Too close for comfort

The role of Contact-Dependent growth Inhibition (CDI) in interbacterial competition and cooperation

MARCUS WÄNESKOG
Abstract


Contact-Dependent growth inhibition (CDI) was discovered in 2005 in the *E. coli* isolate EC93. Since then our knowledge of CDI systems and their impact on bacterial communities have increased exponentially. Yet many biological aspects of CDI systems are still unknown and their impact on complex microbial communities have only just begun to be studied. CDI systems require the function of three proteins; CdiBAI. The outer-membrane transport protein, CdiB, allows for the transportation of the toxin delivery protein CdiA to the cell surface of an inhibitor cell. Through a contact- and receptor-dependent interaction with a target cell the toxic C-terminal domain of CdiA is cleaved off and delivered into the target cell were it mediates a growth arrest. Different CdiA-CT domains encodes for diverse toxic activities, such as nucleases and membrane ionophore toxins. Each unique CdiA-CT toxin has a cognate immunity protein (CdiI) that binds and neutralize against its toxic activity, thus preventing a possible self-inhibition.

In this thesis I have studied the effect of CDI system(s) on both single cell and population level, within both intra- and interspecies bacterial communities. The findings presented here shows that multiple class I *cdiBAI* loci within a cell can function in a synergetic manner and act as versatile interbacterial warfare systems able to inhibit the growth of rival bacteria, even when CdiA expression is low. We also show that class II CdiA receptor-binding domains can mediate broad-range cross-species toxin delivery and growth inhibition, even when a non-optimal target cell receptor is expressed at a low level. Additionally, we show that the *cdiA* gene supports the expression of two separate proteins. The full-length CdiA protein, able to mediate an extracellular toxin delivery, but also the discrete CdiA-CT toxin domain. This stand-alone CdiA-CT expression was stress-dependent and together with its cognate CdiI immunity protein functioned as a selfish-genetic element. Moreover, we show that CDI systems can increase bacterial stress tolerance via an extracellular toxin delivery between kin-cells. This stress tolerance phenotype only occurred under conditions when we also observed a selective degradation of the CdiI immunity protein. Therefore, we suggest that a selective CdiI degradation allows for a sub-population of cells to self-intoxicate, thereby becoming transiently dormant, which confers an increase in stress tolerance. The findings presented in this thesis collectively suggest that CDI systems could function as a pseudo-quorum sensing system able to mediate behavioral changes and stress tolerance within a sub-population of cells in a bacterial community.

Keywords: bacterial interactions, cell-cell binding, contact-dependent growth inhibition, CDI, CdiB, CdiA, CdiI, outer-membrane receptor, BamA, OmpC, OmpF, extracellular toxin delivery, Toxin-Antitoxin, TA-system, persister cells, stress tolerance, stress response, rpoS, rssB, quorum sensing, multicellular behavior

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For my love
Imagination is more important than knowledge. For knowledge is limited, whereas imagination embraces the entire world, stimulating progress, giving birth to evolution.

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


*These authors contributed equally to this work.

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Publications not included in this thesis.


*These authors contributed equally to this work.
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### Abbreviations

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<th>Definition</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>CDI</td>
<td>Contact-dependent growth inhibition</td>
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<td>CDS</td>
<td>Contact-dependent signaling</td>
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<tr>
<td>DAEC</td>
<td>Diffusely adherent <em>E. coli</em></td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EAEC</td>
<td>Enteroaggregative <em>E. coli</em></td>
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<tr>
<td>EHEC</td>
<td>Enterohaemorrhagic <em>E. coli</em></td>
</tr>
<tr>
<td>EIEC</td>
<td>Enteroinvasive <em>E. coli</em></td>
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<tr>
<td>EPEC</td>
<td>Enteropathogenic <em>E. coli</em></td>
</tr>
<tr>
<td>ETEC</td>
<td>Enterotoxigenic <em>E. coli</em></td>
</tr>
<tr>
<td>FHA</td>
<td>Filamentous-hemagglutinin repeats</td>
</tr>
<tr>
<td>HGT</td>
<td>Horizontal gene transfer</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimal inhibitory concentration</td>
</tr>
<tr>
<td>MNEC</td>
<td>Meningitis/sepsis-associated <em>E. coli</em></td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>ORF</td>
<td>Open-reading frame</td>
</tr>
<tr>
<td>PMF</td>
<td>Proton-motive force</td>
</tr>
<tr>
<td>POTRA</td>
<td>Polypeptide-transport-associated</td>
</tr>
<tr>
<td>ppGpp</td>
<td>Guanosine tetraphosphate</td>
</tr>
<tr>
<td>pppGpp</td>
<td>Guanosine pentaphosphate</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>SHX</td>
<td>DL-Serine hydroxamate</td>
</tr>
<tr>
<td>SP</td>
<td>Signal peptide</td>
</tr>
<tr>
<td>T1SS</td>
<td>Type I secretion system</td>
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<tr>
<td>T5SS</td>
<td>Type V secretion system</td>
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<tr>
<td>T6SS</td>
<td>Type VI secretion system</td>
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<tr>
<td>TA</td>
<td>Toxin-Antitoxin</td>
</tr>
<tr>
<td>TPS</td>
<td>Two-partner secretion</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>TSS</td>
<td>Transcriptional start site</td>
</tr>
<tr>
<td>UPEC</td>
<td>Uropathogenic <em>E. coli</em></td>
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Gradualism and punctuated equilibrium are two important evolutionary theories. Gradualism was described by Darwin as incremental fitness increase over time, through natural selection (Darwin, 1859). While punctuated equilibrium was described by Eldredge and Gould as rapid and sudden changes that result in great leaps of fitness increase, followed by long periods of little-to-no increase (Eldredge and Gould, 1972). Unicellular evolution by both gradualism and punctuated equilibrium can be studied in a laboratory setting. However, long-term unicellular evolution has primarily been studied by measuring mutational changes that confer incremental fitness benefits in the form of a faster relative growth rate, i.e. gradualism. In contrast, fast and rapid cell division cannot be considered the most important factor of unicellular evolution since slow growing organisms, such as fungi, often outcompete bacteria even though their average growth rate is often >4-times slower than the growth rate of the average bacteria (Rousk and Baath, 2011). Thus, this results in an experimental bias where small growth rate differences are reported to be the primary driving force of unicellular evolution. This is quite disconcerting since unicellular evolution is just as much a battlefield as it is a metabolic arms race. The ability to kill or inhibit the growth of rival organisms can greatly affect a unicellular organism’s relative fitness. An organism does not necessarily need to be more metabolically fit, compared to its competitors; it simply needs to remove those competitors from its growth niche. The evolution of new and novel growth inhibitory systems that allows an organism to kill or inhibit the growth of any rival organism could create an evolutionary buffer; were a highly aggressive and antagonistic organism could dominate and thrive in a niche even though it lacks metabolic competitiveness. This could potentially allow said organism the opportunity to rapidly evolve new metabolic functions in order to fill the available niche it just created by eliminating all other competitors, i.e. evolution by punctuated equilibrium. The study of growth competition systems should therefore be prioritized to better understand these potentially influential intercellular interactions and their effect on unicellular evolution.

In this thesis I have studied Contact-Dependent growth Inhibition (CDI) using the model organism Escherichia coli to better understand how these potent interbacterial warfare systems can increase bacterial fitness in both heterogeneous and homogenous bacterial populations.
Escherichia coli

*E. coli* (Figure 1) belongs to the *Enterobacteriaceae* bacterial family and is predominately found in the gastrointestinal tract of warm-blooded mammals. Like all other members of the *Enterobacteriaceae* bacterial family, *E. coli* is a Gram-negative facultative anaerobe and is generally considered to be a metabolic generalist capable of utilizing more than 80 different carbon sources (Wagner, 2010). However, there are evidence that suggest that *E. coli* is highly specialized in utilizing gluconate, which allows it to occupy a unique metabolic niche within the highly crowded mucus layer of the mammalian colon (Sweeney *et al.*, 1996). This would make *E. coli* both a metabolic generalist and a specialist, depending on the growth niche it inhabits. *E. coli* is a common commensal of the normal flora, but many pathogenic strains also exist. Some of these strains give rise to mild disease symptoms while others can be lethal. There are eight different recognized *E. coli* pathotypes for humans; enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), uropathogenic *E. coli* (UPEC) and meningitis/sepsis-associated *E. coli* (MNEC) (Kaper *et al.*, 2004). Of these eight pathotypes the six diarrheagenic pathotypes (EPEC, EHEC, ETEC, EAEC, EIEC and DAEC) are the most common intestinal pathotypes in humans. However, UPEC are the most common extraintestinal pathotype in humans (Kaper *et al.*, 2004). The difference between a commensal strain of *E. coli* and a pathogenic strain can be as little as the acquisition of a pathogenicity island (a cluster of genes encoding for virulence factors and other specialized fitness factors) or just the presence of a few genes (Proenca *et al.*, 2017). Most of these virulence and fitness factors are encoded on mobile genetics elements, such as transposons and plasmids. The transfer of which can easily occur by horizontal gene transfer (HGT) mediated by conjugation, phages or simple by an uptake of extracellular DNA (Kaper *et al.*, 2004). Therefore, a commensal *E. coli* strain today might be a pathogenic strain tomorrow. The subtle differences between harmless and harmful *E. coli* makes this organisms just as attractive and relevant to study today as it was more then 130 years ago (Escherich, 1885).
Antagonistic interbacterial interactions

In most environments nutrients are limited, making the ability to inhibit the growth of rival bacteria a beneficial trait. Toward that end, most bacteria utilize numerous ways of killing or arresting the growth of competitor bacteria that are distinct from metabolic abilities that simple allows bacteria to grow faster in a certain niche. These antagonistic bacterial interactions come in many forms and the strong selection pressure that exists for bacteria to outcompete their neighbors have resulted in countless different unique approaches that bacteria utilize to achieve dominance within any given niche.

Acetic acid producing bacteria, such as *Acetobacter* spp. and *Gluconobacter* spp. have the ability to ferment ethanol and simple carbohydrates to produce acetic acid. This lowers the local pH below the viable growth range for most bacteria. *Acetobacter* spp. and *Gluconobacter* however have a great pH and acetic acid tolerance and are still able to grow in this niche, even tough most other bacteria can’t (Madigan, 2015). Lactic acid producing bacteria, such as *Lactobacillus* spp. and *Lactococcus* spp., utilizes a very similar approach by producing lactic acid, also through the fermentation of carbohydrates (Madigan, 2015). Lactic acid also lowers the local pH, which inhibits the growth of many bacteria while not adversely affecting the growth of *Lactobacillus* spp. and *Lactococcus* spp.

Acidic compounds are common metabolic secondary metabolites of most bacteria. A bacterium’s ability to tolerate its own toxic metabolites and weaponize its own metabolic byproducts would obviously confer a strong fitness benefit. However, bacteria can also use growth inhibition systems whose primary, or sole function, is to inhibit competitors.
Secreted antibiotic compounds

Antibiotics are likely the most well known secreted secondary metabolites and antibacterial compounds. The first antibiotic (penicillin G) was discovered in 1929 by Alexander Fleming (Fleming, 1929). Naturally occurring penicillin compounds are produced by many members of the *Penicillium* fungal genus and consists of a group of compounds (penicillin’s G, K, N, O and V) that all have three features in common; a thiazolidine ring, an attached beta-lactam ring, and a side chain (Miller, 2002). Penicillin production allows many *Penicillium* fungal species to effectively outcompete their bacterial competitors (Fleming, 1929).

In 1944 the first bacterially produced antibiotic compound (streptomycin) was discovered to be secreted by *Streptomyces griseus* (Jones et al., 1944). Since then, numerous bacterially produced antibiotics have been discovered. Today almost two-thirds of all known antibiotics are synthesized by members of the *Streptomyces* genus (Berdy, 2012). These include, but are not limited to, streptomycin, chloramphenicol, neomycin, tetracycline and kanamycin. Other bacterial genera able to produce antibiotic compounds are members of the *Amycolatopsis, Micromonospora, Actinomycete* and *Pseudomonas* bacterial genera (Chandra and Kumar, 2017). In *Streptomyces* spp. antibiotic compounds are usually synthesized by a series of proteins, or protein complexes that are encoded in large gene clusters (Martin et al., 2005). These gene clusters also frequently encode for dedicated antibiotic secretion systems, usually ATP-binding cassette(s) (ABC) transporters (Figure 2), and resistance genes that provide resistance against the produced antibiotic compound (Martin et al., 2005).

Conventional scientific opinions view antibiotic secretion/excretion strictly as an antagonistic action with the sole function of inhibiting the growth of competing bacteria. However, this viewpoint has been increasingly challenged. Many scientists are skeptical to if naturally produced antibiotic compounds reach concentrations high enough to effectively inhibit the growth of rival bacteria (minimal inhibitory concentrations (MIC)) *in vivo* or if only sub-MIC concentrations prevail in nature (Abrudan et al., 2015). Social interactions have therefore been suggested as an alternative explanation for the evolutionary purpose of antibiotics. This hypothesis is based on the observation that sub-MIC concentrations of antibiotics have the ability to change both gene expression and morphology of susceptible cells (Abrudan et al., 2015). However, this could very likely just be an indirect effect caused by a general stress response, rather than a specific effect, and thus not a trait selected for by evolution. Moreover, even a sub-MIC concentration of an antibiotic compound would likely have some minor growth retardation effect. The antibiotic producing cell would therefore still outcompete its competitor, in the long-term, while simultaneously not wasting a lot of energy synthesizing large amounts of antibiotics. Furthermore, what speaks against
this communication with antibiotics hypothesis is that antibiotic production seems to be reactive in nature (Abrudan et al., 2015; Kelsic et al., 2015). Antibiotic synthesis in *Streptomyces* spp. is low until cells are challenged by exposure to an antibiotic compound produced by a rival species. This antibiotic exposure results in a counter-response were the antibiotic exposed cell increases its own antibiotic production to counter-inhibit the rival bacteria (Abrudan et al., 2015). This restores the *status quo* and maintains homeostasis of the microbial community. Furthermore, many *Streptomyces* spp. are able to inhibit the production of antibiotics in rival *Streptomyces* spp. and thus reduce the direct threat to themselves (Abrudan et al., 2015). These observations collectively imply that antibiotics are used for more competitive than cooperative behaviors in nature.

Figure 2. ATP-binding cassette(s) (ABC) transporters can move solutes both into and from the cytoplasm (Hollenstein et al., 2007). ABC-transporters can move solutes, transported from the extracellular milieu by outer-membrane proteins (OMP), from the periplasm into the cytoplasm, in an ATP-dependent manner. Compounds, peptides and proteins can also be secreted from the cell by ABC-transporters, after the latter interacts with membrane-fusion proteins (MFP) and an outer-membrane factor (OMF) to form a type I secretion system (T1SS) (Durand et al., 2009). The substrate specificity of T1SS is determined by the ABC-transporter, since both the OMF and MFP can associate with many different ABC-transporters.
Secreted nonribosomal toxic peptides

Nonribosomal processes in bacterial cells can synthesize short peptides of approximately 2-50aa in length, allowing for the synthesis of non-genetically encoded peptides (Schwarzer et al., 2003). The majority of these peptides are typically 3-15aa in size and many have antibacterial properties (Martínez-Núñez and López, 2016; Schwarzer et al., 2003). Vancomycin is a glycosylated nonribosomal peptide produced by Amycolatopsis orientalis (Samel et al., 2008). Bacitracin is a mixture of cyclic peptides produced by a nonribosomal process in Baccilus subtilis (Tay et al., 2010) and tyrocidine are cyclic decapeptides produced by Bacillus brevis (Kohli et al., 2002). All of the above-mentioned nonribosomal toxic peptides are synthesis by a series of enzymes or protein complexes that catalyzes one step at a time in the process until the synthesis of the final product is achieved. This means that there is a great deal of similarities between nonribosomal toxic peptides synthesis and the biochemical synthesis of most conventional antibiotic compounds. This similarity also extends to their method of secretion; by dedicated ABC-transporters (Figure 2) (Kessler et al., 2004). Thus, it could be reasoned that nonribosomal toxic peptides and conventional antibiotic compounds are an example of convergent evolution and whose only difference is there manner of synthesis.

Secreted ribosomal toxic peptides and proteins

Antibiotic compounds and nonribosomal peptides normally require complicated pathways involving multiple enzymes for proper synthesis (Liu et al., 2013; Schwarzer et al., 2003). In contrast, many toxic peptides can be genetically encoded and ribosomally translated before secreted with none or minimal post-translational modifications. This allows for a potentially faster relative evolution of toxic peptides and proteins, which could explain their great diversity (Czaran et al., 2002; Riley and Gordon, 1996).

Genetically encoded and ribosomally translated toxic peptides can be subject to extensive post-translational modifications or be secreted in an unmodified form, usually by dedicated ABC-transporters (Figure 2) (Azpiroz et al., 2001). Examples of the former are some small (<10kDa) E. coli produced microcins, such as microcin M and H47; both of which are post-translationally modified by the attachment of a salmochelin-like siderophore moiety in their C-terminus (Vassiliadis et al., 2010). This allows these microcins to bind to the siderophore receptors; Fiu, Cir and FepA on target bacteria, which results in their uptake. To protect itself from auto-inhibition the microcin producing cell also express an immunity protein that bind the toxic protein and neutralizes its activity (Vassiliadis et al., 2010). But also nisin from Lactococcus lactis, as well as other lantibiotics; produced by a large number of Gram-positive bacteria, such as Streptococcus spp. and
Streptomyces spp., are ribosomally translated peptides that undergo extensive post-translational modifications before being secreted (Sahl and Bierbaum, 1998). These post-translational modifications allow for the modification and addition of unusual amino acids, such as dehydroalanine, dehydrobutyrin and lanthionine residues (Draper et al., 2015). Similarly to microcin producing cells, lantibiotic producing cells either express immunity proteins that bind the toxic peptides, a transport systems that pumps the toxic peptides into the extracellular milieu, or both (Stein et al., 2003).

Examples of secreted ribosomal toxic peptides and proteins that are not post-translationally modified are colicin V and microcin L (Azpiroz and Lavina, 2007). Both colicin V and microcin L are small proteins of <10kDa, both proteins dissipate the proton motive force of susceptible bacteria and both proteins utilizes the siderophore receptor Cir as a receptor (Chehade and Braun, 1988; Morin et al., 2011). Furthermore, both proteins are secreted by dedicated ABC-transporters (Figure 2). This means that colicin V and microcin L share many similarities with microcin M and H47. However, the addition of a C-terminal salmochelin-like siderophore moiety seems to allow microcin M and H47 to bind and be taken up by several different siderophore receptors (Vassiliadis et al., 2010). The lack of post-translational modifications, in the case of colicin V and microcin L appears to restrict the receptor repertoire of these toxic proteins, as they only seem to interact with the siderophore receptor Cir. Thus, post-translational modifications have the ability to greatly affect and modify the function of toxic peptides and proteins.

Besides dedicated ABC-transporters (Figure 2), cells can secrete toxic proteins into the extra-cellular milieu by self-induced cell lysis. Examples of such proteins are the membrane ionophore toxin; colicin E1 and the DNase toxin; colicin E2 (Braun et al., 1994). Colicin E1 and E2 are both organized in a gene cluster encoding three proteins; the toxin protein, a cognate immunity protein that binds and protects against the effect of the toxin and a cell lysis inducing protein. Induction of both toxin and cell lysis protein is dependent on DNA damage, through the activity of the SOS-response pathway. A low level DNA damage results in a very heterogeneous toxin and lysis protein expression within a population, while excessive DNA damage induces a more uniform response resulting in a almost complete population wide expression of both toxin and lysis proteins (Mader et al., 2015; Salles et al., 1987). Because DNA damage correlates with the likelihood of self-induced cell lysis, it could be argued that cells with irreversible damage and with less chance of survival, are the ones that are sacrificed and release toxins. Thus, the sacrificed cells are the ones that are not likely to give rise to evolutionary fit progeny, which means that this can’t be considered a strictly altruistic event. Nevertheless, the effect of this sacrifice on the rest of the population has the potential to be pronounced. Any cell that undergoes self-lysis releases not only toxin molecules but also their entire cellular content,
and thus a large amount of nutrients. The surviving cells can take advantage of these nutrients, since they are immune to the toxin molecule, while other non-immune, non-kin cells will be inhibited or killed. This could greatly change the population dynamics after a stress event and greatly favor the colicin producing population.

Colicins, microcins and lantibiotics are all examples of small toxic peptides and proteins that are exported to the extra-cellular milieu by dedicated ABC-transporters (Figure 2) or by cell lysis. After their secretion/excretion these toxic peptides and proteins diffuse away from the cell until they encounter a susceptible target cell expressing the correct receptor molecule(s). Binding to their cognate receptor(s) results in their uptake, which ultimately allows these molecules to mediate their toxic activity. However, toxic peptides and proteins can also be delivered in a contact-dependent manner, such as the toxic effectors delivered by two-partner secretion (TPS) or type VI secretion systems (T6SS). These toxic peptides and proteins are usually translated as part of a bigger protein and then subject to proteolytic cleavage before being delivered to a target bacterium (Hayes et al., 2014; Meuskens et al., 2019; Navarro-Garcia et al., 2019). What makes these toxic effectors unique is that they require direct cell-cell contact for toxic effector delivery. This is in stark contrast to soluble toxic effectors that can diffuse away from the producing cell, giving rise to millimeter wide zones of inhibition, completely devoid of bacterial growth. Thus, CDI systems do not necessarily affect an entire micro colony but only those bacterial cells that are directly adjacent. The exact effect of this ability is not clearly understood and has only started to be investigated recently.

**TPS systems**

TPS are type V secretion systems, subclass b (Figure 3) (van Ulsen et al., 2014). They are a diverse family of proteins that have been identified bioinformatically in both pathogenic and environmental isolates (Jacob-Dubuisson et al., 2013). The two essential proteins that make up any TPS system are the outer-membrane transport protein TpsB and its delivered exoprotein; TpsA. TpsB are highly conserved proteins belonging to the Omp85-superfamily (Jacob-Dubuisson et al., 2009). TpsA however are very diverse, ranging in size from approximately 700aa to >5500aa in length. Both TpsA and TpsB encode for Sec-signal peptides in their N-terminus and are transported through the inner-membrane by the Sec-translocon (Leyton et al., 2012). In the periplasm TpsB associates with the Bam complex and is inserted into the outer-membrane. The transport of TpsA through the outer-membrane is entirely dependent on the activity of TpsB. TpsB proteins primarily consist of a C-terminally located β-barrel domain that forms a pore in the outer-membrane. Polypeptide-transport-associated (POTRA) domains located in the N-terminal region of TpsB specifically binds, through a unique
protein-protein interaction, the ~300aa long TPS domain located in the N-terminal region of TpsA. This interaction allows for the translocation of TpsA to the cell surface, through the β-barrel pore formed by TpsB (Leyton et al., 2012). TpsA proteins are folded extracellularly and this extracellular folding is believed to provide the necessary energy for TpsA translocation across the outer-membrane (Figure 3).

CDI systems; such as the CdiBAI system of *Enterobacteriaceae* spp. and the BcpAIOB of *Burkholderia* spp., are TPS systems able to deliver toxic effectors to adjacent cells in a receptor- and contact-dependent manner (Anderson et al., 2012; Aoki et al., 2005). Further details on the activity and biological significance of CDI TPS systems expressed by either *Enterobacteriaceae* spp. or *Burkholderia* spp. can be found in the “CDI TPS systems” section of this thesis.

![Figure 3. Simplified schematic view of TpsB and TpsA secretion into the periplasm, and subsequent secretion of TpsA through the outer-membrane by TpsB (Leo et al., 2012). SP; signal peptide, POTRA; polypeptide-transport-associated domain (shown as P in figure), TPS; two-partner secretion domain.](image-url)
The T6SS of Gram-negative bacteria can be a potent interbacterial warfare system. The T6SS of Bacteroides fragilis, Pseudomonas aeruginosa and Vibrio cholera are among the best studied (Navarro-Garcia et al., 2019). In these bacteria the T6SS can act both as an interbacterial warfare system but also as an important transport mechanism for multiple virulence factors (Navarro-Garcia et al., 2019). T6SS is a multi-protein complex consisting of 13-14 core genes assembled into an injection needle with great structural similarity to the T4 bacteriophage tail (Figure 4) (Boyer et al., 2009). The core complex consists of the TssA, E, F, G and K proteins and is anchored to the cell membrane by a membrane complex formed by TssJ, L and M (Cascales and Cambillau, 2012; Silverman et al., 2012). TssB and C form the needle-like complex, while TssD and Hcp form the tail tube. At the tip of the needle-like complex are TssI, VgrG and a PAAR-domain protein, which together forms the tip of the spear-like needle complex (Chen et al., 2019). This domesticated phage machinery have the ability to deliver numerous effector proteins by several independent mechanisms. Effectors can be delivered to an adjacent cell by binding to Hcp, VgrG or PAAR-domain proteins (Chen et al., 2019). Effector proteins can also be co-translated as part of the Hcp, VgrG or PAAR-domain proteins; as an additional C-terminal domain, these hybrid effector proteins are refereed to as evolved Hcp, VgrG and PAAR-domain proteins (Lien and Lai, 2017). Toxic effector proteins are delivered to adjacent cells to by the dynamic action of the contractile sheath, formed by TssB and C, and through this activity the Hcp, VgrG or PAAR-domain proteins, together with their associated effectors, are delivered across the bacterial membrane of a target bacteria (Figure 4) (Basler et al., 2012). To protect against auto-inhibition, all cells with T6SS that deliver effector protein(s) with anti-bacterial activity also express a cognate immunity protein that binds the toxic effector and protects against its activity. After effector delivery to a recipient cell many T6SS components, such as the majority of Hcp proteins, are recycled and re-used. This recycling requires the activity of the AAA+ ATPase protein ClpV, which disassembles many of the T6SS protein-complexes into their constituent subunits (Lien and Lai, 2017). After disassembly, the T6SS puncturing device can re-assemble and be re-used once again for the delivery of additional effector proteins.

Bacteroidales are the most abundant order of bacteria in the intestinal microbiota and many Bacteroidales members utilize T6SS as potent interbacterial warfare systems (Chen et al., 2019). One such species is B. fragilis who’s T6SS is of vital importance for the bacteria’s competitiveness in the intestine. In this environment 1 gram of B. fragilis cells can fire its T6SS >10⁹ times/min (Wexler et al., 2016). This high T6SS activity allows B. fragilis to out-compete both commensal and pathogenic Bacteroides spp. in the mice intestine (Chatzidaki-Livanis et al., 2016; Hecht et al., 2016). Compa-
rably, *V. cholera* also have both intra- and interspecies competitive advantages during environmental and intestinal colonization by using its T6SS (Unterweger *et al.*, 2012; Zhao *et al.*, 2018). T6SS have also been shown to be of great interbacterial competitive advantage for *Salmonella enterica*, *Shigella sonnei* and *Yersinia pseudotuberculosis* (Chen *et al.*, 2019). Moreover, T6SS are not restricted to interbacterial warfare as T6SS can deliver toxic effectors also to unicellular eukaryotes, such as fungi. The first anti-fungal T6SS effectors were identified in *Serratia marcescens*: Tfe1 and Tfe2 (Trunk *et al.*, 2018). Tfe1 causes membrane depolarization, while Tfe2 disrupts nutrient uptake and amino acid metabolism. Since single cell eukaryotes are important members of the intestinal microbiota it is not surprising that bacterial evolution would favor the creation of T6SS effectors able to also target fungal species (Coyne and Comstock, 2019).
Figure 4. Simplified schematic view of a T6SS delivering a toxic effector molecule to a target cell (Navarro-Garcia et al., 2019). T6SS can deliver toxic effectors to both prokaryotic and eukaryotic cells.

Rhs/YD-peptide repeat proteins

*rhs* loci were first identified in *E. coli* as chromosomal regions subject to extensive genetic rearrangements (Lin et al., 1984). Since then, bioinformatic analyses have revealed that *rhs* loci are widespread throughout β-, γ-, and δ-proteobacteria (Hill et al., 1994). Genes encoding for more distantly related YD-peptide repeat proteins have also been found in Gram-positive bacte-
ria, fungi and higher metazoans (Foster, 1993; Tucker et al., 2012). Rhs proteins are comprised of a conserved N-terminal core region and a highly variable C-terminal region (Jackson et al., 2009). These variable C-terminal domains have been shown to encode toxic effectors with diverse toxic activities, such as DNase and tRNase toxins capable of inhibiting the growth of rival bacteria in a contact-dependent manner (Koskiniemi et al., 2013). Rhs/YD-peptide repeat proteins in Gram-negative bacteria can be delivered by the T6SS (Figure 4), similar but not identical to evolved VgrG proteins (Koskiniemi et al., 2013). In the Gram-positive Bacillus subtilis, which lack a T6SS, the Rhs/YD-peptide repeat protein, WapA, appears to be delivered to adjacent cells by the general secretory pathway (Koskiniemi et al., 2013). This further demonstrates the great diversity of toxin delivery mechanisms within bacteria and clearly shows that toxin delivery systems are subject to strong selection pressures. Which could imply that interbacterial warfare systems are a great driving force of unicellular speciation.

Antagonistic interbacterial interactions within biofilms

The production of toxic compounds, peptides and protein can have pronounced effects on the composition and spatial distribution of biofilms (Moons et al., 2009). When the two colicin producing Enterobacteriaceae spp.; Enterobacter agglomerans and Enterobacter gergoviae was co-cultured as a mixed-species biofilm, a thinner and less developed biofilm was observed, compared to the biofilm that was formed by either species when grown alone. Both species established distinct separate homogenous micro colonies within the biofilm over time. This created a very heterogeneous distribution of both species throughout the biofilm structure and prevented each species from establishing in a niche that the other species already occupied. Similar observations have also been made in Pseudoalteromonas tunicata and Serratia plymuthica mixed-species biofilms (Moons et al., 2006; Rao et al., 2005). This heterogeneous spatial distribution is predicted to protect each species from exposure to toxic metabolites, ensuring long-term coexistence. However, this segregated spatial distribution seems to only occur when both species have the ability to inhibit the other. When the ability to inhibit the growth of a rival species is only present in one of two species, that species have a significant growth advantage. When a bacterium possesses a growth inhibition system it also gains the ability to establish in a niche already occupied by a rival bacterial species and over time even displace that rival species (Al-Bakri et al., 2004). This was observed for the toxin producing Pseudomonas aeruginosa when co-cultured together with the toxin susceptible Burkholderia cepacia. Additionally, B. cepacia was entirely incapable of establishing in a niche already occupied by P. aeruginosa (Al-Bakri et al., 2004). Showing that growth inhibition systems can have pronounced effects on biofilm morphology and spatial distribution.
Other growth inhibition systems also known to affect multicellular bacterial behavior are CDI TPS systems. Further details on the biological significance of CDI TPS systems expressed by either Enterobacteriaceae spp. or Burkholderia spp. can be found in the “CDI TPS systems” section of this thesis.

TA-systems

Toxin-Antitoxin (TA) systems are two-component systems were one component is a cytosolic, or membrane bound, toxin protein that is expressed intracellularly. The other component being the toxins cognate antitoxin molecule that protects the toxin-producing cell from a potential unfavorable self-intoxication (Lee and Lee, 2016). This antitoxin molecule can be either an antisense-RNA; that prevents the expression of the toxin protein (type I TA-systems) or a RNA molecule that directly binds the toxin protein and protect against its toxic effect (type III TA-systems). The antitoxin can also be a protein that specifically binds and neutralizes the toxin protein (type II TA-systems), binds and protect the molecular target of the toxin (type IV TA-systems) or degrade the mRNA molecule encoding for the toxin protein (type V TA-systems). Furthermore, this antitoxin molecule is often involved in modulating the effect of its cognate toxin and can under certain circumstances allow the cell to self-intoxicate (Brzozowska and Zielenkiewicz, 2013). This self-intoxication can serve many different purposes.

Type I and II TA-systems often have unstable antitoxin molecules with a shorter half-life than their cognate toxin molecules. These antitoxin molecules; antisense-RNA (type I) or protein (type II) are subject to RNase(s) or proteolytic degradation by cellular protease(s). This allows type I and type II TA-systems to act as selfish-genetic elements where any cell that loses the TA-system(s) self-intoxicates because the less stable antitoxin molecules eventually disappears from the cell (Jensen and Gerdes, 1995). This in turn results in unbound and free toxins capable of inhibiting the growth of the cell. Selfish-genetic elements are useful genetic stabilizers of mobile genetic elements and have also been suggested to act as genome stabilizers. Furthermore, TA-systems can function as protection systems for an entire bacterial colony by limiting the spread of phage particles within a population. (Chopin et al., 2005; Samson et al., 2013). When a cell is infected by a phage that cell undergoes an altruistic self-sacrifice; undergoing cell lysis or growth arrest before new phage particles are produced. Both of these possible outcomes stop the phage infection before it have time to spread to an adjacent cell. TA-systems have also been shown to mediate stress response and persister formation (Ronneau and Helaine, 2019). More detailed description of persister cells can be found in the “Persister cells and other means of antibiotic tolerance” section of this thesis.
Type II TA-systems

The toxins of type II TA-systems (Figure 5), identified to date, can be classified into 12 protein super families and there cognate antitoxin proteins can be classified into 20 different super families, based on there structure and factions (Mruk and Kobayashi, 2014). These TA-systems are often found on plasmids or in prophages, integrons and transposons. This allows type II TA-systems to have a great intra- and intergenome mobility. Moreover, their ability to mediate post-segregational killing or growth inhibition demonstrates a clear evolutionary selection pressure to retain type II TA-systems with mobile genetic elements (Mruk and Kobayashi, 2014).

The first type II TA-system identified was the CcdAB system located on the F-factor of some E. coli strains (Ogura and Hiraga, 1983). The CcdB toxin is a DNA gyrase inhibitor, capable of inhibiting DNA replication. While CcdA is the unstable cognate antitoxin molecule of CcdB, subject to proteolytic degradation by the Lon protease (Vanmelleren et al., 1994). The ccdAB cassette is known to function as a plasmid stabilization element of the F-factor as the loss of the system results in a self-intoxication and growth arrest. Other known toxic activities of type II TA-system toxin molecules include; mRNase (MazEF, RelBE, MqsAR, DinJ-YafQ), tRNase (VapBC), inhibition of translation elongation (Phd-Doc) and inhibition of aminoacyl-tRNA synthetase (HipBA), to mention a few (Mruk and Kobayashi, 2014; Prysak et al., 2009).

The antitoxin protein of type II TA-systems are almost always subject to proteolytic degradation by cellular proteases and thus have a shorter protein half-life than their cognate toxin molecules (Figure 5). Besides CcdA, the antitoxin proteins HipB, RelB and VapB have also been shown to be subject to proteolytic degradation by Lon (Christensen et al., 2001; Hansen et al., 2012; Winther and Gerdes, 2012). The ClpXP proteasomal complex have been shown to degrade the antitoxin Phd, while MqsA have been shown to be subject to proteolytic degradation by both Lon and ClpXP (Kim et al., 2010; Lehnherr and Yarmolinsky, 1995; Wang et al., 2011). The cognate antitoxin ParD of the DNA gyrase inhibitor toxin ParE is also subject to proteolytic degradation by the ClpAP proteasomal complex, while MazE is subject to proteolytic degradation by both Lon and ClpAP (Aizenman et al., 1996; Christensen et al., 2003; Dubiel et al., 2018).

Type II TA-systems often have very similar regulation, with many TA modules being transcriptionally auto-regulated by their antitoxin and toxin complexes, a mode of regulation referred to as conditional cooperativity (Figure 5). This transcriptional auto-regulation of TA-systems by antitoxin and toxin complexes has been demonstrated for relBE, mazEF and ccdAB, to name a few. Numerous other type II TA-systems are also predicted to have some form of auto-regulation (Afif et al., 2001; Li et al., 2008; Marianovsky et al., 2001). This auto-regulation is mediated by the antitoxin-to-toxin ratio,
which determines if a TA-module is transcriptionally repressed or transcriptionally active (Cataudella et al., 2012; Overgaard et al., 2008). In the case of relBE expression, the antitoxin protein RelB forms a homodimer that weakly binds and represses the promoter of relBE. However, when RelB antitoxin molecules bind with RelE toxin molecules a heterotetramer is formed, comprised of two RelB and two RelE molecules. This heterotetramer strongly binds and represses the relBE promoter (Li et al., 2008). Thus, an expression of both toxin and antitoxin molecules are constantly maintained in the cell. The same auto-regulation mechanism has also been shown for the expression of the ccdAB TA-system. A heterotetramer formed by two CcdA antitoxin molecules and two CcdB toxin molecules forms a transcriptional repressor that binds the ccdAB promoter. However, when additional CcdB toxin molecules are present the heterotetramer complex is disrupted and ccdAB transcriptional repression is abolished (Afif et al., 2001). Both relBE and ccdAB auto-regulation seems to be a delicate balancing act which allows the cell to maintain an appropriate ratio between antitoxin and toxin molecules at all times.

The DNA binding ability of type II antitoxin proteins can extend beyond the binding of its own promoter and type II antitoxins can act as transcriptional factors and regulate global transcription (Figure 5). One such antitoxin is MqsA, which binds the promoter of rpoS, encoding the major stationary phase and general stress sigma factor SigmaS (Wang et al., 2011). Through this rpoS repression MqsA regulates a global regulator, which in turn regulates hundreds of genes. The DinJ antitoxin of the DinJ-YafQ type II TA-system have also been shown to effect global gene transcription by binding the promoter of the cold-shock protein CspE, which ultimately leads to an increase in rpoS mRNA translation (Hu et al., 2012; Kim et al., 2010). Type II TA-systems can therefore have major global effects on any cell harboring them. Moreover, since type II TA-system are usually located on mobile genetic elements and are readily spread by HGT they could allow cells to rapidly and substantially change global gene transcription and behavior. This TA-system dependent behavior change could be the first evolutionary step towards a strain divergence and subsequent speciation.

It is important to note that most characterized type II TA-system are not able to kill the TA producing cell, even if the antitoxin molecule is selectively degraded or nonexistent. Instead, TA-systems generally induce a non-growing dormant state that cells can recover from when new antitoxin molecules are expressed. Examples of this include the two mRNase toxins RelE and MazF, who when expressed in E. coli does not kill the cells but rather causes a dormant state that the cells can overcome if the cognate antitoxin is expressed (Pedersen et al., 2002). The same is true for many TA-system that are reported to mediate post-segregational killing. Very little evidence exists to support the claim that selfish-genetic elements and plasmid addiction modules kill the cell that loses them, if they are expressed at native levels.
Instead, most cells intoxicated by TA-systems simple experience a growth arrest and enters a dormant, but viable, state (Song and Wood, 2018). This TA-system induced dormant state, i.e. persister cells, is covered in more detail in the “Persister cells and other means of stress tolerance” section of this thesis.

Figure 5. Overview of a conventional type II TA-system, showing cellular targets and a simplified illustration of the transcriptional and post-translational regulations most type II TA-systems is regulated by. Dotted line; weak repression, TSS; transcriptional start-site.
CDI TPS systems

The proteins essential for CDI TPS (CdiBAI) (Figure 6) were first identified in 2005 in an E. coli isolate, later designated EC93 (Aoki et al., 2005). This E. coli isolate was shown to dominate the urinary tract of several rats in an animal house, which interfered with numerous experiments. Genomic DNA was therefore isolated from EC93 and used to prepare a cosmid library in order to identify the fitness factor(s) that allowed this strain to out-compete rival E. coli and persist within rats (Aoki et al., 2005). Cycling experiments using E. coli lab strains transformed with this cosmid library resulted in the identification of a ≈20kb genome fragment from EC93, which allowed transformed cells to outcompete other E. coli when grown in LB broth. This ≈20kb genome fragment was later shown to contain the cdiBAI gene locus (≈12kb), responsible for this growth inhibitory phenotype. Further investigations also revealed that this growth inhibition was contact-dependent and thus quite different than other previously known toxin delivery and inter-bacterial growth competition systems.

![CDI TPS systems](image)

Prevalence of CDI TPS systems within the bacterial kingdom

Bioinformatics analysis have revealed that CDI TPS systems are widespread among α-, β-, and γ-proteobacteria (Aoki et al., 2010). It is also not uncommon that CDI possessing bacteria within these major bacterial classes have multiple CDI loci (Hayes et al., 2014). This shows that CDI systems are under strong selection pressure, which implies that they are important fitness factors for many different bacterial species living in many different niches. Many opportunistic and obligate pathogenic bacteria, such as; Yersinia pseudotuberculosis, several Burkholderia spp. and Neisseria meningitides,
are known to harbor CDI systems (Poole et al., 2011). Many pathogenic E. coli, such as EC869 (EHEC) also harbors CDI systems. However, the exact effect and possible importance of CDI systems on the pathogenesis of these bacteria have not adequately been studies. While CDI systems are not only found in pathogenic bacteria they are nevertheless an interesting potential fitness factor of these disease causing bacteria and thus a very attractive future area of scientific research.

CDI TPS systems in E. coli

15% of all sequenced E. coli genomes contain a CDI TPS system (Ruhe et al., 2013). CDI TPS (T5SS, subclass b) systems (Figure 3) are comprised of the β-barrel outer-membrane transport protein; CdiB (TpsB), and the contact-dependent toxin delivery protein; CdiA (TpsA) (Figure 6). CdiA proteins are large (250-600 kDa) filamentous exoproteins made up of multiple domains. Some of these domains are highly conserved, such as the N-terminal TPS domain and the filamentous-hemagglutinin repeats (FHA1). While other domains are highly variable and modular, such as the receptor binding domain and the toxic C-terminal domain (polymorphic toxin domain) (Ruhe et al., 2017; Willett et al., 2015). Both CdiB and CdiA are transported through the inner-membrane by the Sec-translocon (Aoki et al., 2005). In the periplasm CdiB associates with the Bam complex and is inserted into the outer-membrane (Figure 3). The N-terminal TPS domain of CdiA then associates with the N-terminal located POTRA domains of CdiB and thru this interaction CdiA is transported through the outer-membrane and presented on the cell-surface (Figure 7) (Ruhe et al., 2018). CdiA proteins encodes toxic C-terminal domains with varies toxic activities. These toxic activates include; membrane ionophore toxins, tRNase, rRNase and DNase toxins (Aoki et al., 2008; Beck et al., 2014; Jones et al., 2017; Michalska et al., 2017; Poole et al., 2011; Schureck and Dunham, 2014; Webb et al., 2013). A receptor-binding domain region, found in the middle of the CdiA protein, mediates the contact-dependent interaction with a target cell (Figure 7) and CdiA proteins are classified according to their receptor-binding domains. So far three receptor-binding domains have been characterized for E. coli CdiA proteins. Class I CdiA proteins bind BamA proteins on target cells, class II binds the OmpC and OmpF outer-membrane complex and class III proteins bind Tsx (Aoki et al., 2008; Beck et al., 2016; Ruhe et al., 2017). CdiA binds to the most variable region of these outer-membrane proteins; the extra-cellular loops. This allows CdiA receptor binding domains to specifically bind to only a sub-section of target cell receptor proteins, while ignoring many other possible receptor variants. Through this receptor-mediated interaction, the toxic C-terminal domain of CdiA is cleaved at a conserved motif (VENN) by a presumed proteolytic autocleavage event and delivered into the periplasm of the target cell. CdiA-CT
toxins with cytoplasmic targets are transported through the inner-membrane by the action of the N-terminal region of the CdiA C-terminal toxic domain, the so-called NT of the CT (Figure 8) (Willett et al., 2015). The C-terminal part of the C-terminal domain (the CT of the CT) of CdiA encodes the toxic function. To protect against auto-inhibition a cognate immunity protein is also expressed intracellularly in any CdiA producing cell (Aoki et al., 2010; Aoki et al., 2005).

Many cdiA-CT polymorphic toxin domains and their cognate immunity genes can often be found alone and separate from the cdiA-NT gene; encoding for the extra-cellular delivery protein (Hayes et al., 2014; Poole et al., 2011). These cdiA-CT and immunity modules are commonly referred to as orphan toxins. When artificially fused to the cdiA-NT, at a conserved motif (VENN), these isolated orphan toxins can be functionally delivered to a neighboring cell (Poole et al., 2011). This indicates that these orphan toxins functions as a potential extended toxin arsenal. This hypothesis is supported by bioinformatic analyses showing different orphan toxins fused to different cdiA-NT delivery genes in other strains and species. However, the exact mechanism for cdiA-CT recombination and switching has not been found in E. coli. Although in both in N. meningitides and Y. pseudotuberculosis large homologies regions (>200bp) can be found between cdiA and orphan toxin modules (Poole et al., 2011). These homologies regions could realistically allow for a high-frequency recombination event between cdiA and many different orphan toxins. This hypothetical cdiA-CT switching could drastically affect the evolutionary fitness of the CdiA producing cell, which could explain why some strains have accumulated >11 orphan toxin and immunity modules (Poole et al., 2011).

CDI TPS systems in Burkholderia spp.

The CDI TPS systems of Burkholderia spp. are very similar, but not identical, to the CDI TPS systems of E. coli. The CDI TPS operon, consisting of the cdiB, cdiA and cdiI genes in E. coli are named; bcpB, bcpA and bcpI, respectively, in Burkholderia. Furthermore, Burkholderia spp. CDI TPS systems also encode a fourth gene; bcpO (Anderson et al., 2012). The protein product of this gene; BcpO, localizes to the outer-membrane, but have an unknown function. The affect of BcpO on the CDI phenotype is enigmatic, as some Burkholderia spp. bcpO mutants have diminished CDI effect, while others show little to no effect (Perault and Cotter, 2018). The exact role and purpose of BcpO in Burkholderia spp. CDI remains to be discovered. Furthermore, the bcpB, bcpA, bcpI and bcpO genes in Burkholderia spp. are organized in a different order; bcpAIOB, than the CDI TPS genes of E. coli (cdIBAI) (Anderson et al., 2012). The evolutionary purpose of this rearrangement, if any, is not known.
So far only one possible receptor of one *Burkholderia* spp. BcpA protein have been eluded. In a genetic screen of CDI resistant mutants of *Burkholderia thailandensis* several different mutations were identified in the LPS pathway that conferred resistance towards *B. pseudomallei* BcpA mediated inhibition, when the bcpAIOB locus of *B. pseudomallei* was expressed in *B. thailandensis* (Koskiniemi et al., 2015). Furthermore, similarly to *E. coli* CdiA-CT domains, the BcpA-CT domain of *B. pseudomallei* encodes a tRNase toxin (Nikolakakis et al., 2012). This demonstrates that *Burkholderia* spp. bcpAIOB systems comprise a unique class of CDI system that share many overlapping functions with the cdiBAI system of *E. coli*.

Figure 7. Simplified schematic view of *E. coli* CdiBA mediated toxin translocation into a target bacterium (Ruhe et al., 2018). P; polypeptide-transport-associated domain, TPS; two-partner secretion domain, FHA-1; filamentous-hemagglutinin repeat region 1, RBD; receptor-binding domain, FHA-2; filamentous-hemagglutinin repeat region 2.
Figure 8. Simplified schematic view of *E. coli* CdiA-CT toxin translocation from the periplasm into the inner-membrane or cytoplasm of a target bacterium.

**CDI TPS systems in *Pseudomonas* spp.**

CDI TPS system(s) of *Pseudomonas aeruginosa* have been identified bioinformatically in all 104 complete *P. aeruginosa* genomes deposited in NCBI (Allen and Hauser, 2019). These CDI TPS loci have all been shown to be in the same genetic locations, in all 104 strains. Furthermore, 81% of these strains also have a second CDI TPS system (Allen and Hauser, 2019). So far only two functional *Pseudomonas* spp. CDI TPS systems have been identified and characterized, both CDI systems are found in the *Pseudomonas aeruginosa* strain PAO1 (Mercy *et al.*, 2016). Bioinformatic analysis of CDI systems in *Pseudomonas* spp. revealed the existent of five different classes of CdiA proteins (Mercy *et al.*, 2016). These CdiA proteins are classified according to conserved protein motifs that precede the toxic, highly variable, C-terminal domains. In *E. coli* CdiA proteins this domain is VENN and is presumed to be a proteolytic auto-cleavage domain, or a proteolytic cleavage site, important for CdiA-CT toxin translocation into a target cell (Ruhe *et al.*, 2013). In *Burkholderia* spp. BcpA proteins this protein motif is NXXLYN and in *Pseudomonas* spp. the five conserved protein motifs are; WVHN (class I), VENN (class II), LYVT (class III), DAMV (class IV), and NEALV (class V) (Mercy *et al.*, 2016). However, only class II and class V CdiA proteins have been identified in *P. aeruginosa*. The exact function of these highly conserved protein motifs in CdiA and BcpA proteins are not completely understood. However, there is some evidence that suggests that they are important for the separation of the toxic C-terminal domain from the N-terminal domains (CdiA-NT and BcpA-NT), and thus essential for extracellular toxin delivery (Mercy *et al.*, 2016). There is also an hypothesis that these conserved motifs act as conserved sites for homologies recombination,
which could allow for CdiA-CT/BcpA-CT interchange and possible allow orphan toxins to be functionally delivered to target cells (Allen and Hauser, 2019). However, this remains to be investigated.

The effect of CDI TPS systems on bacterial populations

CDI TPS systems have been shown in multiple studies and in multiple organisms to affect the behavior of bacterial populations in a manner that is both separate and distinct from its growth inhibitory effect (Garcia et al., 2013; Garcia et al., 2016; Ruhe et al., 2015). One of the strongest observed effects of CDI systems, other than interbacterial growth inhibition, are in biofilm formation, biofilm modulation and cell-cell aggregation (Garcia et al., 2013; Ruhe et al., 2015). For CdiA proteins this auto-aggregation have been demonstrated to be both receptor-dependent and receptor-independent (Figure 9). CdiA-CdiA binding allows for a strong receptor-independent cell-cell aggregation. Because this auto-aggregation can only occur between two CdiA+ cells it means that CdiA proteins can function in a similar way as other auto-aggregation proteins, such as Ag43 (Figure 9) (Danese et al., 2000; Henderson et al., 1997). However, CdiA and BcpA protein can also allow for receptor-dependent cell-cell aggregation (Ruhe et al., 2015). This allows CdiA+ and BcpA+ cells to also bind and aggregate with CdiA- and BcpA- cells (Figure 9). This means that not all cells within a population need a surface expressed CdiA protein for the bacterial population as a whole to aggregate together (Beck et al., 2016; Ruhe et al., 2017). This implies that CdiA/BcpA proteins might be exceptionally well suited to form cross-species and/or cross-strain cellular aggregates, biofilms or biofilm-like structures.

CDI systems have also been shown to mediate biofilm effects in a manner that is independent from their ability to mediate cell-cell aggregation. The CDI system B. thailandensis have been shown to trigger extensive transcriptional changes of biofilm associated genes and significantly effect biofilm production (Garcia et al., 2016). This is especially impressive considering that less than <0.5% of B. thailandensis cells express BcpA proteins within a biofilm (Garcia et al., 2013). This effect occurred even when the cognate immunity protein (BcpI) was constitutively expressed within the population, demonstrating that an auto-inhibition, mediated by the BcpA-CT effector protein was not the cause behind this drastic population-wide transcriptional change. However, two different catalytically inactive BcpA-CT mutants lacking DNase activity, did abolish this effect, indicating that the enzymatic activity of the DNase, but not the growth inhibitory effect of the toxin was essential for this population-wide transcriptional change (Garcia et al., 2016). To reconcile this seemingly contradictory findings the authors propose a hypothesis were the BcpA-CT DNase toxin is able to mediate a sub-lethal effect in the recipient cell. That is to say, that the DNase activity is
not strong enough to kill the cell, due to the presence of the cognate antitoxin (BcpI), but sufficient to mediate a substantial effect on the cell. Another possibility proposed by the authors is that the BcpA-CT toxin has altered substrate specificity when bound to its cognate BcpI antitoxin. This altered substrate specificity was suggested to allow the BcpA-CT toxin to affect the cell in a non-lethal manner, by for example binding and/or cleaving RNA molecules (Garcia et al., 2016). Whatever the exact molecular mechanism behind this CDI mediated transcriptional changes are the phenomenon itself has been named Contact-Dependent Signaling (CDS).

The basic phenomenon of population-wide behavioral changes stimulated by toxic molecules and antibiotics are neither new nor unique. However, the contact-dependent nature of CDI systems provides a new dimension to an old phenomenon. Secreted toxic effectors and antibiotic molecules have been shown to act in a way that is reminiscent of quorum sensing molecules and mediate population-wide behavioral changes through sub-lethal toxic effects (Abrudan et al., 2015). CDS however, has the ability to act on a smaller and more local scale, by mediating CDS only between adjacent cells within multi-cellular bacterial communities. This hypothesis is supported by the finding that CDS do not equally affect bacterial cells within a biofilm (Garcia et al., 2016). The possible evolutionary benefit of this unequal effect could be numerous. It could be an energy reserving strategy restricting the need to produce large amounts of soluble molecules. Or it could be a way to create a “secret handshake” between the producing cell and the recipient cell, since toxic effectors are delivered in a receptor-dependent manner. The recipient cell could therefore be chosen based on the receptor recognized by the CdiA/BcpA protein, allowing for unique behaviors within a sub-population of cells. Conversely, it could be a mechanism to facilitate closer collaboration and cooperation, only between adjacent cells.

CDI systems can also create unique biofilm architectures by mediating kin-selection (Anderson et al., 2014; Blanchard et al., 2014; Bottery et al., 2019). For B. thailandensis CDI TPS systems can function as powerful exclusion mechanisms; any cell lacking a CDI TPS system is prevented from establishing in a niche were a CDI TPS+ population already exists (Anderson et al., 2014). Within E. coli heterogeneous populations CDI TPS systems can, on a single-cell level create distinct barriers between inhibitor (CDI TPS+) and target cells (CDI TPS-), consisting of non-growing, or slow-growing target cells that are in constant contact with inhibitor cells. This barrier of slow-growing CDI TPS- cells limits the area of contact between a CDI TPS+ and a CDI TPS- population (Bottery et al., 2019). Moreover, the most important factors for CDI TPS mediated growth inhibition on single-cell level was shown to be the inhibition rate and toxicity of the CdiA-CT effector. If the rate of inhibition and CdiA-CT toxicity was low the CDI TPS- population could spread and establish a new micro colony, further away from the CDI TPS+ population, and thus avoid growth inhibition.
(Bottery et al., 2019). Additionally, CDI TPS systems were shown to have a fitness cost for the producing cell making a CDI TSP system a fitness liability, unless that fitness cost was counter-balanced by a beneficial growth inhibitory ability, when cells were grown in a mixed bacterial population. This clearly shows that CDI TPS systems can greatly effect the spatial distribution and behavior of bacterial cells within any multi-cellular bacterial population.

Figure 9. Schematic view of possible CdiA-dependent aggregation(s), mediated by either CdiA-to-receptor or CdiA-to-CdiA binding.
Cooperative interbacterial interactions

Interbacterial interactions are not always antagonistic and hostile. The multicellular lifestyle of most bacteria demands a predominately cooperative behavior for the common good of the community. These interactions include genetic exchange, metabolic exchange and cell-cell communications (Madsen et al., 2012; McLean and Kakirde, 2013; Moller et al., 1998). This type of cooperative interactions often comes at a high cost to each individual bacterium. Though, this cost is counter-balanced by the common good and general prosperity it generates for the population as a whole. In these complex and highly structured cooperative communities, any individual bacterial cell that doesn’t work towards the common good, while simultaneously still benefitting from population-wide effects, are referred to as a cheater. Cheaters change their behavior, avoiding the high fitness cost associated with cooperative interactions. However, these bacteria are often penalized by the remainder of the bacterial population through various mechanisms, which restrict the occurrence of cheaters. This maintains a stable population homeostasis and avoids a catastrophic population collapse, also referred to as the tragedy of the commons.

One way to not favor the occurrence and growth of cheaters is to connect multiple beneficial effects to one signal. One example of this is in *P. aeruginosa* biofilms. *P. aeruginosa* express extracellular proteases in a quorum sensing dependent manner (Brint and Ohman, 1995). These proteases are required to break down large polypeptides when *P. aeruginosa* is grown on media where casein is the sole carbon source. However, the expression of these proteases comes at a high cost. Any cell that have a mutation in a quorum sensing pathway will not express and secrete extracellular proteases, even though the rest of the population does. If these mutant cells are grown by themselves they have a severe growth defect, due to the lack of protease expression, but when grown in a mixed population together with WT cells these mutants grow perfectly fine. Cheaters are therefore benefiting from the common goods while not contributing to there production (Diggle et al., 2007). However, if adenosine is added to the media these cheaters are instead heavily disfavored. The uptake and metabolism of adenosine is also linked to quorum sensing, but adenosine utilization is private and not shared among the population (Katzianer et al., 2015). Any cheaters that don’t sense the quorum signaling, allowing them to avoid the costly production of extracellular proteases, also does not benefit from adenosine uptake and utilization. Another similar method that *P. aeruginosa* can utilize to disfavor and even kill cheaters is by synthesizing the toxic compound cyanide (Wang et al., 2015). Both cyanide synthesis and the cyanide degradation protein are expressed in a quorum sensing dependent manner. This results in a lack of cyanide tolerance for any cheater cell that ignores the quorum sensing signal. These methods to restrict the occurrence of cheaters are referred to as meta-
bolic constraint systems and is one of many methods the occurrence of cheaters can be restricted.

Quorum sensing

Many interspecies and intraspecies behaviors are regulated by quorum sensing (Tashiro et al., 2013). Quorum sensing allows some bacteria to sense how many bacterial cells that are within the local environment or how many bacterial cells belongs to the same bacterial family, the same genus or the same species (Figure 10). This allows bacteria to adjust and synchronize behaviors accordingly. It is also not uncommon for a bacterial species to employ multiple different quorum sensing systems to be able to preform multiple levels of quorum sensing (Figure 10) (Tashiro et al., 2013). One such bacterium is P. aeruginosa, which employs at least four different quorum sensing systems; two different interspecies quorum sensing systems, using N-acylhomoserine lactones, one intraspecies quorum sensing system, using 2-alkyl-4-quinolone, and a forth newly discovered system using 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde. The inter- or intraspecies capabilities of this fourth system still remains to be discovered (Lee et al., 2013). These quorum sensing systems effect motility, biofilm and group behaviors in P. aeruginosa bacterial communities, including the synthesis of extracellular antimicrobial chemicals, as well as cell-cell interactions using the T6SS or the creation of membrane vesicles.

Quorum sensing is not only restricted to unique dedicated signaling molecules. The presence of excreted or secreted secondary metabolites and cell wall components can function as indirect quorum sensing molecules, or pseudo-quorum sensing molecules, for many bacteria. Examples of this are many acidic secondary metabolites, such as acetic acid, butyric acid and propionic acid that play an important role in regulating microbial behavior in the intestine (Li et al., 2018). Acetic acid and propionic acid are common secondary metabolites of bacteria belonging to the Bacteroidetes phylum and butyric acid is a common secondary metabolite of bacteria belonging to the Firmicutes phylum (Macfarlane and Macfarlane, 2003). These bacterial phyla’s inhabits the lower intestine of warm-blooded mammals. The concentration gradient of these secondary metabolites act as an important signal for bacteria such as Campylobacter jejuni to locate the lower intestine and regulate expression of virulence factors and genes important for communal behaviors (Luethy et al., 2017). Another important pseudo-quorum sensing molecule is N-acetylglucosamine, which is an important component of the bacterial cell wall peptidoglycan. Excessive extracellular concentration of N-acetylglucosamine functions as a stress signal that presumable indicates that there are a high number of dead cells in the local environment (Naseem et al., 2012). In P. aeruginosa high levels of N-acetylglucosamine results in an expression of phenazine antimicrobial compounds, possible as a defensive
action to fight back against any adjacent antagonistic organism (Korgaonkar and Whiteley, 2011). In *E. coli* N-acetylglucosamine triggers a reduced expression of many genes important for virulence, such as curli and type 1 fimbriae (Barnhart *et al.*, 2006; Sohanpal *et al.*, 2004). This could facilitate increased motility and dispersion, which would make it possible for the bacteria to avoid a hostile organism, or the host’s immune system.

![Figure 10. Simplified illustration of inter- and intraspecies quorum sensing. Quorum sensing molecules produced by the brown species is detected and recognized by both the brown and green species, while quorum sensing molecules produced by the green species can only be detected by the green species.](image)

**Division of labor within a biofilm**

Experimental and laboratory limitations have resulted in a reductionist approach to the study of biofilms. Biofilm or biofilm-like communities are often studied only as homogenous populations. This creates a great bias since biofilms in nature are multicellular, multi-species communities that sometimes even contain several different domains of life, i.e. bacteria, archaea and unicellular eukaryotes. Therefore knowledge obtained from homogenous biofilms should be evaluated based on this lack of diversity and future research should strive to study the heterogeneous and interspecies nature of biofilms as often as possible.

Dental biofilms, also known as dental plagues, are one of the most diverse biofilms known to exist and also one of the most well-studied multicel-
lular bacterial communities (Kolenbrander et al., 2006). Dental biofilms are known to consist of >700 different bacterial species and the bacteria within these multicellular communities exhibit well-studied intraspecies and interspecies communication and cooperation’s (Guo et al., 2014; Hojo et al., 2009; Huang et al., 2011; Kuramitsu et al., 2007). One of the most modest divisions of labor within these biofilms is in initial colonization and aggregation. Only a small fraction of cells and bacterial species within a dental biofilm population specifically binds to a surface, the remaining population instead binds to these initial colonizers and this in turn allows for a three dimensional interspecies biofilm structure to be formed (Rickard et al., 2003). Some bacterial species can even act as a bridge between two different species that normally can’t aggregate together, a phenomenon known as coaggregation (Rickard et al., 2003). This is of significant benefit to the bacterial population as a whole. If all bacteria would directly adhere to a specific surface the biofilm structure would naturally take on a more two dimensional arrangement. By having some bacteria bind specifically to other bacterial cells, rather than to a colonization surface, a more complex biofilm can be formed, which ensure a more robust and durable biofilm. This also allows some bacterial species within dental biofilms to specialize in surface binding while other species can specialize in the synthesis of biofilm components, such as cellulose and amyloid fibers (Rickard et al., 2003). Other forms of division of labor includes the degradation of toxic metabolites and the production of beneficial metabolites only by a sub-population of cells (Stewart and Franklin, 2008). These specialist cells benefit the population as a whole, by purifying the local environment or by providing essential nutrients to a small sub-population of cells that in turn produces other helpful compounds. Some cells are even capable of modifying the local microenvironment by utilizing all the available oxygen, which benefits obligate anaerobic bacteria. These oxygen metabolizers are often found nearby obligate anaerobic bacteria and it is presumed that they are in a local symbiotic relationship (Stewart and Franklin, 2008).

Cooperative behavior have also been shown to be more favorable in bacterial communities were diffusion of common goods are limited (Nadell et al., 2016a). When bacteria grows on media with increasing concentration of agar a greater amount of homogeneous clonal bacterial clusters are formed. Within these clusters bacteria have a higher expression of common goods enzymes. P. aeruginosa produce higher amounts of siderophores when grown in conditions that limits siderophore diffusion and Vibrio cholerae express more chitinase when grown under similar conditions. The proposed evolutionary reason for this is that the producing cell(s) benefit more from common goods enzymes when the product of those enzymes don’t diffuse away from the producing cell(s) (Nadell et al., 2016a). This implies that there is a degree of selfishness also in cooperative bacterial communities. These cooperative bacteria will behave in a manner that benefits the entire
population, but also attempt to derive the greatest possible benefit for themselves.

Division of labor and complex multicellular behavior can also be created within homogenous multicellular communities consisting of only one genotype. Cyanobacteria, such as Anabaena spp., can differentiate in a quorum sensing dependent manner so that approximately 1 bacteria out of every 4-15 cells (depending on species) becomes a nitrogen fixating heterocyst (Flores and Herrero, 2010). The ratio between the vegetative growing, carbon dioxide fixing majority population and the nitrogen fixating minority population is not static, but can change depending on nitrogen availability. This essentially creates two very different cell types, with very different phenotypes, from one genotype. The heterocyst minority population supplies the majority population with much needed nitrogen, while the carbon dioxide fixing majority population supplies the minority population with carbon, in the form of simple sugars. This shows that even homogenous populations can divide labor amongst them and behave in much the same way as interspecies multicellular communities.

Many intraspecies toxin secretion systems are regulated by quorum sensing and predominantly expressed when cells are grown in homogenous intraspecies bacterial communities (Nadell et al., 2016b). The evolution of such intraspecies growth inhibition systems could be highly evolutionary beneficial. Since if a bacterium is in a beneficial and symbiotic relationship with other bacterial species, these species are not an evolutionary threat, they are in fact advantageous. While a closely related bacterium, that is not genetically identical, but able to preform similar activities and functions, would constitute a true evolutionarily competitor. Thus the possession of intraspecies growth inhibitory system(s) would be valuable to outcompete these true competitors from an otherwise harmonies multispecies community.

Exploitation as a form of population bet-hedging

Few single cell organisms cooperate as much as Myxobacteria, even fewer prokaryotic species cooperate as much as Myxobacteria (Cao et al., 2015). Myxobacteria exist not only as a single cell free-living organism, but also as a highly coordinated multicellular community comprised of elaborate multicellular structures. This multicellular existence appears to be highly beneficial for the bacteria, as they are known to dominate in many different environmental niches (Li et al., 2012; Reichenbach, 1999; Singh, 1947). During starvation conditions the unicellular and vegetative growing Myxobacteria, which are dispersed in an environment, swarms and comes together to form a multicellular fruiting body. This migration and subsequent aggregation is mediated by cell-cell communication between kin cells. This swarming activity results in cell-cell aggregation and subsequent formation of a large multicellular structure (Cao et al., 2015). These swarms are mobile hunting
groups that can employ fast movement through coordinated cell-cell interactions, and secrete toxic compounds and lytic enzymes to hunt and consume prey bacteria (Berleman and Kirby, 2009). Myxobacteria can also exchange proteins and lipids by membrane fusion, referred to as outer-membrane exchange. This membrane fusion is triggered by the outer-membrane protein TraA, which both identifies other kin cells and initiates membrane fusion (Pathak et al., 2013). The evolutionary purpose of this rather unique bacterial behavior appears to be for population wide outer-membrane homeostasis and repair of membrane damage. A hypothesis that is supported by the observation that Myxobacteria cells with a lethal lipid A mutation can grow within a population of WT cells, only as long as outer-membrane exchange is possible (Vassallo et al., 2015). However this membrane exchange could also be a mechanism to encourage closer cooperation between kin cells, as TraA can distinguish between kin cells and non-kin cells.

When Myxobacteria swarms are starved the cells undergoes an elaborate differentiation with some cells forming stress resistant spores, while the majority of cells becoming metabolically dormant or undergoes cell lysis (Lee et al., 2012; OConnor and Zusman, 1991). Thus, the population as a whole invests their collective efforts and resources in a just a few cells. These cells (spores) are highly stress resistant and can survive great hardship. However, when a spore experiences a better environment it can germinate and establish itself in this new niche, giving rise to a new population that can repeat the cycle again if conditions deteriorates (Cao et al., 2015). This means that for some Myxobacteria to survive a great many bacterial cells have to be exploited. However, this behavior does benefit the species overall, thus providing a possible evolutionary selection pressure for this behavior. Though it should be noted that the multicellular behavior of Myxobacteria is not limited to just creating spores from a small minority of cells. The multicellular behavior of the Myxobacteria swarms has multiple beneficial effects, which implies that evolution has not solely favored this population-wide exploitation. Nevertheless, this highly elaborate and advanced multicellular behavior of Myxobacteria blurs the line between the definition of what a unicellular and multicellular organism is.
Persister cells and other means of stress tolerance

An important function of division of labor within multicellular communities is for resistance and/or tolerance of antimicrobial agents and antibiotic compounds. Numerous observations have reported an increase in antibiotic tolerance and/or resistance within mixed species biofilms, compared to homogeneous biofilms (Al-Bakri et al., 2005; Burmolle et al., 2006; Kara et al., 2006; Leriche et al., 2003; Whiteley et al., 2001). This increase in tolerance and/or resistance was in some studies connected to a change in the composition of the biofilm extracellular matrix (Flemming and Wingender, 2010). This was also demonstrated with an interdomain biofilm when the pathogenic unicellular eukaryote Candida albicans was co-cultured with the pathogenic Gram-positive bacteria Staphylococcus epidermidis, two pathogenic organisms commonly found in biofilms together (Adam et al., 2002). The extracellular matrix produced by S. epidermidis diminished the uptake of the antifungal drug fluconazole by C. albicans, and an unknown factor produced by C. albicans increased S. epidermidis tolerance to vancomycin. Therefore, it appears that both organisms greatly benefits from this mixed biofilm arrangement. Other methods that bacteria employ to create a higher prevalence of tolerance for the bacterial community as a whole are through physical shielding. Pseudomonas fluorescens is able to grow in multicellular communities exposed to high concentrations of the toxic compound chlorine dioxide when co-cultured together with Bacillus cereus (Lindsay et al., 2002). B. cereus have a higher resistance to chlorine dioxide and can physically shield P. fluorescens by growing around them and surrounding the entire microcolony. This creates a diffusion barrier and limits exposure of P. fluorescens to chlorine dioxide.

Rather than conventional cooperative behaviors, homogeneous bacterial communities can also employ phenotypic switching and heterogeneity to create sub-populations of cells exhibiting different behaviors and increased stress tolerance (Vega and Gore, 2014). These types of phenotypic switching are not always mutually beneficial, like in Cyanobacteria were both heterocyst and vegetatively growing cells benefit from each other, but can be rather one-sided. Phenotypic switching can create a small sub-population of cells that have a much larger resource burden than other bacterial cells. Some examples of this are the expression of antibiotic resistance proteins. Secreted antibiotic resistance enzymes, such as many β-lactamases, can when expressed only by a sub-population of cells confer a benefit for the entire population (Brook, 2009). This cannot be considered a mutually beneficial arrangement, but rather a population wide bet-hedging strategy. These phenotypic switching events are also subject to a great deal of exploitation. Any bacterial cells that do not produce the antibiotic resistance protein have a growth rate advantage. However, if too many cells stops secreting the resistance protein, the entire population will be subject to antibiotic growth
inhibition. Thus, there is an intrinsic selection pressure to maintain an antibiotic resistance homeostasis within the population, a phenomenon known as heteroresistance.

Bacterial cells can also become stress tolerant by utilizing transient phenotypic switching (Balaban et al., 2004). These cells have not acquired a mutation(s) that confer a resistance towards a toxic compound(s), but rather change their behavior to be less susceptible to a toxic compound(s). These bacterial cells are referred to as persister cells and are only temporarily tolerant towards a toxic compound by entering a metabolically dormant state. Many toxic compounds, including antibiotics, affect growing cells to a much higher degree than dormant cell. The reason for this is that antibiotics often target cellular factors and mechanisms required for growth, such as DNA replication, transcription, translation or cell wall synthesis. Thus, by entering a dormant non-replicating state a cell becomes less susceptible to these toxic compounds, because these processes are not active. This hypothesis is supported by experiments showing that fast growing cells are much more susceptible to antibiotic exposure than non-growing, stationary phase cells (Balaban et al., 2004). Furthermore, this phenomenon is not limited to dormant non-replicating cells. S. aureus can stochastically create a sub-population of cells with a much slower growth rate, so called small colony variants (Edwards, 2012). These slow growing cells have a higher tolerance towards gentamycin and can continue to grow even in the presence of the antibiotic, which the fast growing WT cells cannot. Thus, there appear to be a direct connection between growth rate and stress tolerance.

The phenomenon of persister cell formation have been known for more then 80 years and observed in multiple different organisms, both pro- and eukaryotic (Bigger, 1944; Greenwood and Ogrady, 1970; Gunnison et al., 1964; LaFleur et al., 2010; Newsom, 1970; Vega and Gore, 2014). However, the molecular mechanism behind the creation of persister cells is, and has been, under some dispute as of late. There is evidence to suggest that transient stress tolerance can be generated entirely stochastically, such as for the small colony variant in S. aureus, through single cell variation in gene and/or protein expression (Elowitz et al., 2002; Rosenfeld et al., 2005; Swain et al., 2002). These fluctuations and possible metabolic cell-to-cell variations are realistic probabilities for a system so complex as a living cell. Small cell-to-cell variations in any number of possible regulatory proteins or secondary messengers could conceivable effect entire pathways, resulting in the creation of minority populations with different behavior(s). Intrinsic transcriptional and/or translational expression “noise” could also effect the expression of regulatory proteins, which in turn would results in abnormal behavior(s) within a sub-population of cells. Cell-to-cell variations can also be generated by external and/or metabolic factors. Examples of this are persister cell formation in P. aeruginosa were the presence of quorum sensing molecules can greatly effect the frequency of persister cells within a population (Moker et
al., 2010). A similar effect have also been observed for *E. coli*, were the starvation and stationary phase signal molecule indole can give rise to a higher frequency of persister cell formation (Vega *et al*., 2012). Therefore, persister cell formation seems to be both stochastic and deterministic in nature. During optimal conditions only a small fraction of cells are “sacrificed” and enters a dormant state, while under starvation and non-optimal conditions, or conditions when a high cell density have been achieved, a larger fraction of cells enters a dormant state. This bet-hedging strategy could be very beneficial long-term since persister cells are only formed at high frequency if the conditions are not conducive to fast and rapid growth. This means that the population as a whole does not sacrifice very much, while simultaneously safeguarding against a catastrophic population collapse induced by toxic compounds and/or unfavorable environmental conditions.

Early work enriching for *E. coli* mutants with increased persister cell formation revealed SNP’s within the Type II TA-system hipAB (Moyed and Bertrand, 1983) This increase in persister cell formation was later connected to the toxic activity of the HipA toxin (Falla and Chopra, 1998; Korch *et al*., 2003). Several different gain-of-function mutations, especially a variant named HipA7, with two different mutations (G22S and D291A) were shown to give rise to a 1000-fold higher frequency of persister cell formation (Balaban *et al*., 2004). It is important to note that while this mutation(s) was lab-derived, a bioinformatic screen of the genomes of 477 *E. coli* isolates, both commensal and pathogenic, revealed the presence of many of these known gain-of-function mutations in these clinical strains, including the hipA7 allele (Schumacher *et al*., 2015). Additionally, multiple type I and type II TA-systems have been shown to play a role in persister cell formation in many different bacterial species (Ronneau and Helaine, 2019). Persister cell formation mediated by TA-systems can also be both stochastic and deterministic.

Two important stress tolerance pathways in *E. coli* have been linked to TA-system expression. These two pathways are the DNA damage induced SOS-response pathway and the nutrient starvation induced, guanosine pentaphosphate (pppGpp) or guanosine tetraphosphate (ppGpp) stringent response pathway. Additionally, antitoxin molecules from type II TA-systems are also subject to proteolytic degradation by cellular proteases during stress (Gerdes *et al*., 2005), which could result in free toxin molecules in the cell. This would further activate stress response pathways, which could create a feed-forward loop that could give raise to dormant, non-growing cells. These observations collectively imply that TA-systems could play an important role in stress tolerance and/or stress adaptation. Another possible way that TA-system could affect stress tolerance is by manipulation the growth rate of the cell. Many different type II TA-system mutants have been identified that extends the lag-time of the cell (Levin-Reisman *et al*., 2017). This provides
an additional scenario for how TA-systems could increase the frequency of non-growing cells within a population.

The evidence for persister cell formation by stochastic and deterministic cell-to-cell variation in transcription, translation or secondary metabolites, as well as the evidence for persister cell formation by TA-systems, creates a “chicken or the egg” conundrum. Since TA-systems seems to be effected by both the metabolic state and the concentration of secondary messengers within the cell (Ronneau and Helaine, 2019), a relevant question to ask is; does stochastic or deterministic cell-to-cell variations in transcription, translation or secondary metabolites result in TA-system activation, which could amplify, modulate or otherwise affect cellular behavior and give rise to persisters cells (Figure 11). Or does TA-system activation, by either stochastic or deterministic effects, result in cell-to-cell variations in transcription, translation or secondary metabolites, which results in persister cell formation. Or is this a closed circuit where both possibilities are possible and intrinsic fluctuations in either results in an activation or effect on the other?

![Diagram](image)

Figure 11. Simplified illustration of the persister cell induction conundrum. Does abnormal cellular behavior(s) trigger TA-system(s) activation or does the activation of TA-system(s) lead to abnormal cellular behavior(s), which ultimately induces persister cell formation, or does the persister cell induction feed-forward loop truly not have a beginning or end?
Present investigation

In this thesis I have aimed to characterize the effect, limitations and impact of CDI TPS systems on both single cell and population level, within both intra- and interspecies bacterial populations. Toward that end, I have utilized a combination of clinical cdiBAI+ strains and artificial CdiBAI expression constructs to study CDI dependent population effects, single-cell phenotypes and the importance of individual CdiA protein domains and target cell receptors for CdiA mediated toxin delivery. CDI systems are widespread throughout the bacterial kingdom. However, there biological impact on bacterial communities is still inadequately understood. The experimental observations described in this thesis provide important information on the effects and limitations (or lack of limitations) of these interbacterial growth competition systems and aims to clarify the many biological aspects of CDI systems. This hopefully will enable a better understanding of CDI systems and their influence on bacterial communities.

Paper I

**EC93 utilizes two functional CDI TPS systems for antagonistic interbacterial interactions**

CDI TPS systems were first identified in the *E. coli* strain EC93 (Aoki et al., 2005). Initial investigations demonstrated that EC93 was able to inhibit the growth of *E. coli* MG1655 lab strain(s) in liquid media during co-culturing experiments utilizing a CDI TPS (cdiBAIEC93) locus. However, when we competed a ΔcdiAEC93 mutant against MG1655 on solid media we could observe that the growth inhibitory phenotype against MG1655 remained unaffected. We therefore sequenced the genome of EC93 and could bioinformatically identify a second cdiBAI locus in EC93. This second cdiBAI locus was expressed and also capable of mediating antagonistic interbacterial interactions. We compared this second cdiBAI system with the first, previously known, cdiBAI system of EC93 on the basis of receptor specificity, auto-aggregation, toxic activity, toxic potency and relative protein expression. From these investigations we could conclude that both CdiA molecules were class I proteins, utilizing the BamA outer-membrane receptor protein on target cells to deliver their toxic effectors. Both CdiA-CT domains encoded
for toxins able to dissipate the proton-motive force (PMF) of target cells and both toxins required the inner-membrane protein AcrB to mediate their toxic effect. Moreover, we could observe that EC93 outcompeted CDI susceptible target cells by almost 10-fold more when utilizing both CdiBAI loci, compared to only one, demonstrating a strong selection pressure on EC93 to retain and use both systems. These findings collectively show that the possession of multiple growth inhibitory systems can significantly increase bacterial fitness within a population. In contrast, the similarities between the two CdiBAI systems means that the probability of target cell CDI development is not decreased by having two separate CdiBAI systems, since a mutation in either BamA or AcrB could mediate resistance towards both systems. However, the usage of BamA and AcrB as CDI receptors might be evolutionary beneficial. BamA is an essential outer-membrane protein and AcrB have an important function in maintaining low intracellular concentrations of toxic substances (Blair and Piddock, 2009). The loss of AcrB could have a serious fitness cost in vivo. Consequently, the use of BamA and AcrB as CDI receptors might outweigh the potential gain from having two CdiBAI systems with more diverse functionality and receptor recognitions.

**CdiA expression levels are not the most important aspect for CdiA mediated CDI**

By using both sYFP2 transcriptional reporters and direct CdiA protein measurements we could identify that the second CdiA protein of EC93 was expressed at approximately 6-fold lower level than the first CdiA protein, even tough both CdiA proteins were able to mediate equal growth inhibition. During experiments to measure the toxic effect of the two CdiA-CT toxin domains we could observe that, when all other factors were equal and toxic molecules were delivered extracellularly by inhibitor cells, the second CdiA-CT toxin was able to mediate a greater toxic effect than the first CdiA-CT toxin. Thus, we could show that CdiA expression is not the most important factor for CdiA mediated CDI, but rather that it is a combination of CdiA expression and the toxic potency of the CdiA-CT toxin that determines the growth inhibitory phenotype. This observation could have a greater impact on the CDI field than one might think. CdiA expression in many clinical bacterial strains is usually very low, or below the detection limit for most assays. This is commonly used as an explanation for the lack of CdiA mediated CDI phenotypes in clinical strains. However, we show that even a low level of CdiA expression can mediate a strong growth inhibition. Therefore, CdiA expression alone should not be the only considered factor to predict CdiA activity in a particular bacteria or condition. Furthermore, from our observations we hypothesize that CDI TPS mediated growth inhibition have evolved to favor an optimal amount of growth inhibition, i.e. that a potent toxin is delivered less efficiently than a weak toxin, to achieve the same relative amount of growth inhibition. This could imply that there is a possible
fitness cost associated with toxin self-delivery, or that the delivery of more potent toxin molecules does not provide an additional evolutionary benefit during CdiA mediated CDI. Thus, identifying the evolutionary purpose, if any, behind this effect might provide important insight into the purpose of CDI TPS systems.

Paper II

CDI TPS systems increase bacterial stress tolerance in clonal bacterial populations

Expression of TA-systems have previously been shown to result in a higher fraction of stress tolerant, so called, persister cells within a population (Balaban et al., 2004). CDI TPS systems share many similarities with conventional type II TA-systems. But in contrast to type II TA systems, CDI TPS systems allow for toxin delivery between cells. This extracellular delivery means that CDI TPS mediated phenotypes could be cell-density dependent, which would provide a new level of complexity to the toxin induced stress tolerance phenomenon. To investigate if CDI TPS systems affected bacterial stress tolerance we exposed different E. coli cdiBAI+ strains to antibiotics. The absence of the first cdiBAI_EC93 locus in EC93 reduced bacterial survival by 5-10-fold, 3h post-treatment by ciprofloxacin. Moreover, when we inserted the first cdiBAI_EC93 locus of EC93 into the chromosome of MG1655 we could observe a 5-10-fold increase in survival, 3h post-treatment by ciprofloxacin. Demonstrating that the strain background and gene context is inconsequential for this effect, and that the cdiBAI_EC93 system alone is sufficient to mediate an increase in bacterial stress tolerance in clonal bacterial populations. We could also show that this increase in stress tolerance was dependent on extracellular toxin delivery. Cells unable to receive CdiA toxin(s), because of a non-permissive CdiA target receptor (BamA from Salmonella), cells expressing a CdiA protein without a CdiA-CT toxin, or cells exposed to antibiotics at a low-cell density, before cell-cell toxin delivery could occur, did not exhibit a increases in stress tolerance. This CDI TPS dependent stress tolerance was also observed with the delivery of multiple CdiA-CT toxins; such as a membrane ionophore or two different tRNase toxins, and when treated with other antibiotics, such as cefotaxime. These results further demonstrate that CDI TPS systems are not restricted to only antagonistic interactions. But that CDI TPS systems can mediate cell-cell aggregation and biofilm phenotypes (Ruhe et al., 2015), change transcriptional expression within a bacterial population (Garcia et al., 2016) and increase bacterial stress tolerance (Paper II).
CdiA-CT toxin activity and CdiI proteolytic degradation forms the basis of a feed-forward loop

Extra-cellular CdiA toxin delivery within a clonal bacterial population increased bacterial stress tolerance, even when cells were immune towards CDI mediated growth inhibition. How could an extracellular toxin delivery have an effect on a cell immune towards that toxin? We hypothesized that the CdiI immunity proteins were unstable and subject to proteolytic degradation under certain conditions, similar to type II TA-system antitoxin molecules. When we activated the stringent response, by treating cells with DL-Serine hydroxamate (SHX) (a serine analogue that inhibits tRNA charging by serine-tRNA synthetase(s)), we could observe that the UPEC536 CdiI immunity protein was rapidly degraded. We could also show that this proteolytic degradation was dependent on the Lon protease and that the CDI TPS mediated antibiotic tolerance phenotype was dependent on both the stringent response alarmone (p)ppGpp and the Lon protease. Furthermore, when we expressed the CdiA1-CT toxin of EC93 intracellularly within target cells, or extracellularly delivered the toxin to target cells, we could observe (p)ppGpp induction, activation of the rpoS promoter and activation of a SigmaS dependent promoter (osmY). This clearly showed that the CdiA1-CT toxin of EC93 could cause both a (p)ppGpp induction and a general stress response in target cells, besides also causing a growth arrest. These observations together could create the basis of a possible feed-forward loop, were the CdiA-CT toxin(s) are able to generate a general stress response within the cell, in the absence of sufficient cognate immunity protein(s). While simultaneously the cognate immunity protein(s) of CdiA-CT toxins would be subject to stress induced proteolytic degradation. Furthermore, it is not difficult to imagine that when CdiA-CT toxins are being delivered between cells there is a small minority of cells that receive a higher number of toxin molecules than the rest. These cells could then trigger a CdiA-CT mediated stress response, which would cause the degradation of CdiI immunity proteins. This feed-forward loop could potentially give rise to a small number of non-growing cells that would be resistant towards antibiotic treatment, or other toxic molecules. Moreover, these cells could possible wake up from this dormant state by expressing more CdiI immunity proteins, presumably after the CdiA-CT toxin have undergone proteolytic degradation or natural protein decay/inactivation. A hypothesis that is supported by the observation that cells inhibited by the CdiA-CT toxins of EC93, either from intracellular expression or extracellular delivery, can wake up after a period of dormancy (Aoki et al., 2005; Aoki et al., 2009).

We hypothesize that CDI TPS systems have the ability to function as a cell-density-dependent pseudo-quorum sensing system, since the number of persister cells will increase, due to extracellular toxin delivery, within a dense bacterial population. This could constitute a viable bet-hedging strate-
gy. Because within any homogenous, or clonal population, the need to safeguard against a catastrophic population collapse would be greater than the benefit gained from fast and rapid growth, since one bacterial genotype already dominate that specific niche. A bacterial population could therefore resolve to grow fast, when few kin-cells are around, and achieve a greater ability to withstand stress, when many kin-cells are nearby. This type of bet-hedging strategy is not unheard of, both *E. coli* and *P. aeruginosa* form more non-growing, stress tolerant persister cells when exposed to quorum sensing molecules (Moker *et al.*, 2010; Vega *et al.*, 2012).

**Paper III**

**Transcriptional start sites (TSS) within the *cdiA-CT* toxin domain allows for functional CdiA-CT and CdiI immunity proteins to be expressed**

Multiple, so-called orphan toxins modules can frequently be found downstream of most *cdiBAI* loci (Poole *et al.*, 2011). These modules are comprised of two open-reading frames (ORF); a *cdiA-CT* domain, encoding for a toxic effector, and an ORF encoding for the cognate immunity protein (*cdiI*) of this effector. These orphan toxins can be functionally delivered to a neighboring cell, if they are recombined to the *cdiA-NT* domain at a conserved motif. This *cdiA-CT* toxin recombination would presumable be an evolutionary beneficial event, since it would allow for an additional toxin to be utilized in CDI TPS mediated interbacterial warfare. However, how these orphan modules are retained within the genome, when not used for interbacterial warfare, is not clear. This conundrum is further amplified by the observation that some bacterial strains, such as *E. coli* EC869, have 11 different, bioinformatically identified, orphan toxin modules (Poole *et al.*, 2011). Consequently, a single recombination event has the ability to result in the loss of 11 different potential CdiA-CT toxin effectors (one main toxin and 10 orphan toxins). Thus, these orphan toxin modules should be subject to some kind of selection pressure, in order to be stably maintained in the genome.

When we bioinformatically investigated these orphan toxin modules we could identify several potential TSS and <100bp downstream of these we could also pinpoint the presence of putative ribosome-binding site(s) (RBS) and potential in-frame start codon(s). To investigate if any transcriptional activity originated from these *cdiA-CT* domains we isolated four different *cdiA-CT* and immunity modules, taken from three different *E. coli* strains and *Enterobacter cloacae*, from their native context and cloned them onto a low-copy plasmid. By using sYFP2 transcriptional reporters we could show that all four CdiA-CT and immunity modules had internal TSS. Furthermore, these TSS allowed for a sufficiently high CdiI expression to provide
the cell with immunity toward extracellular CdiA-CT toxin delivery. Since we had previously (Paper II) demonstrated that CdiI immunity proteins could be subject to proteolytic degradation we hypothesized that also a functional CdiA-CT toxin was expressed intracellularly and that these cdiA-CT and immunity modules constituted a selfish-genetic element. To test this hypothesis we competed cells that retained the cdiA-CT and immunity modules on a low-copy plasmid, against cells that selectively lost the system, by virtue of unstable plasmid propagation. Cells that lost cdiA-CT and cdiI containing plasmids were outcompeted by cells retaining the system(s) by 2-3-fold over six generations of growth. Moreover, when we introduced an inactivating point mutation in the cdiA-CT toxin domain this selfish-genetic element phenotype was abolished. Thus, we could conclude that an internal functional CdiA-CT toxin and CdiI immunity protein expression occurs in the cell, providing an immunity towards extracellular CdiA-CT toxin delivery and a selfish-genetic element phenotype. This clearly demonstrated that cdiA-CT orphan toxin modules are subject to several different selection pressures, which should result in their continued retention within a bacteria’s genome. This stable propagation of orphan toxin modules could have great evolutionary importance, since it would allow these potential toxin effectors to be preserved within the genome until they, in the future, possible recombines to the cdiA delivery gene and is used in interbacterial warfare.

Intracellular cdiA-CT and cdiI gene expression is stress induced

We observed that the loss of cdiA-CT and cdiI modules from the genome of E. coli resulted in a fitness cost. However, we could only observe this selfish-genetic element phenotype when cells were grown in starvation inducing media (minimal media). Therefore, we were curious to investigate if this was due to an increase in CdiA-CT expression during starvation and/or stress conditions, or if this was entirely due to the selective proteolytic degradation of the CdiI immunity protein, during starvation and/or stress conditions. To answer the former we studied CdiA-CT expression mediated by the global general stress sigma factor, SigmaS, by utilizing an E. coli MG1655 rpoS (the gene encoding for SigmaS) null mutant or an rssB null mutant. RssB is the proteolytic chaperone protein responsible for mediating the post-translational degradation of SigmaS, by the ClpXP proteasomal complex during optimal, non-stress conditions (Muffler et al., 1996; Schweder et al., 1996; Zhou and Gottesman, 1998). Thus, an rssB null mutant has higher intracellular levels of SigmaS during optimal growth and very high levels during stress. When we manipulated the levels of SigmaS within the cell we could determine that cdiA-CT expression correlated perfectly with SigmaS dependent-transcriptional activity. This expression pattern followed the same general trend as the SigmaS dependent osmY promoter. However, the expression of the known type II TA-system, ccdAB, also followed this trend. Since ccdAB have not been reported to have a SigmaS dependent expression
we cannot disregard that the effect we observe is an indirect global effect, rather than a direct effect, mediated by direct binding of SigmaS on the cdiA-CT promoter(s). Another possibility is that cdiA-CT domain(s) and known type II TA-system(s), such as ccdAB, both have SigmaS dependent expression, but that this observation have been missed previously. Nevertheless, the observation that cdiA-CT expression follows the same trend as ccdAB expression and appears to respond to elevated levels of SigmaS, either directly or indirectly, implies that cdiA-CT and immunity modules constitutes a novel, stress induced TA-system. However, from these results we cannot determine if it is an increased expression of CdiA-CT or a selective degradation of CdiI, during growth in minimal media, or indeed both, that creates this selfish-genetic element. Thus, we plan to answer this remaining question by separating CdiA-CT+I expression from CdiI degradation and studying which factor mediates this phenotype.

From our findings in Paper II and Paper III a possible additional inter- and intracellular feed-forward loop becomes apparent from three distinct observations. 1) The extracellular delivery of CdiA-CT toxins have the ability to increase the expression of (p)ppGpp, as well as the expression and transcriptional activity of SigmaS in target cells. 2) Intracellular CdiA-CT and CdiI immunity protein expression is positively effected by SigmaS activity. 3) CdiI is degraded by the Lon protease during nutrient stress. This implies that extracellular delivery of CdiA-CT toxins might result in an increase in intracellular CdiA-CT expression during a stress condition (Figure 12). Thus, this could be the main underling mechanism for how CdiA+ bacterial cells can self-intoxicate via an extracellular CdiA toxin delivery (Paper II). This intracellular feed-forward loop could potentially amplify a mild stress effect that an unequal extracellular CdiA toxin delivery could trigger within a population. Consequently, the bacterial cells that receive a few toxic molecules more than the rest of the population would be stressed and thus express more intracellular CdiA-CT toxins, which together with the proteolytic degradation of CdiI would cause an even greater stress event, which could increase the intracellular CdiA-CT expression even further. Hence, CDI TPS dependent persister cell formation would be regulated not only by how many toxin molecules are delivered between cells, but also by the metabolic state of the cell. It is therefore not implausible that both of these factors combined determines the fraction of cells within a population that enters a dormant and stress tolerant state, in vivo. However, this remains to be investigated.
Figure 12. Simplified illustration of how extracellular CdiA-CT delivery could mediate an intracellular CdiA-CT+I expression, forming the basis for a cell-density-dependent pseudo-quorum sensing feed-forward system able to mediate an increase in stress tolerance.

Paper IV

Class II CdiA receptor-binding domains allow for broad range cross-species toxin delivery

Class II CdiA proteins utilize the OmpC and OmpF receptor proteins to delivery toxic effectors to target cells and previous studies have shown that Class II CdiA receptor binding domains have a narrow range of possible target cell receptors, resulting in intra-strain specific growth inhibition (Beck et al., 2016). This was not an unexpected finding since the sequence of OmpC outer-membrane loops are very diverse, due to strong selection for immunogenic variation to avoid targeting by the host immune system or bacterial phages (Liu et al., 2012; Silva et al., 2016; Singh et al., 2000; Stenkova et al., 2016). The authors therefore proposed that class II CdiA protein(s) have evolved to favor a bacteria’s own OmpC sequence, and to a lesser extent OmpF sequence, at the cost of not recognizing other strains and species OmpC and OmpF variants (Beck et al., 2016). However, we could bioinformatically identify almost identical class II CdiA receptor-binding domains in many different bacterial strains and species with very diverse OmpC sequences. This implied that either very small amino acid substitutions is required to change receptor specificity, or class II CdiA receptor-binding domains does not have the same receptor limitations that were previously reported.

When we replaced the *ompC* ORF of *E. coli* MG1655 with the *ompC* ORF of two other *E. coli* strains or two other *Enterobacteriaceae* spp. (*Salmonella* and *E. cloacae*) we could observe that all OmpC sequences allowed
for class II CdiA mediated toxin delivery. However, we could observe a difference in the efficiency of CdiA mediated growth inhibition and it appeared that some OmpC variants functioned as more optimal receptor than others. When we competed cdiBAI+ E. coli MG1655 strains against different clinical isolates we could also observe strong CdiA mediated growth inhibition, as long as we accounted for various other fitness factors these clinical strains expressed, such as capsule and T6SS. This clearly demonstrates that Class II CdiA receptor-binding domains allows for broad-range cross-species growth inhibition.

Since our results contradicted previous reports we were curious how this phenotype could have been missed by others. We could infer that the previous study investigating class II CdiA receptor limitations only expressed low levels of OmpC in target cells (Beck et al., 2016). When we did the same we also observed a severe limitation in class II CdiA mediated growth inhibition, in liquid media. However, this limitation was only conditional. When inhibitor and target cells were co-cultured on solid media low level OmpC expression did not constitute a limiting factor for toxin delivery by class II CdiA receptor-binding domains. We could also observe that when competitions were preformed on solid media there was no difference in the efficiency of growth inhibition, regardless of which OmpC variant the target cells expressed. Even a low expression of a non-optimal receptor was sufficient for class II CdiA mediated growth inhibition on solid media. This implies that in real-life conditions, such as in biofilms and biofilm-like bacterial communities, there will be little-to-no receptor limitation for class II CdiA mediated toxin delivery. Thus, class II CdiA systems might play a significant role in shaping Enterobacteriaceae spp. interspecies bacterial communities.

**OmpF is a beneficial, but not essential receptor protein for class II CdiA mediated toxin delivery**

The previous study investigating class II CdiA receptor limitations reported an essential role of both OmpC and OmpF in class II CdiA-receptor binding and toxin delivery (Beck et al., 2016). However, when we measured OmpF expression in our MG1655 target cells we could observe that OmpF abundance in liquid media was below the detection limit for our WB assay. Thus, we speculated that OmpF expression might not be essential for class II CdiA mediated toxin delivery. To test this we created ompF null mutants expression different OmpC variants. In liquid media only optimal OmpC receptors allowed for toxin delivery, and subsequent growth inhibition. However, on solid media all OmpC variants, with the exception of Salmonella spp. OmpC, allowed for toxin delivery and growth inhibition by class II CdiA receptor-binding domains, even when OmpF was absent. This demonstrated two things; OmpF is not a essential receptor protein for class II CdiA mediated toxin delivery, but it is a beneficial receptor protein when the OmpC variant is non-optimal or the growth conditions are non-optimal, i.e. turbu-
lent liquid media growth vs. static solid media. From these results we hypothesized that the beneficial effect of OmpF was its modulating effect on CdIA-OmpC binding. Thus, we measured the relative cell-cell binding of CdIA-OmpC in the presence or absence of OmpF. Our findings perfectly supported our hypothesis and we could observe that OmpC and OmpF expression allowed for a stronger binding to CdIA, than just OmpC by itself. The exact in vivo implication, if any, for this finding is not simple to elicit. However, since OmpC is the most abundant outer-membrane protein in E. coli the requirement of only OmpC as a CdIA target cell receptor should have numerous advantages. Especially since we have shown that OmpF expression can be very low in many different conditions (Paper IV). Additionally, the beneficial, but not essential role of OmpF as a CdIA target cell receptor could imply that OmpC and OmpF expression plays a role in influencing CdIA receptor binding and cell-cell aggregation by allowing some cells to preferentially aggregate under certain conditions. However, the lack of limitations for growth inhibition we observe on solid media could imply that there is only an evolutionary benefit from having the ability to mediate broad-range cross-species toxin delivery and that a bias in receptor specificity might not have a effect in vivo. Alternatively, their might never be an in vivo condition were CdIA is expressed simultaneously as a bias in CdIA receptor binding would have an effect.

Concluding remarks

In Paper I we could observe that a low-level of CdIA expression can still mediate a strong growth inhibitory phenotype. From this result it becomes apparent that many previous conclusions of CDI TPS systems should be re-evaluated. Many clinical E. coli strains, such as UPEC536, have been reported to not have a active CDI TPS system in LB media (Aoki et al., 2010). The lack of detectible CdIA expression was attributed to this absence of CdIA mediated growth inhibition. However, it is equally possible that surface proteins known to limit and prevent CdIA mediated CDI, such as fimbriae and capsule, or maybe even motility and/or the spatial distribution of bacterial cells when grown on lab media could be the real reason for this lack of a CdIA mediated growth inhibition. Comparably, the CDI TPS system of B. thailandensis (BcpAIOB) have only been reported to be expressed at detectable levels within <0.5% of WT cells in a population of B. thailandensis (Anderson et al., 2012). But a WT strain could still outcompete a bcpAIOB mutant during co-culturing on solid media by almost 1000-fold. It is entirely possible that a low level of expression within the entire population facilitates this growth inhibition, rather than a CDI activity mediated by only <0.5% of cells. The low level expression of many cdIBAI and bcpAIOB loci should
therefore be evaluated further in a model system that are known to not provide any limitations for CDI TPS mediated growth inhibition.

Our findings from **Paper II** and **Paper III** could lay the foundation for a conditional pseudo-quorum sensing system responsive to both cell-density; because of extracellular toxin delivery, and cellular stress and/or metabolism; because of SigmaS dependent expression. This might explain how CDI TPS system(s) could realistically function as CDS systems within clonal bacterial populations. The extracellular delivery of toxic molecules could result in an intracellular CdiA-CT toxin expression and when a cell is stressed a proteolytic degradation of CdiI immunity proteins could occur. This might allow a CDI TPS system to change the behavior of only a sub-population of bacterial cells, within a bacterial population. In **Paper II** we have demonstrated that one effect of this is an increase in stress tolerance. While the CDI TPS system in *B. thailandensis* greatly effect biofilm formation, implying that they might change global expression on a scale that allows for a change in growth style, i.e. a sessile (biofilm) growth, instead of a motile one. However, it is plausible that CDI TPS system dependent population effects are not limited to just an increase in stress tolerance and biofilm formation. It is also possible that different toxic molecules give different effects when delivered between kin-cells. A hypothesis that is supported by the observation that BepA-CT toxins from *B. pseudomallei* do not increase biofilm formation in a population of *B. thailandensis*, even though the native BepA-CT toxin of *B. thailandensis* does (Garcia *et al.*, 2016). Thus, it appears that the CDS “message” and effect is unique to each individual BepA-CT toxin. The population effects of different CdiA-CT toxins should therefore be investigated. Furthermore, extracellular CdiA toxin delivery could possible give rise to an increase in intracellular CdiA-CT expression. However, the intracellular CdiA-CT expression can also occur entirely independent of CdiBAI expression or CdiA mediated extracellular toxin delivery, in a stress dependent manner (**Paper III**). Consequently, the intracellular CdiA-CT toxin expression, by itself, could serve an important evolutionary purpose. Many type II TA-systems increase stress tolerance within a bacterial population (Ronneau and Helaine, 2019). A stress induced intracellular CdiA-CT and CdiI expression might form the basis of a type II TA-system that could increase stress tolerance within a bacterial population, even in the absence of extracellular toxin delivery. Thus, the effect of CdiA-CT intracellular expression during stress should be investigated further and compared to other known type II TA-systems.

Our work in **Paper IV** demonstrates that class II CdiA receptor-binding domains are capable of broad-range cross-species toxin delivery. This finding has numerous possible implications for how CDI TPS systems could affect the growth and spatial distribution of bacteria within multi-species bacterial communities. Furthermore, the broad-range receptor specificity and toxin delivery of class II CdiA receptor-binding domains opens up the possi-
bility that CDS is not limited to intraspecies communication, but rather that some CDI TPS systems could be utilized for cross-species communication and behavior manipulation. This aspect of CDS has never been openly raised within the CDS field, but merits a greater consideration in the future.

From our findings in Paper II, Paper III and Paper IV a final question becomes apparent; is the evolutionary purpose of CDI TPS systems to mediate antagonistic or cooperative interactions between bacteria? Is the receptor promiscuity of the class II CdiA receptor-binding domains an evolutionary mechanism to retain self-receptor recognition, even when the receptor (OmpC) is subject to extensive immunogenic variation, or is the promiscuity of class II CdiA-OmpC binding a mechanism that allows for broad-range cross-species growth inhibition (Paper IV). What speaks to the former is that CdiA mediated interactions between kin-cells have multiple beneficial effects. Both CdiA mediated cell-cell aggregation and CDI TPS toxins can greatly effect biofilm formation (Garcia et al., 2016; Ruhe et al., 2015), as well as stress tolerance (Paper II), in clonal bacterial populations. Thus, being able to aggregate and deliver toxic effectors to kin-cells should be evolutionary favorable. On the other hand, the ability to mediate broad-range cross-species growth inhibition would very likely be evolutionarily beneficial in a mixed species bacterial community. Moreover, the connection between stress and CdiA-CT and CdiI expression (Paper III) might indicate a possible link between CDI TPS systems and stress adaptation. Further implying that CDI TPS systems are important biological systems used to manipulate the behavior(s) of bacteria, rather than just strictly utilized for antagonistic interbacterial interactions. The reported effects of CDI TPS systems on bacterial communities also raise the question if narrow (class I), or broad-range (class II) CdiA receptor-binding specificity have an, as of yet, unknown effect on bacterial growth and spatial distribution in mixed species bacterial communities. Moreover, revealing the narrow or broad-range receptor recognition of class III CdiA receptor-binding domains, that recognize the Tsx outer-membrane protein (Ruhe et al., 2017), could clarify if receptor-binding promiscuity is the norm for E. coli CdiA proteins. It is also plausible that different CdiA receptor-binding domains have evolved for different purposes and that they have been evolutionarily optimized for specific bacterial lifestyles and growth niches.

The expression of CDI TPS systems should be investigate further as this would better indicate when and were CDI TPS systems are used in vivo and for what purpose. Additionally, the heavy reliance on lab media and lab conditions within the CDI TPS field severely limits the real-life relevance of most findings and can in some instances results in incorrect conclusions. The broad-range cross-species toxin delivery capability of class II CdiA receptor-binding domains was previously missed because experiments were exclusively preformed in rich (LB) liquid media (Beck et al., 2016). Studying CDI TPS systems under many different conditions is therefore of paramount im-
portance to illicit the real-life effects and significance of these biological systems. Conversely, many scientific findings, including those of this thesis, can only provide indications of the real-life effect of CDI TPS systems and we cannot say with certainty that the effect(s) we observe in the lab would correspond to those bacterial behaviors that transpires in vivo. However, this issue should be resolved as soon as the CDI field moves forward and begins to investigate the real-life impact and effect of CDI TPS systems in vivo.
Future perspectives

Since the discovery of the first cdiBAI loci in EC93 (Aoki et al., 2005), numerous studies have demonstrated the significant influence that CDI TPS systems can have on bacterial communities and cellular behavior(s) (Bottery et al., 2019; Garcia et al., 2016). Yet there are still many areas of CDI research that is almost completely overlooked. Most CDI TPS systems have predominantly been studied on population level. The effect of CDI toxin delivery is measured by a change in the ratio between two populations after co-incubation. CdiA mediated cell-cell aggregation is often measured by the ability of CdiA+ cells to aggregate to such a degree that cellular flocculation becomes visually observable with the naked eye. CDS, CdiA expression, CdiA toxin delivery and CdiI immunity degradation are also predominantly studied on population level. However, complex systems able to mediate differential expression within a small sub-population of cells, such as CDI TPS systems, should not be predominantly studied on population level. The CDI field as a whole should aim to move towards a single-cell approach when studying CDI dependent effects. Our finding that CDI systems can be used in bet-hedging strategies (Paper II) implies that also other CDI effects, such as CdiA dependent transcriptional changes or proteolytic degradation of CdiI immunity protein(s) is likely effecting a small sub-population of cells to a much higher degree then the majority of the population. Eliciting the behavioral changes and response mechanism(s) that this minority population exhibit would go a long way to understanding the purpose and multidimensional influence that CDI TPS systems might have in vivo. Furthermore, a better understanding of how different CDI TPS systems and CDI toxins effect populations under different environmental conditions would go a long way to determine the true nature of CDI system(s); i.e. its antagonistic, cooperative or dual role in vivo.

An additional area of neglect within the CDI TPS field is the post-translational regulation and modification of CDI TPS systems and CdiI immunity proteins. Our finding that CdiI immunity protein(s) are subject to proteolytic degradation (Paper II) raises the question of when and how this proteolytic degradation is initiated in vivo. Determining under what condition(s) CdiI proteins are degraded could clarify under which conditions CDI mediated effects would be more influential and result in more drastic changes in bacterial behavior(s). Additionally, many extracellularly secreted toxin proteins are known to undergo post-translational modifications that can
greatly influence their functionality (Vassiliadis et al., 2010). Thus, the existence of any CdiA protein modifications and their possible effect on CdiA mediated toxin delivery would also constitute an attractive area of future research.

Many pathogenic bacteria are known to possess multiple interbacterial competition systems (Poole et al., 2011). Though CDI TPS systems are not exclusively found in pathogenic bacteria, they are nevertheless an interesting potential fitness factor of these disease-causing organisms. The study of which could improve our understanding of how pathogenic bacteria communicate and interact with commensal bacteria before, during and/or after an infection. The study of CdiA-receptor binding could tell us more about which bacterial species that a certain pathogen interacts with and why. Lastly, a greater understanding of these type of microbial interactions could eventually allow us to design and engineer a probiotic bacteria expressing CDI TPS system(s) that could be ingested and allowed to pre-colonize the gastrointestinal tract of a patient, thereby functioning as a prophylactic barrier against colonization by pathogenic bacteria. This is not a novel idea, nor is it without precedent or scientific merit. One example of a gastrointestinal tract pre-colonized probiotic bacterium known to decrease the likelihood of infection and/or colonization by certain pathogenic bacteria is the E. coli strain Nissle 1917 (Schutz, 1989). Consequently, this would be an appealing new avenue of CDI research and could hypothetically play a significant role in a possible post-antibiotic era.


Våra resultat från artikel II och artikel III antyder att CdiA toxinleverans mellan celler kan fungera som ett sätt att reglera genuttryckoch beteende, beroende på vilka och hur många celler som finns i omgivningen. I homogena populationer med hög celldensitet så sker leverans av toxorer vid mycket högre grad, vilket resulterar i en större andel av celler som ej växer. Dessa celler skulle vara mer stresstoleranta än sina syskonceeller och fraktionen icke växande celler skulle därför påverkas av hur många celler som finns i närheten. Således både mekanismen för hur celler slutar växa samt hur celler bestämmer sig för när de ska sluta växa skulle följaktligen regleras av samma system.

Slutligen i artikel IV så har vi visat att somliga CdiA proteiner (klass II) kan leverera toxinmolekyler till en stor mångfald av olika bakteriella arter. Vi har också kunnat visa att även en låg mängd av en icke optimal receptor kan tillåta CdiA toxinleverans, under vissa konditioner. Dessa resultat anly-
der att CdiA toxinleveranser antagligen sker i större utsträckning och i mer omfattande mening inom bakteriella kolonier, än vad tidigare har antagits.

Dessa upptäckter tillsammans visar att kontaktberoende tillväxtinhiberingssystem är multifunktionella interbakteriella krigssystem som kan leverera toxiska molekyler till en mångfald av olika arter, under många olika känslor. Utöver detta så kan de även fungera som multifunktionella kommunikationssystem, samt stimulera till ökad stresstolerans under förhållanden där många celler förekommer i samma omgivning. Betydelsen av kontaktberoende tillväxtinhiberingssystem för bakteriella samspel och för multicellulära bakteriella beteenden bör således vara enorm. Dock krävs ytterligare forskning för att bättre förstå dessa system samt deras fullständiga påverkan på mikrobiella populationer.
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