkholderia cenocepacia populations. Res. Microbiol. **156**(10): 974–983. doi:10.1016/j.resmic.2005.05.007.

Bisicchia, P., Noone, D., Lioliou, E., Howell, A., Quigley, S., Jensen, T., Jarmer, H., and Devine, K.M. 2007. The essential YycFG two-component system controls cell wall metabolism in Bacillus subtilis. Mol. Microbiol. **65**(1): 180–200. doi:MMI5782 [pii].

Bloodworth, R.A.M., Gislason, A.S., and Cardona, S.T. 2013. Burkholderia cenocepacia conditional growth mutant library created by random promoter replacement of essential genes. MicrobiologyOpen **2**(2): 243–258. doi:10.1002/mbo3.71.

Borland, S., Oudart, A., Prigent-Combaret, C., Brochier-Armanet, C., and Wisniewski-Dyé, F. 2015. Genome-wide survey of two-component signal transduction systems in the plant growth-promoting bacterium Azospirillum. BMC Genomics **16**: 833. doi:10.1186/s12864-015-1962-x.

Boyle-Vavra, S., Yin, S., Jo, D.S., Montgomery, C.P., and Daum, R.S. 2013. VraT/YvqF Is Required for Methicillin Resistance and Activation of the VraSR Regulon in Staphylococcus aureus. Antimicrob. Agents Chemother. **57**(1): 83–95. doi:10.1128/AAC.01651-12.

Bretl, D.J., Bigley, T.M., Terhune, S.S., and Zahrt, T.C. 2014. The MprB extracytoplasmic domain negatively regulates activation of the Mycobacterium tuberculosis MprAB two-component system. J. Bacteriol. **196**(2): 391–406. doi:10.1128/JB.01064-13.

Breton, Y.L., Belew, A.T., Valdes, K.M., Islam, E., Curry, P., Tettelin, H., Shirtliff, M.E., El-Sayed, N.M., and McIver, K.S. 2015. Essential Genes in the Core Genome of the Human Pathogen *Streptococcus pyogenes*. Sci. Rep. **5**: srep09838. doi:10.1038/srep09838.

Brown, E.D., and Wright, G.D. 2016. Antibacterial drug discovery in the resistance era. Nature **529**(7586): 336–343. doi:10.1038/nature17042.

Burkholder, W.H. 1950. Sour skin, a bacterial rot of onion bulbs. Phytopathology **40**: 115–117.

Burns, J.L., Wadsworth, C.D., Barry, J.J., and Goodall, C.P. 1996. Nucleotide sequence analysis of a gene from Burkholderia (Pseudomonas) cepacia encoding an outer membrane lipoprotein involved in multiple antibiotic resistance. Antimicrob. Agents Chemother. **40**(2): 307–313.

Buroni, S., Matthijs, N., Spadaro, F., Van Acker, H., Scoffone, V.C., Pasca, M.R., Riccardi, G., and Coenye, T. 2014. Differential Roles of RND Efflux Pumps in Antimicrobial Drug Resistance of Sessile and Planktonic Burkholderia cenocepacia Cells. Antimicrob. Agents Chemother. **58**(12): 7424–7429. doi:10.1128/AAC.03800-14.

Buroni, S., Pasca, M.R., Flannagan, R.S., Bazzini, S., Milano, A., Bertani, I., Venturi, V., Valvano, M.A., and Riccardi, G. 2009. Assessment of three Resistance-Nodulation-Cell Division drug efflux transporters of Burkholderia cenocepacia in intrinsic antibiotic resistance. BMC Microbiol. **9**: 200. doi:10.1186/1471-2180-9-200.

Cardona, S.T., Selin, C., and Gislason, A.S. 2015. Genomic tools to profile antibiotic mode of action. Crit. Rev. Microbiol. **4**: 465–472. doi:10.3109/1040841X.2013.866073.

Cardona, S.T., and Valvano, M.A. 2005. An expression vector containing a rhamnose-inducible promoter provides tightly regulated gene expression in Burkholderia cenocepacia. Plasmid **54**(3): 219–228. doi:10.1016/j.plasmid.2005.03.004.

Chen, H., Xiong, Z., Liu, K., Li, S., Wang, R., Wang, X., Zhang, Y., and Wang, H. 2016. Transcriptional profiling of the two-component regulatory system VraSR in Staphylococcus aureus with low-level vancomycin resistance. Int. J. Antimicrob. Agents **47**(5): 362–367. doi:10.1016/j.ijantimicag.2016.02.003.

Chen, J., Li, S., Xu, B., Su, C., Jiang, Q., Zhou, C., Jin, Q., Zhao, Y., and Xiao, M. 2017. Characterization of Burkholderia sp. XTB-5 for Phenol Degradation and Plant Growth Promotion and Its Application in Bioremediation of Contaminated Soil. Land Degrad. Dev. **28**(3): 1091–1099. doi:10.1002/ldr.2646.

Chen, L. 2017. Notes from the Field: Pan-Resistant New Delhi Metallo-Beta-Lactamase-Producing Klebsiella pneumoniae — Washoe County, Nevada, 2016. MMWR Morb. Mortal. Wkly. Rep. **66**. doi:10.15585/mmwr.mm6601a7.

Cheng, A.T., Ottemann, K.M., and Yildiz, F.H. 2015. Vibrio cholerae Response Regulator VxrB Controls Colonization and Regulates the Type VI Secretion System. PLoS Pathog. **11**(5): e1004933. doi:10.1371/journal.ppat.1004933.

Chiarini, L., Bevivino, A., Dalmastri, C., Tabacchioni, S., and Visca, P. 2006. *Burkholderia cepacia* complex species: health hazards and biotechnological potential. Trends Microbiol. **14**(6): 277–286. doi:S0966-842X(06)00099-0 [pii]; 10.1016/j.tim.2006.04.006 [doi].

Christen, B., Abeliuk, E., Collier, J.M., Kalogeraki, V.S., Passarelli, B., Coller, J.A., Fero, M.J., McAdams, H.H., and Shapiro, L. 2011. The essential genome of a bacterium. Mol. Syst. Biol. **7**: 528. doi:10.1038/msb.2011.58.

Christiansen, M.T., Kaas, R.S., Chaudhuri, R.R., Holmes, M.A., Hasman, H., and Aarestrup, F.M. 2014. Genome-Wide High-Throughput Screening to Investigate Essential Genes Involved in Methicillin-Resistant Staphylococcus aureus Sequence Type 398 Survival. PLOS ONE **9**(2): e89018. doi:10.1371/journal.pone.0089018.

Coenye, T., and Vandamme, P. 2003. Diversity and significance of Burkholderia species occupying diverse ecological niches. Environ. Microbiol. **5**(9): 719–729. doi:10.1046/j.1462-2920.2003.00471.x.

Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M.A., Rajandream, M.-A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J.E., Taylor, K., Whitehead, S., and Barrell, B.G. 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature **393**(6685): 537–544. doi:10.1038/31159.

Coyne, S., Courvalin, P., and Périchon, B. 2011. Efflux-Mediated Antibiotic Resistance in Acinetobacter spp. Antimicrob. Agents Chemother. **55**(3): 947–953. doi:10.1128/AAC.01388-10.

Dance, D.A.B. 2000. Ecology of Burkholderia pseudomallei and the interactions between environmental Burkholderia spp. and human–animal hosts. Acta Trop. **74**(2): 159–168. doi:10.1016/S0001-706X(99)00066-2.

Delaune, A., Poupel, O., Mallet, A., Coic, Y.M., Msadek, T., and Dubrac, S. 2011. Peptidoglycan crosslinking relaxation plays an important role in *Staphylococcus aureus* WalKR-dependent cell viability. PloS One **6**(2): e17054. doi:10.1371/journal.pone.0017054 [doi].

DeVito, J.A., Mills, J.A., Liu, V.G., Agarwal, A., Sizemore, C.F., Yao, Z., Stoughton, D.M., Cappiello, M.G., Barbosa, M.D.F.S., Foster, L.A., and Pompliano, D.L. 2002. An array of target-specific screening strains for antibacterial discovery. Nat. Biotechnol. **20**(5): 478–483. doi:10.1038/nbt0502-478.

Dorr, T., Alvarez, L., Delgado, F., Davis, B.M., Cava, F., and Waldor, M.K. 2016. A cell wall damage response mediated by a sensor kinase/response regulator pair enables beta-

lactam tolerance. Proc. Natl. Acad. Sci. U. S. A. **113**(2): 404–409. doi:10.1073/pnas.1520333113 [doi].

Drevinek, P., and Mahenthiralingam, E. 2010. Burkholderia cenocepacia in cystic fibrosis: epidemiology and molecular mechanisms of virulence. Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis. **16**(7): 821–830. doi:10.1111/j.1469-0691.2010.03237.x.

Dubrac, S., Bisicchia, P., Devine, K.M., and Msadek, T. 2008. A matter of life and death: cell wall homeostasis and the WalKR (YycGF) essential signal transduction pathway. Mol. Microbiol. **70**(6): 1307–1322. doi:10.1111/j.1365-2958.2008.06483.x [doi].

Dubrac, S., Boneca, I.G., Poupel, O., and Msadek, T. 2007. New insights into the WalK/WalR (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell wall metabolism and biofilm formation in Staphylococcus aureus. J. Bacteriol. **189**(22): 8257–8269. doi:JB.00645-07 [pii].

Dubrac, S., and Msadek, T. 2004a. Identification of genes controlled by the essential YycG/YycF two-component system of Staphylococcus aureus. J. Bacteriol. **186**(4): 1175–1181.

Dubrac, S., and Msadek, T. 2004b. Identification of genes controlled by the essential YycG/YycF two-component system of Staphylococcus aureus. J. Bacteriol. **186**(4): 1175–1181.

Duque, C., Stipp, R.N., Wang, B., Smith, D.J., Höfling, J.F., Kuramitsu, H.K., Duncan, M.J., and Mattos-Graner, R.O. 2011. Downregulation of GbpB, a component of the VicRK regulon, affects biofilm formation and cell surface characteristics of Streptococcus mutans. Infect. Immun. **79**(2): 786–796. doi:10.1128/IAI.00725-10.

Eberl, L., and Vandamme, P. 2016. Members of the genus Burkholderia: good and bad guys. F1000Research **5**: 1007. doi:10.12688/f1000research.8221.1.

Fabret, C., and Hoch, J.A. 1998a. A two-component signal transduction system essential for growth of Bacillus subtilis: implications for anti-infective therapy. J. Bacteriol. **180**(23): 6375–6383.

Fabret, C., and Hoch, J.A. 1998b. A two-component signal transduction system essential for growth of Bacillus subtilis: implications for anti-infective therapy. J. Bacteriol. **180**(23): 6375–6383.

Farizano, J.V., Pescaretti, M. de las M., López, F.E., Hsu, F.-F., and Delgado, M.A. 2012. The PmrAB system-inducing conditions control both lipid A remodeling and O-antigen length distribution, influencing the Salmonella Typhimurium-host interactions. J. Biol. Chem. **287**(46): 38778–38789. doi:10.1074/jbc.M112.397414.

Ferretti, J.J., McShan, W.M., Ajdic, D., Savic, D.J., Savic, G., Lyon, K., Primeaux, C., Sezate, S., Suvorov, A.N., Kenton, S., Lai, H.S., Lin, S.P., Qian, Y., Jia, H.G., Najar, F.Z., Ren, Q., Zhu, H., Song, L., White, J., Yuan, X., Clifton, S.W., Roe, B.A., and McLaughlin, R. 2001. Complete genome sequence of an M1 strain of Streptococcus pyogenes. Proc. Natl. Acad. Sci. **98**(8): 4658–4663. doi:10.1073/pnas.071559398.

Figurski, D.H., and Helinski, D.R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. U. S. A. **76**(4): 1648–1652.

Flannagan, R.S., Linn, T., and Valvano, M.A. 2008. A system for the construction of targeted unmarked gene deletions in the genus Burkholderia. Environ. Microbiol. **10**(6): 1652–1660. doi:10.1111/j.1462-2920.2008.01576.x.

Folescu, T.W., da Costa, C.H., Cohen, R.W.F., Neto, O.C. da C., Albano, R.M., and Marques, E.A. 2015. Burkholderia cepacia complex: clinical course in cystic fibrosis patients. BMC Pulm. Med. **15**. doi:10.1186/s12890-015-0148-2.

Fritsch, F., Mauder, N., Williams, T., Weiser, J., Oberle, M., and Beier, D. 2011. The cell envelope stress response mediated by the LiaFSRLm three-component system of Listeria monocytogenes is controlled via the phosphatase activity of the bifunctional histidine kinase LiaSLm. Microbiology **157**(2): 373–386. doi:10.1099/mic.0.044776-0.

Fukuchi, K., Kasahara, Y., Asai, K., Kobayashi, K., Moriya, S., and Ogasawara, N. 2000. The essential two-component regulatory system encoded by yycF and yycG modulates expression of the ftsAZ operon in Bacillus subtilis. Microbiol. Read. Engl. **146 (Pt 7)**(Pt 7): 1573–1583. doi:10.1099/00221287-146-7-1573 [doi].

Gao, B., Lara-Tejero, M., Lefebre, M., Goodman, A.L., and Galán, J.E. 2014. Novel Components of the Flagellar System in Epsilonproteobacteria. mBio **5**(3): e01349-14. doi:10.1128/mBio.01349-14.

Gardete, S., Wu, S.W., Gill, S., and Tomasz, A. 2006. Role of VraSR in antibiotic resistance and antibiotic-induced stress response in Staphylococcus aureus. Antimicrob. Agents Chemother. **50**(10): 3424–3434. doi:10.1128/AAC.00356-06.

Gislason, A.S., Choy, M., Bloodworth, R.A.M., Qu, W., Stietz, M.S., Li, X., Zhang, C., and Cardona, S.T. 2017. Competitive Growth Enhances Conditional Growth Mutant Sensitivity to Antibiotics and Exposes a Two-Component System as an Emerging Antibacterial Target in *Burkholderia cenocepacia*. Antimicrob. Agents Chemother. **61**(1). doi:10.1128/AAC.00790-16.

Golding, G.R., Bryden, L., Levett, P.N., McDonald, R.R., Wong, A., Graham, M.R., Tyler, S., Domselaar, G.V., Mabon, P., Kent, H., Butaye, P., Smith, T.C., Kadlec, K., Schwarz, S., Weese, S.J., and Mulvey, M.R. 2012. Whole-Genome Sequence of Livestock-Associated ST398 Methicillin-Resistant Staphylococcus aureus Isolated from Humans in Canada. J. Bacteriol. **194**(23): 6627–6628. doi:10.1128/JB.01680-12.

Gooderham, W.J., and Hancock, R.E.W. 2009. Regulation of virulence and antibiotic resistance by two-component regulatory systems in Pseudomonas aeruginosa. FEMS Microbiol. Rev. **33**(2): 279–294. doi:10.1111/j.1574-6976.2008.00135.x.

Gotoh, Y., Eguchi, Y., Watanabe, T., Okamoto, S., Doi, A., and Utsumi, R. 2010. Two-component signal transduction as potential drug targets in pathogenic bacteria. Curr. Opin. Microbiol. **13**(2): 232–239. doi:10.1016/j.mib.2010.01.008; 10.1016/j.mib.2010.01.008.

Gottschalk, S., Bygebjerg-Hove, I., Bonde, M., Nielsen, P.K., Nguyen, T.H., Gravesen, A., and Kallipolitis, B.H. 2008. The two-component system CesRK controls the transcriptional induction of cell envelope-related genes in Listeria monocytogenes in response to cell wall-acting antibiotics. J. Bacteriol. **190**(13): 4772–4776. doi:10.1128/JB.00015-08.

Guest, R.L., and Raivio, T.L. 2016. Role of the Gram-Negative Envelope Stress Response in the Presence of Antimicrobial Agents. Trends Microbiol. **24**(5): 377–390. doi:http://dx.doi.org/10.1016/j.tim.2016.03.001.

Guglierame, P., Pasca, M.R., De Rossi, E., Buroni, S., Arrigo, P., Manina, G., and Riccardi, G. 2006. Efflux pump genes of the resistance-nodulation-division family in Burkholderia cenocepacia genome. BMC Microbiol. **6**: 1–14. doi:10.1186/1471-2180-6-66.

Hallez, R., Mignolet, J., Mullem, V.V., Wery, M., Vandenhaute, J., Letesson, J.-J., Jacobs-Wagner, C., and Bolle, X.D. 2007. The asymmetric distribution of the essential histidine kinase PdhS indicates a differentiation event in *Brucella abortus*. EMBO J. **26**(5): 1444–1455. doi:10.1038/sj.emboj.7601577.

Hamid, S., Amin, M.T., Alazba, A.A., and Manzoor, U. 2014. Simultaneous Degradation of Trichloroethylene and Toluene by Burkholderia cepacia G4 and the Effect of Biotransformation on Bacterial Density. CLEAN – Soil Air Water **42**(7): 998–1003. doi:10.1002/clen.201300201.

Hardt, P., Engels, I., Rausch, M., Gajdiss, M., Ulm, H., Sass, P., Ohlsen, K., Sahl, H.-G., Bierbaum, G., Schneider, T., and Grein, F. 2017. The cell wall precursor lipid II acts as a molecular signal for the Ser/Thr kinase PknB of Staphylococcus aureus. Int. J. Med. Microbiol. IJMM **307**(1): 1–10. doi:10.1016/j.ijmm.2016.12.001.

Harris, T.L., Worthington, R.J., Hittle, L.E., Zurawski, D.V., Ernst, R.K., and Melander, C. 2014. Small molecule downregulation of PmrAB reverses lipid A modification and breaks colistin resistance. ACS Chem. Biol. **9**(1): 122–127. doi:10.1021/cb400490k.

Held, M.R., Begier, E.M., Beardsley, D.S., Browne, F.A., Martinello, R.A., Baltimore, R.S., McDonald, L.C., Jensen, B., Hadler, J.L., and Dembry, L.-M. 2006. Life-Threatening Sepsis Caused by Burkholderia cepacia From Contaminated Intravenous Flush Solutions Prepared by a Compounding Pharmacy in Another State. Pediatrics **118**(1): e212–e215. doi:10.1542/peds.2005-2617.

Henst, C.V. der, Beaufay, F., Mignolet, J., Didembourg, C., Colinet, J., Hallet, B., Letesson, J.-J., and Bolle, X.D. 2012. The Histidine Kinase PdhS Controls Cell Cycle Progression of the Pathogenic Alphaproteobacterium Brucella abortus. J. Bacteriol. **194**(19): 5305–5314. doi:10.1128/JB.00699-12.

Hirakawa, H., Nishino, K., Hirata, T., and Yamaguchi, A. 2003. Comprehensive studies of drug resistance mediated by overexpression of response regulators of two-component signal transduction systems in Escherichia coli. J. Bacteriol. **185**(6): 1851–1856.

Holden, M.T., Titball, R.W., Peacock, S.J., Cerdeno-Tarraga, A.M., Atkins, T., Crossman, L.C., Pitt, T., Churcher, C., Mungall, K., Bentley, S.D., Sebaihia, M., Thomson, N.R., Bason, N., Beacham, I.R., Brooks, K., Brown, K.A., Brown, N.F., Challis, G.L., Cherevach, I., Chillingworth, T., Cronin, A., Crossett, B., Davis, P., DeShazer, D., Feltwell, T., Fraser, A., Hance, Z., Hauser, H., Holroyd, S., Jagels, K., Keith, K.E., Maddison, M., Moule, S., Price, C., Quail, M.A., Rabbinowitsch, E., Rutherford, K., Sanders, M., Simmonds, M., Songsivilai, S., Stevens, K., Tumapa, S., Vesaratchavest, M., Whitehead, S., Yeats, C., Barrell, B.G., Oyston, P.C., and Parkhill, J. 2004. Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. Proc. Natl. Acad. Sci. U. S. A. **101**(39): 14240–14245. doi:10.1073/pnas.0403302101.

Holden, M.T.G., Seth-Smith, H.M.B., Crossman, L.C., Sebaihia, M., Bentley, S.D., Cerdeño-Tárraga, A.M., Thomson, N.R., Bason, N., Quail, M.A., Sharp, S., Cherevach, I., Churcher, C., Goodhead, I., Hauser, H., Holroyd, N., Mungall, K., Scott, P., Walker, D., White, B., Rose, H., Iversen, P., Mil-Homens, D., Rocha, E.P.C., Fialho, A.M., Baldwin, A., Dowson, C., Barrell, B.G., Govan, J.R., Vandamme, P., Hart, C.A., Mahenthiralingam, E., and Parkhill, J. 2009. The Genome of Burkholderia cenocepacia J2315, an Epidemic Pathogen of Cystic Fibrosis Patients. J. Bacteriol. **191**(1): 261–277. doi:10.1128/JB.01230-08.

Hong, H.-J., Hutchings, M.I., Buttner, M.J., and Biotechnology and Biological Sciences Research Council, UK. 2008. Vancomycin resistance VanS/VanR two-component systems. Adv. Exp. Med. Biol. **631**: 200–213.

Hooven, T.A., Catomeris, A.J., Akabas, L.H., Randis, T.M., Maskell, D.J., Peters, S.E., Ott, S., Santana-Cruz, I., Tallon, L.J., Tettelin, H., and Ratner, A.J. 2016. The essential genome of Streptococcus agalactiae. BMC Genomics **17**. doi:10.1186/s12864-016-2741-z.

Hutchings, M.I., Hong, H.-J., and Buttner, M.J. 2006. The vancomycin resistance VanRS two-component signal transduction system of Streptomyces coelicolor. Mol. Microbiol. **59**(3): 923–935. doi:10.1111/j.1365-2958.2005.04953.x.

Isles, A., Maclusky, I., Corey, M., Gold, R., Prober, C., Fleming, P., and Levison, H. 1984. Pseudomonas cepacia infection in cystic fibrosis: an emerging problem. J. Pediatr. **104**(2): 206–210.

Jacobs, C., Domian, I.J., Maddock, J.R., and Shapiro, L. 1999. Cell cycle-dependent polar localization of an essential bacterial histidine kinase that controls DNA replication and cell division. Cell **97**(1): 111–120.

Jansen, A., Türck, M., Szekat, C., Nagel, M., Clever, I., and Bierbaum, G. 2007. Role of insertion elements and yycFG in the development of decreased susceptibility to vancomycin in Staphylococcus aureus. Int. J. Med. Microbiol. IJMM **297**(4): 205–215. doi:10.1016/j.ijmm.2007.02.002.

Jones, A., Dodd, M., Govan, J., Barcus, V., Doherty, C., Morris, J., and Webb, A. 2004. Burkholderia cenocepacia and Burkholderia multivorans: influence on survival in cystic fibrosis. Thorax **59**(11): 948–951. doi:10.1136/thx.2003.017210.

Khatiwara, A., Jiang, T., Sung, S.-S., Dawoud, T., Kim, J.N., Bhattacharya, D., Kim, H.-B., Ricke, S.C., and Kwon, Y.M. 2012. Genome Scanning for Conditionally Essential Genes in Salmonella enterica Serotype Typhimurium. Appl. Environ. Microbiol. **78**(9): 3098–3107. doi:10.1128/AEM.06865-11.

Klein, B.A., Tenorio, E.L., Lazinski, D.W., Camilli, A., Duncan, M.J., and Hu, L.T. 2012. Identification of essential genes of the periodontal pathogen Porphyromonas gingivalis. BMC Genomics **13**: 578. doi:10.1186/1471-2164-13-578.

Kolar, S.L., Nagarajan, V., Oszmiana, A., Rivera, F.E., Miller, H.K., Davenport, J.E., Riordan, J.T., Potempa, J., Barber, D.S., Koziel, J., Elasri, M.O., and Shaw, L.N. 2011. NsaRS is a cell-envelope-stress-sensing two-component system of Staphylococcus aureus. Microbiol. Read. Engl. **157**(Pt 8): 2206–2219. doi:10.1099/mic.0.049692-0.

Kuroda, M., Kuroda, H., Oshima, T., Takeuchi, F., Mori, H., and Hiramatsu, K. 2003. Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in Staphylococcus aureus. Mol. Microbiol. **49**(3): 807–821.

Larimer, F.W., Chain, P., Hauser, L., Lamerdin, J., Malfatti, S., Do, L., Land, M.L., Pelletier, D.A., Beatty, J.T., Lang, A.S., Tabita, F.R., Gibson, J.L., Hanson, T.E., Bobst, C., Torres, J.L.T. y, Peres, C., Harrison, F.H., Gibson, J., and Harwood, C.S. 2004. Complete genome sequence of the metabolically versatile photosynthetic bacterium Rhodopseudomonas palustris. Nat. Biotechnol. **22**(1): 55–61. doi:10.1038/nbt923.

Laub, M.T., Chen, S.L., Shapiro, L., and McAdams, H.H. 2002. Genes directly controlled by CtrA, a master regulator of the Caulobacter cell cycle. Proc. Natl. Acad. Sci. U. S. A. **99**(7): 4632–4637. doi:10.1073/pnas.062065699.

Li, L., Wang, Q., Zhang, H., Yang, M., Khan, M.I., and Zhou, X. 2016. Sensor histidine kinase is a beta-lactam receptor and induces resistance to beta-lactam antibiotics. Proc. Natl. Acad. Sci. U. S. A. **113**(6): 1648–1653. doi:10.1073/pnas.1520300113 [doi].

Li, W., Roberts, D.P., Dery, P.D., Meyer, S.L.F., Lohrke, S., Lumsden, R.D., and Hebbar, K.P. 2002. Broad spectrum anti-biotic activity and disease suppression by the potential biocontrol agent Burkholderia ambifaria BC-F. Crop Prot. **21**(2): 129–135. doi:10.1016/S0261-2194(01)00074-6.

Livak, K.J., and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods San Diego Calif **25**(4): 402–408. doi:10.1006/meth.2001.1262.

Loutet, S.A., and Valvano, M.A. 2010. A Decade of Burkholderia cenocepacia Virulence Determinant Research. Infect. Immun. **78**(10): 4088–4100. doi:10.1128/IAI.00212-10.

Luciani, A., Villella, V.R., Esposito, S., Brunetti-Pierri, N., Medina, D.L., Settembre, C., Gavina, M., Raia, V., Ballabio, A., and Maiuri, L. 2011. Cystic fibrosis: a disorder with defective autophagy. Autophagy **7**(1): 104–106.

Macfarlane, E.L.A., Kwasnicka, A., and Hancock, R.E.W. 2000. Role of Pseudomonas aeruginosa PhoP-PhoQ in resistance to antimicrobial cationic peptides and aminoglycosides. Microbiology **146**(10): 2543–2554. doi:10.1099/00221287-146-10-2543.

Mahenthiralingam, E., Coenye, T., Chung, J.W., Speert, D.P., Govan, J.R.W., Taylor, P., and Vandamme, P. 2000. Diagnostically and Experimentally Useful Panel of Strains from the Burkholderia cepacia Complex. J. Clin. Microbiol. **38**(2): 910–913.

Mahenthiralingam, E., Urban, T.A., and Goldberg, J.B. 2005. The multifarious, multireplicon *Burkholderia cepacia* complex. Nat. Rev. **3**(2): 144–156.

Maier, T., Schmidt, A., Güell, M., Kühner, S., Gavin, A.-C., Aebersold, R., and Serrano, L. 2011. Quantification of mRNA and protein and integration with protein turnover in a bacterium. Mol. Syst. Biol. **7**: 511. doi:10.1038/msb.2011.38.

Marchand, I., Damier-Piolle, L., Courvalin, P., and Lambert, T. 2004. Expression of the RND-type efflux pump AdeABC in Acinetobacter baumannii is regulated by the AdeRS two-component system. Antimicrob. Agents Chemother. **48**(9): 3298–3304. doi:10.1128/AAC.48.9.3298-3304.2004.

Marigliano, A., D'Errico, M.M., Pellegrini, I., Savini, S., Prospero, E., and Barbadoro, P. 2010. Ultrasound echocardiographic gel contamination by Burkholderia cepacia in an Italian hospital. J. Hosp. Infect. **76**(4): 360–361. doi:10.1016/j.jhin.2010.07.017.

Martin, M., Christiansen, B., Caspari, G., Hogardt, M., von Thomsen, A.J., Ott, E., and Mattner, F. 2011. Hospital-wide outbreak of Burkholderia contaminans caused by prefabricated moist washcloths. J. Hosp. Infect. **77**(3): 267–270. doi:10.1016/j.jhin.2010.10.004.

Martin, P.K., Li, T., Sun, D., Biek, D.P., and Schmid, M.B. 1999. Role in cell permeability of an essential two-component system in Staphylococcus aureus. J. Bacteriol. **181**(12): 3666–3673.

Martínez, B., Zomer, A.L., Rodríguez, A., Kok, J., and Kuipers, O.P. 2007. Cell envelope stress induced by the bacteriocin Lcn972 is sensed by the Lactococcal two-component system CesSR. Mol. Microbiol. **64**(2): 473–486. doi:10.1111/j.1365-2958.2007.05668.x.

Martinez, J.L., and Rojo, F. 2011. Metabolic regulation of antibiotic resistance. FEMS Microbiol. Rev. **35**(5): 768–789. doi:10.1111/j.1574-6976.2011.00282.x;

Mavrianos, J., Berkow, E.L., Desai, C., Pandey, A., Batish, M., Rabadi, M.J., Barker, K.S., Pain, D., Rogers, P.D., Eugenin, E.A., and Chauhan, N. 2013. Mitochondrial Two-Component Signaling Systems in Candida albicans. Eukaryot. Cell **12**(6): 913–922. doi:10.1128/EC.00048-13.

Miller, V.L., and Mekalanos, J.J. 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in Vibrio cholerae requires toxR. J. Bacteriol. **170**(6): 2575–2583.

Möker, N., Brocker, M., Schaffer, S., Krämer, R., Morbach, S., and Bott, M. 2004. Deletion of the genes encoding the MtrA–MtrB two-component system of Corynebacterium glutamicum has a strong influence on cell morphology, antibiotics susceptibility and expression of genes involved in osmoprotection. Mol. Microbiol. **54**(2): 420–438. doi:10.1111/j.1365-2958.2004.04249.x.

Moskowitz, S.M., Ernst, R.K., and Miller, S.I. 2004. PmrAB, a Two-Component Regulatory System of Pseudomonas aeruginosa That Modulates Resistance to Cationic Antimicrobial Peptides and Addition of Aminoarabinose to Lipid A. J. Bacteriol. **186**(2): 575–579. doi:10.1128/JB.186.2.575-579.2004.

Moule, M.G., Hemsley, C.M., Seet, Q., Guerra-Assunção, J.A., Lim, J., Sarkar-Tyson, M., Clark, T.G., Tan, P.B.O., Titball, R.W., Cuccui, J., and Wren, B.W. 2014. Genome-wide saturation mutagenesis of *Burkholderia pseudomallei* K96243 predicts essential genes and novel targets for antimicrobial development. mBio **5**(1): e00926-913. doi:10.1128/mBio.00926-13.

Muthaiyan, A., Silverman, J.A., Jayaswal, R.K., and Wilkinson, B.J. 2008. Transcriptional profiling reveals that daptomycin induces the Staphylococcus aureus cell wall stress stimulon and genes responsive to membrane depolarization. Antimicrob. Agents Chemother. **52**(3): 980–990. doi:10.1128/AAC.01121-07.

Naveed, M., Hussain, M.B., Zahir, Z.A., Mitter, B., and Sessitsch, A. 2014. Drought stress amelioration in wheat through inoculation with Burkholderia phytofirmans strain PsJN. Plant Growth Regul. **73**(2): 121–131. doi:10.1007/s10725-013-9874-8.

Ng, W.-L., Kazmierczak, K.M., and Winkler, M.E. 2004. Defective cell wall synthesis in Streptococcus pneumoniae R6 depleted for the essential PcsB putative murein hydrolase or the VicR (YycF) response regulator. Mol. Microbiol. **53**(4): 1161–1175. doi:10.1111/j.1365-2958.2004.04196.x.

Ng, W.-L., Robertson, G.T., Kazmierczak, K.M., Zhao, J., Gilmour, R., and Winkler, M.E. 2003. Constitutive expression of PcsB suppresses the requirement for the essential VicR (YycF) response regulator in Streptococcus pneumoniae R6. Mol. Microbiol. **50**(5): 1647–1663.

Okada, A., Igarashi, M., Okajima, T., Kinoshita, N., Umekita, M., Sawa, R., Inoue, K., Watanabe, T., Doi, A., Martin, A., Quinn, J., Nishimura, Y., and Utsumi, R. 2010. Walkmycin B targets WalK (YycG), a histidine kinase essential for bacterial cell growth. J. Antibiot. (Tokyo) **63**(2): 89–94. doi:10.1038/ja.2009.128.

Ortega, X.P., Cardona, S.T., Brown, A.R., Loutet, S.A., Flannagan, R.S., Campopiano, D.J., Govan, J.R.W., and Valvano, M.A. 2007. A Putative Gene Cluster for Aminoarabinose Biosynthesis Is Essential for Burkholderia cenocepacia Viability. J. Bacteriol. **189**(9): 3639–3644. doi:10.1128/JB.00153-07.

Pechter, K.B., Gallagher, L., Pyles, H., Manoil, C.S., and Harwood, C.S. 2016. Essential Genome of the Metabolically Versatile Alphaproteobacterium Rhodopseudomonas palustris. J. Bacteriol. **198**(5): 867–876. doi:10.1128/JB.00771-15.

Pedrosa, F.O., Monteiro, R.A., Wassem, R., Cruz, L.M., Ayub, R.A., Colauto, N.B., Fernandez, M.A., Fungaro, M.H.P., Grisard, E.C., Hungria, M., Madeira, H.M.F., Nodari, R.O., Osaku, C.A., Petzl-Erler, M.L., Terenzi, H., Vieira, L.G.E., Steffens, M.B.R., Weiss, V.A., Pereira, L.F.P., Almeida, M.I.M., Alves, L.R., Marin, A., Araujo, L.M., Balsanelli, E., Baura, V.A., Chubatsu, L.S., Faoro, H., Favetti, A., Friedermann, G., Glienke, C., Karp, S., Kava-Cordeiro, V., Raittz, R.T., Ramos, H.J.O., Ribeiro, E.M.S.F., Rigo, L.U., Rocha, S.N., Schwab, S., Silva, A.G., Souza, E.M., Tadra-Sfeir, M.Z., Torres, R.A., Dabul, A.N.G., Soares, M.A.M., Gasques, L.S., Gimenes, C.C.T., Valle, J.S., Ciferri, R.R., Correa, L.C., Murace, N.K., Pamphile, J.A., Patussi, E.V., Prioli, A.J., Prioli, S.M.A., Rocha, C.L.M.S.C., Arantes, O.M.N., Furlaneto, M.C., Godoy, L.P., Oliveira, C.E.C., Satori, D., Vilas-Boas, L.A., Watanabe, M.A.E., Dambros, B.P., Guerra, M.P., Mathioni, S.M., Santos, K.L., Steindel, M., Vernal, J., Barcellos, F.G., Campo, R.J., Chueire, L.M.O., Nicolás, M.F., Pereira-Ferrari, L., Silva, J.L. da C., Gioppo, N.M.R., Margarido, V.P., Menck-Soares, M.A., Pinto, F.G.S., Simão, R. de C.G., Takahashi, E.K., Yates, M.G., and Souza, E.M. 2011. Genome of Herbaspirillum seropedicae Strain SmR1, a Specialized Diazotrophic Endophyte of Tropical Grasses. PLOS Genet. **7**(5): e1002064. doi:10.1371/journal.pgen.1002064.

Perry, B.J., Akter, M.S., and Yost, C.K. 2016. The Use of Transposon Insertion Sequencing to Interrogate the Core Functional Genome of the Legume Symbiont Rhizobium leguminosarum. Front. Microbiol. **7**: 1873. doi:10.3389/fmicb.2016.01873.

Pinedo, I., Ledger, T., Greve, M., and Poupin, M.J. 2015. Burkholderia phytofirmans PsJN induces long-term metabolic and transcriptional changes involved in Arabidopsis thaliana salt tolerance. Front. Plant Sci. **6**: 466. doi:10.3389/fpls.2015.00466.

Plocinska, R., Purushotham, G., Sarva, K., Vadrevu, I.S., Pandeeti, E.V.P., Arora, N., Plocinski, P., Madiraju, M.V., and Rajagopalan, M. 2012. Septal localization of the Mycobacterium tuberculosis MtrB sensor kinase promotes MtrA regulon expression. J. Biol. Chem. **287**(28): 23887–23899. doi:10.1074/jbc.M112.346544.

Podnecky, N.L., Rhodes, K.A., and Schweizer, H.P. 2015. Efflux pump-mediated drug resistance in Burkholderia. Front. Microbiol. **6**. doi:10.3389/fmicb.2015.00305.

Pogliano, J., Lynch, A.S., Belin, D., Lin, E.C., and Beckwith, J. 1997. Regulation of Escherichia coli cell envelope proteins involved in protein folding and degradation by the Cpx two-component system. Genes Dev. **11**(9): 1169–1182.

Pope, C.E., Short, P., and Carter, P.E. 2010. Species distribution of Burkholderia cepacia complex isolates in cystic fibrosis and non-cystic fibrosis patients in New Zealand. J. Cyst. Fibros. **9**(6): 442–446. doi:10.1016/j.jcf.2010.08.011.

Powell, J.E., Leonard, S.P., Kwong, W.K., Engel, P., and Moran, N.A. 2016. Genome-wide screen identifies host colonization determinants in a bacterial gut symbiont. Proc. Natl. Acad. Sci. **113**(48): 13887–13892. doi:10.1073/pnas.1610856113.

Pritchett, C.L., Little, A.S., Okkotsu, Y., Frisk, A., Cody, W.L., Covey, C.R., and Schurr, M.J. 2015. Expression analysis of the Pseudomonas aeruginosa AlgZR two-component regulatory system. J. Bacteriol. **197**(4): 736–748. doi:10.1128/JB.02290-14 [doi].

Purushotham, G., Sarva, K.B., Blaszczyk, E., Rajagopalan, M., and Madiraju, M.V. 2015. Mycobacterium tuberculosis oriC sequestration by MtrA response regulator. Mol. Microbiol. **98**(3): 586–604. doi:10.1111/mmi.13144.

Quon, K.C., Marczynski, G.T., and Shapiro, L. 1996a. Cell cycle control by an essential bacterial two-component signal transduction protein. Cell **84**(1): 83–93. doi:S0092-8674(00)80995-2 [pii].

Quon, K.C., Marczynski, G.T., and Shapiro, L. 1996b. Cell cycle control by an essential bacterial two-component signal transduction protein. Cell **84**(1): 83–93. doi:S0092-8674(00)80995-2 [pii].

Raivio, T.L., and Silhavy, T.J. 1997. Transduction of envelope stress in Escherichia coli by the Cpx two-component system. J. Bacteriol. **179**(24): 7724–7733.

Ratjen, F., and Döring, G. 2003. Cystic fibrosis. Lancet Lond. Engl. **361**(9358): 681–689. doi:10.1016/S0140-6736(03)12567-6.

Reik, R., Spilker, T., and LiPuma, J.J. 2005. Distribution of Burkholderia cepacia Complex Species among Isolates Recovered from Persons with or without Cystic Fibrosis. J. Clin. Microbiol. **43**(6): 2926–2928. doi:10.1128/JCM.43.6.2926-2928.2005.

Remmele, C.W., Xian, Y., Albrecht, M., Faulstich, M., Fraunholz, M., Heinrichs, E., Dittrich, M.T., Müller, T., Reinhardt, R., and Rudel, T. 2014. Transcriptional landscape and essential genes of Neisseria gonorrhoeae. Nucleic Acids Res. **42**(16): 10579–10595. doi:10.1093/nar/gku762.

Resch, U., Tsatsaronis, J.A., Rhun, A.L., Stübiger, G., Rohde, M., Kasvandik, S., Holzmeister, S., Tinnefeld, P., Wai, S.N., and Charpentier, E. 2016. A Two-Component Regulatory System Impacts Extracellular Membrane-Derived Vesicle Production in Group A Streptococcus. mBio **7**(6): e00207-16. doi:10.1128/mBio.00207-16.

Rosconi, F., Vries, S.P.W. de, Baig, A., Fabiano, E., and Grant, A.J. 2016. Essential genes for in vitro growth of the endophyte Herbaspirillum seropedicae SmR1 revealed by transposon insertion sequencing. Appl. Environ. Microbiol.: AEM.02281-16. doi:10.1128/AEM.02281-16.

Salama, N.R., Shepherd, B., and Falkow, S. 2004. Global Transposon Mutagenesis and Essential Gene Analysis of Helicobacter pylori. J. Bacteriol. **186**(23): 7926–7935. doi:10.1128/JB.186.23.7926-7935.2004.

Sánchez, P., Linares, J.F., Ruiz-Díez, B., Campanario, E., Navas, A., Baquero, F., and Martínez, J.L. 2002. Fitness of in vitro selected Pseudomonas aeruginosa nalB and nfxB multidrug resistant mutants. J. Antimicrob. Chemother. **50**(5): 657–664.

Schell, M.A., Zhao, P., and Wells, L. 2011. Outer membrane proteome of Burkholderia pseudomallei and Burkholderia mallei from diverse growth conditions. J. Proteome Res. **10**(5): 2417–2424. doi:10.1021/pr1012398.

Shah, I.M., and Dworkin, J. 2010. Induction and regulation of a secreted peptidoglycan hydrolase by a membrane Ser/Thr kinase that detects muropeptides. Mol. Microbiol. **75**(5): 1232–1243. doi:10.1111/j.1365-2958.2010.07046.x [doi].

Sham, L.-T., Barendt, S.M., Kopecky, K.E., and Winkler, M.E. 2011. Essential PcsB putative peptidoglycan hydrolase interacts with the essential FtsXSpn cell division protein in Streptococcus pneumoniae D39. Proc. Natl. Acad. Sci. U. S. A. **108**(45): E1061-1069. doi:10.1073/pnas.1108323108.

Sherrard, L.J., Tunney, M.M., and Elborn, J.S. 2014. Antimicrobial resistance in the respiratory microbiota of people with cystic fibrosis. The Lancet **384**(9944): 703–713. doi:10.1016/S0140-6736(14)61137-5.

Shi, Y., Cromie, M.J., Hsu, F.-F., Turk, J., and Groisman, E.A. 2004. PhoP-regulated Salmonella resistance to the antimicrobial peptides magainin 2 and polymyxin B. Mol. Microbiol. **53**(1): 229–241. doi:10.1111/j.1365-2958.2004.04107.x.

Silhavy, T.J., Kahne, D., and Walker, S. 2010. The Bacterial Cell Envelope. Cold Spring Harb Perspect Biol **2**(5). doi:10.1101/cshperspect.a000414.

Silver, L.L. 2011. Challenges of antibacterial discovery. Clin. Microbiol. Rev. **24**(1): 71–109.

Skerker, J.M., and Laub, M.T. 2004. Cell-cycle progression and the generation of asymmetry in Caulobacter crescentus. Nat. Rev. Microbiol. **2**(4): 325–337. doi:10.1038/nrmicro864.

Skerker, J.M., Prasol, M.S., Perchuk, B.S., Biondi, E.G., and Laub, M.T. 2005. Two-component signal transduction pathways regulating growth and cell cycle progression in a bacterium: a system-level analysis. PLoS Biol. **3**(10): e334. doi:10.1371/journal.pbio.0030334.

Sousa, S.A., Feliciano, J.R., Pita, T., Guerreiro, S.I., and Leitão, J.H. 2017. Burkholderia cepacia Complex Regulation of Virulence Gene Expression: A Review. Genes **8**(1). doi:10.3390/genes8010043.

Sousa, S.A., Ramos, C.G., and Leitao, J.H. 2011. *Burkholderia cepacia* Complex: Emerging Multihost Pathogens Equipped with a Wide Range of Virulence Factors and Determinants. Int. J. Microbiol. **2011**: 607575. Epub 2010 Aug 3. doi:10.1155/2011/607575.

Srinivasan, V.B., Vaidyanathan, V., Mondal, A., and Rajamohan, G. 2012. Role of the Two Component Signal Transduction System CpxAR in Conferring Cefepime and Chloramphenicol Resistance in Klebsiella pneumoniae NTUH-K2044. PLOS ONE **7**(4): e33777. doi:10.1371/journal.pone.0033777.

Stephenson, K., and Hoch, J.A. 2004. Developing inhibitors to selectively target two-component and phosphorelay signal transduction systems of pathogenic microorganisms. Curr. Med. Chem. **11**(6): 765–773.

Stock, A.M., Robinson, V.L., and Goudreau, P.N. 2000. Two-component signal transduction. Annu. Rev. Biochem. **69**: 183–215. doi:10.1146/annurev.biochem.69.1.183.

Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., Brinkman, F.S.L., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G.K.-S., Wu, Z., Paulsen, I.T., Reizer, J., Saier, M.H., Hancock, R.E.W., Lory, S., and Olson, M.V. 2000. Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. Nature **406**(6799): 959–964. doi:10.1038/35023079.

Su, F., Jacquard, C., Villaume, S., Michel, J., Rabenoelina, F., Clément, C., Barka, E.A., Dhondt-Cordelier, S., and Vaillant-Gaveau, N. 2015. Burkholderia phytofirmans PsJN reduces impact of freezing temperatures on photosynthesis in Arabidopsis thaliana. Front. Plant Sci. **6**: 810. doi:10.3389/fpls.2015.00810.

Sun, H., Yang, Y., Xue, T., and Sun, B. 2013. Modulation of cell wall synthesis and susceptibility to vancomycin by the two-component system AirSR in Staphylococcus aureus NCTC8325. BMC Microbiol. **13**: 286. doi:10.1186/1471-2180-13-286.

Svensson, S.L., Davis, L.M., MacKichan, J.K., Allan, B.J., Pajaniappan, M., Thompson, S.A., and Gaynor, E.C. 2009. The CprS sensor kinase of the zoonotic pathogen *Campylobacter jejuni* influences biofilm formation and is required for optimal chick colonization. Mol. Microbiol. **71**(1): 253–272. doi:10.1111/j.1365-2958.2008.06534.x [doi].

Svensson, S.L., Hyunh, S., Parker, C.T., and Gaynor, E.C. 2015. The Campylobacter jejuni CprRS two-component regulatory system regulates aspects of the cell envelope. Mol. Microbiol. **96**(1): 189–209. doi:10.1111/mmi.12927.

Taylor, R.C., Robertson, B.-J.M.W., Markillie, L.M., Serres, M.H., Linggi, B.E., Aldrich, J.T., Hill, E.A., Romine, M.F., Lipton, M.S., and Wiley, H.S. 2013. Changes in translational efficiency is a dominant regulatory mechanism in the environmental response of bacteria. Integr. Biol. **5**(11): 1393–1406. doi:10.1039/C3IB40120K.

Tettelin, H., Masignani, V., Cieslewicz, M.J., Donati, C., Medini, D., Ward, N.L., Angiuoli, S.V., Crabtree, J., Jones, A.L., Durkin, A.S., DeBoy, R.T., Davidsen, T.M., Mora, M., Scarselli, M., Ros, I.M. y, Peterson, J.D., Hauser, C.R., Sundaram, J.P., Nelson, W.C., Madupu, R., Brinkac, L.M., Dodson, R.J., Rosovitz, M.J., Sullivan, S.A., Daugherty, S.C., Haft, D.H., Selengut, J., Gwinn, M.L., Zhou, L., Zafar, N., Khouri, H., Radune, D., Dimitrov, G., Watkins, K., O'Connor, K.J.B., Smith, S., Utterback, T.R., White, O., Rubens, C.E., Grandi, G., Madoff, L.C., Kasper, D.L., Telford, J.L., Wessels, M.R., Rappuoli, R., and Fraser, C.M. 2005. Genome analysis of multiple pathogenic isolates of Streptococcus agalactiae: Implications for the microbial "pan-genome." Proc. Natl. Acad. Sci. U. S. A. **102**(39): 13950–13955. doi:10.1073/pnas.0506758102.

Thein, M., Sauer, G., Paramasivam, N., Grin, I., and Linke, D. 2010. Efficient subfractionation of gram-negative bacteria for proteomics studies. J. Proteome Res. **9**(12): 6135–6147. doi:10.1021/pr1002438.

Theocharis, A., Bordiec, S., Fernandez, O., Paquis, S., Dhondt-Cordelier, S., Baillieul, F., Clément, C., and Barka, E.A. 2012. Burkholderia phytofirmans PsJN primes Vitis vinifera L. and confers a better tolerance to low nonfreezing temperatures. Mol. Plant-Microbe Interact. MPMI **25**(2): 241–249. doi:10.1094/MPMI-05-11-0124.

Torbeck, L., Raccasi, D., Guilfoyle, D.E., Friedman, R.L., and Hussong, D. 2011. Burkholderia cepacia: This Decision Is Overdue. PDA J. Pharm. Sci. Technol. **65**(5): 535–543. doi:10.5731/pdajpst.2011.00793.

Tseng, S.-P., Tsai, W.-C., Liang, C.-Y., Lin, Y.-S., Huang, J.-W., Chang, C.-Y., Tyan, Y.-C., and Lu, P.-L. 2014. The Contribution of Antibiotic Resistance Mechanisms in Clinical Burkholderia cepacia Complex Isolates: An Emphasis on Efflux Pump Activity: e104986. PLoS One San Franc. **9**(8). doi:http://dx.doi.org.qe2a-proxy.mun.ca/10.1371/journal.pone.0104986.

Ussery, D.W., Kiil, K., Lagesen, K., Sicheritz-Pontén, T., Bohlin, J., and Wassenaar, T.M. 2009. The Genus Burkholderia: Analysis of 56 Genomic Sequences. **6**: 140–157. doi:10.1159/000235768.

Van, V.T., Berge, O., Kê, S.N., Balandreau, J., and Heulin, T. 2000. Repeated beneficial effects of rice inoculation with a strain of Burkholderia vietnamiensison early and late yield

components in low fertility sulphate acid soils of Vietnam. Plant Soil **218**(1–2): 273–284. doi:10.1023/A:1014986916913.

Velikova, N., Bem, A.E., van Baarlen, P., Wells, J.M., and Marina, A. 2013. WalK, the Path towards New Antibacterials with Low Potential for Resistance Development. ACS Med. Chem. Lett. **4**(10): 891–894. doi:10.1021/ml400320s.

Velikova, N., and Wells, J.M. 2016. Rationale and Prospects of Targeting Bacterial Two-component Systems for Antibacterial Treatment of Cystic Fibrosis Patients. Curr. Drug Targets. doi:CDT-EPUB-73545 [pii].

Venter, H., Mowla, R., Ohene-Agyei, T., and Ma, S. 2015. RND-type drug efflux pumps from Gram-negative bacteria: molecular mechanism and inhibition. Front. Microbiol. **6**. doi:10.3389/fmicb.2015.00377.

Vogel, C., and Marcotte, E.M. 2012. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nat. Rev. Genet. **13**(4): 227–232. doi:10.1038/nrg3185.

Wang, N., Ozer, E.A., Mandel, M.J., and Hauser, A.R. 2014. Genome-wide identification of Acinetobacter baumannii genes necessary for persistence in the lung. mBio **5**(3): e01163-1114. doi:10.1128/mBio.01163-14.

Watanabe, T., Okada, A., Gotoh, Y., and Utsumi, R. 2008. Inhibitors targeting two-component signal transduction. Adv. Exp. Med. Biol. **631**: 229–236. doi:10.1007/978-0-387-78885-2_16.

Weber, B.S., Ly, P.M., Irwin, J.N., Pukatzki, S., and Feldman, M.F. 2015. A multidrug resistance plasmid contains the molecular switch for type VI secretion in Acinetobacter baumannii. Proc. Natl. Acad. Sci. U. S. A. **112**(30): 9442–9447. doi:10.1073/pnas.1502966112.

Wong, Y.-C., Abd El Ghany, M., Naeem, R., Lee, K.-W., Tan, Y.-C., Pain, A., and Nathan, S. 2016. Candidate Essential Genes in Burkholderia cenocepacia J2315 Identified by Genome-Wide TraDIS. Front. Microbiol. **7**. doi:10.3389/fmicb.2016.01288.

Worthington, R.J., Blackledge, M.S., and Melander, C. 2013. Small-molecule inhibition of bacterial two-component systems to combat antibiotic resistance and virulence. Future Med. Chem. **5**(11): 1265–1284. doi:10.4155/fmc.13.58; 10.4155/fmc.13.58.

Wu, C.-J., Huang, Y.-W., Lin, Y.-T., Ning, H.-C., and Yang, T.-C. 2016. Inactivation of SmeSyRy Two-Component Regulatory System Inversely Regulates the Expression of SmeYZ and SmeDEF Efflux Pumps in Stenotrophomonas maltophilia. PLOS ONE **11**(8): e0160943. doi:10.1371/journal.pone.0160943.

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T., and Arakawa, M. 1992. Proposal of Burkholderia gen. nov. and transfer of seven species of the genus Pseudomonas homology group II to the new genus, with the type species Burkholderia cepacia (Palleroni and Holmes 1981) comb. nov. Microbiol. Immunol. **36**(12): 1251–1275.

Yamaguchi, H., Furuhata, K., Fukushima, T., Yamamoto, H., and Sekiguchi, J. 2004. Characterization of a new Bacillus subtilis peptidoglycan hydrolase gene, yvcE (named cwlO), and the enzymatic properties of its encoded protein. J. Biosci. Bioeng. **98**(3): 174–181. doi:S1389-1723(04)00262-2 [pii].

Yan, M., Yu, C., Yang, J., and Ji, Y. 2011. The essential two-component system YhcSR is involved in regulation of the nitrate respiratory pathway of Staphylococcus aureus. J. Bacteriol. **193**(8): 1799–1805. doi:10.1128/JB.01511-10.

Yoon, E.-J., Courvalin, P., and Grillot-Courvalin, C. 2013. RND-Type Efflux Pumps in Multidrug-Resistant Clinical Isolates of Acinetobacter baumannii: Major Role for AdeABC Overexpression and AdeRS Mutations. Antimicrob. Agents Chemother. **57**(7): 2989–2995. doi:10.1128/AAC.02556-12.

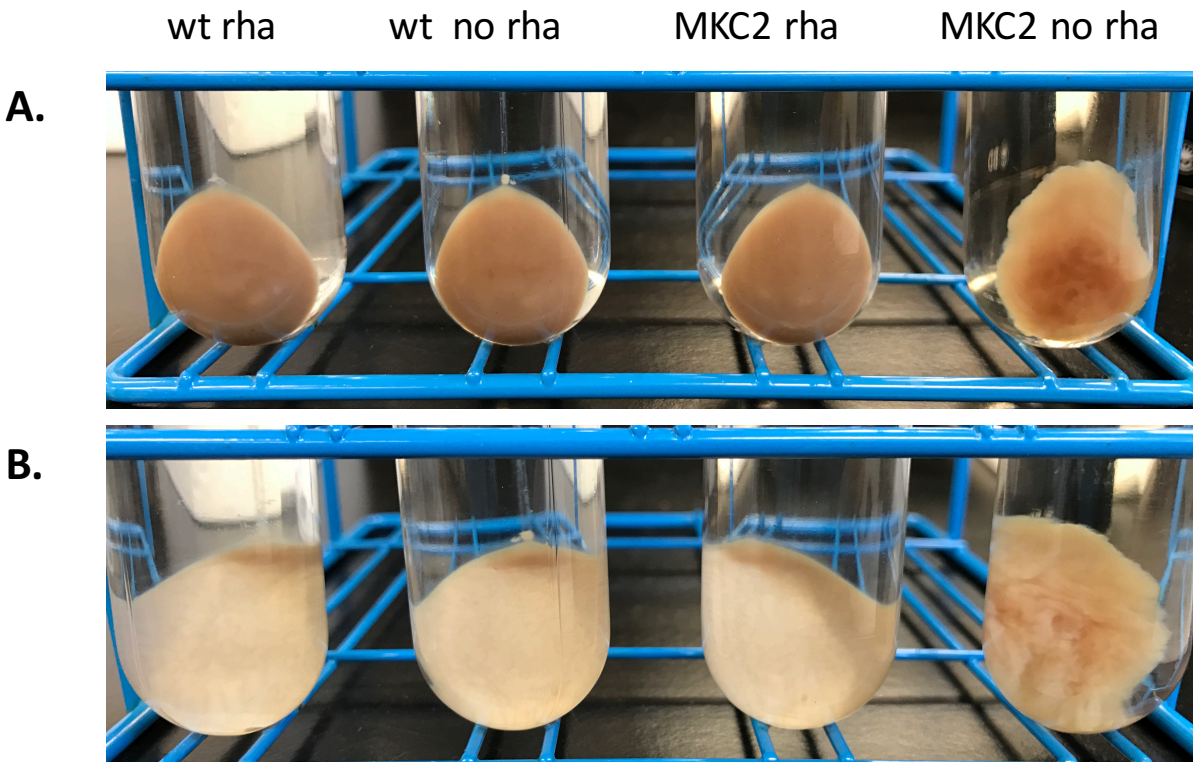
Young, J.P.W., Crossman, L.C., Johnston, A.W., Thomson, N.R., Ghazoui, Z.F., Hull, K.H., Wexler, M., Curson, A.R., Todd, J.D., Poole, P.S., Mauchline, T.H., East, A.K., Quail, M.A., Churcher, C., Arrowsmith, C., Cherevach, I., Chillingworth, T., Clarke, K., Cronin, A., Davis, P., Fraser, A., Hance, Z., Hauser, H., Jagels, K., Moule, S., Mungall, K., Norbertczak, H., Rabbinowitsch, E., Sanders, M., Simmonds, M., Whitehead, S., and Parkhill, J. 2006. The genome of Rhizobium leguminosarum has recognizable core and accessory components. Genome Biol. **7**: R34. doi:10.1186/gb-2006-7-4-r34.

Zahrt, T.C., and Deretic, V. 2000. An essential two-component signal transduction system in Mycobacterium tuberculosis. J. Bacteriol. **182**(13): 3832–3838.

Zhang, Y.J., Ioerger, T.R., Huttenhower, C., Long, J.E., Sassetti, C.M., Sacchettini, J.C., and Rubin, E.J. 2012. Global Assessment of Genomic Regions Required for Growth in Mycobacterium tuberculosis. PLOS Pathog. **8**(9): e1002946. doi:10.1371/journal.ppat.1002946.

Zlosnik, J.E.A., Zhou, G., Brant, R., Henry, D.A., Hird, T.J., Mahenthiralingam, E., Chilvers, M.A., Wilcox, P., and Speert, D.P. 2014. Burkholderia Species Infections in Patients with Cystic Fibrosis in British Columbia, Canada. 30 Years' Experience. Ann. Am. Thorac. Soc. **12**(1): 70–78. doi:10.1513/AnnalsATS.201408-395OC.

Appendix



Appendix Figure 1. Photograph after lysozyme treatment and first ultracentrifugation step in the cell fractionation protocol. A) View of the four pellets after ultracentrifugation and before resuspension in Buffer 2. B) 180° rotated view from Panel A to highlight the differences in the appearances of the pellet of the MKC2 no rha sample compared to the other three samples.

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