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The effects of internally expressed Contact-Dependent growth Inhibition (CDI) toxins in bacteria

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Abstract
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Bacteria, both pathogenic and non-pathogenic, have developed multiple forms of competition mechanisms to combat each other including, but not limited to, Contact-Dependent growth Inhibition (CDI) systems, Type VI Secretion Systems and the associated Rearrangement hotspot (Rhs) toxin system. These systems usually confers a great fitness advantage as they allow for precise delivery of toxic molecules into competing bacteria whilst sister cells are protected from auto-inhibition by producing a cognate immunity protein. Delivery between sister cells may serve as a form of "self-recognition" whilst maintaining selection pressure for these genes within the population. How these genes are maintained in conditions where delivery does not occur has until now not been fully understood.

This thesis describes secondary functions, maintained selection pressure and regulation of Rhs and CDI systems in three parts. In Paper I, we made a novel discovery that *rhs* toxin and immunity genes from *Salmonella enterica* serovar Typhimurium are transcribed from internal transcriptional start sites independent of the full length delivery gene. This results in functional cytosolic proteins capable of regulating proliferation and growth rate of *S*. Typhimurium during infection of RAW264.7 cells. In Paper II, we continued our work from Paper I and studied growth effects *in vitro* as well as regulation of the internal expression. Our findings show that Rhs causes a small fitness cost also in rich laboratory medium and is regulated by alternate sigma factor RpoS, two-component system PhoP/Q and DNA binding protein H-NS. In Paper III, we made a discovery similar to our findings in Paper I and II by observing internally transcribed toxin and immunity genes of multiple CDI systems from *E. coli* regulated by RpoS. We propose that CDI toxin-immunity pairs function as selfish genetic elements that maintain gene selection whilst simultaneously retaining the ability to protect the cell from externally delivered toxins.

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Till minne av Kicki

If nobody has told you this yet today,
you're doing a great job.

-Scott Klopfenstein

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Stårsta, M**.†, Hammarlöf, D.L.†, Wäneskog, M., Schlegel, S., Xu, F., Gynnå, A.H., Borg, M., Herschend, S., Koskiniemi, S., (2020). RHS-elements function as type II toxin-antitoxin modules that regulate intra-macrophage replication of *Salmonella* Typhimurium. PLOS Genetics.

II Lee D.†, **Stårsta, M**.†, Schlegel, S., Kjellin, J., Hammarlöf, D.L. and Koskiniemi, S. Global regulators H-NS, RpoS and PhoP/Q regulate expression of Rhs Toxin-Antitoxin system. *Manuscript.*

III **Stårsta, M**.†, Wäneskog, M.†, Eriksson, H., Ghosh, A., Schlegel S. and Koskiniemi S. Contact-dependent growth inhibition modules contain intracellularly expressed selfish genetic elements. *Manuscript.*

† These authors contributed equally to this work

Contents

Abbreviations

ATR	Acid Tolerance Response
CDI	Contact-Dependent growth Inhibition
CDS	Contact-Dependent Signalling
CT	C-terminal Toxin
EC93	*E. coli* 93
H-NS	Histone-like Nucleoid Structuring protein
HGT	Horizontal Gene Transfer
IM	Inner Membrane
M-cell	Microvilli cell
mRNA	Messenger RNA
OD	Optical Density
ORF	Open Reading Frame
PAAR	Proline-Alanine-Alanine-Asparagine domain
PP	Payer's Patch
qPCR	Quantitative Polymerase Chain Reaction
RBD	Receptor Binding Domain
Rhs	Rearrangement hotspots
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SCV	*Salmonella* Containing Vacuole
SHX	DL-Serine Hydroxamate
SPI	*Salmonella* Pathogenicity Island
sRNA	Small RNA
T5SS	Type V Secretion System
T6SS	Type VI Secretion System
TA	Toxin-Antitoxin
tRNA	Transfer RNA
ts	Temperature sensitive
UPEC	Uropathogenic *E. coli*
UTI	Urinary-tract Infection
wt	Wild type

Introduction

If the COVID-19 pandemic has taught us anything, it is that a single untreatable pathogen may have a huge impact on humanity. For reference, the bacterium *Yersinia pestis* was responsible for killing a third of the total population on earth during The Plague in the middle ages. Had this pandemic occurred in modern times, we may have been able to prevent it by using antibiotics. However, pathogens have adapted to humanity's increased dependence on these drugs and accumulated several resistance mechanisms. Because of this, we are in dire need of new treatments of bacterial infections. Bacteria have also evolved countless virulence factors to hide from, disrupt or directly kill immune cells (Madigan et al., 2012; Ventola, 2015). Therefore, understanding bacterial behaviour before, during and after infection could prove to be key aspects in discovering new drug targets. Hopefully this would enable the development of new therapeutics for treating or preventing infections that our immune system cannot clear on its own. Furthermore, bacteria must not only survive the immune system but must also compete with other bacteria, both in the environment and within the host. This may be done in several ways such as by producing a protective biofilm, secreting toxic compounds or using other, more precise means of competition such as Contact-Dependent growth Inhibition (CDI). CDI systems may give a bacterium an advantage over its competitors by allowing delivery of toxins into target cells but have also been described to be directly and indirectly important for other parts of bacterial biology including biofilm formation, persister formation, motility and virulence (Aoki et al., 2005; Pukatzki et al., 2006; MacIntyre et al., 2010; Garcia et al., 2013; Melvin et al., 2017; Ghosh et al., 2018). Thus, due to their importance in bacterial biology, these systems may be future drug targets to limit virulence or to develop probiotic strains to outcompete pathogens. Therefore, studying CDI systems are of great interest as we may yet discover more secondary functions to help us understand their full potential.

In this thesis I present experimental evidence that Rhs and CDI systems have a secondary function of regulating growth of pathogenic bacteria, both *in vivo* and *in vitro*, by expressing toxin-immunity pairs internally, independent of toxin delivery. I identify alternate sigma factor RpoS to be a key activator of these systems. Furthermore, I propose that this internal expression maintains selection pressure of these advantageous competition systems even in the absence of inter-bacterial delivery of toxins.

Microbiome

Microorganisms can be found occupying countless different niches on our planet and have evolved astonishing ways of surviving in extreme environments such as on aquatic thermal vents, exposure to high levels of radioactivity (*Deinococcus radiodurans*) and even within immune cells (*Salmonella enterica, Mycobacterium Tuberculosis*) (Winn et al., 1986; Smith, 2003; Cox and Battista, 2005; Haraga et al., 2008). The microbiome, or microbiota, is a collective name for the complex composition of organisms that reside on and inside the human body which is unique for every individual (Zhu et al., 2015). The many species may serve various important purposes. For example, intestinal bacteria benefits the host by producing important nutrients such as vitamin K and B12 which humans are incapable of (Blount, 2015). Also patients treated with probiotic *Lactobacilli* have shown improvements in stress-related mental disorders such as anxiety or depression due to the bacteria interacting with neurotransmitters in the intestine which affects the central nervous system (Bravo et al., 2011). The benefit that is perhaps most relevant for this thesis is a process called colonization resistance. This is the ability of the microbiome to occupy the same environmental niche as pathogens which protects the host by limiting infections (van der Waaij et al., 1971). Interactions between pathogenic bacteria and commensal bacteria are further discussed in later sections.

Some species within the microbiota are so called opportunistic pathogens meaning if given the chance they may cause disease. *Staphylococcus aureus*, which normally resides on the skin, can cause lethal infections if they enter the blood stream (Tong et al., 2015). *Clostridioides difficile* infection may arise if the balance in the intestinal normal flora is disrupted, commonly resulting in diarrhoea but occasionally also multi-organ failure and even death (Mullish and Williams, 2018). Please note that *C. difficile* infection is commonly abbreviated as CDI, however, in this thesis that particular abbreviation is reserved for Contact-Dependent growth Inhibition (page 28). Alterations in the microbiota, caused by antibiotic treatment or certain pathogens (*e.g. S. enterica),* may result in antibiotic associated diarrhoea or inflammatory bowel disease caused by persistent infection (Mullish and Williams, 2018; Schultz et al., 2018). Inoculating patients with microbiota from healthy individuals have shown improvements on *C. difficile* infections and to some extent on inflammatory bowel disease (Lopez and Grinspan, 2016). Collectively, the microbiome plays important roles in many aspects of mammalian health suggesting that dysbiosis caused by invading pathogens, antibiotic treatments or both may result in complex, unpredictable complications.

Salmonella enterica serovar Typhimurium

Salmonella around the world

Salmonella enterica serovar Typhimurium (hereafter referred to as *S.* Typhimurium or *Salmonella* unless otherwise is specified) is a zoonotic pathogen causing non-typhoidal gastro-intestinal disease in humans (Haraga et al., 2008). Symptoms include abdominal pains, vomiting and diarrhoea that may result in dehydration (Coburn et al., 2007; Aranda-Michel and Giannella, 1999). *Salmonella* as a genus is listed by The World Health Organization as one of four major causes of diarrheal diseases world-wide (WHO, 2018). In a study published in The Lancet Infectious Diseases, the authors estimate the global burden of non-typhoidal *Salmonella* infections in 2017 to be more than half a million cases out of which more than a tenth had lethal outcomes (Stanaway et al., 2019). In Sweden the number of documented *Salmonella* spp. infections were less than 2000 cases in 2019 as reported by the Public Health Agency of Sweden. About one third of these cases were caused *S.* Typhimurium, including its monophasic variant, with the majority of infections occurring abroad. The only exception to this trend in recent years was in 2020 when travelling was limited due to restrictions in regards to the COVID-19 pandemic resulting in less cases contracted abroad (Folkhälsomyndigheten, 2021). Interestingly, *S.* Typhimurium may infect a wide array of different species of both mammals and birds. In fact, birds have been linked to seasonal outbreaks of *S.* Typhimurium in cats and humans in Sweden. For obvious ethical reasons, *in vivo* studies of *S.* Typhimurium are commonly performed in mice rather than humans. In this infection model, however, the pathogen causes typhoidal-like symptoms which are commonly associated with *Salmonella enterica* serovar Typhi in humans. As opposed to Typhimurium, Typhi infections are typically more host-specific, however, mice infected with Typhimurium do not develop diarrhoea which is typical of Typhi infections in humans. Thus, the murine model may not be representative for studying either of the two serovars. Instead, bovine infection models have proven to be a more representative way to study *S.* Typhimurium pathogenicity (Santos et al., 2001; Söderlund et al., 2019).

Early stages of infection

S. Typhimurium enters its host orally by contaminated food or water and must survive defence mechanisms such as gastric acid and bile (Garcia-del Portillo et al., 1993; Prouty et al., 2004). The bacterium endures pH levels as low as 3.3 through its acid tolerance response (ATR) involving numerous acid shock proteins. The ATR is also necessary for later stages of infection, including invasion (J. W. Foster, 1993; Garcia-del Portillo et al., 1993). In the small intestine the average concentration of bile is ~0.4%. However, the minimal

inhibitory concentration of bile for *S*. Typhimurium is 18%, but the pathogen been observed to survive at concentrations higher than 60%. Thus, *Salmonella* is actually far more tolerant to bile than necessary (van Velkinburgh and Gunn, 1999; Hofmann and Eckmann, 2006). Both ATR and bile resistance are dependent on activation by the two-component system PhoP/Q, amongst other regulators, which is also an important regulator of virulence genes in later stages of infection (Bearson et al., 1998; van Velkinburgh and Gunn, 1999; Miller et al., 1989). Once the bacterium reaches the small intestine, more specifically the Payer's patches (PP) , the bacterium must compete with the normal flora. In fact, disrupting the normal flora with antibiotics renders the host more susceptible to *Salmonella* infections (Carter and Collins, 1974; Garner et al., 2009). *S*. Typhimurium has developed multiple ways of competing with commensal bacteria. Demand for iron is high in the intestine but *Salmonella* can efficiently scavenge iron through the iron chelator Salmochelin which is recognized by outer membrane protein IroN (Hantke et al., 2003) Additionally, *S*. Typhimurium is dependent on its Type VI Secretion System (T6SS, described in more detail in its own section; page 34) encoded on *Salmonella* Pathogenicity Island VI (SPI6). This system mediates inter-bacterial delivery of toxins allowing *S*. Typhimurium to kill or inhibit commensal bacteria to enable its own colonization in the intestine (Figure 1 & Figure 5) (Liu et al., 2013; Mulder et al., 2012; Sana et al., 2016).

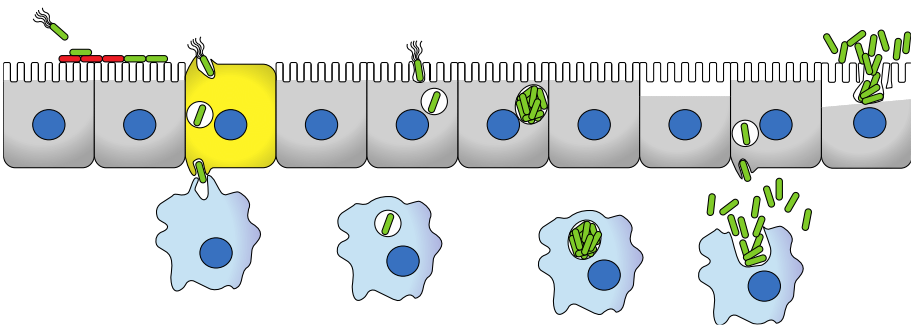

Figure 1, Schematic illustration of S. Typhimurium infection and cellular invasion. Salmonella (green) competes with the current occupants of the niche (red) e.g. by. using its SPI-6 encoded type VI secretion system. The SPI-1 encoded Type III secretion system is used to trigger phagocytosis by M-cells (portrayed in yellow), epithelial cells (gray) or macrophages (blue) where the bacterium may replicate.

Salmonella, the master of puppets

Upon successfully establishing itself in the intestine, *S.* Typhimurium utilizes a Salmonella Pathogenicity Island 1 (SPI-1) encoded Type III secretion system ($T3SS^{SPI-1}$) to trigger phagocytosis by epithelial cells or microvilli cells (M-cells) of the PP (Galán, 2001; Haraga et al., 2008; Jones et al., 1994). After M-cell uptake, the bacterium can exit the infected cell on the other side of the epithelial layer where it may trigger $T3SS^{SPI-1}$-dependent phagocytosis by macrophages where it may replicate (Figure 1) (Haraga et al., 2008). Once internalized, *S.* Typhimurium will provoke an immune reaction, through the translocation of flagellin into host cytosol, to induce inflammation in the intestine (Sun et al., 2007). This innate immune response will kill much of the commensal bacteria already colonizing the area, sometimes permanently altering the microbiome, whilst *Salmonella* persists, thus aiding its colonization. In fact, *S.* Typhimurium that are incapable of provoking inflammation have been found to be outcompeted by the normal flora in mice (Stecher et al., 2007; Haraga et al., 2008) suggesting that this mechanism is essential for *Salmonella* colonization in addition to T6SS. The bacterium resides inside the macrophage phagosome, commonly referred to as the *Salmonella* Containing Vesicle (SCV) for 3-4 h before it begins to replicate (Mills and Finlay, 1998). During SCV growth *S.* Typhimurium must to cope with the defence mechanisms of the host cell such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Figure 2). This can occur in two different scenarios. In scenario A, *Salmonella* uses a second T3SS, encoded on *Salmonella* Pathogenicity Island 2 (SPI-2), to block the phago-lysosomal fusion thus protecting the pathogen from the hazardous ROS and RNS (Vazquez-Torres et al., 2000; Chakravortty et al., 2002). In scenario B, if the phago-lysosomal fusion is successful, the bacterium can utilize a protein called superoxide dismutase to neutralize bactericidal compounds (De Groote et al., 1997) (Figure 2). *Salmonella* can kill the macrophage by the secretion of effector SipB which results in cell death (Hernandez et al., 2003). The released pathogens may then re-infect the basolateral surface of the epithelium to eventually reach the intestinal lumen again (Haraga et al., 2008). Furthermore, *S.* Typhimurium is capable of infecting dendritic cells and thus interfere with the recruitment of the adaptive immune system; a processes which cannot be observed for serovars Typhi or Enteritidis (Bueno et al., 2008). Although it is not commonly associated with the disease, *S.* Typhimurium may cause systemic infection (bacteremia) in patients with compromised immune system (Gordon et al., 2008) as typically extracellular replication of this pathogen is limited by neutrophils (Conlan, 1996).

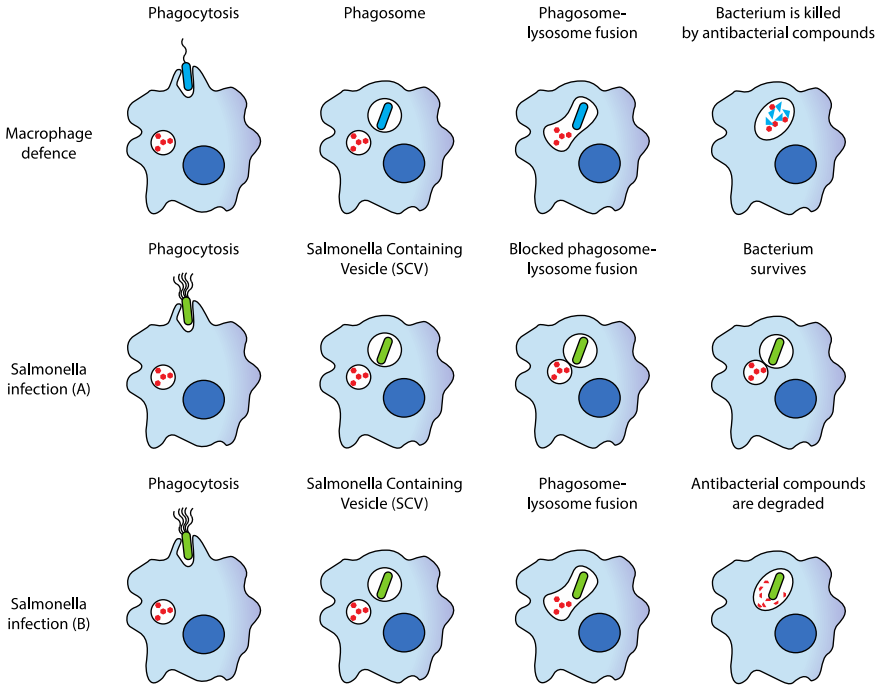

Figure 2, schematic illustration of the macrophage defence system and coping mechanisms of S. Typhimurium. ***Top panel****; The macrophage phagocytoses bacterium followed by the phagosome fusing with the lysosome which exposes the bacterium to antibacterial compounds that ultimately kills the pathogen thus neutralizing the threat.* ***Middle panel****; Salmonella infection scenario (A), following phagocytosis the invading bacterium can block fusion of the Salmonella Containing Vesicle (SCV) to the lysosome (by using its SPI-2 encoded T3SS) thus the pathogen survives.* ***Bottom panel****; Salmonella infection scenario (B), where successful fusion of the SCV and lysosome occurs, S. Typhimurium is capable of neutralizing the antimicrobial compounds using superoxide dismutases.*

Uropathogenic Escherichia coli (UPEC)

Escherichia coli, first discovered in 1884, is the most studied organism on our planet. The species is extremely versatile and can employ pathogenic, commensal or even probiotic lifestyles. *E. coli* belongs to the *Enterobacteriaceae* family of bacteria and is a common cause of gastro-intestinal and urinary-tract infections (UTIs) (Blount, 2015; Kaper et al., 2004; Wassenaar, 2016). Whilst several pathotypes have been described to cause intestinal-, invasive and extra-intestinal infections in humans including; Enterohaemorrhagic *E. coli* (EHEC), Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and enteroinvasive *E. coli* (EIEC); this thesis focuses on Uropathogenic *E. coli* (UPEC) (Kaper et al., 2004).

UPEC is the most common cause of UTIs worldwide. Older, perhaps outdated, estimates report 150 million cases of UTIs each year out of which 85% are caused by UPEC (Terlizzi et al., 2017). Like other types of *E. coli*, the uropathogen reside in the intestine but upon spreading to the urethra, they may establish an infection in the bladder (Yamamoto et al., 1997). The lifestyle in the urinary tract requires the ability to adhere strongly to epithelial cells by using type 1 pili to avoid getting flushed out by the flow of urine. Experiments in mice have shown that UPEC is capable of invading the epithelium and grow intracellularly. Invasion of host cells through endocytosis is thought to be triggered by the type 1 pili-associated adhesin FimH (Martinez et al., 2000). Once internalized, the pathogen resists being exocytosed from the host cell by escaping into the cytoplasm where they employ a biofilm-like lifestyle to evade host defences (Silverman et al., 2013). During late stages of intracellular growth as the host cell bursts, UPEC may form highly motile cells that may infect new cells or long filamentous cells that are capable of subverting the innate defences, *i.e.* phagocytosis (Lewis et al., 2016). Persistent UTIs may result in spread to the kidneys causing pyelonephritis which could be life-threatening if untreated (Marrs et al., 2005). Despite successful treatment by antibiotics, infection relapse is not uncommon due to sister cells still residing within the intestine or hiding within the bladder epithelium (Spaulding et al., 2017). Furthermore, antibiotic resistance is becoming and increasing problem for treatment of these pathogens (Bunduki et al., 2021).

The increasing difficulties in treating bacterial infections

The "post-antibiotic era"

To this day, antibiotics remains the single most important way of treating acute bacterial infections. These drugs are essential for many parts of the healthcare systems including surgery. Despite impressive progress on treatments for viral infections during the COVID-19 pandemic, treatment of bacterial infections is becoming more and more difficult due to an emerging resistance development. In Europe alone, an estimated 33 000 people die each year from infections of multi-resistant bacteria (Cassini et al., 2019; Mahase, 2021). Resistance may come from enzymes degrading the antibiotic, such as beta-lactamases or from efflux pumps that pump out the antibiotic before it can react. Resistance to some types antibiotics only require a single point mutation in the gene encoding the target of the compound, such as the *rpoB*(A1547G) and *rpsL*(A128G) mutations in *E. coli* resulting in resistance to rifampicin and streptomycin respectively (Barrick et al., 2010; Du et al., 2018; Jacoby, 2009; Via et al., 2010). Furthermore, environmental stress can result in the formation of antibiotic persistent or tolerant cells. These heterogenous subpopulations consists of dormant or slow growing cells respectively that are not susceptible to antibiotic treatment and can repopulate once the antibiotic disappears (Balaban et al., 2019, 2004). The importance of these cells during infection is, however, debated within the field. This topic is further discussed in the Type II Toxin-Antitoxin systems section below (page 26).

Resistance genes present on plasmids may spread between different species resulting in bacteria that are resistant despite not previously encountering the antibiotic and a single plasmid may encode resistance to several different antibiotics. For example, in 2005 there was an outbreak of multi-resistant *K. pneumoniae* at Akademiska sjukhuset in Uppsala, Sweden. The isolated resistance plasmid pUUH239.2 from this strain was in possession of resistance against β-lactams, aminoglycosides, tetracyclines, trimethoprim, sulphonamides and macrolides. Additionally genes encoding resistance to quaternary ammonium compounds and even heavy metal ions (silver, copper and arsenic) could be detected (Sandegren et al., 2012). This would suggest that selection for this plasmid could occur even in the absence of antibiotics. There may, however, be ways of at least slowing down the spread of resistance genes. A recent study by the Public Health Agency of Sweden (Folkhälsomyndigheten) in collaboration with Linköping University found that treating UTIs with the narrow-spectrum antibiotic temocillin reduced the development of antibiotic resistance in the microbiota without compromising treatment efficiency (Edlund et al., 2022). Thus this suggests that carefully selecting which antibiotic to use may prove useful to slow down resistance

development. Nevertheless, new treatment options for bacterial infections are becoming increasingly important.

Fight bacteria with bacteria

Multiple ideas exist for hindering infections by enteric pathogens, however, the examples mentioned below are strictly prophylactic and cannot substitute antibiotics during acute infections. As mentioned in the Microbiome section (page 14) and in the *Salmonella enterica* serovar Typhimurium section (page 15); the commensal microbiota plays an important role in limiting establishment of pathogens in the intestine through a phenomenon known as colonization resistance (van der Waaij et al., 1971; Stecher et al., 2007; Garner et al., 2009). Certain probiotic strains such as *E. coli* Nissle 1917 have been proven capable of successfully outcompeting other *E. coli* (Wassenaar, 2016). In relation to this, a live attenuated vaccine against *Vibrio cholerae* has been proven successful in rabbits where an avirulent strain colonizes the intestine thus occupying the same niche or "colonization vacancies" as the pathogen which limits infection (Fu et al., 2018). Vaccines against *S.* Typhimurium are also being explored. Already in the 1980s, experiments in mice showed that individuals infected with avirulent *phoP* mutants of *S.* Typhimurium had an increased protection against infections by wild type bacteria one month later (Miller et al., 1989). Despite these results being more than 30 years old, no vaccine against *S.* Typhimurium for humans exists today (Sanapala et al., 2018). In a recent study an inactivated vaccine, utilizing so called "ghost bacteria", was shown to provide full protection in mice (Rabea et al., 2022). Another possible strategy is to reduce virulence in pathogenic bacteria. Virulence factors such as the T3SS in *S.* Typhimurium, which is essential for early stages of infection, can be targeted by Tanic acid rendering the cells unable to infect HeLa cells. The compound inhibits the genes encoding the system on SPI-1 (Shu et al., 2022), preventing *Salmonella* invasion of the epithelium. Hence, it is likely that inhibiting virulence rather than directly killing the bacteria could result in less selection for compensatory mutations as the pathogens may be cleared by the immune system or the normal flora instead of the drug. Mutations and gene selection is further discussed in the next section.

Selection of genes

Gaining beneficial genes

New genetic material can be obtained by bacteria through transformation, conjugation or phage transduction in a process called horizontal gene transfer (HGT) (Burmeister, 2015). According to Darwin's theory on evolution; the individual with the highest fitness will outcompete the others through natural selection (Darwin, 1859, 2003). Of course, horizontally obtained genes may not always be beneficial for the cell and may even result in a decrease in fitness likely resulting in that individual being outcompeted. However, in the event of an acquired gene being beneficial for the cell, the obtained increase in "relative fitness" will likely result in that genotype being favoured and overtaking the population (Campbell et al., 2008). Beneficial genes may include the ability to utilize new nutrients, virulence factors, competition systems or resistance to toxic compounds, to name a few, which can enable the cell to occupy a certain niche more efficiently or outcompete other organisms (Aoki et al., 2005; Burmeister, 2015; Caro-Quintero and Konstantinidis, 2015). Some genes or mutations may, however, cause a fitness cost despite giving the bacterium an advantage. It is thus important that the genetic alteration gives an overall bigger increase in fitness than decrease which is usually referred to as "trade-off". For example, in the antibiotic resistance section of this thesis (page 20), I briefly discuss the *rpoB*(A1547G) mutation in *E. coli*. This single point mutation results in a 20% decrease in fitness but confers resistance towards the antibiotic rifampicin. Thus, selection pressure to maintain the element is upheld in presence of the drug in a process called "directional selection" (Barrick et al., 2010; Campbell et al., 2008). Theoretically, if exposure to the antibiotic stops, the directional selection pressure changes and the bacteria that acquire a new mutation that restores their native *rpoB* gene, hence losing the now costly mutation, will have a higher relative fitness due to their increased rate of growth. However, in the presence of the antibiotic the cells will likely accumulate other compensatory mutations to cope with the fitness cost caused by *rpoB* and thus the resistance may be maintained even without selection.

Losing "unnecessary" genes

Bacterial pathogens, such as *S. enterica* are capable of causing disease by having co-evolved with humans and/or other hosts for ages. Therefore, to survive the hostile environment of the host, selection of beneficial genes must be upheld. However, in order to adapt to a new host, genetic alterations may be necessary even resulting in gene loss. As briefly discussed in the *S.* Typhimurium section of this thesis (page 15), different serovars of *S. enterica* have different levels of host specificity. This is possible by the pathogens

having undergone so called "host adaptations". Pathogenicity in *S. enterica* is associated with the acquisition of mobile genetic elements, *i.e.* SPIs. A noticeable difference in the conservation of SPIs between different serovars relevant for this thesis is SPI-6 and SPI-19 which both encode Type VI Secretion Systems (T6SS) and Rhs elements that are important for virulence (see pages 15, 34 and 37 for more details). Genetical analysis suggest that these SPIs were common in the ancestral strain of S. enterica and have since been lost in some serovars. SPI-19 is for example not present in *S.* Typhimurium. (Liu et al., 2013; Chaudhuri et al., 2013; Langridge et al., 2015; Sana et al., 2016; Ray et al., 2022). Genes that are deemed unnecessary or even costly by evolution are more prone to accumulate inactivating mutations, such as premature stop codons, turning them into pseudogenes as there is no selection to maintain them; in fact, deletion of unnecessary genes can even provide a fitness advantage (Koskiniemi et al., 2012). Thus, it seems the importance and fitness cost of genes encoding T6SS or Rhs vary between different hosts which explains why they are not found in all types of *S. enterica*. (Langridge et al., 2015; Ray et al., 2022). Interestingly, some genes, such as toxin-antitoxin pairs, are described "selfish genetic elements" meaning that they uphold their own selection by creating an "artificial" selection pressure where losing the genes comes at high costs (Ogura and Hiraga, 1983; Fraikin et al., 2020). These systems are described further in the next section.

New phenotypes through gene duplications

In addition to HGT and mutations, new phenotypes may arise from a gene duplication event which may occur naturally for any gene at a 1/1000 frequency in a bacterial population (Reams et al., 2010). An example of this, relevant for this thesis, is the Rhs elements in *S.* Typhimurium (described in more detail on page 37). Within this locus there are two ORFs encoding effectors, one full-length ORF described as functional, and a shorter, highly homologous ORF previously described as "non-functional". In the event of a duplication of the full-length *rhs* gene, the homologous "non-functional" *rhs* gene can recombine into the duplication to restore its functionality. Thus, the bacterium is now in possession of a second functional Rhs system without sacrificing the original function (Figure 6). The cell that undergoes this event gains a fitness advantage as it is now capable of delivering a second type of toxic molecule into other bacteria. However, this is also yet another example of trade-off as the evolved strain has a slower growth rate (Koskiniemi et al., 2014). The shorter ORF is here described as "non-functional" if it does not undergo this recombination event, however, functionality and selection of these genetic elements are discussed in Papers I & II of this thesis (page 39 & 41).

Toxin-Antitoxin (TA) systems

The eight types of TA systems

Toxin-antitoxin (TA) systems are typically defined as a toxin with a given function and an antitoxin that neutralizes the molecular function of the former (Harms et al., 2018). These systems are particularly common in pathogens and are involved in several different biological functions including plasmid addiction, persistence biofilm formation, growth regulation and phage protection (Stewart et al., 2005; Norton and Mulvey, 2012; Bukowski et al., 2013; Guegler and Laub, 2021). There are eight types of identified TA systems, and below is a brief description of the different types before shifting focus to the Type II TA systems (Singh et al., 2021). Rather than classifying TA systems according to Toxin activities or properties, they are characterized by the type of antitoxin which for the types described here are either RNA based or protein based (summarized in Table 1). Type I systems encode a small RNA (sRNA) that binds to the toxin messenger RNA (mRNA) molecule thus inhibiting translation (Gerdes et al., 1986). Type II TA systems instead encodes a protein that binds directly to the toxin molecule and blocks its function (Figure 3) (Ogura and Hiraga, 1983). Similar to Type II, the antitoxin of Type III systems binds to the toxin molecule to block its function but in this case the antitoxin is instead an RNA molecule (Blower et al., 2012). The Type IV systems use a protein that acts as an antagonist by binding to the target of the toxin to block activity (Masuda et al., 2012). In Type V systems the toxin mRNA can be degraded by the antitoxin protein (Wang et al., 2012). Type VI systems takes this one step further as the toxin protein is targeted for proteolytic degradation after interacting with the antitoxin protein (Aakre et al., 2013). Reminiscent of Type II, the Type VII antitoxin protein destabilizes the protein toxin in the presence of oxygen (Wang et al., 2021). Type VIII systems differs from all other TA systems in that both the toxin and the antitoxin are sRNAs. The toxin sRNA interferes with regulation of numerous genes whilst the antitoxin sRNA interferes with the toxin (Choi et al., 2018). Because this thesis focuses on the similarities between Type II TA systems and CDI/Rhs systems, the remainder of this section will focus on this type as the others are outside the scope of this thesis.

Table 1, summary of the eight different types of TA systems as defined by the mechanism of the antitoxin to neutralize the activity of the toxin.

TA Type	Antitoxin	Antitoxin function
I	sRNA	Binds to toxin mRNA to block translation.
II	Protein	Binds to toxin to block its function.
III	sRNA	Binds to toxin to block its function.
IV	Protein	Binds to toxin target to block toxin function.
V	Protein	Degrades toxin mRNA to block translation.
VI	Protein	Binds to toxin to make it a target for proteolytic degradation.
VII	Protein	Binds to toxin and destabilizes it.
VIII	sRNA	Binds to toxin (sRNA) to block its function.

Type II TA addiction modules

As mentioned above, the molecular function of Type II (TA) toxins are neutralized by an unstable antitoxin protein (Harms et al., 2018). The gene encoding the antitoxin is usually found upstream of the toxin gene with a few exceptions such as the ShpAB system in *S.* Typhimurium in which the order is reversed (Harms et al., 2018; Slattery et al., 2013). Type II TA systems have been found to play several important roles in bacterial physiology. The first described function was as "addiction modules" by the CcdAB system where *ccdA* and *ccdB* encode the antitoxin and the cognate toxin respectively from the F-plasmid in *E. coli*. Cells that loose a plasmid encoding a TA system will also loose the antitoxin gene (Ogura and Hiraga, 1983). Due to a difference in protein stability between toxin and antitoxin, the latter may be targeted for proteolytic degradation, *e.g.* by the Lon protease as in the case of CcdA, resulting in free toxin molecules (Van Melderen et al., 1994; Fraikin et al., 2020). The toxin can then interact with its molecular target (DNA gyrase in the case of CcdB) unhindered ultimately killing the cell or inhibiting growth. This phenomenon is commonly referred to as post-segregational killing/distortion or plasmid addiction (Ogura and Hiraga, 1983; Bernard and Couturier, 1992).

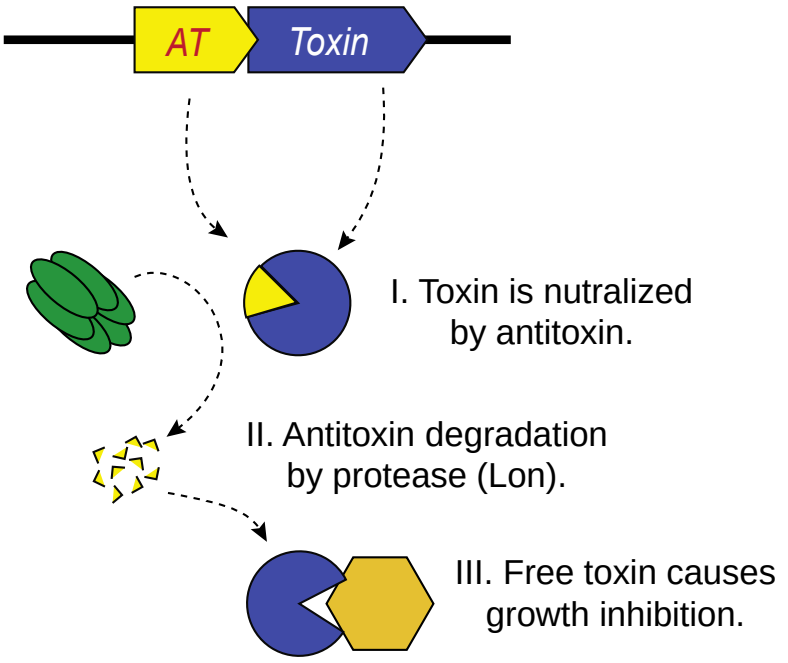

Figure 3, Schematic representation of a general type II toxin-antitoxin system. I, Antitoxin binds to the toxin and blocks its activity. II, Antitoxin is targeted for proteolytic degradation by protease (such as the Lon protease). III, Toxin without bound antitoxin interacts with its molecular target and causes growth inhibition (or cell death).

Type II TA antibiotic persisters

Antibiotic persisters were first described in 1944. In an attempt to prove that penicillin was bactericidal it was discovered that a distinct subpopulation of *Staphylococci* could survive penicillin treatment. Importantly, antibiotic persistence is not to be confused with antibiotic tolerance, hetero-resistance or persistent infections (see persister definition on the next page) (Bigger, 1944; Balaban et al., 2019). Studies suggest that Type II TA systems are implicated in persistence towards antibiotic treatments. In the case of *S.* Typhimurium, the environmental stress of the SCV, such as nutrient depravation and acidification, likely causes protease-dependent degradation of the antitoxin which results in a non-growing subpopulation (Helaine et al., 2014). Treatment of growing cultures with a bactericidal antibiotic, such as ampicillin, results in the death of the vast majority of the population. However, the antibiotic does not affect the small heterogenous subpopulation of non-growing cells. Once the antimicrobial compound is removed, the dormant bacteria may resume growth and thus re-establish the population (Balaban et

al., 2004). The clinical importance of this phenomenon is, however, debated. *In vivo* experiments have shown that Type II TA system ShpAB indeed gives rise to antibiotic persisters during intra-macrophage growth of *S.* Typhimurium in mice. Furthermore, this system has a negative impact on replication suggesting a role in growth regulation or a fitness trade-off (Helaine et al., 2014).

The role of Type II TA systems in persister formation is, however, somewhat controversial. Due to a ϕ80 phage contamination, two highly cited papers from the Gerdes lab, showing that deleting all ten type II TA systems in *E. coli* affects persister formation and that the persister phenotype is caused by the stringent response inducing Lon-dependent degradation antitoxins, were retracted. Attempts to replicate these results in a phage free strain were unsuccessful even by other labs (Ramisetty et al., 2016; Maisonneuve et al., 2018; Goormaghtigh et al., 2018). Due to inconsistencies, controversy and confusion within the field, a collective effort was made at the end of the last decade to properly define antibiotic persistence. In order to not further distort this definition, the direct quote from the published article is presented below (Balaban et al., 2019).

> "Antibiotic persistence is a population-level phenomenon that historically has been derived from the observation of biphasic killing curves, indicating the presence of two subpopulations, consisting of cells that are killed fast by the antibiotic and tolerant cells that may survive. By definition, the term antibiotic persistence is always connected with a heterogeneous population, in which only a part of the population consists of tolerant cells."
>
> - Balaban et al., 2019

Persisters may also be formed by other factors such as other types of TA systems or by the CdiBAI system in *E. coli* which is discussed in the next section (Ghosh et al., 2018).

Contact-Dependent Growth Inhibition (CDI)

Discovery of a novel type of competition system in *E. coli*

Contact-Dependent growth Inhibition (CDI) as a phenomenon was first characterized in the *E. coli* isolate EC93, a uropathogen in rats that had a remarkable ability to outcompete other strains of *E. coli*. It was found that this isolate carries a then previously unidentified system for inter-cellular competition dubbed CdiBAI (Aoki et al., 2005). This system distinguishes itself from other forms of bacterial competition systems such as colicins, which are secreted into the surrounding environment and taken up by unsuspecting targets, in that it requires direct cell-cell contact to allow for delivery (Aoki et al., 2005; Cascales et al., 2007). The system functions as a two partner type V secretion system (T5SS) where CdiB forms a β-barrel transport protein that delivers CdiA onto the cell surface the latter of which forms an extracellular stick-like structure (Aoki et al., 2005; Hayes et al., 2010). Additionally CdiA contains a polymorphic toxic C-terminal domain, commonly referred to as C-terminal Toxin (CT) which can be delivered into neighbouring cells causing inhibition of growth (Aoki et al., 2008; Webb et al., 2013). A defining trait for CdiA in *E. coli* is the VENN motif which is present in the pre-toxin domain and marks the beginning of the CT (Aoki et al., 2010). The CDI+ cells are protected from auto-inhibition by the cognate CdiI protein encoded from the *cdiI* gene located downstream from *cdiB* and *cdiA* (Figure 4) (Aoki et al., 2005). Several reports for different systems have shown that CdiI binds directly to the toxic CT to block its activity (Aoki et al., 2010; Morse et al., 2012; Nikolakakis et al., 2012). Since its discovery, genes encoding CDI systems have been identified in several species across the α-, β-, and γ-proteobacteria classes (Aoki et al., 2010). Despite the wide distribution, most of the studies have been done on *E. coli* CdiBAI.

Classification by receptor and toxin function

CDI systems in *E. coli* may be classified by their receptor binding domain (RBD) present on CdiA responsible for binding to a specific outer membrane protein on the target cell. Class I RBD binds to BamA, class II to OmpC/F, class III to Tsx and class IV to LPS. Potential Class V systems have also been identified but are not yet well studied (Aoki et al., 2008; Beck et al., 2016; Ruhe et al., 2017; Halvorsen et al., 2021). Different classes of RBDs have different levels of specificity regarding binding as Class I only recognizes BamA from closely related species whilst Class II RBDs are more promiscuous allowing for a broader toxin delivery (Ruhe et al., 2017; Virtanen et al., 2019). In addition to class, CDI systems may be categorized according to toxin function. The CT is highly variable which allows for different toxin activities in different strains or species. The two main CDI systems in EC93

both have membrane ionophore activity causing membrane depolarization thus disrupting the proton-motive force (Aoki et al., 2009; Wäneskog et al., 2021); UPEC536 instead deploys a tRNA anticodon nuclease which disrupts translation (Aoki et al., 2010; Diner et al., 2012) whilst the Orphan11 toxin in EC869 is a DNase (Morse et al., 2012) to name a few. Each polymorphic toxin also requires its own unique cognate immunity protein (Aoki et al., 2010).

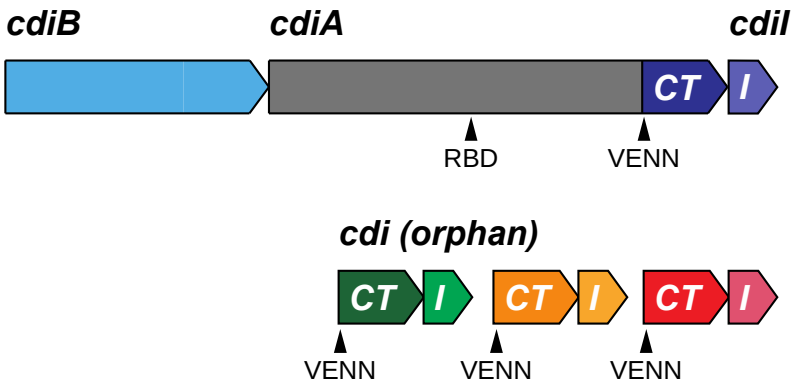

Figure 4, Schematic illustration of a cdiBAI locus with adjacent orphan modules (genes are not to scale). Receptor Binding Domain (RBD), Valine-Glutamate-Asparagine-Asparagine (VENN) motif and polymorphic C-terminus Toxin (CT) domains with its cognate immunity gene (I) are marked at their approximate locations.

Orphan modules

Some strains encode multiple functional *cdiBAI* loci capable of delivering toxins in different conditions such as in EC93 (Wäneskog et al., 2021). However, so called orphan modules are commonly found downstream of the *cdiBAI* locus which encode unique, functional toxin-immunity pairs (Figure 4). These orphan CTs may possess different toxin functions from the delivered main toxin but lack the delivery mechanism of CdiA. Orphan modules are often present as main toxins in other species or strains (Poole et al., 2011). An example of this is the Orphan1 (O1) in EC93 which is homologous to *cdiA-CT*^{UPEC536} and have the same toxic function suggesting a common ancestry. The immunity proteins are however not homologous but somewhat surprisingly CdiI^{UPEC536} protects against the EC93$_{O1}$ but not vice versa (Poole et al., 2011). Some strains may harbour several different orphan genes such as EC869 which has eleven different orphan toxin-immunity pairs encoded on its chromosome in addition to its main *cdiA+I* (Poole et al., 2011). The purpose of orphan genes is, however, not yet fully understood. It has been hypothesized that these modules are acquired by horizontal gene transfer

(HGT) and could provide an alternate arsenal of effector molecules for the cell to use. This would, however, require the orphan module to recombine into *cdiA* which would cause a deletion of *cdiA-CT+*I_{main} making the cell susceptible to toxin delivery from its neighbours that did not undergo this recombination event (Poole et al., 2011; Ruhe et al., 2014). Whilst this phenomenon has not been demonstrated experimentally, it is possible to restore the function of orphan toxin delivery by generating CdiA-CT_{Orphan} chimeras (Aoki et al., 2010; Poole et al., 2011). This does, however, not prove that this event would ever occur in nature and thus the function of orphan modules remains elusive.

Non-competitive functions of CDI

The function of CDI systems are not limited to kin selection and bacterial warfare but have also been found to be important for more cooperative behaviours (Aoki et al., 2005; Ruhe et al., 2013). In *Burkolderia thailandensis* CDI has been shown to be of importance for inter-bacterial communication where toxin delivery is capable of altering gene expression in recipient cells. Somewhat paradoxically the recipient cells will downregulate their *cdi* genes in response to toxin delivery thus functioning as a highly specific form of inter-cellular autoregulation. This phenomenon has been dubbed contact-dependent signalling (CDS) (Garcia et al., 2016). Moreover, early studies on CDI found that inhibition by CdiA-CT^{EC93} was reversible as target cells could resume growth 6 h after receiving the toxin (Aoki et al., 2009). Thus, it is possible that toxin delivery may alter gene expression in the target cell suggesting that this system could possibly mediate a type of CDS similar to *B. thailandensis*. Indeed, Ghosh et al. discovered that delivery of CdiA-CT^{EC93} or CdiA-$CT^{UPEC536}$ increases the levels of global transcription factor RpoS by inducing the stringent response in target cells, ultimately resulting in the formation of a subpopulation of antibiotic persister cells (described in more detail on page 26). This suggests that CDI systems in *E. coli* can be used as a highly specific bet hedging strategy in dense populations (Ghosh et al., 2018).

Despite being commonly found in pathogens, the function of CDI systems in pathogenesis is unclear (Aoki et al., 2010). Toxin delivery to eukaryotic hosts have not yet been observed but several reports describe the role of CDI systems in processes important for virulence. The HecA CDI system in plant pathogen *Erwinia chrysanthemii* for example, is necessary for aggregation and is required for pathogenesis in plants (Rojas et al., 2002). Similarly, deleting the CDI systems in *Pseudomonas* impairs virulence in a lettuce model through an unknown mechanism in addition to repressing swarming motility (Melvin et al., 2017). Biofilm formation is an important function for many bacteria as it protects the population from threats such as toxic compounds or competing microbes (López et al., 2010). In *B. thailandensis* the CDI system has been found to be important for biofilm formation independent of cellular

inhibition (Garcia et al., 2013). Furthermore, CdiA in EC93 is important for cell-cell adhesion independent of toxin delivery or receptor interaction. Instead, the CdiA filaments bind to each other resulting in auto-aggregation and by extent formation of biofilm (Ruhe et al., 2015). However, unpublished data from the Low lab suggests that functional CdiA-CT is important for biofilm formation in UPEC536 through an unknown mechanism (Unpublished data, see Diner et al., 2012). On the contrary, the presence of a CDI system in opportunistic human pathogen *Acinetobacter baumannii DSM30011* appears to hinder biofilm formation and adhesion to eukaryotic cells (Roussin et al., 2019). Thus, whilst much remains to be studied concerning CDI systems, their importance in multiple biological functions are not to be neglected.

Type VI Secretion System

Discovery of the sixth type of secretion system

Shortly after the discovery of CDI systems a new type of secretion system, capable of delivering toxins in a contact-dependent manner, was reported. Even before it was given its current name, the Type VI secretion system (T6SS) and its role in virulence had unknowingly been studied in other organisms such as *S.* Typhimurium and *Edwardsiella tarda* (Folkesson et al., 2002; Parsons and Heffron, 2005; Srinivasa Rao et al., 2004).The name was first coined in 2006 after the discovery of this system in *V. cholerae* due to its importance in evading phagocytosis (Pukatzki et al., 2006). A second report of this secretion system, this time in in *P. aeruginosa*, was published shortly after (Mougous et al., 2006). Complete T6SS related gene clusters have since been identified in both pathogenic and non-pathogenic Gram negative bacteria (Boyer et al., 2009). Some bacteria, such as *P. aeruginosa, Yersinia pestis and B. pseudomallei* (Mougous et al., 2006; Boyer et al., 2009), can encode several different T6SSs whilst others, such as *V. cholerae* and *S.* Typhimurium, only encode a single system (Blondel et al., 2009; Pukatzki et al., 2006). Remarkably, T6SS is capable of delivering effector molecules into both eukaryotic and bacterial cells functioning both as an important virulence factor and as a competitive system capable of shaping microbial communities (Pukatzki et al., 2006; MacIntyre et al., 2010).

Assembly of the secretion apparatus

In contrast to the CdiBAI system in *E. coli*, which only requires three genes to function (Figure 4) (Aoki et al., 2005), the T6SS is a complex machinery that is encoded by at least 13 genes, commonly referred to as core genes (Cascales, 2008). Structurally, the T6SS machinery consists of the membrane

complex, the tail spike and the baseplate (Figure 5). The membrane complex sits in the inner membrane and is composed of TssJ, TssL and TssM (*vasK*). It anchors the baseplate and serves as a pore, or channel, through which the tail spike can travel (Durand et al., 2015). The tail spike largely consists of the Haemolysin co-regulated protein (Hcp) which forms hexamers, shaped like hexagons, stacking on top of each other to assemble a tube-like structure. This architecture bears great resemblance to the T4 phage tail spike to which it shares a common evolutionary origin (Mougous et al., 2006; Brunet et al., 2014; Leiman et al., 2009). At the pole of the tube that interacts with the membrane complex, the valine–glycine repeat protein G (VgrG) trimer forms a tip-like structure. On top of VgrG sits the Pro-Ala-Ala-Arg (PAAR) domain protein which forms a cone-like structure that has been described to "sharpen" the tail-spike (Shneider et al., 2013) (this elaborated upon on page 36). The baseplate, formed by TssEFGK forms a complex around VgrG and attaches to the base of the inner membrane complex, facing the cytoplasm, where it serves as a platform on which the Hcp tube can assemble. The baseplate triggers a conformational change of TssBC (VipA/B in *V. cholerae*), which forms a sheath around the Hcp tube, causing a contraction that pushes the tail spike through the membrane complex, into the neighbouring cell (Brackmann et al., 2018; Brunet et al., 2015b; Cherrak et al., 2018). The ClpV protease recognizes the contracted structure and disassembles the apparatus mere seconds after launch and membrane and baseplate components are recycled (Basler and Mekalanos, 2012) (Figure 5).

T6SS have been found to deliver a cocktail of different effector molecules into target cells including PAAR-domain RHS toxins (described separately in section below; page 35) (Hernandez et al., 2020). It was initially hypothesized that effector molecules would travel from the cytoplasm through the Hcp tube (Mougous et al., 2006) similar to substances traveling through a syringe. Later studies, however, revealed that effector molecules most likely associate with the Hcp lumen, VgrG or PAAR previous to assembly of the T6SS apparatus (Hernandez et al., 2020). Whilst VgrGs are important for system function; studies in *V. cholerae* have shown that catalytically active toxins are not required for assembly of the T6SS. In fact, swapping the active site of VgrG3, which has lysozyme activity, with the active site from a VgrG from *S. enterica* serovar Arizonae, which has nuclease activity, results in a fully functional secretion apparatus. Furthermore, the toxic domain in a PAAR protein may also be substituted with the above mentioned *S.* Arizonae nuclease domain and retain its function (Ho et al., 2017).

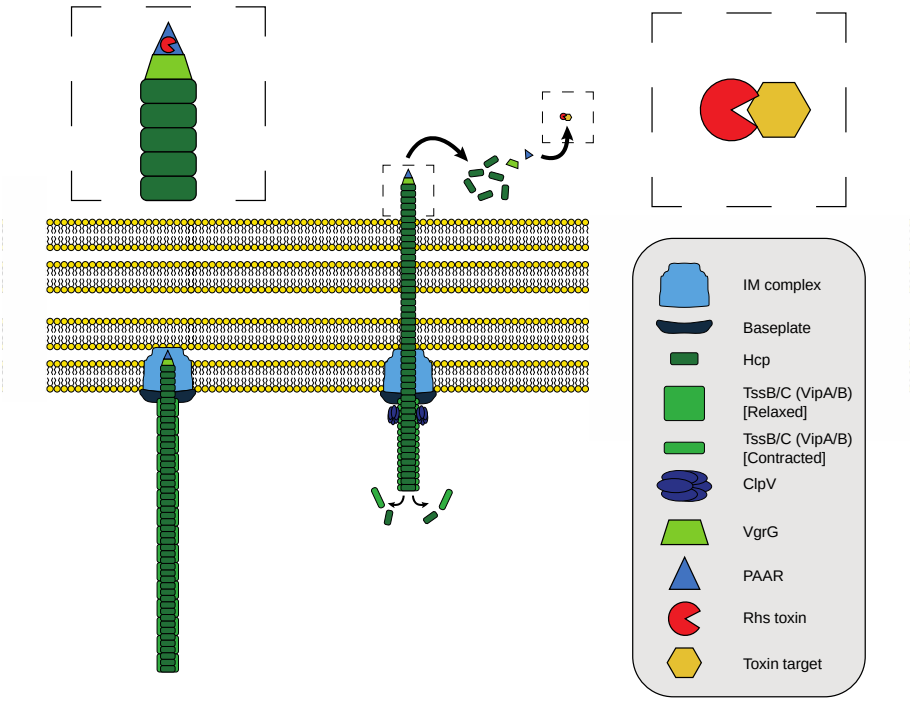

Figure 5, schematic illustration of Type VI Secretion System dependent delivery of Rhs toxin across inhibitor cell membrane into target cell. Left; assembled secretion machinery ready to fire with Rhs toxin encapsulated within the PAAR on top of VgrG. Right; Contraction of TssB/C (VipA/B) pushes the Hcp tube (tail spike) through the inner membrane (IM) complex and into neighbouring cell where the Rhs toxin may reach is molecular target and cause inhibition. The ClpV protease disassembles the complex after launch.

T6SS role in virulence and bacterial antagonism

Genes in the T6SS have been associated with virulence even before the secretion apparatus was identified. In 2004, before the system was given its current name, the homologous Evp (*E. tarda* virulence protein) system was seen to be important for virulence in the fish pathogen *E. tarda*. The study identified that this system appeared to function differently from other previously identified secretion systems (Srinivasa Rao et al., 2004). Even in the initial report, T6SS was found to be of importance for *V. cholerae* (strain V52) virulence in a *Dictyostelium discoideum* infection model. *V. cholerae* cells without a functioning T6SS were less capable of surviving *D. discoideum* phagocytosis and displayed impaired cytotoxicity towards mammalian J774 macrophages (Pukatzki et al., 2006). On the contrary, *P. aeruginosa* instead uses its T6SS to deliver effectors into epithelial cells that causes changes in the microtubule system which triggers endocytosis (Sana et al., 2015).

As mentioned in previous sections (page 14 & 15), an important aspect of bacterial infections is the ability of a pathogen to compete with the commensal flora of the host (van der Waaij et al., 1971; Garner et al., 2009). As opposed to the CDI system in *E. coli* which commonly delivers bacteriostatic molecules in a receptor specific manner (Aoki et al., 2008; Poole et al., 2011; Aoki et al., 2010; Ruhe et al., 2013), T6SS enables a bacterial cell to deliver a cocktail of both bacteriostatic and bactericidal toxins in a receptor-independent manner (Hernandez et al., 2020). *V. cholerae* V52 has been shown to use its T6SS system to outcompete numerous different strains of Gram-negative bacteria including *E. coli* K-12, *S.* Typhimurium, *Citrobacter rodentium*, EHEC, and EPEC *in vitro*. In fact, contact-dependent delivery of T6SS effectors results in complete lysis of *E. coli* cells (MacIntyre et al., 2010). *In vivo* studies have shown that *V. cholerae* utilizes T6SS to outcompete commensal bacteria in the intestine allowing for more efficient colonization (Fu et al., 2018; Zhao et al., 2018). Additionally, T6SS-dependent killing of *E. coli* likely induces the innate immune system due to the release of Microbe-Associated Molecular Patterns (MAMPs) ultimately, resulting in more severe disease in mice (Zhao et al., 2018). Despite its aggressive phenotype, *V. cholerae* V52 is not capable of outcompeting *P. aeruginosa in vitro* (MacIntyre et al., 2010). This is likely because of a phenomenon known as "bacterial duelling" or "tit-for-tat". *P. aeruginosa* has a potent T6SS that is activated if the bacterium is provoked. Thus, if *V. cholerae* attacks, *P. aeruginosa* will retaliate with its own effectors and kill the attacker. Interestingly, this response is not directed in a random direction. Instead, the T6SS is formed adjacent to the location where the attack occurred to correctly aim the counterattack towards the offender. *V. cholera* without a functional T6SS are, however, left unharmed suggesting that *P. aeruginosa* primarily uses this system in self-defence (Basler and Mekalanos, 2012; Basler et al., 2013). Furthermore, T6SS activity in *P. aeruginosa* may also be induced by membrane stress (Stolle et al., 2021). Similar to *V. cholerae*, *Shigella sonnei* requires a T6SS to compete with the intestinal flora in order to establish itself within the a murine host (Anderson et al., 2017). Non-pathogenic bacteria may also utilize T6SS for intra-bacterial antagonism such as the symbiont *V. fischeri* which uses its T6SS to eliminate competing strains of the same species to remain within its host (Speare et al., 2018).

T6SS in *S.* Typhimurium virulence

As mentioned in the *Salmonella* section (Page 15), *S.* Typhimurium uses its T6SS during intestinal colonization to compete with the normal flora. VasK (*sciS*) have been suggested to control virulence by limiting intracellular replication in late stages of J774 macrophage infection as well as reducing lethality in mice (Parsons and Heffron, 2005). This is somewhat contradicted by later studies where T6SS genes have been shown to be important for intra-

macrophage replication and systemic infection in mice (Mulder et al., 2012; Liu et al., 2013) which is supported further by Pezoa et al., who reported that the system is important for gastrointestinal colonization and systemic spread in chickens (Pezoa et al., 2013). Early work on genes located on *Salmonella* Pathogenicity Island 6 (SPI-6), formally known as *S. enterica* centisome 7 island (SCI), in *S.* Typhimurium showed that deleting the entire gene cluster affects invasion of Hep2 epithelial cells (Folkesson et al., 2002). This effect was, however, likely caused by an unknown factor as deleting only T6SS associated genes do not affect invasion in Hep2 or HeLa cells (Mulder et al., 2012). Furthermore T6SS is required for *S. enterica* Serotype Gallinarum intra-macrophage survival (Blondel et al., 2013). However, as mentioned in the "Selection of genes" section (Page 22), T6SSs on SPI-6 or SPI-19 appears to be of varying importance for serovars of *S. enterica* with different host specificities (Langridge et al., 2015). Whilst the exact function of T6SS during *S.* Typhimurium infection remains somewhat unclear and contradictive, several known virulence regulators and conditions have been suggested in mediating expression levels including SPI-2 activator SsrB; DNA binding protein H-NS; two-component system PhoP/Q; the ferric uptake regulator (Fur); Stationary phase sigma unit RpoS; and bile salts (Parsons and Heffron, 2005; Blondel et al., 2013; Brunet et al., 2015a; Wang et al., 2019; Sana et al., 2016).

Rearrangement hotspots (Rhs)

History of the elusive Rhs system

The *rhs*-elements were first observed four decades ago due to their ability to promote recombination in the chromosome of *E. coli* K-12 where duplications frequently ($2x10^{-4}$/cell/generation) occur between two *rhs* loci, *rhsA* and *rhsB*, in a *recA*-dependent manner (Lin et al., 1984). Later studies described the encoded protein to be divided into four domains in *Enterobacteriaceae*; The N-terminal domain which is variable, but conserved within subspecies; a GC-rich, variable core domain with a conserved secondary structure containing Tyr-Asp (YD)-repeats; a highly conserved PxxxxDPxGL motif; and a highly variable C-terminal. (Feulner et al., 1990; Jackson et al., 2009; Poole et al., 2011). Notably, *E. coli* MG1655 is in possession of 4-5 Rhs systems, RhsA-E, with RhsE being labelled as a "relic" or non-functional gene. (Jackson et al., 2009).

rhs genes have been found to be under high selection and homologous proteins can be found in Gram positive bacteria, such as the Wall associated protein A (WapA) in *Bacillus subtilis*, and even in eukaryotes (teneurin family of proteins) but the function remained unknown for decades after its initial discovery (S. J. Foster, 1993; Minet and Chiquet-Ehrismann, 2000; Petersen

et al., 2007). *In vivo* studies in calves showed that *rhsA* mutants of *E. coli* O26:H were deficient in colonization and Rhs in *Myxococcus xanthus* was predicted to be secreted and involved in social motility (van Diemen et al., 2005; Youderian and Hartzell, 2007). In 2011, Poole et al. reported similarities between the highly variable C-terminal regions of Rhs and the CT domains found in CDI systems (page 28). Thus, they hypothesized that these systems may be functionally analogous and could detect that Rhs systems from plant pathogen *Dickeya dadantii* functioned as toxin-immunity pairs which had previously been proposed for RhsA in *E. coli* K-12 (Poole et al., 2011; Vlazny and Hill, 1995). Following this discovery, it was reported that RhsT of *P. aeruginosa* could be translocated into eukaryotic cells where it causes inflammasome mediated death (Kung et al., 2012). Additionally, Koskiniemi et al. discovered that Rhs toxins in plant pathogen *D. dadantii* can be delivered by the T6SS into neighbouring cells in a contact-dependent manner causing inhibition of growth. Similarly, the Rhs homolog in *B. subtilis*, WapA, was found to inhibit neighbouring cells, likely transported through a general secretion pathway (Koskiniemi et al., 2013).

The co-dependency of Rhs/PAAR and T6SS

The original model for Rhs delivery suggests that the YD-repeats in the core domain folds into a β-cage or cone-like structure with the C-terminal toxin (CT) encapsulated within. The PAAR domain stabilizes the VgrG trimer of the T6SS forming a sharp tip on the injectosome (Figure 5) (Shneider et al., 2013; Busby et al., 2013; Donato et al., 2020). In a recent study, however, RhsA in *P. protegens* was characterized as a large "cocoon-like" structure that was hanging beside the VgrG-PAAR tip and is stabilized by the EagR chaperone protein. In the latter model the CT is suggested to be autoproteolytically cleaved within the cocoon and remain unfolded whilst a plug seals the structure. Upon delivery the plug is removed and the CT escapes and folds into an active toxin within the target cell (Günther et al., 2022). Rhs/PAAR toxins have been shown to be important for functional T6SS assembly with some species being in possession of multiple cognate Rhs/PAAR and VgrG pairs which are specific to each other (Whitney et al., 2014; Cianfanelli et al., 2016; Donato et al., 2020). Work done on *Serratia marcescens* have shown that T6SS requires at least one PAAR protein for functionality, however, in this organism VgrG1 uses a PAAR protein (Paar1) without an adjacent Rhs toxin which allows for Rhs-independent T6SS assembly. Interestingly, VgrG2 has multiple compatible PAARs with different Rhs toxins functions allowing for a wider selection of effectors, but only with VgrG2 as they cannot be loaded onto Paar1. (Cianfanelli et al., 2016). Furthermore, a study on *Agrobacterium tumefaciens* suggests that a functional PAAR associated toxin effector a requirement for assembly of the T6SS as inactive toxin mutants are incapable of launching the secretion

apparatus. This is possibly to prevent cells from firing empty tail spikes (Wu et al., 2020). Similar results have been shown in *Enterobacter cloacae* where the Rhs protein (not just the PAAR) greatly enhances T6SS assembly. Despite this, the actual CT is not a requirement (Donato et al., 2020). In some cases, *rhs* genes are not limited to T6SS gene clusters. As shown in *E. cloacae*, where *rhsB* is not adjacent to any T6SS genes, the toxin is still dependent on this system for delivery despite its chromosomal location (Whitney et al., 2014). On the contrary, *V. cholerae* is not in possession of Rhs yet its T6SS remains fully functional (Zheng et al., 2011). Furthermore, despite encoding several Rhs systems; *E. coli* K-12 is not in possession of a T6SS (Donato et al., 2020). This suggests an alternative function or mode of delivery for these systems or perhaps they are only maintained because of the previously mentioned high selection pressure for these genes (Petersen et al., 2007). Thus, despite much progress within in the field of Rhs, the roles of the first ever discovered systems remains elusive.

Rhs in *S.* Typhimurium

The *rhs* loci in *S.* Typhimurium is present on SPI6 adjacent to the T6SS cluster of genes (Folkesson et al., 2002; Blondel et al., 2009; Jackson et al., 2009). Phylogenetically, based on the core domain, this system belongs to Rhs Clade II making it more similar to systems found in *Shigella flexneri*, *Yersinia pestis* and *Serratia marcescens* compared to the previously mentioned systems in *E. coli* and *D. dadantii* which belong to Clades I and IV respectively (Jackson et al., 2009). The main locus consists of the core gene where the CT encodes a toxin directly followed by *rhsI* encoding a cognate immunity protein reminiscent of the gene orientation of *cdiAI*. Similar to CDI systems in *E. coli*, *rhs* in *S.* Typhimurium contains an orphan module; a second truncated locus where the majority of the core delivery gene is missing (Figure 6, top panel) (Poole et al., 2011; Koskiniemi et al., 2014). The orphan gene carries a 95% homologous domain to the main core gene. This homology does not include the CT which instead requires its own unique immunity gene thus suggesting a different toxin function and/or target. In the event of a gene duplication of the main *rhs* locus, the homology in the orphan gene allows for recombination into the core domain generating two full length loci capable of delivery by T6SS (Figure 6) (Koskiniemi et al., 2014). Interestingly, in the original study by Lin et al., no *rhs*-elements could be observed in *S.* Typhimurium LT2. A possible explanation for this oversight is that the authors defined *rhs*-elements by their ability to recombine into each other which, as mentioned above, likely only occurs in the event of a rhs^{main} gene duplication in *S.* Typhimurium. Another possibility is that the methods used to detect these elements in *E. coli* were simply not directly transferable to *Salmonella* (Lin et al., 1984).

Similar to systems from other species, Rhs has been associated with virulence according to some studies. A deletion of the orphan toxin impairs

systemic infection of *S.* Typhimurium in mice (Liu et al., 2013) and experiments in cattle and pigs have shown that Rhs is crucial to establish infection (Chaudhuri et al., 2013). Because relatively little work has been done on Rhs (and T6SS) in *S.* Typhimurium; not much is known about toxin function, molecular targets, protein structure or its role in bacterial physiology.

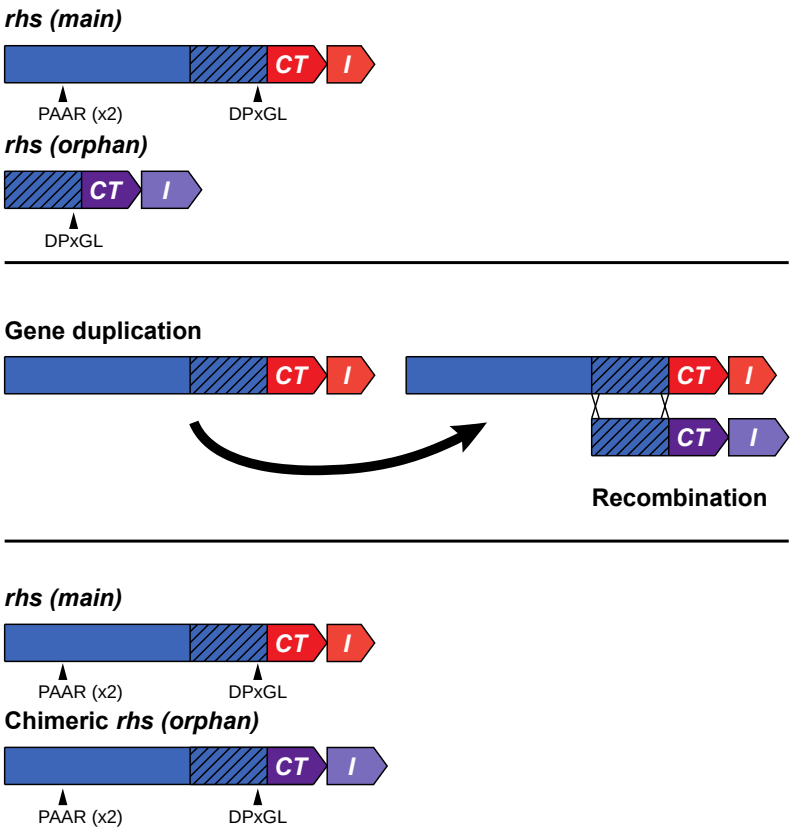

Figure 6, schematic representation of rhs locus duplication event in S. Typhimurium as proposed by Koskiniemi et al., 2014 (figure is not to scale). ***Top panel****, rhs main and orphan loci. Approximate location of PAAR and DPxGL motifs along with homologous domain (striped), polymorphic C-terminus Toxin (CT) domain with its cognate immunity gene (rhsI) are marked at their approximate locations.* ***Middle panel****, gene duplication of the main locus resulting in recombination of homologous domain (striped).* ***Lower panel****, chimeric full length orphan locus capable of delivery is formed after recombination. Figure is modelled after figure 5A in Paper I (Stårsta et al., 2020. PLoS Gen.).*

Present investigation

The initial aim of this thesis was to study the role of Contact-Dependent growth Inhibition (CDI) systems during bacterial pathogenesis. CDI in this context is used as a collective name for the Rhs system in *S.* Typhimurium and the CdiBAI system in *E. coli*. During the course of the experiments, we discovered that toxin and immunity genes in Rhs could be expressed internally, independent of delivery. We therefore studied this phenomenon in parallel with virulence as they likely overlap. Thus, the new aim became to study the effects of internally expressed CDI toxins in pathogenic bacteria. In this thesis I describe novel secondary functions of CDI systems in *S.* Typhimurium and *E. coli*. In paper I we discovered that Rhs in *S.* Typhimurium harbours an internal expression which enables it to function as a Type II TA system capable of regulating intra-macrophage growth. In paper II we followed up on the findings in paper I and studied *in vitro* effects and the regulation of Rhs by known virulence regulators. In paper III we examined the possibility of CdiBAI systems in *E. coli,* having an internal expression similar to our discovery in paper I. Our findings show that these systems indeed also possess internally expressed C-terminal toxins and immunities that are functioning as selfish genetic elements and are capable of regulating *E. coli* growth *in vitro* and protect against delivered toxin.

Paper I

rhsCT+I are expressed independently of the full-length *rhs* gene

A previously published genome wide study on the *S.* Typhimurium 4/74 (SL1344) transcriptome revealed hypothetical transcriptional start sites (TSSs) upstream of the toxin (*rhsCT*) and immunity (*rhsI*) in both the main and the orphan loci of *rhs* (Kröger et al., 2013). We measured the relative expression of the toxin genes by qPCR and could observe that they were indeed 100-fold more highly expressed compared to the N-terminal delivery domain ($rhs^{delivery}$) for cells grown to mid-exponential phase in LB. Additionally, *Salmonella* grown for 8 h within RAW264.7 macrophage-like monocytes displayed 5- to 9-fold higher expression for $rhsCT^{main}$ and $rhsCT^{orphan}$ respectively relative to $rhs^{delivery}$. We could identify the presence of four putative promoters within the *rhs* gene in addition to the 5´ promotor upstream of the *rhs* locus. We studied expression of each of the five promotors

(named P1-5) by cloning them upstream of a transcriptionally fused YFP marker and measured fluorescence by which we could detect that P2 gave the strongest signal. This promotor is located in a domain upstream of *rhsCT* that is homologous between *rhs*^{main} and *rhs*^{orphan} and thus occurs twice. We identified a short internal ORF within the *rhs*^{main} and *rhs*^{orphan} coding sequence, and could verify that the proteins encoded from these internal ORFs were functional by overexpressing the P2 transcript from an arabinose inducible promotor. This resulted in impaired growth in arabinose compared to cells grown in glucose, whereas cells encoding the cognate immunity protein were unaffected in both conditions. We then confirmed RhsCT+I^{orphan} molecular interactions by co-immunoprecipitation, showing that internally expressed RhsCT and RhsI comprise a bonafide TA-system.

Rhs toxins regulate growth during intra-macrophage growth

A previous study on Type II Toxin-Antitoxin (TA) systems in *Salmonella* discovered that some of these modules are involved in regulating intra-macrophage replication as well and the process of dormancy following phagocytosis (Helaine et al., 2014). By infecting RAW264.7 cells we discovered that *rhs* mutants of *S.* Typhimurium where the complete system was knocked out proliferated twice as much during intracellular growth compared wild type (wt). Furthermore by using a previously described method named fluorescence dilution (FD) (Helaine et al., 2014), which allowed us to determine relative growth rate and the percentage of growing cells, we could detect that cells lacking the entire *rhs* locus grew 30% faster compared to wt. Strangely, we could observe that cells lacking only RhsCT+I^{main} were 25% less dormant but grew 15% slower compared to wt which cannot be explained by our results. As previous studies have shown that some Type II antitoxins and CdiI from UPEC536 are degraded by the Lon protease (Van Melderen et al., 1994; Smith and Rawlings, 1998; Ghosh et al., 2018) we were interested to study if a similar observation could be made for RhsI. Cells were exposed to SHX, which induces the stringent response, and protein levels were visualized by Western blot. Indeed, we could observe degradation of both RhsI^{main} and Rhs^{orphan}. When repeating the experiment in a Δ*lon* background RhsI^{orpahn} was protected from degradation whilst RhsI^{main} was still degraded but at a slower rate suggesting that several proteases may target the latter. Thus, based on our results we concluded that *rhsCT+I* can encode functional toxin-immunity pairs independent of the delivery mechanism and that these systems impair intracellular growth likely due to protease-dependent degradation of RhsI, reminiscent of Type II TA systems.

Paper II

Rhs causes a small effect on fitness *in vitro* unrelated to expression levels

Our findings in Paper I describe that *rhs* genes can be expressed independently of toxin delivery from internal transcriptional start sites both *in vivo* and *in vitro*. Based on these results, we were interested to study if these toxins confer a fitness cost in laboratory medium and how the gene expression is regulated. We competed *S.* Typhimurium wt against *rhs* mutants in rich laboratory medium (LB) and in medium that induces SPI-2 (InSPI-2). Based on these experiments we could determine a small 50% increase in fitness for mutants lacking both the *rhs* main and orphan genes but only in rich medium. This is surprising as qPCR data show a 5- to 9-fold increase in expression of these genes in during mid-exponential phase of growth in InSPI-2 and in macrophages relative to LB. Furthermore, InSPI-2 is designed to mimic the intracellular environment of the macrophage; an environment where we could observe a 100% increase in growth for a $\Delta rhs^{complete}$ mutant in Paper I.

***rhs* genes are activated by RpoS and PhoP/Q and repressed by H-NS**

As mentioned above, the expression of *rhs* is higher in cells grown in InSPI-2 or within macrophages relative to LB cultures. Therefore, we were interested to study if *rhs* expression is affected by known virulence factors. To screen how *rhs* genes are regulated *in vitro* we constructed transcriptional fusions to $rhsI^{main/orphan}$ and made gene knockouts of previously characterized virulence regulators. By measuring these constructs with flowcytometry (MACS) we could determine which regulators to study further. We complemented this screening with qPCR from exponentially growing cells. Our results indicate that alternate sigma factor RpoS, a regulator of several stationary phase genes, activates expression of *rhs* in rich medium but has little to no effect in InSPI-2. Additionally, the two-component system PhoP/Q, known to regulate several virulence genes during infection including *vasK* (*sciS*) in the T6SS locus (Miller et al., 1989; Parsons and Heffron, 2005), also acts as an activator of *rhs* in InSPI-2 according to our qPCR data. This is, however, contradicted by the MACS data which indicates that PhoP/Q instead acts as a repressor on *rhs*. A key difference between these experiments is that the qPCR analyses exponentially growing cells whilst the MACS measurements were performed on stationary phase cultures suggesting that growth phase could be of importance. Finally, in a previous study it was shown that the histone-like nucleoid structuring protein (H-NS) suppresses SPI-6 where the *rhs* genes are located. Both our qPCR data and MACS data are in agreement with this statement as Δ*hns* mutants show a strong increase in expression of *rhs*. To study if expression levels of *rhs* have any impact on growth we replaced the P2 promotor in $rhsCT^{main}$ with two constitutive promotors of different strengths; one weaker and one stronger than P2. When we repeated the competition experiment in LB with these strains we could not observe any

differences in growth suggesting that any differences in fitness caused by Rhs in different conditions are not due to changes in gene expression but are instead caused by an unidentified factor, possibly the stability of RhsI. Thus, our results indicate that expression levels do not correlate with observed fitness effects. Our findings in Paper I show that both immunity proteins are degraded by the Lon protease. We were interested to see if RhsI is stabilized in the presence of its cognate toxin. Western blot data shows that RhsI indeed is stabilized by RhsCT. Collectively, our results from Paper II show that *rhs* genes are activated by RpoS and possibly PhoP/Q whilst H-NS acts as a repressor. Additionally, the presence of *rhs* toxins confers a small fitness cost *in vitro* independent of internal expression levels, possibly caused by the unstable RhsI protein. We hypothesize that the function of the internally expressed toxin is to stabilize the immunity protein to protect the bacterium from sister cell delivery of RhsCT.

Paper III

cdiA-CT+I can be expressed internally similar to *rhsCT+I*

CDI systems in *E. coli* are commonly followed by one or more orphan modules. Previous studies have shown that these systems are functional but cannot be delivered due to lacking a full length *cdiA* gene (Poole et al., 2011). The purpose of these modules and how they are maintained on the chromosome is poorly understood. Based on the many similarities between Rhs and the CdiBAI system in we hypothesized that the latter also could contain internal ORFs encoding toxin-antitoxin modules. By cloning the *cdiA-CT+I* from uropathogens EC93 or UPEC536 upstream of a fluorescent marker on a pSC101 vector (here referred to as a CT-vector) we could observe expression from one or more promotors, both in cells grown in rich or minimal medium. These expression levels were comparable to the well characterized Type II TA system CcdAB, which we used as a positive control throughout this paper. We could observe that expression in rich medium increased over time whilst cells grown in minimal medium had high expression levels already in early stages of growth with a relatively low increase over time. We therefore hypothesized that the internal expression could be regulated by RpoS which is induced upon nutrient stress and regulates many stationary phase genes (Lange and Hengge-Aronis, 1991). Additionally, we bioinformatically characterized three internal TSSs and made knockouts of these in the CT-vectors accordingly in order to study the origin(s) of the fluorescent signal. Flowcytometry analysis revealed that the expression in an accumulative signal from all three promotors and that RpoS may play a small role in regulating these.

Internally expressed toxin and immunity are functional and protect from delivered toxins and function as selfish genetic elements

We studied the if these transcripts encode functional immunity proteins by performing a competition assay where *E. coli* with the CT-vector were competed against an inhibitor strain capable of delivering the corresponding toxin. We could observe that the CT-vector could nearly or fully abolish the inhibitory phenotype of CdiA-CT^{EC93} and CdiA-CT^{UPEC536} respectively. Next, we deleted the immunity gene from the CT-vectors and grew the cells in stress-inducing minimal medium to study if also the toxin was functional. Our results show that cells that were lacking the cognate immunity gene reached a lower final OD_{600} after 24 h suggesting that the toxin is functional. By growing EC93 transformed with the CT-vectors in minimal medium, we could observe a 10% fitness cost of CdiA-CT^{EC93} and CdiA-CT^{UPEC536} in a Δ*rpoS* background which made us ask how selection for these genes is maintained under in conditions where delivery does not occur. Type II TA systems, such as CcdAB, have previously been described to function as selfish genetic elements capable of killing or inhibiting growth of the cell (Ogura and Hiraga, 1983). We were interested to see if CDI systems could function in a similar manner. Upon transforming EC93 with temperature sensitive (ts) CT-vectors and competing these against the stable CT-vectors at high temperature in minimal medium, we could observe that CdiA-CT may function as selfish genetic elements upon plasmid loss. Finally, we observe that the internally expressed toxin-immunity pairs appear to be a common feature amongst CDI system even in different species. In conclusion, our findings in paper III shows that *cdiA-CT-I* contain internal transcriptional start sites which encode functional toxin-immunity pairs that, despite causing a small growth defect, are beneficial for the cell by providing protection from delivered toxin and are maintained in the genome by acting as selfish genetic elements.

Future perspectives

Rhs affects intra-macrophage growth, but how?

In Paper I, we presented evidence that Rhs in *S.* Typhimurium has a negative impact on fitness during intra-macrophage growth. Of course, with the currently available information we can only speculate over why this occurs. The fact that Rhs associates with the T6SS could mean that it could be involved in regulating growth and limiting virulence as suggested by previous work on the latter system (Parsons and Heffron, 2005). A possible explanation for this could be that hyper-virulent strains of pathogens likely have a limited capability of transmission due to them killing the host more quickly. Rhs in *S.* Typhimurium SL1344 have been found to be of importance for infections in pigs and cattle (Chaudhuri et al., 2013). Therefore, further *in vivo* experiments could be relevant to study any potential effects of Rhs on colonization, infection and systemic spread. Furthermore, our results only indicate that the observed effect in macrophages is caused at least partly by internally expressed toxin, but does not rule out possible effects of inter-bacterial toxin delivery via the T6SS. Because T6SS have previously been associated with *Salmonella* intra-macrophage survival, one can thus hypothesize that this effect is because the cells actually deliver Rhs toxins within the SCV. In regards to CDI systems being common amongst pathogens, they could serve as future drug targets to dampen virulence or impair the ability to colonize the intestine. Other virulence factors such as the T3SS in *S.* Typhimurium which is essential for early stages of infection can be targeted by Tanic acid rendering the cells unable to infect HeLa cells. This effect is possible by the compound inhibiting the genes encoding the system on SPI-1 (Shu et al., 2022). Therefore, targeting species specific parts of CDI systems or the T6SS either directly or by altering gene expression could severely decrease the fitness of pathogens in early stages of infection.

Under which conditions can we observe delivery of Rhs?

In paper II, the regulation of the internal expression of *rhs* is linked to global virulence regulators RpoS, PhoP/Q and H-NS. Finding a phenotype for these internally expressed toxins *in vitro* did, however, prove itself to be difficult as we could only show a 50% increase over 24 h in a $\Delta rhsCT{+}I^{main+orphan}$ mutant but not in a $\Delta rhs^{comlete}$ strain. This might be explained by the $\Delta rhs^{comlete}$ strain not being able to correctly assemble its T6SS due to it lacking a PAAR domain protein to stabilize the VgrG trimer which could indicate that these systems

are active in this condition (Shneider et al., 2013; Donato et al., 2020). Furthermore, the toxic effect and conditions where Rhs is delivered are somewhat unclear. Studies on T6SS in *S.* Typhimurium have shown that the system can be activated when cells are grown on 0.05% bile (through an unknown factor), in the absence of iron due to the ferric uptake regulator (Fur) being a repressor of *clpV* or in an *hns* mutant background (Brunet et al., 2015a; Sana et al., 2016; Wang et al., 2019). These studies did, however, focus on T6SS activity as a whole rather than Rhs specifically and our efforts to reproduce these results have been unsuccessful. The T6SS activity in *P. aeruginosa* has been shown to be passive but is activated if the cell is provoked by another T6SS from a neighbouring cell (Basler et al., 2013). It is possible that the system in *Salmonella* is activated in a similar manner. Thus, its main function may be as a defence mechanism as it has other efficient ways of competing with the normal flora. Furthermore, it is unclear if the internally expressed immunity protein can protect against delivered toxin similar to what we observed for CDI in paper III. In a previous report, Koskiniemi et al., observed that evolved strains of *S.* Typhimurium LT2 could inhibit the parental strain, likely through delivery of $RhsCT^{orphan}$. However, the inhibition phenotype could be rescued by expressing $RhsI^{orphan}$ from a plasmid thus they hypothesized that the orphan genes are not expressed (Koskiniemi et al., 2014). In paper II we have shown that this is in fact not the case as we can observe both transcriptional and translational expression. However, we also showed that RhsI in the absence of is highly unstable a which may explain how inhibition is possible as it may simply be a matter of shifting the toxin-immunity balance. Finding a stable condition to study Rhs delivery without having to rely on unstable evolved strains is thus of key interest to study immunity function, toxin mechanism and molecular targets of Rhs.

What is the purpose of orphan toxins?

Koskiniemi et al., have previously shown that Rhs orphan modules can recombine into the main locus without losing the original system in the event of a gene duplication allowing for two functional systems. This is, however, a rare event and revertants are common but the question remains why the orphan genes are incapable of delivery to begin with (Koskiniemi et al., 2014). In both Paper II and III we hypothesize that orphan modules may merely exist to protect the cell from delivery by other strains or species with the function of the toxin may simply be to maintain selection of the immunity. This is in contrast to a common theory that these modules serve as alternate effectors for delivery. As we can observe a fitness cost for these systems due to the internal expression there must be a trade-off as to how many orphan modules a cell can acquire and still benefit from them. A bioinformatical approach to look for orphan toxins functioning as main toxins in other strains/species and comparing the habitat and lifestyle of these bacteria could reveal an explanation as to how these systems were first acquired. A similar approach

was taken in what is likely the most cited study in this thesis (Poole et al., 2011) but the distribution of orphan genes needs to be further explored to understand the evolutionary aspects. An interesting question from this is whether the number of orphan modules or their toxin function can be linked to a certain niche. A somewhat cynical but perhaps plausible explanation is that orphan modules are nothing more than selfish genetic elements and any other functions are coincidental.

In conclusion

As we keep finding new roles for various CDI systems, we may ask in what other important aspects of bacterial lifestyle that these systems might play a role and how universal these effects are. Can they be used as drug targets or perhaps be used to arm a potent strain of probiotics? These questions are not answered in this thesis but hopefully they will be answered someday.

Svensk sammanfattning (Swedish summary)

Rhs (Rearrangement hotspots) och CDI (Contact-Dependent growth Inhibition) är båda molekylära system som möjliggör för en bakterie att leverera ett toxiskt protein till kringliggande celler genom komplexa leveransmekanismer. Bakterien och dess systerceller är skyddade från detta toxin genom ett immunitetsprotein som neutraliserar den toxiska funktionen. Dessa system har visats vara mycket fördelaktiga då de tillåter immuna bakterier att konkurrera ut sina motståndare genom att hämma deras tillväxt. I denna avhandling beskrivs i tre artiklar hur jag och mina kollegor upptäckte att både toxin och immunitet kan tillverkas i cellen oberoende av leveransproteinet för båda systemen samt vilka funktioner dessa proteiner har i bakterier när de inte levereras.

I Artikel I (Paper I) gjorde vi det ursprungliga fyndet att gener som kodar för Rhs toxin och immunitet i den patogena bakterien *Salmonella enterica* serovar Typhimurium kan uttryckas oberoende av resterande delar av systemet. Genom att infektera makrofager (en typ av immunceller) från möss (RAW264.7 celler) visar vi att mutanter som saknar Rhs har en 30% snabbare tillväxt och hinner föröka sig dubbelt så mycket under 16 h relativt till *Salmonella* med Rhs intakt. Vidare visar vi att under konditioner när *S.* Typhimurium känner av svält bryter de ner immunitetsproteinet med hjälp av proteaset Lon. Vi resonerar att detta sannolikt är orsaken till att vi ser den tidigare nämnda effekten på tillväxt då bakterier med minskade nivåer av immunitet kommer påverkas mer av det toxiska proteinet. Denna nya karaktärisering av Rhs liknar den beskrivning som tidigare gjorts för många så kallade typ II toxin-antitoxinsystem. Slutligen visar vi genom en bioinformatisk analys att de promotorer som orsakar det oberoende uttrycket är välkonserverade bland Rhs från flera typer av bakterier. Vi föreslår därför att Rhs i *S.* Typhimurium har en sekundär funktion som ett typ II toxin-antitoxinsystem utöver sin tidigare beskrivna roll i bakteriella interaktioner.

I Artikel II (Paper II) fortsätter vi att studera Rhs i *S.* Typhimurium men denna gång var vi intresserade av vilka faktorer som påverkar uttrycket av dessa gener samt hur detta system påverkar tillväxt *in vitro* (utanför sin normala kontext, dvs. i provrör). Genom att jämföra tillväxten för *Salmonella* med och utan Rhs i rikt tillväxtmedium (LB) kan vi observera att detta system återigen medför en viss åverkan på tillväxt. Mutanter som saknar Rhs växer ungefär 50% bättre under loppet av 24 h men när vi upprepar detta experiment i ett medium (InSPI-2) som liknar miljön som *Salmonella* befinner sig i under

intracellulär tillväxt (i makrofager) ser vi ingen effekt. Trots detta visar mätningar av genuttryck med qPCR och flödescytometri att *rhs* uttrycks mer i InSPI-2. Vi visar att tre så kallade transkriptionsfaktorer som tidigare visats vara associerade med virulensgener reglerar uttrycket av *rhs in vitro*. RpoS och delvis också PhoP/Q ökar genuttrycket medan H-NS minskar det. Genom att ersätta promotorn som driver toxin och immunitet med en stark och en svag promotor visar vi att uttrycket av *rhs* i sig inte spelar någon roll för effekten av systemet *in vitro*.

I Artikel III (Paper III) visar vi att våra resultat från Rhs i Artikel I stämmer överens med CDI-system i *E. coli*. Våra data visar att toxin och immunitet även i dessa system kan uttryckas oberoende av leveransmekanismen. Genom att tävla bakterier med toxin och immunitet, men som inte kan leverera toxinet, mot en stam som kan leverera toxin ser vi att dessa är skyddade från hämningar i tillväxt. Dock kommer dessa system med en liten tillväxtskostnad under näringsfattiga förhållanden vilket väcker frågan hur selektionstrycket för dessa gener upprätthålls i förhållanden när bakterien inte utsätts för levererat toxin. När vi klonar toxin och immunitet på en temperaturkänslig plasmid, som går förlorad i höga temperaturer, ser vi att när cellerna gör sig av med CDI generna i 42°C försämras deras tillväxt också kraftigt jämfört med celler som behåller plasmiden. Detta fenomen kallas ”plasmid addiction” (plasmidberoende) och har sedan länge beskrivits för typ II toxin-antitoxinsystem. När generna för toxin och antitoxin/immunitet går förlorade finns det fortfarande fungerande protein i cellen. Antitoxinet/immuniteten är dock mindre stabilt än toxinet och kan brytas ned av proteas såsom det tidigare nämnda Lon. Detta gör att precis som i Artikel I finns det sannolikt mer toxin än immunitet vilket har en kraftig åverkan på tillväxt. Vi kan därför föreslå att även CDI-system i *E. coli* har en sekundär effekt som liknar typ II toxin-antitoxinsystem utöver sin roll som ett kontaktberoende tillväxthämmande system.

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