Aus dem Max von Pettenkofer-Institut für Hygiene

und Medizinische Mikrobiologie

Institut der Ludwig-Maximilians-Universität München

Vorstand: Prof. Dr. med Sebastian Suerbaum

Chitinase A and Cytolysin A: Important virulence factors of *Salmonella enterica,* secreted by type 10 secretion systems

Dissertation
zum Erwerb des Doktorgrades der Naturwissenschaften
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von

Lena Krone

aus

Bocholt

Jahr

2024

Mit Genehmigung der Medizinischen Fakultät

der Universität München

Betreuerin:	Prof. Dr. rer. nat. Christine Josenhans
Zweitgutachter:	Prof. Dr. rer. nat. Nicolai Siegel
Dekan:	Prof. Dr. med. Thomas Gudermann
Tag der mündlichen Prüfung:	15. Juli 2025

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1. List of abbreviations

ChiA	chitinase A
ClyA	cytolysin A
E. coli	*Escherichia coli*
GlcNAc	β 1,4-N-acetylglucosamine
iNTS	invasive non-typhoidal *Salmonella*
L. monocytogenes	*Listeria monocytogenes*
L. pneumophila	*Legionella pneumophila*
M cells	microfold cells
MLN	mesenteric lymph nodes
NTS	non-typhoidal *Salmonella*
OMVs	outer membrane vesicles
PBMCs	peripheral blood mononuclear cells
PMN	polymorphonuclear leukocytes
S. marcescens	*Serratia marcescens*
S. Paratyphi A	*Salmonella enterica* serovar Paratyphi A
S. Typhi	*Salmonella enterica* serovar Typhi
S. Typhimurium	*Salmonella enterica* serovar Typhimurium
SCV	*Salmonella*-containing vacuole
Sec pathway	general secretion pathway
SPI	*Salmonella* pathogenicity island
SPI1-T3SS	type 3 secretion system encoded on *Salmonella* pathogenicity island 1
T10SS	type 10 secretion system
T1SS	type 1 secretion systems
T3SS	type 3 secretion systems
T4SS	type 4 secretion system
T6SS	type 6 secretion system
TaiA	Typhi associated invasin A

Tat machinery	twin-arginine translocation machinery
Y. entomophaga	*Yersinia entomophaga*

2. Abstract

Salmonella enterica is a foodborne pathogen which is spread by contaminated food or water. The infection by *Salmonella* leads to millions of illnesses and death cases per year. It is a facultative intracellular pathogen, which is able to invade into host cells and to survive and proliferate within the cells by translocation of effector proteins and secretion of toxins. During invasion, a membrane compartment emerges, which is restructured to a *Salmonella*-containing vacuole (SCV). While non-typhoidal serovars typically result in self-limiting gastroenteritis, typhoidal serovars have the capability to spread from the initial intestinal infection site to secondary sites within the human body. This leads to systemic disease and long-term bacterial persistence. *S. enterica* harbors several virulence factors crucial for colonizing the host and infiltrating diverse host target cells. We first focused on the pathogenicity factor chitinase A (ChiA). It was known that ChiA functions as an enzyme, breaking down chitin by cleaving its β1-4 glycosidic bonds. Our hypothesis was that ChiA also plays an important role for *S. enterica* virulence in a chitin-free environment such as the human host. It was our aim to elucidate the effect of ChiA on *Salmonella* invasion into human cells and further to clarify the effect of transcriptional regulators and other proteins encoded in the *chiA* operon on ChiA regulation and secretion. We found that the deletion of *chiA* leads to reduced invasion into human host target cells, comparable with data of other studies, published at about the same time. Intriguingly, our studies revealed that ChiA-mediated invasion occurs specifically in polarized human intestinal epithelial cells, whereas non-polarized or non-intestinal epithelial cells did not show reduced invasion phenotypes. In addition, we found that both cell-associated and free mucus lead to ChiA expression and secretion. This finding aligns with other studies showing that host cell glycans, which have structures similar to mucus, are targets of *Salmonella* chitinases. Furthermore, we unraveled the regulation of ChiA expression by a transcriptional regulator (STM0017) that we named ChiR. Finally, we showed that ChiA is actively secreted by a type 10 secretion system (T10SS) encoded within the *chiA* operon. Former studies have shown that the secretion of cargo proteins by T10SS in Gram-negative bacteria is facilitated by specified peptidoglycan hydrolases. They showed that these peptidoglycan hydrolases accumulate within the periplasm by traversing the inner membrane with the help of holins. Subsequently, the hydrolases cleave the peptidoglycan layer which enables the secretion of proteins through the bacterial cell wall. However, the final release mechanism through the bacterial outer membrane is currently unknown and needs further investigations in the future. In a second study, we focused on cytolysin A (ClyA), a barely described toxin known to be encoded only in strictly human-adapted typhoidal *Salmonella* serovars such as Typhi and Paratyphi A. ClyA is a pore-forming toxin and its homologue in *Escherichia coli* is well-described regarding its pore formation characteristics and regulation of expression. Using *S. enterica* serovar Paratyphi A, we demonstrated that ClyA represents a mainly intracellularly induced toxin that is exclusively expressed and secreted from bacteria residing within the SCV. Since its expression within the SCV correlates with the activity of a T10SS, which is responsible for the secretion of typhoid toxin, we hypothesized that the same secretion system also secretes ClyA. Using a TtsA peptidoglycan hydrolase mutant, we showed that ClyA secretion is completely abolished in this

T10SS deficient mutant, thereby verifying our hypothesis. Once secreted, ClyA exhibit specific cytolytic activities towards macrophage-like THP-1 and U937 cells, but lacked activity towards epithelial cells such as HeLa, CaCo-2 and HT29-MTX cells. We postulate that during infection, ClyA is mainly produced within infected epithelial cells and aids *Salmonella* infection by lysing attracted macrophages after its release from epithelial producer cells. However, this assumption and the mechanistic details regarding the susceptibilities of different cell types need to be investigated in further studies.

In summary, this work elucidates the expression, regulation, and secretion of ChiA and ClyA, two important yet barely described virulence factors of *S. enterica*. Additionally, it demonstrates the relevance of two similar but slightly different T10SS and their impacts on the pathogenicity of *S. enterica*.

3. Zusammenfassung

Salmonella enterica ist ein pathogenes Bakterium, das über kontaminierte Lebensmittel und Wasser verbreitet wird. Die Infektion mit Salmonellen führt zu Millionen von Krankheits- und Todesfällen pro Jahr. Es handelt sich um ein fakultativ intrazelluläres Bakterium, welches in der Lage ist, in Wirtszellen zu invadieren, in diesen zu überleben und zu proliferieren. Dieses wird unter anderem durch Translokation von Effektorproteinen und die Sekretion von Toxinen ermöglicht. Während der Invasion entsteht ein Membrankompartiment, welches zur SCV ("*Salmonella*-containing vacuole") umstrukturiert wird. Während nicht-typhoide Serovare normalerweise zu einer selbstlimitierenden Gastroenteritis führen, können die typhoiden Serovare im Körper verbreitet werden und weitere Organe infizieren. Diese Dissemination führt unbehandelt zu einer systemischen, persistierenden Infektion. Salmonellen verfügen über verschiedene Virulenzfaktoren, die elementar für die Kolonisierung des Wirts und die Infiltration diverser Zielzellen sind. Wir fokussierten uns zunächst auf den Pathogenitätsfaktor Chitinase A (ChiA). Es war bekannt, dass es sich bei ChiA um ein Enzym handelt, das die glykosidischen β1-4-Verbindungen des Chitins spalten kann. Unsere Hypothese war, dass ChiA auch eine Rolle für die Virulenz von *S. enterica* im menschlichen Wirt, einer Umgebung ohne Chitin, spielt. Es war unser Ziel, den Effekt von ChiA auf die Invasion von menschlichen Wirtszellen durch Salmonellen aufzudecken und des Weiteren die Auswirkungen von Transkriptionsfaktoren und weiteren, im *chiA*-Operon kodierten Proteinen auf die Regulation und Sekretion von ChiA aufzuklären. Wir fanden heraus, dass die Deletion von ChiA zu einer reduzierten Invasion in humane Wirtszellen führt. Diese Ergebnisse waren vergleichbar mit den Daten anderer Studien, die etwa zur gleichen Zeit publiziert wurden. Interessanterweise zeigten unsere Studien, dass die ChiA-vermittelte Invasion speziell bei polarisierten humanen intestinalen Epithelzellen auftritt, wohingegen nicht-polarisierte oder nicht-intestinale Epithelzellen keinen reduzierten Invasionsphänotypen zeigen. Wir fanden heraus, dass sowohl zell-assoziierter als auch freier Mukus zur Expression und Sekretion von ChiA führen. Dieses passt zu den Ergebnissen anderer Studien, die zeigten, dass Wirtszellglykane, die eine ähnliche Struktur wie Mukus haben, Ziel von Chitinasen sind. In unserer Studie konnten wir zudem aufdecken, dass die Expression von ChiA durch einen Transkriptionsfaktor reguliert wird (STM0017). Diesen nannten wir ChiR. Zuletzt konnten wir zeigen, dass ChiA aktiv über ein Typ-10-Sekretionssystem (T10SS) sekretiert wird, welches im *chiA*-Operon kodiert wird. Vorherige Studien zeigten bereits, dass bei Gram-negativen Bakterien die Sekretion von Proteinen durch ein T10SS, durch eine spezielle Peptidoglykan-Hydrolase vermittelt wird. Sie zeigten zudem, dass diese Peptidoglykan-Hydrolasen im Periplasma akkumulieren, nachdem sie die innere Membran mit Hilfe von Holinen passiert haben. Die Peptidoglykan-Hydrolasen spalten die Peptidoglykan-Schicht, wodurch die Sekretion von Porteinen durch die bakterielle Zellwand ermöglicht wird. Die finale Freisetzung durch die äußere Membran ist aktuell noch nicht bekannt und erfordert weitere Untersuchungen. In einer zweiten Studie fokussierten wir uns auf Cytolysin A. Dabei handelt es sich um ein kaum beschriebenes Toxin, für das bekannt ist, dass es nur in human-adaptierten typhoiden *Salmonella* Serovaren wie Typhi und Paratyphi A kodiert ist. Es wurde gezeigt, dass ClyA ein

porenformendes Toxin ist. In *Escherichia coli* ist dessen Homolog bezüglich der Porenbildung und Regulation der Expression gut in der Literatur beschrieben. Anhand von *S. enterica* serovar Paratyphi A, demonstrieren wir in dieser Arbeit, dass ClyA ein hauptsächlich intrazellulär induziertes Toxin ist, das von Bakterien in der SCV exprimiert und sekretiert wird. Da die Expression in der SCV mit der Aktivität des T10SS, welches für die Sekretion des Typhus-Toxins verantwortlich ist, korreliert, verfolgten wir die Hypothese, dass dieses Sekretionssystem auch ClyA sekretiert. Anhand einer Mutante mit Defekt in der TtsA Peptidoglykan-Hydrolase konnten wir zeigen, dass die Sekretion von ClyA in der Mutante ohne dieses T10SS vollständig eliminiert ist. Das bestätigt unsere Hypothese. Nach der Sekretion zeigt ClyA eine zytolytische Aktivität, die spezifisch für makrophagen-ähnliche Zellen, wie THP-1 and U937 Zellen ist, wohingegen eine Aktivität gegenüber Epithelzellen wie HeLa, CaCo-2 und HT29-MTX Zellen fehlt. Aus diesem Grund postulieren wir, dass ClyA während der Infektion hauptsächlich in infizierten Epithelzellen produziert wird. Das könnten den Salmonellen helfen, nach dem Verlassen der Epithelzellen die angezogenen Makrophagen zu lysieren. Diese Annahme, sowie die mechanistischen Details zur Spezifität von ClyA für verschiedene Zelltypen müssen in weiteren Studien untersucht werden.

Zusammenfassend wird in dieser Dissertation, die Expression, Regulation und Sekretion von ChiA und ClyA, zwei wichtigen Virulenzfaktoren von *S. enterica* erforscht. Zudem zeigt diese Arbeit die Relevanz von zwei ähnlichen, aber dennoch unterschiedlichen T10SS, sowie ihren Einfluss auf die Pathogenität von *S. enterica.*

4. Introduction

4.1 *Salmonella enterica* serovars and their emergence as human pathogens

Salmonella enterica (*S. enterica*) is a Gram-negative, rod shaped bacterium that is facultative anaerobe and belongs to the family of *Enterobacteriaceae* (Jajere, 2019). *S. enterica* is divided up into six subspecies, which contain more than 2500 different serovars classified with the White-Kauffmann-Le Minor scheme (Grimont & Weill, 2007). *Salmonella* serovars are classified as non-typhoidal and typhoidal serovars according to the pathology in the human host. Non-typhoidal serovars (NTS) as for example *S. enterica* serovar Typhimurium (*S.* Typhimurium) and serovar Enteritidis (*S.* Enteritidis) have a broad host specificity, whereas typhoidal serovars as for example *S. enterica* serovar Typhi (*S.* Typhi) or Paratyphi A (*S.* Paratyphi A) are specialized to the human host (Gal-Mor *et al*, 2014). NTS are mostly described to cause self-limiting gastroenteritis and diarrhea in healthy and immune-competent people (Feasey *et al*, 2012; Gal-Mor *et al.*, 2014). However, there are also invasive non-typhoidal *Salmonella* (iNTS) infection causing systemic disease, which mainly occur in African countries (Feasey *et al.*, 2012). The different pathogenicity compared with NTS strains are a result of distinct virulence proteins and not clearly understood (Suez *et al*, 2013). Typhoidal *Salmonella* serovars cause enteric fever (typhoid and paratyphoid fever) and systemic infection (Besser, 2018; Bhan *et al*, 2005; Gal-Mor *et al.*, 2014). They are more common in Southeast Asia (Besser, 2018; World Health Organization, 2015)

There are different estimates, how many cases and deaths worldwide per year emerge. The World Health Organization estimated in 2016 180 million of illnesses and 298 thousand deaths annually caused by *S. enterica* infection (World Health Organization, 2015). For gastroenteritis caused by infection with NTS 93.9 million cases and 155 thousand deaths in year 2010 were estimated by Majowicz *et al* (2010). For enteric fever on the other hand are estimated 27 million cases and 200 thousand death per year (Buckle *et al*, 2012; Crump *et al*, 2004). A more recent study published estimations of 14.3 million illnesses and 135.9 thousand deaths in 2017 (GBD 2017 Typhoid and Paratyphoid Collaborators, 2019). The spread of antimicrobial resistances is an emerging threat and complicates the treatment of *S. enterica* infection (El-Sharkawy *et al*, 2017; Gokul *et al*, 2010; Menezes *et al*, 2010; Ramatla *et al*, 2021).

Up to now, only vaccination against typhoid fever is available, whereas vaccination against non-typhoidal serovar infection has not been developed so far. Against typhoid fever Ty21a as oral vaccination and Vi polysaccharide as parenteral vaccination are commonly used. The recommended vaccination according to the World Health Organization is a Vi tetanus toxoid conjugate vaccine (Milligan *et al*, 2018).

The high number of cases shows the importance of studies like ours on *S. enterica* to prevent and fight against *S. enterica* infection and thousands of deaths every year.

4.2 *S. enterica* infection mechanism

The course of infection differs immensely between the serovars Typhimurium and Paratyphi or Typhi. Animal models used to the study the different course of infections are mice or cattle. *S.* Typhimurium leads to systemic disease in mice, comparable to typhoid fever in human, and to enteric infection in cattle, comparable to gastroenteritis in human (Coombes *et al*, 2005; Santos *et al*, 2001; Watson *et al*, 1995). To study enterocolitis in mice, a pre-treatment of the animals with streptomycin is possible. It lowers the colonization with commensal bacteria and facilitates *S.* Typhimurium colonization and in turn development of colitis (Barthel *et al*, 2003).

S. Typhi and *S.* Paratyphi A are human pathogens and not able to colonize commonly used in-bred mice. A recent study shows susceptibility of collaborative cross mouse strains, with a higher genetic variability as in-bred mice, for *S.* Typhi (Alugupalli *et al*, 2023). Some studies were also conducted with humanized mice. Therefore, immunodeficient mice are modified and cells of the human immune system are transplanted. Some of the key virulence factors could be identified using this model (Firoz Mian *et al*, 2011; Pearson *et al*, 2008; Song *et al*, 2010). The host restrictions of typhoidal *S. enterica* make it hard to draw conclusions for the exact infection process for typhoidal *S. enterica* infections of the human host.

4.2.1 Invasion and intracellular survival of typhoidal and non-typhoidal *Salmonella* serovars in the human host

Once *S. enterica* is ingested by contaminated food and water, it passes the stomach and reaches the small intestine. The small intestine and the colon are covered with a mucus layer (Ermund *et al*, 2013; Furter *et al*, 2019; Johansson *et al*, 2008). *S. enterica* enters and transverses the mucus with the aid of motility (Furter *et al.*, 2019). One pathogenicity factor that is expressed and secreted upon contact with intestinal epithelial cells and mucus is the Chitinase A (ChiA). Its expression, secretion and impacts on the pathogenicity of *Salmonella* is described and discussed in chapter 4.2.4.1 and in publication I.

Once it has crossed the protecting mucus layer*, S. enterica* reaches the enterocytic cell layer and adheres to and invades into the intestinal epithelial cells. *S. enterica* can adhere to the intestinal cells by various adhesins like pili and fimbriae (Rehman *et al*, 2019; Wagner & Hensel, 2011). It is reported that *S. enterica* invades preferably in microfold cells (M cells) in the Peyer's patches, but also invades into other enterocytes (Jones *et al*, 1994; Kohbata *et al*, 1986). *S. enterica* secretes effector proteins via a type 3 secretion system which is encoded on *Salmonella* pathogenicity island 1 (SPI1-T3SS). These effectors lead to the rearrangement of the cytoskeleton of the host cells and the formation of membrane ruffles that subsequently leads to the uptake of the bacteria into the host cells (Finlay *et al*, 1991; Francis *et al*, 1993; Garcia-del Portillo & Finlay, 1994; Hayward & Koronakis, 1999).

Once *S. enterica* has invaded the epithelial cells, a specific intracellular membrane compartment matures (Alpuche-Aranda *et al*, 1994). It acquires lysosomal glycoproteins and lysosomal

enzymes but is distinct from compartments arising in the traditional endocytic and phagocytic pathways (Garcia-del Portillo & Finlay, 1995; Steele-Mortimer *et al*, 1999). It is specifically modified to a *Salmonella*-containing vacuole (SCV) by effector proteins translocated via a second T3SS encoded on SPI2 (Hensel *et al*, 1997). The effector proteins (Figueira & Holden, 2012; Haraga *et al*, 2008) have various effects such as maintaining membrane integrity (Beuzon *et al*, 2000), formation of *Salmonella*-induced tubules (Garcia-del Portillo *et al*, 1993; Stein *et al*, 1996), positioning of the SCV (Deiwick *et al*, 2006; Ramsden *et al*, 2007a; Ramsden *et al*, 2007b), and modifications of the host immune response (Gotoh *et al*, 2004; Haraga & Miller, 2003). That contributes to the ability of *S. enterica* to survive and replicate within the SCV. However, *S. enterica* is also able to escape from the SCV into the host cell cytoplasm and to strongly replicate there (Knodler, 2015). This phenotype has been observed for paratyphoidal and non-typhoidal subpopulations in epithelial host cells (Brumell *et al*, 2002; Cohen *et al*, 2022; Knodler *et al*, 2014), but not in macrophages or fibroblasts (Beuzon *et al*, 2002). The bacteria might destabilize the SCV membrane by translocation of effector proteins (Beuzon *et al.*, 2000; Chandrasekhar *et al*, 2023), however, a T3SS and flagella independent factor has also been discussed (Knodler *et al.*, 2014).

The intravacuolar conditions lead to induction of virulence genes via different two-component systems such as the PhoP/Q regulon (Alpuche Aranda *et al*, 1992; Deiwick *et al*, 1999). The PhoP/Q system is one of the core virulence regulators and influences expression of many genes which were acquired by horizontal gene transfer (Groisman, 2001; Ilyas *et al*, 2017). Some typhoidal specific genes (CdtB, ClyA and STY1499) are also regulated by PhoP/Q (Charles *et al*, 2009; Fowler & Galan, 2018). The expression, regulation and secretion of ClyA (also known as HlyE) will be described in detail in chapter 4.2.4.2 and publication II.

By transcytosis through intestinal epithelial cells or M cells, *S. enterica* can reach the lamina propria (Muller *et al*, 2012). On the one hand *S. enterica* can be carried to the lamina propria by dendritic cells (Chieppa *et al*, 2006), but on the other hand the bacteria can disrupt tight junctions which leads to intercellular bacterial passage (Kohler *et al*, 2007). In the lamina propria *S. enterica* is phagocytosed by lamina propria phagocytes (Hapfelmeier *et al*, 2005; Muller *et al.*, 2012).

The interaction of *S. enterica* with phagocytes was studied in detail, however, the differences between the *Salmonella* serovars and the different types of macrophages make it hard to derive the situation in the human host. Within macrophages, a SCV is formed and SPI2-T3SS effector proteins are translocated (Alpuche-Aranda *et al.*, 1994; Cirillo *et al*, 1998; Hensel *et al*, 1998) that results in *S. enterica* ability to replicate within infected macrophages. In human macrophages, *S.* Paratyphi and *S.* Typhi can replicate better than *S.* Typhimurium and *S.* Enteritidis. That might enable the bacteria to survive and to spread from the primary intestinal infection site to a secondary infection site within the human body (Schwan *et al*, 2000). However, most of the studies were conducted with *S.* Typhimurium. The role of SPI2-T3SS effector proteins for survival and replication of typhoidal *Salmonella* serovars within human phagocytes were recently discussed due to inconsistent data (Thurston & Holden, 2023). Whereas some data show that SPI2-T3SS effector proteins have an impact on proliferation of typhoidal *Salmonella* in

macrophages (Hamblin *et al*, 2023; Khan *et al*, 2003; Stratford *et al*, 2005) other studies do not confirm these results (Forest *et al*, 2010; Reuter *et al*, 2021; Sabbagh *et al*, 2012). Further studies are therefore needed to unravel additional unknown processes (Thurston & Holden, 2023). *S. enterica* is also capable to kill macrophages by inducing cell death through different complex pathways dependent (Fink & Cookson, 2007; Hersh *et al*, 1999; Lin *et al*, 2020; Lundberg *et al*, 1999) and independent (Rolli *et al*, 2010; van der Velden *et al*, 2000) of the T3SS. Hueffer & Galan (2004) reviewed some of the pathways and described them as a balanced interplay between *S. enterica* and host cells and a balance between survival and pro-apoptotic pathways (Hueffer & Galan, 2004). The bacteria are transported by dendritic cells to the mesenteric lymph nodes (MLN); however, they can also travel autonomously with the lymph (Bravo-Blas *et al*, 2019; Voedisch *et al*, 2009).

4.2.2 *Salmonella enterica* serovar Typhimurium leads to self-limiting gastroenteritis in the human host

Systemic spread of *S.* Typhimurium can be prevented by the MLN and the lymphoid tissue in the gut (Voedisch *et al.*, 2009). Thus, *S.* Typhimurium infection is limited to the intestine. The effector proteins translocated by *S.* Typhimurium lead to induction of fluid secretion (diarrhea) by alteration of the chloride efflux (Galyov *et al*, 1997; Zhang *et al*, 2002), disruption of tight junctions (Boyle *et al*, 2006; Jepson *et al*, 1995), transmigration of PMN (polymorphonuclear leukocytes) across the intestinal epithelial layer (Criss *et al*, 2001; Lee *et al*, 2000; McCormick *et al*, 1993) and extrusion of infected epithelial cells (Wallis *et al*, 1986). Furthermore, inflammation is mediated by host factors as a response to bacterial invasion in general (Eckmann *et al*, 1993; Jung *et al*, 1995). Thus, *S.* Typhimurium is fought against by the immune system and self-limiting in immune competent people. In immune compromised humans *S.* Typhimurium infection can lead to systemic disease comparable to systemic spread of *S.* Typhi.

4.2.3 Establishment of systemic infection with typhoidal serovars

Typhoidal *S. enterica* can infect neutrophils and macrophages and are transported to the liver and spleens, where they reside and survive within macrophages and neutrophils (Geddes *et al*, 2007; Richter-Dahlfors *et al*, 1997; Salcedo *et al*, 2001). However, *S. enterica* can also be directly transported from the gut to the bloodstream by CD18 positive phagocytes, which also affects systemic spread to the liver and spleen (Vazquez-Torres *et al*, 1999). From the liver, *S. enterica* is able to colonize the gall bladder, where they can proliferate inside cells or extracellularly, leading to a local inflammation (Menendez *et al*, 2009). Possible gallbladder stones are a substrate for biofilm formation and a cause for chronical carriage by protection of the bacteria from high bile concentrations and antibiotic treatment. Here, *S. enterica* can persist asymptomatically (Crawford *et al*, 2010; Gonzalez-Escobedo & Gunn, 2013; Prouty *et al*, 2002). Within macrophages, epithelial cells and biofilms *S. enterica* is able to form persister cells, which might also be a strategy for persistent infection (Helaine *et al*, 2014; Luk *et al*, 2021). During

chronic carrier state, *S. enterica* can be shed for years by stool or urine of infected people (Crump, 2019; Hoffman *et al*, 2023).

Thus, there are huge differences in the symptoms of disease after infection with typhoidal and non-typhoidal serovars, which might be a result of typhoid specific genes.

4.2.3.1 The role of typhoidal specific proteins

The role of typhoid specific proteins for the establishment of systemic disease in the human host is discussed in many publications. *S.* Typhimurium and *S.* Typhi share 89% of the genes, 600 genes are unique to *S.* Typhi and about 5% of the genes in *S.* Typhi are pseudogenes. The most of them are intact in *S.* Typhimurium (McClelland *et al*, 2001; Parkhill *et al*, 2001; Sabbagh *et al*, 2010).

There are differences in the presence of *Salmonella* pathogenicity islands (SPIs) between the two serovars. SPI7, 15, 17 and 18 are specific for *S.* Typhi, however, differences in genes can also be found in shared SPIs. For example, some of the well-studied SPI1-T3SS and SPI2-T3SS effectors are missing in *S.* Typhi (Sabbagh *et al.*, 2010). In contrast, *S.* Typhi harbors some additional virulence factors:

On SPI7 the Vi-capsule is encoded (Hashimoto *et al*, 1993). The capsule consists of α-1,4-linked N-acetylgalactosaminuronate (Szu & Bystricky, 2003). It has different functions and leads for example to a higher resistance against the host immune system and to a suppressed inflammatory response in the host (Haneda *et al*, 2009; Hirose *et al*, 1997; Raffatellu *et al*, 2005).

A further typhoid specific virulence factor is the typhoid toxin. It is a A_2B_5 toxin and consists of the subunits CdtB, PltA and PltB (Song *et al*, 2013). The subunit CdtB has a cell-distending impact on target cells and arrests the cell cycle (Haghjoo & Galan, 2004; Lara-Tejero & Galan, 2000, 2002). Whereas in mice an effect of the purified toxin on the general health status could be observed (weight loss, lethargic, death) (Song *et al.*, 2013), a human challenge experiment indicated no direct impact of typhoid toxin on the infection rate and early typhoid fever symptoms in typhi infected volunteers (Gibani *et al*, 2019). The typhoid toxin secretion depends on a T10SS, that will be described in detail in chapter 4.3.1.1.

In addition to these well studied virulence factors, the barely described SPI18 encodes for two typhoidal specific proteins, the invasion TaiA and Cytolysin A (ClyA, also known as HlyE) (Faucher *et al*, 2009; Oscarsson *et al*, 2002), which will be described in chapter 4.2.4.2 and publication II.

There are many serovar specific differences and a lot of specific genes encoding for proteins that functions are completely unknown. Here we focused on two virulence factors of *S. enterica.* One that is well conserved in both non-typhoidal and typhoidal serovars (ChiA) and one that is specific for typhoidal Salmonella serovars (ClyA).

4.2.4 Relevant but barely described pathogenicity factors of *S. enterica*

4.2.4.1 Chitinase A, an important pathogenicity factor

Chitinases are glycoside hydrolases and belong to the glycoside hydrolase family 18 and 19. They cleave chitin that consists of β 1,4-N-acetylglucosamine (GlcNAc) and can be found in the cell wall of fungi and shells of arthropods and crustacea. Some of the chitinases work in cooperation with chitin-binding-proteins, which have carbohydrate-binding modules and help to cleave the chitin (Frederiksen *et al*, 2013). In environmental bacteria such as *Vibrio* spp., chitinases are important for utilization of chitin as a carbon source (Keyhani & Roseman, 1999). But also other pathogenic bacteria, as for example *Legionella pneumophila* (*L. pneumophila*), *Listeria monocytogenes* (*L. monocytogenes*), *Pseudomonas aeruginosa*, *Serratia marcescens* (*S. marcescens*), *Escherichia coli* (*E. coli)* or *Enterococcus faecalis* encode for chitinases (Frederiksen *et al.*, 2013). Mutation of the chitinases in *L. pneumophila* and *L. monocytogenes* for example lead to a reduced recovery from mice organs (Chaudhuri *et al*, 2010; DebRoy *et al*, 2006) whereas a chitinase mutant in *E. coli* shows a reduced adhesion to epithelial cells (Tran *et al*, 2011).

In *S.* Typhimurium, two chitinases are known and have been described. The chitinase A (STM0018, ChiA) and the chitinase STM0233 (Arabyan *et al*, 2017; Devlin *et al*, 2022). Furthermore, a putative chitinase and a prophage chitinase has been reported but not further elucidated (Arabyan *et al.*, 2017). ChiA is also conserved and expressed in other *Salmonella* serovars such as the strictly human adapted Typhi serovar (Faucher *et al*, 2006). ChiA in principle is a chitinolytic active enzyme capable to cleave chitin substrates (Larsen *et al*, 2011). However, during microarray studies it was found that *chiA* is also expressed by the bacteria within eukaryotic cells during the course of infection (Eriksson *et al*, 2003; Harvey *et al*, 2011; Hautefort *et al*, 2008; Wright *et al*, 2009).

4.2.4.2 Cytolysin A, an intracellularly secreted toxin of typhoidal *Salmonella* serovars

The intravacuolar conditions lead to induction of virulence genes via different two-component systems such as the PhoP/Q regulon (Alpuche Aranda *et al.*, 1992; Deiwick *et al.*, 1999). One toxin in typhoidal *Salmonella* serovars is cytolysin A (ClyA). It is encoded on SPI18 together with the putative invasin TaiA (Typhi associated invasin A). Non-typhoidal serovars such as Typhimurium or Enteritidis do not encode for ClyA (Oscarsson *et al.*, 2002; von Rhein *et al*, 2009). *E. coli* possesses a homologue, also known as SheA or HlyE with 91% amino acid identity to *S.* Typhi (Oscarsson *et al.*, 2002). ClyA is 34 kDa pore-forming toxin characterized in *E. coli* as a hemolysin (del Castillo *et al*, 1997). For the *E. coli* ClyA, structural analyses, the assembly of the pore as dodecamer and the membrane embedding were studied in detail (Benke *et al*, 2015; Fahie *et al*, 2013; Mueller *et al*, 2009; Peng *et al*, 2019; Roderer & Glockshuber, 2017).

In *S. enterica,* ClyA expression is mainly regulated under intracellular conditions (Murase, 2022) via *crp* and *rpoS* (Faucher *et al.*, 2009; Fuentes *et al*, 2009; Jofre *et al*, 2014), *phoP* (Faucher *et al.*, 2009; Jofre *et al.*, 2014), *fis* (DNA-binding protein) (Jofre *et al.*, 2014), *slyA* (Lithgow *et al*, 2007; Ludwig *et al*, 1999; Oscarsson *et al*, 1996; Wyborn *et al*, 2004). It is known that there is in vivo relevance for ClyA during human infection. ClyA antibodies can be detected within antisera after typhoid and paratyphoid infection (von Rhein *et al*, 2006). In immunoaffinity-based mass spectrometric analyses, ClyA was also identified as immunoreactive protein of *S.* Typhi (Safi *et al*, 2023). Only little was known about the in vivo function of ClyA in typhoidal *S. enterica* during infection and how it is secreted. A putative effect on pathogenicity of S. *enterica*; the invasion and intracellular survival ability in different host cells was tested before, but the results were conflicting (Faucher *et al.*, 2009; Fuentes *et al*, 2008). However, there was some evidence that not only *E. coli* ClyA, but also heterologously expressed *S. enterica* ClyA has a cell lysis effect (Oscarsson *et al.*, 2002; von Rhein *et al.*, 2009; Wai *et al*, 2003).

The different virulence proteins are secreted by various secretions systems in *S. enterica.*

4.3 Secretion systems in Gram-negative bacteria

Many bacterial virulence factors are secreted by dedicated secretion systems. Gram-negative bacteria possess three different barriers: the inner membrane, the outer membrane and in between the peptidoglycan layer. Depending on the target and fate of the substrate, which can be associated with the bacterial outer membrane, released into the extracellular space or injected into eukaryotic or bacterial cells, secretion machineries can span the inner and outer membrane or only the outer membrane (Costa *et al*, 2015; Gerlach & Hensel, 2007). The substrates are secreted either in a one-step or two-step secretion mechanism (Rego *et al*, 2010) overcoming the different barriers of the Gram-negative cell wall.

4.3.1 Secretion systems in *S. enterica*

To overcome the inner membrane of the bacterial cell wall in a two-step mechanism, proteins can be transported via the general secretion pathway (Sec pathway). The Sec translocon mediates the secretion of unfolded proteins and is furthermore involved in the insertion of membrane proteins into the cytoplasmic membrane. The Sec translocon consists mainly of protein targeting components as for example the chaperone SecB, a motor protein (SecA) and a membrane integrated protein conducting channel (SecY, SecE and SecG). Further proteins interact with the translocating proteins as for example YidC and SecDF. Proteins secreted by the Sec system can either be translocated co-translational or post-translational. The proteins are targeted to the Sec translocon by N-terminal signal sequences (signal peptides). They are typically 20 amino acids long, have a tripartite structure and a cleavage site for the signal peptidase which cleaves the signal sequence during or after translocation (Natale *et al*, 2008). Via the twin-arginine translocation machinery (Tat machinery) folded proteins or cofactor containing proteins are transported across the cytoplasmic membrane. The TAT translocon consists of two or three

membrane spanning subunits (TatA and TatC or TatA, TatB and TatC) and is driven by the proton-motive-force. Proteins transported by the Tat machinery also possess a signal sequence with a similar structure as for the Sec signal sequence but containing a twin-arginine motif pattern (Natale *et al.*, 2008).

To overcome the peptidoglycan and the outer membrane, *S. enterica* has various secretion systems. *S. enterica* possesses type 1 secretion systems (T1SS), type 3 secretion systems (T3SS), type 4 secretion systems (T4SS) and type 6 secretion system (T6SS) (Bao *et al*, 2020). Recently, a type 10 secretion system was discovered in *S.* Typhi (Geiger *et al*, 2018; Palmer *et al*, 2021).

4.3.1.1 Type 10 secretion system (T10SS)

In recent studies the T10SS, has been discovered. It is not a multimeric protein complex and was initially described in *S.* Typhi where it is responsible for the secretion of intravacuolar expressed typhoid toxin (Geiger *et al*, 2020; Geiger *et al.*, 2018; Hodak & Galan, 2013). In *S. marcescens,* a similar secretion system has been described, responsible for the secretion of a chitinase (Hamilton *et al*, 2014). This novel secretion system was later named as type 10 secretion system (T10SS) (Hamilton *et al*, 2014; Palmer *et al.*, 2021).

The typhoid toxin secreting T10SS in *S.* Typhi (Figure 1) consists of a holin, a peptidoglycan hydrolase (muramidase) TtsA and a LD-transpeptidase YcbB (Geiger *et al.*, 2020; Geiger *et al.*, 2018; Hodak & Galan, 2013). Holin and peptidoglycan hydrolases are bacteriophage proteins that are important for the phage release during the lytic cycle of bacteriophage infection (Wang *et al*, 2000; Young *et al*, 2000). The detailed mechanism of typhoid toxin secretion by the T10SS has been described by Geiger *et al.* (2018). The LD-transpeptidase is transported to the periplasm through the Sec machinery. It modifies the peptidoglycan at the bacterial poles and changes canonical 4-3 (DD) crosslinks of the peptidoglycan layer into non-canonical 3-3 (LD) crosslinks. The peptidoglycan hydrolase TtsA reaches the peptidoglycan through a holin and specifically cleaves the 3-3 (LD) crosslinks in the modified peptidoglycan. The assembled typhoid toxin, which consists of the subunits CdtB, PltA and PltB is transported to the periplasm through the Sec-machinery. It first accumulates on the inner cis side of the periplasm at the bacterial poles. Once the peptidoglycan layer is cleaved by TtsA it can cross the layer to the outer trans side of the periplasm beneath the outer membrane. The final release of the toxin through the outer membrane layer is currently unknown (Geiger *et al.,* 2018). But it could be shown that sub-inhibitory concentrations of antimicrobial peptides or bile salts can trigger this final release substantially (Geiger *et al.*, 2018; Hodak & Galan, 2013). In an additional study the final release via outer membrane vesicles (OMVs) has been discussed (Guidi *et al*, 2013). Due to its locally restricted and 3-3 (LD) crosslinks specific activity, TtsA does not lyse bacteria during the secretion process. Therefore, typhoid toxin secretion by the T10SS is an active non-lytic secretion mechanism (Geiger *et al.*, 2018). The composition of additional T10SS in other bacteria will be discussed in chapter 8.3.1.

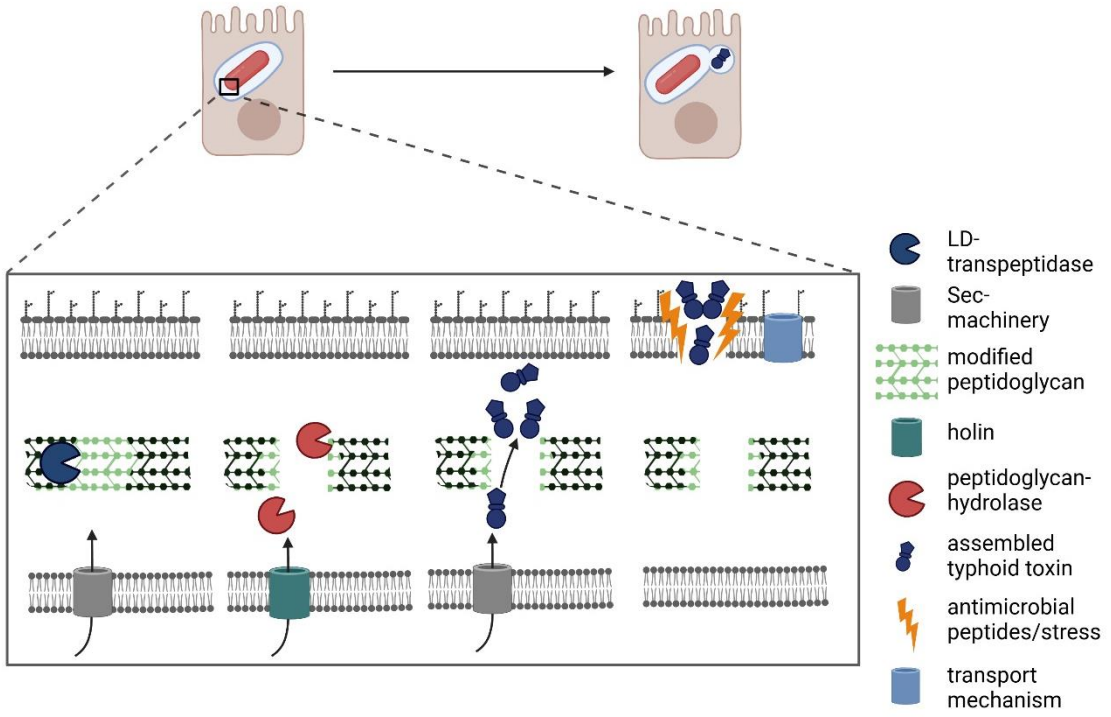

Figure 1: A T10SS is responsible for the secretion of the typhoid toxin in *S.* Typhi. Detailed description in the text**.** Created with BioRender.com

5. Hypothesis and aims of this work

In *S. enterica* much is known about the well-described T3SS and its secreted effector proteins and adhesion factors. But in addition, there are much more virulence factors secreted by other secretion systems using different secretion mechanisms.

Recent studies focused on T10SS and their cargo proteins in *S. enterica* (Geiger *et al.*, 2020; Geiger *et al.*, 2018; Hodak & Galan, 2013). It has been elucidated before, that T10SS are often encoded within an operon together with its cargo protein. Within the chitinase operon a peptidoglycan-hydrolase and a holin have been found (Hodak & Galan, 2013). So far, it has not been analyzed whether the chitinase is secreted depending on the present holin and peptidoglycan-hydrolase and how gene expression within the *chiA* operon is regulated. Thus, the aims were to investigation these open questions.

From the literature, it was known, that ChiA is expressed within different host cells (Eriksson *et al.*, 2003; Harvey *et al.*, 2011; Hautefort *et al.*, 2008; Wright *et al.*, 2009). Starting from the chitin cleavage activity of ChiA and the substrate similarities of chitin compared to β1-4 glycosidic bonds of glycosylated proteins and lipids in host cells (Frederiksen *et al.*, 2013) and mucus (Bansil & Turner, 2018), it was assumed that these host cell structures can induce ChiA expression upon contact with host cells and lead to increased invasion. The aim of this work was therefore to elucidate the induction of ChiA upon contact with various host cells and potentials impacts on ChiA-depending invasion rates in target cells.

The second study aimed to find additional cargo proteins secreted by the T10SS in typhoidal *Salmonella* serovars. This secretion system was initially investigated in studies exploring the secretion of typhoid toxin in *S.* Typhi (Geiger *et al.*, 2020; Geiger *et al.*, 2018; Hodak & Galan, 2013). The hypothesis was, that additional proteins which are expressed under similar conditions, are secreted via this T10SS. The focus was on ClyA that is regulated by PhoP under the same intracellular conditions as typhoid toxin (Faucher *et al.*, 2009; Jofre *et al.*, 2014).

A further aim of this study was to explore the impact of ClyA on the pathogenicity of typhoidal *S. enterica* serovars. Activity details were only known for a ClyA homologue in *E. coli* capable to induce pores in host cell membranes (Benke *et al.*, 2015; Ludwig *et al.*, 1999; Peng *et al.*, 2019). The specific aim here was to elucidate the cytolytic activities of paratyphoidal ClyA on various human target cells and, consequently, its contribution to the high virulence of typhoidal *S. enterica* serovars in the human host.

6. Publication I

Krone, L., Faass, L., Hauke, M., Josenhans, C., and Geiger, T. (2023). Chitinase A, a tightly regulated virulence factor of *Salmonella enterica* serovar Typhimurium, is actively secreted by a Type 10 Secretion System. PLoS Pathog 19, e1011306. https://doi.org/10.1371/journal.ppat.1011306

6.1 Contributions to Publication I

In this work, I was involved in the process of conceptualization. Together with T. Geiger, the experimental setup was discussed and developed. Under review of literature, we developed and discussed hypothesizes together, I developed a workflow and verified the hypothesis with the different experiments. With exception of RNA extraction and RT-qPCR I established all the methods for our laboratory. The methodology RNA extraction and RT-qPCR were established by the co-authors C. Josenhans, M. Hauke and L. Faass, who also helped me to perform this experiment. I evaluated all the data, validated them and finally visualized them for the publication. I wrote a first draft of the methodology paragraph and was involved in review and editing the draft, written by T. Geiger.

7. Publication II

Krone, L., Mahankali, S. and Geiger, T. Cytolysin A is an intracellularly induced and secreted cytotoxin of typhoidal *Salmonella*. *Nat Commun* 15, 8414 (2024). https://doi.org/10.1038/s41467-024-52745-0

7.1 Contributions to Publication II

In this work, I was involved in the process of conceptualization. Together with T. Geiger, the experimental setup was discussed and developed. Under review of literature, we evolved and discussed hypothesizes together and proofed them with the different experiments. I established methods for our laboratory, evaluated half of the data, validated them and finally visualized them for the publication. S. Mahankali was involved in Western blot analyses, LDH release assays and CFU invasion assays for revision of the manuscript. I wrote a first draft of the methodology and results paragraph, contributed to the discussion by review of literature and was involved in editing the final draft of T. Geiger.

8. Discussion

Pathogen-host cell interaction is a complex interplay. In our studies, we could get new insights into the induction and secretion of important virulence factors of *S. enterica* in response to different human cell types. The two factors investigated in the present thesis, ChiA and ClyA, are very different pathogenicity factors, which are important at different stages of *S enterica* infection.

8.1 ChiA is an extracellularly expressed virulence factor of *S. enterica,* specifically induced by polarized intestinal epithelial cells and mucus

In the human body, different tissues contain different layers of various cell types. In the small intestine the majority of cells are absorptive enterocytes that form the intestinal barrier. But there are also additional, more specialized cells, such as secreting goblet and Paneth cell or M cells that are able to transport luminal agents through the epithelial layer (Peterson & Artis, 2014). In our study (Publication I) we tested ChiA induction in a cell culture model upon contact to CaCo-2 cells and HT29-MTX cells. CaCo-2 cells are human epithelial colorectal adenocarcinoma cells comparable to absorptive enterocytes, but slightly heterogeneous (Lea, 2015; Rousset, 1986), whereas HT29-MTX cells are homogenous mucus producing goblet cells (Lesuffleur *et al*, 1990). Due to their heterogeneity CaCo-2 cells can also express mucins and a thin mucus layer could be found on this cell type (Navabi *et al*, 2013; van Klinken *et al*, 1996). The expression of the virulence factor ChiA is induced upon contact to polarized epithelial cells, but not in contact to non-polarized intestinal epithelial cells or HeLa cells. We could also show that addition of a crude mucus extract is also sufficient to induce ChiA production. It remained open, if mucus is the only stimulus for ChiA expression or if further changes during cell polarization have a similar or amplifying effect.

During polarization of CaCo-2 cells to enterocytes cellular structures change enormously. Besides formation of an apical brush border which consists of microvilli, the formation of tight junctions between neighboring cells and expression of enzymes that are characteristic for enterocytes (Engle *et al*, 1998) change, as well as the glycome (Link-Lenczowski *et al*, 2019; Park *et al*, 2015). It was previously shown that sialyation and fucosylation are upregulated during CaCo-2 cell polarization (Link-Lenczowski *et al.*, 2019). Furthermore, an increase in GlcNAc containing oligosaccharides could be detected (Park *et al.*, 2015).

It has not been studied if special glycans induce ChiA expression and secretion. But, interestingly, in recent studies about ChiA, it was shown, that *S.* Typhimurium interacts with the host cell glycome with the aid of ChiA by an alteration of Lewis X/A-containing glycans (Devlin *et al.*, 2022) and removal of sialic acid residues from host cells (Chandra *et al*, 2022). They finally showed, that the modification of the host cell glycome enables the bacteria to invade more efficiently into intestinal epithelial cells by binding with fimbriae to the altered structures (Chandra *et al.*, 2022).

Intriguingly, mucus mainly consists of various mucins, glycoproteins that are bound to variable, O-linked glycans (Johansson *et al.*, 2008) which supports the idea, that special glycans induce ChiA expression and secretion. The expression of the different mucins varies during differentiation of intestinal epithelial cells (van Klinken *et al.*, 1996) which might influence differences in ChiA expression upon contact to polarized and non-polarized cells and in turn the infection process.

Concluding from our results and the different other studies ChiA is induced upon contact with cellular structures, which are remodeled during polarization of intestinal epithelial cells. It could be assumed that ChiA expression is induced by both the glycome modification and the secreted mucus of intestinal epithelial cells. However, we and others could not prove, if a special glycan structure, cleaved carbohydrates, amino acids or whole proteins are sensed and subsequently induce ChiA expression. In additional experiments we did not see induction of ChiA expression by the addition of N-acetylglucosamine (GlcNAc) (data not shown), which is the smallest component of chitin (Frederiksen *et al.*, 2013). In future studies it would be interesting to investigate the exact ChiA inducing signal and how it can be sensed. That would also enable us to have a deeper understanding of the importance of ChiA for the pathogenicity of *S. enterica.*

8.1.1 ChiA is important for the pathogenicity of *S. enterica*

In our study on ChiA (Publication I), we used various cell culture models and focused on the interaction with polarized intestinal epithelial cell lines. The cell culture models enabled us to get insight in the expression and regulation of ChiA and to find out about the relevance for invasion into these cells. We could clearly show that a mutant with a defect in *chiA* is impaired in invasion into polarized intestinal epithelial cells. These data are complementary to recently published data of Devlin *et al.* (2022) and Chandra *et al.* (2022) who showed similar results for the effect on invasion into different cell lines.

Besides cell culture experiments they further focused on the in vivo relevance of ChiA for *S.* Typhimurium infection and proved it with a mouse infection model. It was shown that ChiA and a second chitinase STM0233 are well expressed during mouse infection (Devlin *et al.*, 2022). Furthermore, a reduced invasion into intestinal epithelial cells and a delayed systemic spread of a ChiA mutant has been observed (Devlin *et al.*, 2022). That had a negative impact on the amount of recovered bacteria from the liver, spleen and mesenteric lymph nodes, resulting in increased survival rates of infected mice and altered humoral and adapted immune responses (Chandra *et al.*, 2022; Devlin *et al.*, 2022). The multiple effects of ChiA at different stages of the infection might base on the more complex in vivo situation. Mucus for example is also present in the stomach (Phillipson *et al*, 2008), and the host glycome is remodeled by the microbiome (Bry *et al*, 1996; Goto *et al*, 2014), which might influence ChiA expression and secretion. Besides that, other cell types with glycosylated surface proteins might also be modified by ChiA with an effect on later infection stages and the systemic spread. However, this hypothesis and the synergetic effects with further pathogenicity factors have to be investigated in continuative studies.

8.2 ClyA is an intracellularly expressed virulence factor of typhoidal *S. enterica*

The second factor I studied in this thesis was the cytolysin A (ClyA) of typhoidal *Salmonella* (Publication II). After invasion into the intestinal epithelial cells a SCV is formed and within the SCV the PhoP/Q regulon is activated (Alpuche-Aranda *et al.*, 1994; Alpuche Aranda *et al.*, 1992; Deiwick *et al.*, 1999). This does not only lead to expression and secretion of SPI2-T3SS effector proteins (Figueira & Holden, 2012; Haraga *et al.*, 2008; Hensel *et al.*, 1997), but also to the induction of ClyA which has been also confirmed in our present studies.

8.2.1 ClyA expression and secretion is induced by intravacuolar conditions

We could show by fluorescence microscopy, that ClyA is mainly expressed by *S.* Paratyphi A within the SCV. In this regard, *S.* Paratyphi A differs immensely from two *S.* Typhi strains, which were described to express ClyA under extracellular conditions. On blood agar plates, a ClyA-dependent hemolytic phenotype was shown for *S.* Typhi strain STH2379 (Oscarsson *et al.*, 2002) as well as the attenuated vaccination strain Ty21a (Fuentes *et al.*, 2008). Both strains have in common that they are RpoS-deficient (Kopecko *et al*, 2009; Valenzuela *et al*, 2014). Notably, RpoS has been shown to be important for the upregulation of ClyA (Fuentes *et al.*, 2009; Jofre *et al.*, 2014). This is contradicting to the phenotypes observed by us. However, an introduction of RpoS into the mutated strain *S.* Typhi Ty21a did not show an abolished hemolytic phenotype in previous studies (Oscarsson *et al.*, 2002). Thus, it is not known why these strains express and secrete ClyA under extracellular conditions in large amounts. However, we could see expression of *clyA* mainly in bacteria within the SCV in HeLa epithelial cells and macrophage-like THP-1 cells and not in bacteria within the cytosol. Comparative whole genome sequencing analysis of these two strains and different *S.* Paratyphi A strains as well as quantitative analysis of ClyA expression under different conditions may shed more light on the various regulation mechanisms of ClyA.

8.2.2 ClyA is a pore-forming toxin and kills specifically macrophage-like cells such as THP-1 and U937.

ClyA is strongly induced under intravacuolar conditions, which indicates a lack of impact on *S.* Paratyphi A invasion. Indeed, we performed gentamicin protection assays and could not detect an effect of ClyA on invasion of *S.* Paratyphi A in human cell lines as for example THP-1 cells, HeLa cells, CaCo-2 and HT-29 MTX cells. However, we could see a statistically significant effect of ClyA on invasion into PBMCs isolated from human blood. We further found that ClyA has no impact on the intracellular survival of S. Paratyphi A nor its escape from the SCV to the cytosol. We hypothesize that the difference in ClyA's effect on the invasion of *S.* Paratyphi A into stable cell lines versus primary PBMCs is due to a low basal expression of ClyA outside of cells. This expression is below the detection limit of Western blot analyses and fluorescence microscopy but

is sufficient to induce cell lysis in highly sensitive, isolated primary PBMCs and/or assist the bacteria in invading these primary immune cells. However, more in-depth studies are necessary to investigate this phenomenon and confirm the hypothesis.

From the pore-forming characteristic of ClyA shown in previous studies (Mueller *et al.*, 2009; Roderer & Glockshuber, 2017), we rather concluded an impact of ClyA upon contact with different cell types during the process of *S.* Paratyphi A infection. To elucidate the role of ClyA during the infection process, we analyzed the toxicity of ClyA towards different human cell types. Interestingly, cell lines differed substantially in their susceptibility to ClyA.

We observed a cell killing effect of ClyA only on macrophage-like cell lines such as THP-1 and U937 cells, while ClyA had no effect on epithelial HeLa cells and intestinal epithelial CaCo-2 cells in a polarized or non-polarized state (Publication II). Furthermore, no effect on mucus producing HT29-MTX cells could be observed. These results are different from the outcome of a *E. coli* ClyA study, where also epithelial cells can be killed by *E. coli* ClyA (Wai *et al.*, 2003). It has to be considered that the amount of ClyA is not determined in our study nor in the work of Wai *et al.* (2003) It has to be investigated in further studies and by quantitative comparative analysis, why there are differences in the specificity for different cell types. Interestingly, we could show that *S.* Paratyphi A ClyA is produced in macrophage-like THP-1 cells and epithelial HeLa cells. That leads to the question, why only the macrophage like cells were susceptible to *S.* Paratyphi A ClyA induced lysis in our work.

The cholesterol-binding feature of ClyA (Oscarsson *et al*, 1999) and stabilization of the ClyA pore by cholesterol (Sathyanarayana *et al*, 2018) explains, why bacterial cells are not susceptible to the cytotoxic effect of ClyA. It might be possible that different membrane compositions, as for example the amount of the sterols in different eukaryotic host cell types, also affect susceptibility to ClyA by influencing ClyA insertion or membrane integrity.

The complex reasons for the differences in cytotoxic effect on the cells could be elucidated with analysis of the membrane composition using various cell types at different states of differentiation. It might give a hint, if the membrane composition could be correlated with the susceptibility to ClyA. It has to be considered, that the lipidome is not stable in the different cell types. The cholesterol content for example is changed during polarization of epithelial cells (Sampaio *et al*, 2011) and during differentiation of THP-1 monocytes to macrophages (Gaus *et al*, 2005). Furthermore, bacterial infection affects the cholesterol mechanism as it is for example known for different intracellular pathogens (Samanta *et al*, 2017) which also might change ClyA susceptibility.

Various other receptors in eukaryotic membranes, such as different lipids, glycosylphosphatidylinositol-anchored proteins (Diep *et al*, 1998; Hong *et al*, 2002), other glycosylated proteins and membrane proteins in general are also reported as receptors for other pore-forming toxins (Dal Peraro & van der Goot, 2016; Los *et al*, 2013). Reasons for differences in sensitivity for ClyA could be unraveled by screens for interaction partners of ClyA, besides

cholesterol. To find out about the binding to specific receptors would maybe enable us to understand the details of the effect of ClyA on the target cells.

8.2.3 The mechanism how ClyA kills macrophage-like cells is evasive and has to be elucidated in further studies

Our (Publication II) and other former studies could not clearly answer the question by which mechanisms ClyA kills cells. Whereas erythrocytes are lysed through osmotic lysis after pore formation, the cell death of nucleated cells is more complex (Aroian & van der Goot, 2007). Different pore-forming toxins may lead to cell death via different pathways, as for example apoptosis (Bantel *et al*, 2001; Genestier *et al*, 2005; Haslinger *et al*, 2003), necrosis (Craven *et al*, 2009; Warny & Kelly, 1999) or pyroptosis (Fink & Cookson, 2006), which depends on the toxin, the concentration of the toxin (Walev *et al*, 1993) and the target cell type. Pore-forming toxins for example may lead to the influx of calcium ions or the efflux of potassium ions, which can have different effects on the nucleated cells. Both can trigger apoptosis besides other effects on the cells (Bortner *et al*, 1997; Giorgi *et al*, 2012).

Two previous studies about *E. coli* ClyA deal with this question. It was reported for purified *E. coli* ClyA that the intracellular calcium concentration in primary epithelial cells of rats is changing upon contact to ClyA, which leads to cell death (Wai *et al.*, 2003). It was elucidated for ClyA in a different study that sublytic concentrations of ClyA lead to oscillation of calcium ions with an effect on the intracellular signaling and expression of proinflammatory cytokines (Soderblom *et al*, 2005). If the effect of *S.* Paratyphi A ClyA might be similar has to be studied.

Interestingly, it was reported for other pore-forming toxins that they could have different cell killing mechanisms in contact to different cell types. It was for example reported for the pore-forming α-hemolysin of *Staphylococcus aureus* that this is able to induce pyroptosis in macrophages, mediated by caspase-1 (Craven *et al.*, 2009; Fernandes-Alnemri *et al*, 2007), whereas it leads to caspase-mediated apoptosis in lymphocytes (Bantel *et al.*, 2001) and caspase-2 dependent apoptosis in epithelial cells (Imre *et al*, 2012). That shows the complexity of the effects of pore-forming toxins. The difference in susceptibility for ClyA might be a further example for multiple effects of a pore-forming toxin on different cell types. Comprehensive studies with various cell types in different states and distinct toxin concentrations might provide additional information.

A further aspect which must be considered is that differences in response to pore-forming toxins can be explained by different abilities to cope with pores in the plasma membrane. It is reported that cells evolved repair mechanisms, depending on the toxin concentration, the pore size and the duration of the toxin exposure (Bischofberger *et al*, 2009). In dependence on Calcium ion influx or also independently from that, cells can for example remove the toxins from the plasma membrane by endocytosis and subsequent degradation by autophagy (Husmann *et al*, 2009; Idone *et al*, 2008). That was for example shown for the pore-forming streptolysin O (Idone *et al.*, 2008) or the alpha-toxin of *S. aureus* (Husmann *et al.*, 2009). Thus, for ClyA, epithelial cells and

phagocytic cells might cope differently with the activity of the pore-forming toxin, which makes it more difficult to elucidate the mode of action of ClyA.

Thus, further investigations on the cell killing mechanism of ClyA are necessary to understand its complexity, which would enable us to better understand the consequence of the cell killing ability of ClyA for the pathogenicity of *S. enterica* in the human body.

8.2.4 The relevance of ClyA for *Salmonella* infection is not clearly understood yet

We showed that ClyA, which is expressed and secreted during HeLa cells infection, is sufficient to kill THP-1 macrophages (Publication II). Now it would be interesting to find out more about the spatiotemporal expression and secretion of ClyA in vivo in order to conclude, if ClyA prevents the uptake of the bacteria by macrophages in the lamina propria after production of ClyA within the intestinal epithelial cells. However, up to now, in vivo animal models for infection with the strictly human adapted *S.* Typhi and *S.* Paratyphi A have not been found. Humanized mice with transplanted human immune cells could be used to identify single virulence factors or to describe parts of the disease (Firoz Mian *et al.*, 2011; Pearson *et al.*, 2008; Song *et al.*, 2010). Thus, mostly cell culture models are used to study the interaction of typhoidal *S. enterica* serovars and macrophages. These interactions are very complex and described as a balanced interaction by Hueffer & Galan (2004). On the one hand, *S. enterica* is able to survive in the SCV within macrophages (Alpuche-Aranda *et al.*, 1994; Cirillo *et al.*, 1998; Hensel *et al.*, 1998), and on the other hand, *S. enterica* is also capable to kill macrophages by inducing cell death through different pathways dependent (Fink & Cookson, 2007; Hernandez *et al*, 2003; Hersh *et al.*, 1999; Lin *et al.*, 2020; Lundberg *et al.*, 1999) and independent (Rolli *et al.*, 2010; van der Velden *et al.*, 2000) of the T3SS.

ClyA is only encoded by typhoidal *Salmonella* serovars (von Rhein *et al.*, 2009). The interaction between typhoidal *Salmonella* serovars and human macrophages and the effect of the T3SS effectors has recently been discussed as described above (Thurston & Holden, 2023). It was shown by Reuter *et al.* (2021) and Forest *et al.* (2010) that SPI2-T3SS play a less important role for replication of typhoidal *S. enterica* in human macrophages than for replication of non-typhoidal *Salmonella* serovars. They assume that less intracellular expression and activity is important for persisting and recurring infections (Reuter *et al.*, 2021). That would fit to the results that *S.* Typhimurium SPI2-T3SS effector proteins contribute to enterocolitis in mice (Coburn *et al*, 2005; Hapfelmeier *et al.*, 2005). Thus, a reduced expression of SPI2-T3SS effector proteins in *S.* Typhi could be a reason for less inflammation during the infection. However, the five SPI2-T3SS effector proteins SifA, SpvB, SseF, SseJ, and SteA were also shown to contribute to a cytotoxic effect on macrophages (Matsuda *et al*, 2019). Thus, ClyA might be an additional typhoidal virulence factor, which leads to the death of macrophages, when SPI2-T3SS effectors are less expressed.

We could show that ClyA is produced immediately after bacterial uptake into the host cells (Publication II), although we did not investigate, whether it is secreted immediately. We showed that TtsA is active in a major proportion of intracellular bacteria at early timepoints, that may lead to early ClyA secretion. There might be another important role of ClyA, produced in different epithelial cells at the systemic site of infection. However, that ClyA leads to deep tissue infection in mice, when heterologously expressed in *S.* Typhimurium (Fuentes *et al.*, 2008) would favor the assumption that ClyA leads to the release of *S. enterica* from infected macrophages once they reach deep tissues during systemic spread. It is described that ClyA is only present in typhoidal serovars (von Rhein *et al.*, 2009), however, it could also be found in some invasive non-typhoidal *Salmonella* strains (iNTS) (Suez *et al.*, 2013). These iNTS strains also lead to bacteremia and extraintestinal disease, although they are classified a non-typhoidal serovars. The presence of ClyA in the genome of these strains could indicate the importance of ClyA for establishment of a systemic disease.

8.3 T10SS as a universal secretion mechanism important for secretion of ChiA and ClyA

Despite being completely different virulence factors for *S. enterica* we could show that both, ChiA and ClyA, are secreted dependent on a T10SS (Publications I and II). Those results emphasize the importance of this secretion system in *S. enterica*. The fact that a T10SS is expressed in extracellular bacteria in contact with host cells at early stages of infection (ChiA) but also at later stages of infection when bacteria are already within host cells (ClyA) shows the universality of this secretion system.

8.3.1 Various T10SS differ in their composition

During the last years, holin/peptidoglycan hydrolase dependent secretion systems could be discovered in different bacteria. The presence and function of these secretion mechanisms were studied in *S. marcescens* (Hamilton *et al.*, 2014; Palmer *et al.*, 2021), *Yersinia entomophaga* (*Y. entomophaga)* (Feldmuller *et al*, 2024; Sitsel *et al*, 2024; Springer *et al*, 2018)*, Clostridia* spp. (Govind & Dupuy, 2012; Mehner-Breitfeld *et al*, 2018; Saadat & Melville, 2021; Vidor *et al*, 2022; Wydau-Dematteis *et al*, 2018) and *Salmonella* serovars (Geiger *et al.*, 2020; Geiger *et al.*, 2018; Hodak & Galan, 2013). However, the secretion mechanisms differ in the presence or lack of some proteins, in the transport of the cargo proteins through the inner membrane and the final release through the outer membrane.

In *S. enterica,* we focused on two different T10SS with different components (Publications I and II). Within the ChiA operon, we could identify a holin, a peptidoglycan hydrolase, a regulator and the cargo protein ChiA. For ClyA, the typhoid toxin secretion mechanism could be identified. Here, TtsA, a peptidoglycan hydrolase is present (Geiger *et al.*, 2018; Hodak & Galan, 2013). A specific holin could not be identified so far. Hodak & Galan (2013) assumed that holins, which are located

outside of the typhoid toxin operon may play a role in toxin secretion. They showed in their study that the coexpression of TtsA and different holins in remote operons leads to an enhanced effect of TtsA. The holin, which is responsible for the secretion of ChiA (STY0015) was for example also tested and in vitro an involvement in transport of TtsA to the periplasm was observed (Hodak & Galan, 2013). If STY0015 plays a role in the secretion of the typhoid toxin and ClyA in vivo was not clarified. We could show in our work, that TtsA and the holin in the chitinase operon are expressed under very different conditions, but we did not examine, if the holin STM0015 is also expressed within the SCV at later timepoints.

The T10SS responsible for secretion of the chitinase in *S. marcescens* or for secretion of the Tc toxin, a toxin with insecticidal function, in *Y. entomophaga* consist of different components. In addition to the cargo proteins, the holin, transcriptional regulators and the peptidoglycan hydrolase, spanins are present (Hamilton *et al.*, 2014; Palmer *et al.*, 2021; Sanger *et al*, 2023; Sitsel *et al.*, 2024; Springer *et al.*, 2018). Spanins are bacteriophage proteins which are involved in the lytic release of bacteriophages from bacteria by fusion of the inner and outer membrane (Abeysekera *et al*, 2022; Cahill & Young, 2019). The presence of spanins in addition to the holin and the peptidoglycan-hydrolase might correlate with a lytic release of the cargo proteins.

8.3.2 ChiA and ClyA are secreted in a non-lytic manner

In a study of *Y. entomophaga* and *S. marcescens* the expression and secretion of T10SS cargo proteins was observed in a very small subpopulation of bacteria which were lysed because of the activity of the T10SS (Feldmuller *et al.*, 2024; Sitsel *et al.*, 2024). In *S. enterica* we clearly showed a non-lytic secretion mechanism for ChiA and ClyA. In both studies (Publications I and II) we proved that bacteria do not undergo cell lysis during the process of protein secretion. As an example, for secretion of ChiA, we showed that there is no difference in the number of colony-forming units for ChiA secreting and non-secreting bacteria. Additionally, there is no difference in their viability (propidium iodide staining) and a cytoplasmic control protein RecA was not released into the bacterial supernatant during ChiA secretion. For ClyA secretion, we similarly showed that only periplasmic contents (for example the periplasmic protein MalE) but not cytoplasmic contents such as RecA were secreted, which indicates a non-lytic secretion process. These results confirm the study by Geiger *et al.* (2018) for the secretion of typhoid toxin via the same T10SS (Geiger *et al.*, 2018). Furthermore, we showed that both of the investigated proteins are expressed in the majority of the bacteria and not only in a small subpopulation as it was shown for *Y. entomophaga* (Feldmuller *et al.*, 2024; Sitsel *et al.*, 2024) and *S. marcescens* (Costa *et al*, 2019; Feldmuller *et al.*, 2024; Hamilton *et al.*, 2014).

8.3.3 Details of the T10SS mechanisms which remain to be elucidated in further studies

Once the cargo proteins are transcribed, they are transported by the T10SS in different, not fully understood ways. The transport through the inner membrane is only elucidated for some of the

cargo proteins of the T10SS. For ClyA and ChiA, no signal peptide for the Sec machinery is present, whereas other proteins such as the typhoid toxin are translocated by the Sec-machinery through the inner membrane into the periplasm (Haghjoo & Galan, 2004). Interestingly, for the chitinase secretion in *S. marcescens*, only for some of the cargo proteins of the same T10SS a signal peptide for Sec secretion has been described (Hamilton *et al.*, 2014). The phenomena that proteins are transported through the inner membrane without a signal peptide for the Sec or the Tat secretion system, was called non-classical protein secretion by Bendtsen *et al* (2005). They analyzed different proteins in Gram-negative and Gram-positive bacteria, which are secreted in an unknown way, and which do not have a signal peptide for the Sec or Tat translocon and compared their sequences. However, they could not find a shared sequence (Bendtsen *et al.*, 2005).

When the proteins passed the inner membrane, they are at the cis side of the peptidoglycan. The peptidoglycan is cleaved by the corresponding peptidoglycan hydrolase. It was shown before for *S.* Typhi that the peptidoglycan hydrolase TtsA can only cleave the peptidoglycan after it was modified by a LD-transpeptidase YcbB. YcbB changes 4-3 (DD) crosslinks to 3-3 (LD) crosslinks (Geiger *et al.*, 2020; Geiger *et al.*, 2018; Hodak & Galan, 2013). It would further be interesting to find out whether LD transpeptidases, subsequently 3-3 cross-linked peptidoglycan is also relevant for ChiA secretion and its peptidoglycan hydrolase. Up to now, it is unknown how the LD-transpeptidase for secretion of the typhoid toxin and ClyA is regulated. It was only shown before that YcbB and TtsA are active at the bacterial cell poles (Geiger *et al.*, 2018), but not, how large the area of the modified peptidoglycan and the cleavage sites are. The assembled typhoid toxin has a length of approximately 90 Å (Song *et al.*, 2013), whereas the assembled ClyA pore is larger (130 Å high and a maximum diameter of 105 Å) (Mueller *et al.*, 2009). So, it would be interesting to elucidate the size of the space which is created by the different peptidoglycan hydrolases and how it is regulated.

After cleavage of the peptidoglycan layer, the proteins can pass the layer to the trans side of the peptidoglycan. An interesting question is, if secretion by the non-lytic T10SS is specific or if the whole content of the cis side of the peptidoglycan layer which is present at this time is transported to the trans side of the peptidoglycan. We could show, that ClyA is secreted by the same secretion system as the typhoid toxin and both proteins are regulated by PhoP/Q under intravacuolar conditions (Faucher *et al.*, 2009; Fowler & Galan, 2018). If all periplasmic proteins that are present at the time of T10SS activity would be secreted, a strict regulation and great amounts of these different potential periplasmic cargo proteins would be necessary.

Consequently, the secretion by the T10SS might lead to disrupted cell walls. Thus, it would be interesting, if the cleavage of the peptidoglycan is a reversible process and if the peptidoglycan layer is repaired. Murein hydrolases are for example important for cell division, assembly of pili, flagella or other secretion systems (Vollmer *et al*, 2008; Weiss, 2004). The disrupted cell wall might be repaired in a similar way as the cell wall in the process for cell division.

8.3.4 The final release of both secreted virulence proteins, ClyA and ChiA, could be mediated by OMVs

Once the proteins reach the trans side of the peptidoglycan layer, they must ultimately be released through the outer membrane of the bacteria. A lytic mechanism, which was ruled out by us for secretion of ClyA and ChiA, would explain the final release of the cargo proteins through the bacterial outer membrane, which so far cannot be explained with our secretion model (Sitsel *et al.*, 2024).

In our studies, we performed ultracentrifugation of the cytoplasmic fraction of infected THP-1 cells (publication II). Our results indicated that ClyA is present in a vesicle-associated or membrane-bound form, rather than as soluble proteins. Whether these intracellular conditions promote the formation of bacterial OMVs and the subsequent release of ClyA via this mechanism or whether these vesicles originate from the host cells (SCV) as it was shown for typhoid toxin (Spano *et al*, 2008) remains to be investigated in future studies.

For ClyA, the final release within OMVs could be observed in vitro (Wai *et al.*, 2003). It is noteworthy to say that in this publication the vaccination strain Ty21a was used (Germanier & Fuer, 1975; Oscarsson *et al.*, 2002). Ty21a has a mutation that leads to the accumulation of galactose-I-phosphate and uridine diphosphate galactose which leads to bacteria lysis (Germanier & Fuer, 1975). It can be assumed that the release of ClyA by OMVs is caused by an impaired cell wall stability of these bacteria, which could be a stress response to cell wall stress conditions. That the formation of OMVs is often described as a stress response of Gram-negative bacteria to various cell wall stress conditions (McBroom & Kuehn, 2007) would be in favor for that. However, for the typhoid toxin a final release in OMVs was also shown (Guidi *et al.*, 2013). It is important to mention that in this study of Guidi *et al.* (2013) only the toxin itself and not TtsA, which is essential for secretion of the protein, was expressed heterologously in *S.* Typhimurium (Guidi *et al.*, 2013; Hodak & Galan, 2013), which might lead to a difference in the whole secretion mechanism. The typhoid toxin and ClyA are both expressed within the SCV (Spano *et al.*, 2008). It was shown for *S.* Typhimurium that OMVs could also be found within the SCV in murine macrophages (Yoon *et al*, 2011) and in CaCo-2 cells (Guidi *et al.*, 2013). In the study of Guidi *et al.* (2013) a possible mechanism was shown, how an intravacuolar expressed toxins can have an effect on the neighboring cells when released in OMVs. Thus, while the final release of toxins via OMVs could potentially affect target cells, whether ClyA and ChiA are truly secreted through bacterial OMVs still needs to be investigated in future studies.

9. Short Summary

ClyA and ChiA were barely described virulence factors of *S. enterica.* We could show that both are highly expressed and secreted during the process of infection, but under very different conditions. While ChiA is expressed and secreted extracellularly upon contact to polarized intestinal epithelial cells with a mucus layer and relevant for invasion into these cells, ClyA is mainly expressed and secreted intracellularly and as an active cytolysin it has cytotoxic effects on macrophages. Both proteins are relevant for the infection process when tested in cell culture models and both can also be found as functional proteins in typhoidal *Salmonella* serovars, while pseudogenization and gene deletion in human adapted serovars such as *S.* Paratyphi and *S.* Typhi is common (Baumler & Fang, 2013; Sabbagh *et al.*, 2010), which indicates an importance of present genes for human infection. We could show in our studies, that ClyA and ChiA are secreted in dependence on two different T10SS. The recently published data and our investigations show the universality and importance of the secretion mechanism for the pathogenicity of different pathogenic bacteria, such as different *Salmonella* serovars. Figure 2 summarizes the most important information of our studies.

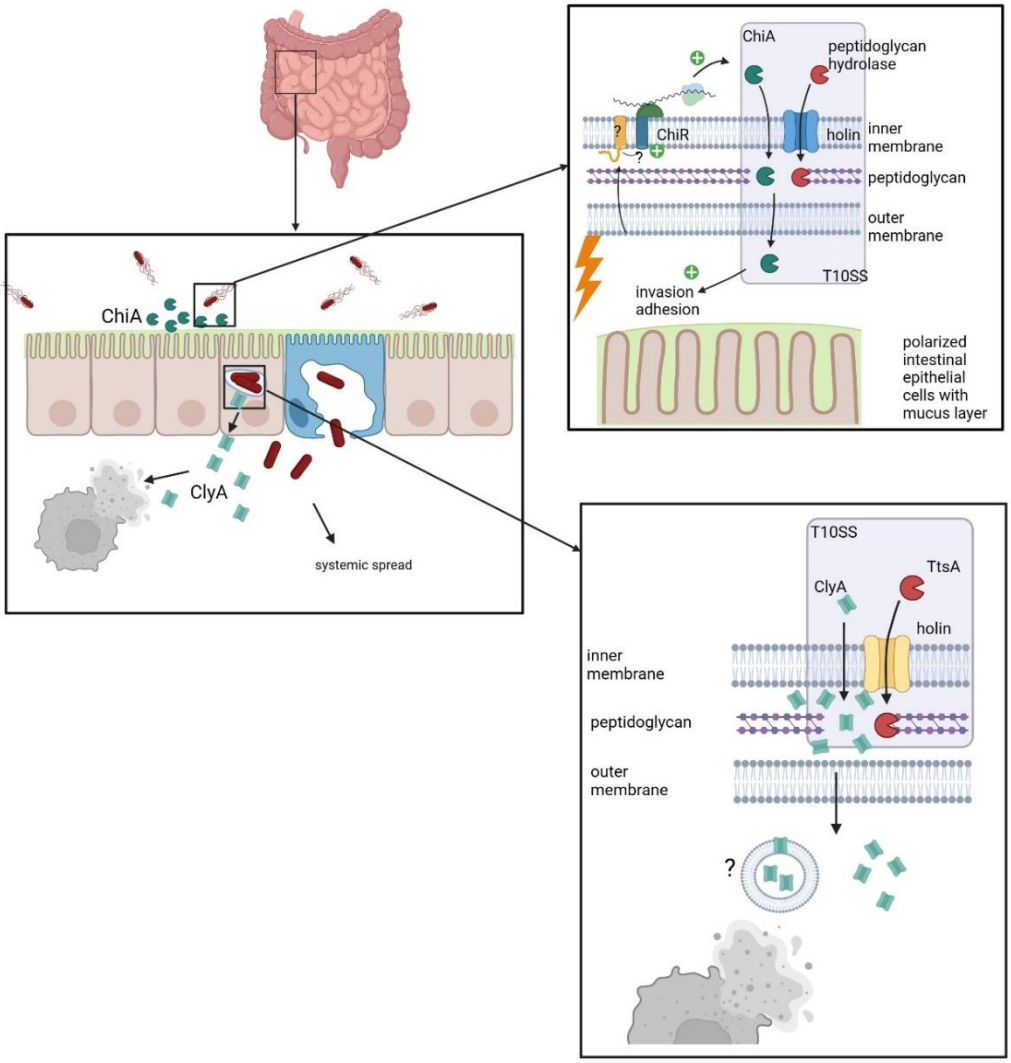

Figure 2 Graphical Summary. *S. enterica* passes the stomach and reaches the small intestine. Due to contact to polarized intestinal epithelial cells with an attached mucus layer, the expression of ChiA is upregulated via the transcriptional regulator ChiR. It leaves the bacterial cell through a T10SS. For the T10SS, the peptidoglycan hydrolase is transported through a holin to the periplasm and cleaves the peptidoglycan. ChiA passes the inner membrane through a currently unknown mechanism. It is transported through the cleaved peptidoglycan and leaves the periplasm through an undistinguished mechanism. In the extracellular space, it contributes to the invasion of *S. enterica.* Once *S. enterica* invaded into the intestinal epithelial cells, a SCV is formed and restructured by the bacteria. The intravacuolar conditions induce expression of ClyA. ClyA is transported through the bacterial cell wall into the extracellular space through a further T10SS, also known for the secretion of the typhoid toxin. Again, a peptidoglycan hydrolase called TtsA reaches the peptidoglycan layer presumably through a holin, cleaves the peptidoglycan and ClyA can pass the cell wall similarly to ChiA. The final release might be mediated by OMVs or a different unknown mechanism. Outside of the bacteria in the lamina propia phagocytes might be lysed by the pore-forming toxin. Created with BioRender.com, adapted from publication I and publication II.

10. Outlook

In our studies, we could unravel important details for the regulation, expression, secretion and effect of the pathogenicity factors ClyA and ChiA. Further experiments are necessary to answer remaining questions about molecular mechanisms.

For ChiA, the inducing component, also present in polarized cell culture, could not be identified in detail. It would be interesting to find out the exact signal and how it is transduced from the cell surface to the transcriptional regulator ChiR. In the case of ClyA, in addition to details about the transport and export mechanisms, it will be necessary to find out the molecular mechanism by which the macrophage-like cells are killed. That might give an explanation why macrophage-like cells are susceptible and epithelial cells not. The information in which cells and tissues ClyA is expressed in vivo could clarify the role of ClyA for pathogenicity of typhoidal *Salmonella* serovars.

For both pathogenicity factors details about the T10SS remained open. It has to be figured out for both secretion paths how the cargo proteins are transported through the inner and outer membrane. There is evidence suggesting that OMVs may play a role in the final release of *S.* Paratyphi A ClyA, but this needs to be confirmed by further experiments.

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List of publications

Thesis publications:

Krone, L., Mahankali, S. and Geiger, T. (2024). Cytolysin A is an intracellularly induced and secreted cytotoxin of typhoidal *Salmonella*. *Nat Commun* 15, 8414. https://doi.org/10.1038/s41467-024-52745-0

Krone, L., Faass, L., Hauke, M., Josenhans, C., and Geiger, T. (2023). Chitinase A, a tightly regulated virulence factor of Salmonella enterica serovar Typhimurium, is actively secreted by a Type 10 Secretion System. PLoS Pathog 19, e1011306. https://doi.org/10.1371/journal.ppat.1011306

Research articles before and outside of thesis:

Galeev, A., Suwandi, A., Bakker, H., Oktiviyari, A., Routier, F.H., Krone, L., Hensel, M., and Grassl, G.A. (2020). Proteoglycan-Dependent Endo-Lysosomal Fusion Affects Intracellular Survival of *Salmonella* Typhimurium in Epithelial Cells. Front Immunol 11, 731. 10.3389/fimmu.2020.00731.

Noster, J., Persicke, M., Chao, T.C., Krone, L., Heppner, B., Hensel, M., and Hansmeier, N. (2019). Impact of ROS-Induced Damage of TCA Cycle Enzymes on Metabolism and Virulence of *Salmonella enterica* serovar Typhimurium. Front Microbiol 10, 762. 10.3389/fmicb.2019.00762.

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Krone, L., Geiger, T. (2023). Comprehensive investigations into cytolysin A, a major virulence factor of typhoidal *Salmonella enterica* serovars. DGHM Jahrestagung 2023, Lübeck (Poster)

Krone, L., Faass, L., Hauke, M., Josenhans, C., and Geiger, T. (2023). Chitinase A, a tightly regulated virulence factor of *Salmonella enterica* serovar Typhimurium, is actively secreted by a Type 10 Secretion System. GRC Salmonella: From Basic Biology to Complex Pathogen-Host Interactions, Lucca, Italy (Poster)

Krone, L., Faass, L., Hauke, M., Josenhans, C., and Geiger, T. (2022). The expression and secretion of chitinase A (ChiA) – a novel virulence factor of *Salmonella* Typhimurium, DGHM Jahrestagung 2022, Berlin (Talk)

Danksagung

Vielen Dank an die Gutachter und die Prüfungskommission für die Prüfung dieser Dissertation. Bis zur Entstehung war es ein langer Weg. Auf diesem Weg haben mich viele Menschen tatkräftig unterstützt und begleitet.

Mein größter Dank gilt Tobias Geiger. Tobias, danke für deine Betreuung, die Unterstützung, die gute Atmosphäre mit der Möglichkeit zu wachsen und auch der Erlaubnis, Fehler zu machen. Danke für deine Geduld, dein Verständnis und die gute, gemeinsame Zeit.

Zudem möchte ich mich recht herzlich bei Christine Josenhans bedanken. Danke, für Ihre Begleitung auf dem Weg zur Dissertation, für den Input und die Betreuung und für Ihren Einsatz für uns Doktoranden im Rahmen des intramuralen Graduiertenprogramms „Infection Research on Human Pathogens@MvPI“.

Danke auch an Prof. Suerbaum, als Leiter des Instituts, für die Möglichkeit, am Max von Pettenkofer-Institut zu arbeiten und zu promovieren, sowie für die unentwegte strukturelle und finanzielle Unterstützung des MvPI-Graduiertenprogramms von 2019-ongoing.

Srujita, thank you for the time we worked together. I didn’t have to feel lonely anymore.

A big thank to all the kind people in the institute, who adopted me from time to time, who were there, when I had questions or problems. Thank you for the time we spent together and for the nice time in the lunchbreaks and at festivities.

Neben den lieben Menschen im Institut hatte und habe ich in meinem privaten Umfeld mir sehr wichtige Menschen, die mich in den vergangenen Jahren unterstützt haben, mich ermutigt haben und einfach für mich da waren und sind. Danke, an meine Eltern und meine Schwester Christine, an Jan und Finja mit Chili für eure Unterstützung. Ich kann gar nicht in Worte fassen, wie wichtig ihr seid. Ein großer Dank gilt auch meinen lieben Freunden, insbesondere Janina, die auch in der Ferne da ist.

Übereinstimmungserklärung

LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

Dekanat Medizinische Fakultät
Promotionsbüro

Erklärung zur Übereinstimmung der gebundenen Ausgabe der Dissertation mit der elektronischen Fassung

Krone, Lena

Name, Vorname

Hiermit erkläre ich, dass die elektronische Version der eingereichten Dissertation mit dem Titel:

Chitinase A and Cytolysin A: Important virulence factors of Salmonella enterica, secreted by type 10 secretion systems

in Inhalt und Formatierung mit den gedruckten und gebundenen Exemplaren übereinstimmt.

München, 16.07.2025	Lena Krone
Ort, Datum	Unterschrift Doktorandin/Doktorand