From Department of Microbiology, Tumor and Cell Biology
Karolinska Institutet, Stockholm, Sweden

ADAPTATION OF *SALMONELLA ENTERICA* TO ANTIBIOTICS AND INNATE IMMUNITY EFFECTORS

Kim Vestö

Karolinska Institutet

Stockholm 2021

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2021

ISBN 978-91-8016-123-7

Adaptation of *Salmonella enterica* to antibiotics and innate immunity effectors

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Kim Vestö

The thesis will be defended in public at lecture hall **CMB**, Berzelius väg 21, Solna

Friday the 26th of March 2021 at 9.00

Principal Supervisor:
Professor Mikael Rhen
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Co-supervisor(s):
Senior researcher Edmund Loh
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Opponent:
Associate professor Lotte Jelsbak
Roskilde University
Department of Science and Environment

Examination Board:
Associate professor Peter Bergman
Karolinska Institutet
Department of Laboratory Medicine

Associate professor Elena Vikström
Linköpings Universitet
Department of Biomedical and Clinical Sciences

Associate professor Anna Smed-Sörensen
Karolinska Institutet
Department of Medicine, Solna

POPULAR SCIENCE SUMMARY OF THE THESIS

Bacteria are of extreme importance for the planet and humanity. Most bacteria are harmless and are part in various activities ranging from helping plants to grow to breaking down our food in our intestines. However, a handful of bacteria are not harmless and will cause an infection if they get access to the human body. Some of these infections are very serious as exemplified by bacterial growth in the lungs or the urinary tract, which can even allow for bacteria to enter the blood and possibly lead to death of the infection person. When one is unfortunate enough to experience a serious bacterial infection one can usually find help in antibiotics. Antibiotics are medicines that are designed to kill bacteria or stop bacteria from growing, which is how they work as treatment for infections. However, the consequence of extensive use of antibiotics can lead to bacteria becoming resistant to antibiotics. This means that if one gets and infection caused by a resistant bacteria, the bacteria will not be affected when given antibiotic treatment.

This is exactly what has happened with many infections today, leaving very few treatment options left. One such infection is called typhoid fever which is caused by a bacterium called *Salmonella.* Typhoid fever is most common in low- and middle-income countries in Africa and Asia and one gets it by eating or drinking the food that has been contaminated with the bacterium. After this *Salmonella* will enter the body and make its way to the liver, spleen and bone marrow. This makes the person very ill and can even lead to death. In order to find new treatments to the infections *Salmonella* cause one has to gain knowledge about the biology of the bacterium by for example studying antibiotic resistance or the ability of the bacterium to grow within cells. This is analogous to the fact that if your car breaks down it will be very hard for you to fix it if you don't understand how the motor of the car works. For this in this thesis I have studied a variant of *Salmonella* that does not cause typhoid fever in humans, but causes a typhoid fever-like illness in mice. This *Salmonella* enables easier access to experiments due to it being less dangerous than the variant of *Salmonella* that causes typhoid fever.

As such to try to increase our knowledge about the biology of *Salmonella* we discovered a new gene involved in antibiotic resistance towards a specific antibiotic, namely vancomycin. This gene of *Salmonella* is called *mepS* and the protein for

which the gene codes for functions in the turnover of the bacterial cell wall, a structure not existing in humans. In this we show that if one removes *mepS* from *Salmonella* the bacterium becomes sensitized to vancomycin. Further experiments confirmed that *mepS* was required for *Salmonella* to be resistant to vancomycin. In this way we have increased the knowledge about what is required by *Salmonella* to survive the antibiotic and possibly found a new target for future antibiotic treatment in the form of *mepS*.

As for *Salmonella*s ability to grow within cells we found that a gene named *prc*, which codes for a protein whose function is to degrade other proteins, is needed for the bacterium to be able to fully survive. In this we also show that the importance of *prc* for growth within cells is dependent on an other gene called *pbp3sal*, which codes for a protein needed in building the cell wall when growing within mouse cells. In addition we show the same requirement for *prc* in a mouse infection model further highlighting the importance of Prc in *Salmonella*s ability to cause disease. This adds to our knowledge of which genes are required for *Salmonella*s ability to cause disease and suggests that the proteins in question could be targets for future antibiotic treatment.

The genes needed for *Salmonella* to be able to grow within cells is one aspect of how *Salmonella* causes disease. An other aspect is the fact that the cells are not happy with *Salmonella* being inside them. This results in that when the cell feels that *Salmonella* has entered them it will try to kill the bacterium in many different ways. In this we have studied how a specific protein called iNOS works in response to a *Salmonella* infection of individual cells. The function of iNOS is to produce nitric oxide, a molecule that when further reacting with strong oxidants becomes very toxic, that will destroy various parts of the bacteria resulting in growth arrest or death. We find that when *Salmonella* has successfully grown within a cell, that cell is often lacking oxygen and iNOS is not present. This we suggest could be due to the general stress *Salmonella* causes to the cell when growing within it, leading to a reduction in protein synthesis and hence lack of iNOS. These results begin to highlight new aspects of *Salmonella* infections on the level of individual infected cells.

ABSTRACT

Salmonella enterica is a bacterial pathogen causing major morbidity and mortality in low- and middle-income countries. The bacteria can cause a wide range of disease, ranging from the severe systemic disease typhoid fever to localized gastroenteritis. Characteristics of typhoid fever, caused by *Salmonella enterica* serovar Typhi (*S*. Typhi), include the bacteria's ability to proliferate within host cells, intrinsic resistance to selected antibiotics, and emerging acquired antibiotic resistance. As *S*. Typhi is strictly human adapted and highly pathogenic one often uses *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) as a model organism for understanding details of typhoid fever. Using *S.* Typhimurium this thesis reveals genetic details governing intrinsic antibiotic resistance and virulence in *in vitro* and *in vivo* infection models, as well as details the interplay between the pathogen and phagocytic cells.

In this we have discovered a new genetic determinant for intrinsic vancomycin resistance coding for muramyl endopeptidase MepS (also known as Spr), an enzyme tasked with cleaving cell wall, and that MepS is functionally connected to the periplasmic protease Prc (also known as Tsp) in this matter. Vancomycin is an antibiotic that inhibits cell wall synthesis, but is not effective against Gram-negative enteric bacteria. This has been thought to be due to the relative impermeability of the outer membrane resulting in vancomycin not being able to access its target the cell wall (due to its large size). However, we present results that adds to this in showing that the outer membrane is not the only factor resulting in intrinsic vancomycin resistance.

With regard to intracellular pathogenesis of *S.* Typhimurium in mouse infection models we show in this thesis that the periplasmic protease Prc is required for the full fitness of the bacterium when in macrophages and mice. This requirement is dependent on the cell wall synthesizing enzyme $PBP3_{SAL}$ highlighting the possible role of Prc in regulation of bacterial proliferation during intracellular phases of infection. As for further aspects of intracellular pathogenesis of *S.* Typhimurium in macrophages we show that the presence of *S.* Typhimurium in single cells correlates with hypoxia and lack of iNOS, an innate immunity effector tasked with killing invading organisms by producing reactive nitrogen species. We suggest this

correlation to be a result of general shut-off of protein synthesis due to hypoxia generated by the presence of *S.* Typhimurium proliferating within the macrophage.

All these result add to the basic knowledge of both determinants for intrinsic antibiotic resistance and aspects governing intracellular pathogenesis of *S.* Typhimurium with regards to both genes involved and effect on innate immunity effectors. We believe the results presented in this thesis is a good starting point for further studies regarding further mechanistical studies into the phenomena described.

LIST OF SCIENTIFIC PAPERS

I. Vestö K., Huseby D.L., Snygg I., Wang H., Hughes D., and Rhen M. (2018). Muramyl endopeptidase Spr contributes to intrinsic vancomycin resistance in *Salmonella enterica* serovar Typhimurium. *Front. Microbiol.* 9:2941. doi: 10.3389/fmicb.2018.02941

II. Vestö K., Frederiksen R.F., Snygg I., Fahlgren A., Fällman M., and Rhen M. Genetic and phenotypic characterization of periplasmic protease Prc in *Salmonella enterica* serovar Typhimurium reveals connection to alternative peptidoglycan synthase PBP3sal in *in vitro* and *in vivo* infection models. *Manuscript*

III. Wrande M., Vestö K., Puiac Banesaru S., Anwar N., Nordfjell J., Liu L., McInerney G.M., and Rhen M. (2020). Replication of *Salmonella enterica* serovar Typhimurium in RAW264.7 phagocytes correlates with hypoxia and lack of iNOS expression. *Front. Cell. Infect. Microbiol.* 10:537782. doi: 10.3389/fcimb.2020.537782

CONTENTS

LIST OF ABBREVIATIONS

S. enterica	*Salmonella enterica*
S. Typhi	*Salmonella enterica* serovar Typhi
S. Paratyphi	*Salmonella enterica* serovar Paratyphi
S. Typhimurium	*Salmonella enterica* serovar Typhimurium
S. Enteritidis	*Salmonella enterica* serovar Enteritidis
ST313	Sequence Type 313
ROS	Reactive oxygen species
T3SS-1	Type 3 secretion system-1
SPI-1	*Salmonella* pathogenicity island-1
SCV	*Salmonella*-containing vacuole
T3SS-2	Type 3 secretion system-2
SPI-2	*Salmonella* pathogenicity island-2
RNS	Reactive nitrogen species
iNOS	Inducible nitric oxide synthase
SIF	*Salmonella*-induced filaments
E. coli	*Escherichia coli*
IVA	*In vivo* assembly
MIC	Minimum inhibitory concentration
MOI	Multiplicity of infection
CFU	Colony-forming unit
PBP	Penicillin-binding protein

1 INTRODUCTION

This thesis was written during the fall and winter of 2020/2021. The year 2020 will be remembered as the year of the SARS-CoV-2/COVID-19 pandemic. As of writing this it is now over a year since the first descriptions of "undiagnosed pneumonia" emerged from China on the 30th of December 2019, a message disseminated by ProMED, a surveillance system for infectious diseases. Yet it is still less than a year from WHO announcing the outbreak of SARS-CoV-2 being a pandemic the 11th of March 2020. Due to the pandemic research has come to the forefront both in media and in the eye of the public. In this we have seen a global response towards an infectious agent on a massive scale; immense investments in both treatment and prevention from national and private entities alike. By far the biggest achievement during the pandemic is the fact that the first vaccine against the disease SARS-CoV-2 causes, i.e. COVID-19, has already gained licensure in the form of emergency use authorization licensure in December of 2020. This is an unbelievable achievement for humanity.

However, for those who have been studying and teaching about infectious diseases for a big part of their academic life this achievement is slightly bittersweet. The reason for this comes from the fact that even though it might have seemed like COVID-19 was the only infectious disease in existence during 2020, this is not the case. Various infectious diseases have been causing a massive burden on human health year in, year out, even during the pandemic of 2020 and will continue to do so after the pandemic. Hence, a hopeful optimist might look at the response mustered by humanity towards the pandemic and ask "could we mobilize the same resources used to combat SARS-CoV-2 in order to combat other infectious diseases such as malaria or tuberculosis?". To this a cynic might answer "we can, we could have, we haven't, and we probably will not". I'm not certain whether I'm the hopeful optimist or the cynic, or something in between, but I hope that the pandemic of 2020 will be a year when we realize we really can have an impact on infectious diseases and reduce the mortality caused by them if we wish to do so.

On this list of infectious diseases addressed with the same vigor as SARS-CoV-2 I hope to see infections caused by the bacterium *Salmonella enterica*. The burden of these infections are substantial globally and with increasing antibiotic resistance the

treatment options are quickly becoming limited. This thesis aims to advance our understanding of fundamental aspects of antibiotic resistance and pathogenesis of *Salmonella*. This has been done by studying genetic requirements of the bacterium for both antibiotic resistance, intracellular pathogenesis, and adaptation to innate immunity of the host phagocytes.

2 LITERATURE REVIEW

2.1 *Salmonella*

Salmonella is a genus of Gram-negative bacteria known to have the ability to infect great many species of animal with a large amount of morbidity and mortality in humans (1–3). The genus is further divided into two species; *Salmonella bongori* and *Salmonella enterica* (*S. enterica*) with *S. enterica* being responsible for all the human pathology caused by the genus. The clinical manifestations of infections caused by different serovars of *S. enterica* vary a lot and can be divided into three distinct types of infection. These infections are typhoid fever, invasive non-typhoidal salmonellosis, and non-typhoidal salmonellosis all of which will be described in the following sections. The common denominator for all the different types of diseases caused by *S. enterica* is that they are consequences of an individual ingesting foodstuff or water contaminated with the bacterium. Hence, the majority of the burden of disease caused by *S. enterica* globally can mostly be attributed simply to the lack of clean water and sanitation as is the case for low- to middle-income countries (4–8). Even in countries where infrastructure for safe water and sanitation are more developed outbreaks of *S. enterica* can still arise from almost any kind of contaminated foodstuff ranging from eggs and seafood to ice cream and sprouts (9–14).

2.2 Typhoid fever

Out of the three diseases typhoid fever is the most severe. It manifests as an invasive systemic disease in immunocompetent individuals with one publication estimating a case-fatality ratio of 0.95%, and much higher in non-treated individuals, resulting in a total of 135,900 deaths from 14 million cases yearly (1) with an other publication estimating the total number of infections to 17.8 million solely in low- and middle-income countries (15). However, the true nature of these numbers is hard to precisely estimate as highlighted by the 95%-confidence interval being 7 to 48 million cases annually (15). Regardless, it is well established that the highest burden of disease lies in central Africa and eastern/southeastern Asia with the majority of infection occurring in children under 5 years of age (1,15–17). The serovars of *S. enterica* causing typhoid fever are *S. enterica* serovar Typhi (*S.* Typhi), *S. enterica* serovar Paratyphi (*S.* Paratyphi) A, B and C, with the disease caused by the serovar

Paratyphi sometimes distinguished as paratyphoid fever due to them causing a slightly less severe disease compared to serovar Typhi (18,19).

Once an infection with *S.* Typhi is initiated by ingesting the bacterium it passes through the esophagus into the stomach and progress towards the small intestine. From hereon the exact details of the pathogenesis of typhoid fever in humans are uncertain due to *S.* Typhi being human-specific pathogen. This facts makes studies both ethically and practically difficult to conduct. Instead, the details of the pathogenesis are mostly inferred from animal models, such as mouse models, where a typhoid fever-like illness can be manifested using *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) as the model organism. In these host-pathogen pairs of mouse and *S*. Typhimurium it has been shown that following entry into the small intestine the bacterium will adhere to the epithelial mucosa of the intestine (20,21). From here the bacteria will invade the host by entering intestinal epithelial cells (20,22,23) and/or M cells, cells part of the intestine whose function is to sample the lumen of the intestine (24–27), to traverse deeper into the host (22,28,29).

Following traversion of the intestine through M cells *Salmonella* will be within Peyer's patches, an immune organ of which M cells are part of (30,31). There it is believed that the bacterium will make use of CD18+ phagocytes, amongst them macrophages, monocytes and most notably dendritic cells, as Trojan horses to travel through the body first via the mesenteric lymph nodes (32–35). From the mesenteric lymph nodes the bacteria will find itself seeded into various organs such as liver, spleen, and gallbladder where the bacteria can be found within cells, such as macrophages resident of the organ (36–44). This seeding most likely happens via a primary bacteremia (32,45) that may go unnoticed by the infected individual. From the organs bacteria will be seeded into the bloodstream a second time resulting in the clinical symptoms associated with typhoid fever over a week from the initial ingestion of the bacteria (18,19,46).

Typhoid fever is characterized by "influenza-like symptoms" in the form of general malaise and fever, but also by specific signs in both an enlarged liver and spleen, tender abdomen, and the possibility of having coating on the tongue (18,19,47). As the disease progresses the fever will increase with some individuals also getting skin

lesions in the form of rose spots (18,19,47). More severe complications can manifest in the form of gastrointestinal bleeding, intestinal perforation, confusion, and inflammation of several organs such as the liver and heart (18,19,47). While most individuals who recover completely clear the bacteria, still a few percentage continue excreting *S.* Typhi months after the clinical disease has resolved with some even becoming chronic carriers with the bacteria most likely residing long-term in the gallbladder (48–52).

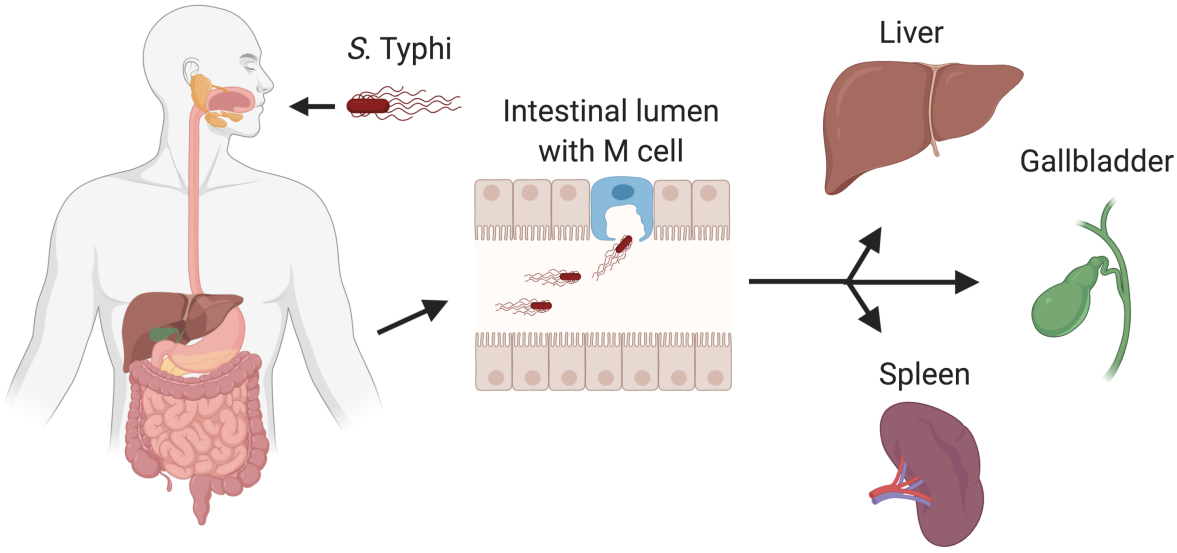

FIGURE 1 Illustration of the pathogenesis of typhoid fever. Following ingestion of *S.* Typhi the bacteria will travel to the intestine, across the intestinal barrier, and disseminate throughout the body.

During the years the treatment regimen for typhoid fever has changed from antibiotic to antibiotic due to emergence of antibiotic resistance rendering a prevailing regimen useless. The original choice for treatment from 1950's onward was the protein synthesis inhibitor chloramphenicol (53), but the use of this antibiotic was affected from the 1970's on due to the discovery of *S.* Typhi strains harbouring chloramphenicol resistance (54–56). The treatment options were then expanded to include the folate synthesis inhibitors trimethoprim and sulfonamide in addition to the cell wall synthesis inhibitor ampicillin (57). However a few decades later *S.* Typhi resistant to all of these antibiotics was widespread (58,59). Today the main treatment for typhoid fever is protein synthesis inhibitor azithromycin, DNA gyrase inhibitor ciprofloxacin and cell wall synthesis inhibitor ceftriaxone (60–62), although for how long is unclear since both ciprofloxacin- and ceftriaxone-resistant *S.* Typhi has been discovered (63–67).

With treatment options for typhoid fever becoming scarce the role of vaccines is ever more important in preventing typhoid fever. As of today there are two vaccines available, one of them being Ty21a, a vaccine consisting of an attenuated *S.* Typhi ingested orally, and Vi, a vaccine consisting of the outermost capsule of *S.* Typhi given intramuscularly (68,69). The immune response mounted following vaccination with Ty21a even results in cross-protection against paratyphoid fever (70,71). However, these vaccines haven't been widely adopted due to issues with long-term protection towards disease, resulting in the need for revaccinations approximately every third year, and subpar efficacy in the most vulnerable group of individuals namely young children (68,69).

Due to these concerns the search for a typhoid fever vaccine continues with two main vaccine candidates arriving with the concept of making the Vi capsule more immunogenic by conjugating the polysaccharide with a strong antigen (69). One of the conjugate vaccine candidates aimed at better efficacy and safety for children consists of the Vi capsular polysaccharide conjugated to an inactive variant of exotoxin A from *Pseudomonas aeruginosa*. This vaccine candidate is termed Vi-rEA and has showed efficacy in young children, yet is still not licensed in any country (69,72–74). However, a conjugate vaccine that is licensed, albeit only in India while also being recommended by the WHO for endemic areas, consists of the Vi capsular polysaccharide conjugated with a tetanus toxoid named Vi-TT (69). This conjugate Vi-TT vaccine has been evaluated in children from the age of 6 months and shown to be superiorly efficacious to the current Vi capsular vaccine in this susceptible group of young children with similar results in adults in a human challenge model (75–77). Additionally, an aspect that might complicate the effectiveness of such vaccines is the fact that *S.* Typhi lacking the Vi-antigen have already been documented (78) and could possibly be selected for with an vaccine that is not targeting any antigens of said *S.* Typhi.

2.3 Invasive non-typhoidal salmonellosis

In the last few decades a different type of disease caused by *S. enterica* has emerged in the form of invasive non-typhoidal salmonellosis. This disease is not cause by the human-specific *S.* Typhi or *S.* Paratyphi, but instead by non-typhoidal serovars of *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) and *S.* Typhimurium (79–81). With invasive non-typhoidal salmonellosis still being a fairly recently recognized disease the estimates of the burden it causes range a lot. A study from 2015 estimated that amount of cases each year is 3,4 million with approximately 600,000 deaths globally (82), while a study from 2019 estimates there to be around 500,000 cases each year with approximately 80,000 deaths globally (2). Even though invasive non-typhoidal salmonellosis might be causing mortality parallel to that of typhoid fever, the pathogenesis differs significantly. The infection simply results in a bloodstream infection, often without any associated gastroenteritis, following the ingestion of the bacteria. Originally one observed the ability of such non-typhoidal *Salmonella* serovars to cause invasive disease mainly in AIDS patients (83), but also in malnourished individuals (81), with all this making *Salmonella* the most common non-malarial bloodstream infection on the African continent (79).

As *Salmonella* has previously been thought to cause either typhoid fever or gastroenteritis the discovery of a bloodstream infection stands out. The search for what makes *S.* Typhimurium sometimes cause invasive disease has identified the reason to be a specific genotype of *S.* Typhimurium in the form of sequence type 313 (ST313). This genotype was originally identified through sequencing (84) and is at present almost exclusively found in Africa with increased burden especially in children (79–81). A suggested reason for the emergence of *S.* Typhimurium ST313 as the causative agent for invasive non-typhoidal salmonellosis is that it could be more invasive than non-ST313 variants of *S.* Typhimurium (85).

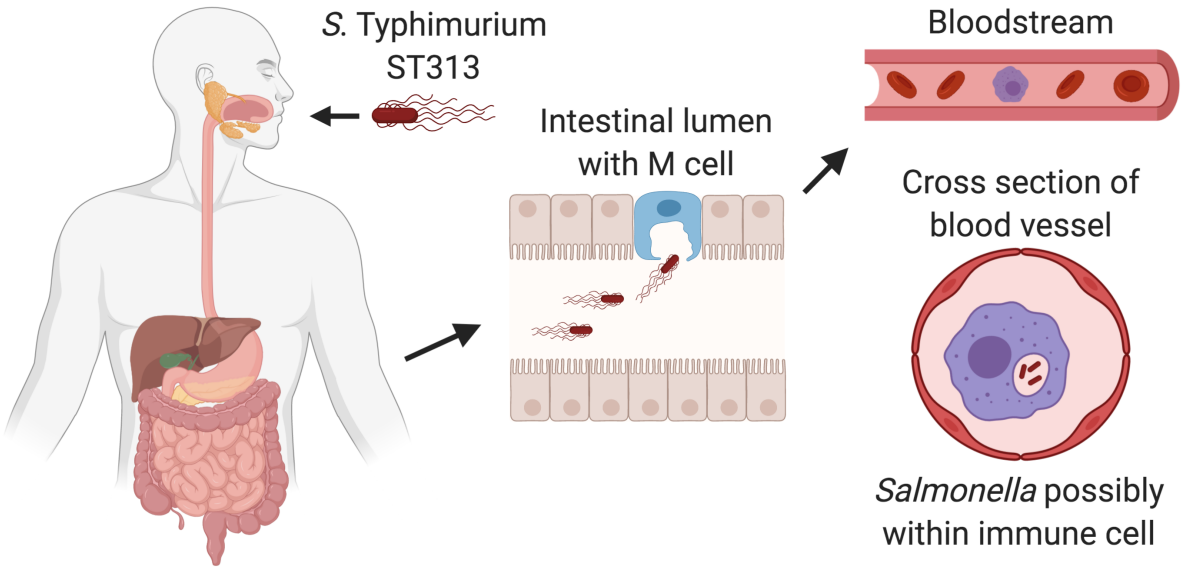

FIGURE 2 Illustration of the pathogenesis of invasive non-typhoidal salmonellosis. Following ingestion of *S.* Typhimurium ST313 the bacteria will travel to the intestine, across the intestinal barrier, and into the bloodstream where it possibly resides within immune cells.

As to the reason for this increased invasiveness one has managed to identify at least one contributing factor in *S.* Typhimurium ST313 by showing it to have enhanced ability to survive within macrophages while also managing to trigger less inflammatory reactions from the host cells (86,87). Both of these details can be seen as beneficial in causing a more systemic disease, compared to non-ST313 variants of *S.* Typhimurium. Additional details on the invasiveness of *S.* Typhimurium ST313 and its' evolution have recently been revealed by studies that have determined that ST313 has evolved from non-invasive *S.* Typhimurium strains by reductive evolution, i.e. genes being mutated into pseudogenes or mutations resulting in up/down-regulation of genes (88). The mutations resulting in enhanced invasiveness can seemingly be pinpointed to changes in the genetic region for the MacAB protein. These proteins make up the MacAB-TolC outer-membrane efflux pump tasked with transporting antimicrobial compounds out of the bacterium (89,90) and resisting oxidative stress (91). Thus, an up-regulation of *macAB* transcription could be one of the reason for ST313s enhanced ability to survive within macrophages (92). How this enhanced ability to survive within macrophages correlates to the mortality caused by seemingly a bloodstream infection has not been studied, but one could possibly expect ST313 to reside within host cells when in blood as is the case for *S.* Typhi during typhoid fever (93–95).

2.4 Non-typhoidal salmonellosis

The third type of disease caused by *Salmonella* is non-typhoidal salmonellosis. This disease is what most people in the Nordic countries associate *Salmonella* with as it is characterized by profuse diarrhea due to gastroenteritis (96). Non-typhoidal salmonellosis is often caused by the same serovars of *Salmonella* as the ones causing invasive non-typhoidal salmonellosis, i.e. *S.* Typhimurium and *S.* Enteritidis. Yet the pathogenesis of the invasive and non-invasive non-typhoidal salmonellosis differs greatly. While non-typhoidal salmonellosis is restricted to the intestine with systemic involvement being rare it still causes a large burden globally; in 2010 the burden was estimated to be 94 million cases of gastroenteritis resulting in approximately 150,000 deaths each year (3).

Following ingestion and arrival to the intestine *S.* Typhimurium will establish itself by using H_2 as a source in intermediate metabolism aiding the bacteria in the initial colonization of the lumen of the intestine (97). From here non-typhoidal salmonellae will not invade deeper tissue, but instead will what seems like actively trigger an inflammatory response from the body by using its needle-like appendage in the form of a secretion system to translocate proteins into enterocytes (98–100). The action of translocating proteins into the enterocytes is thought to be one of the reason leading to the enterocytes recruiting large amounts neutrophils to the site, a hallmark for disease caused by non-typhoidal salmonellae, resulting in inflammation that gives rise to the profuse diarrhea (101–104).

While it seems counterintuitive to actively trigger inflammation several publications have highlighted possible reasons for this. First, after the initial colonization the amount of *S.* Typhimurium is too low so the inflammation aids in flushing out the competing microbiota that is indirectly protecting the host due to the colonization resistance (105–107). Second, the inflammation and influx of neutrophils creates a very hostile environment for any organism present in the intestine by leading to for example lack of oxygen. This environment is taken advantage by *S.* Typhimurium as the bacterium is one of few bacteria with the ability to switch its respiration to one based on tetrathionate. Tetrathionate is a byproduct of oxidation of thiosulphate, created due to reactive oxygen species (ROS) from the inflammation, that can be used by salmonellae as a terminal electron acceptor (108). This ability results in an

overgrowth of *S.* Typhimurium in the gut as it the access to tetrathionate itself aids the bacteria to then use ethanolamine as a carbon source (109). This gives *S.* Typhimurium the opportunity to occupy the space left behind by the microbiota that was flushed out by the inflammation, a detail that can also be seen by the fact that the infection results in the reduction of both amount and complexity of the microbiota in the intestine (106). From hereon *S.* Typhimurium will continue multiplying in the intestine, but is restricted from proceeding further into the host by the immune response and ending up being flushed out of the intestine a few days later.

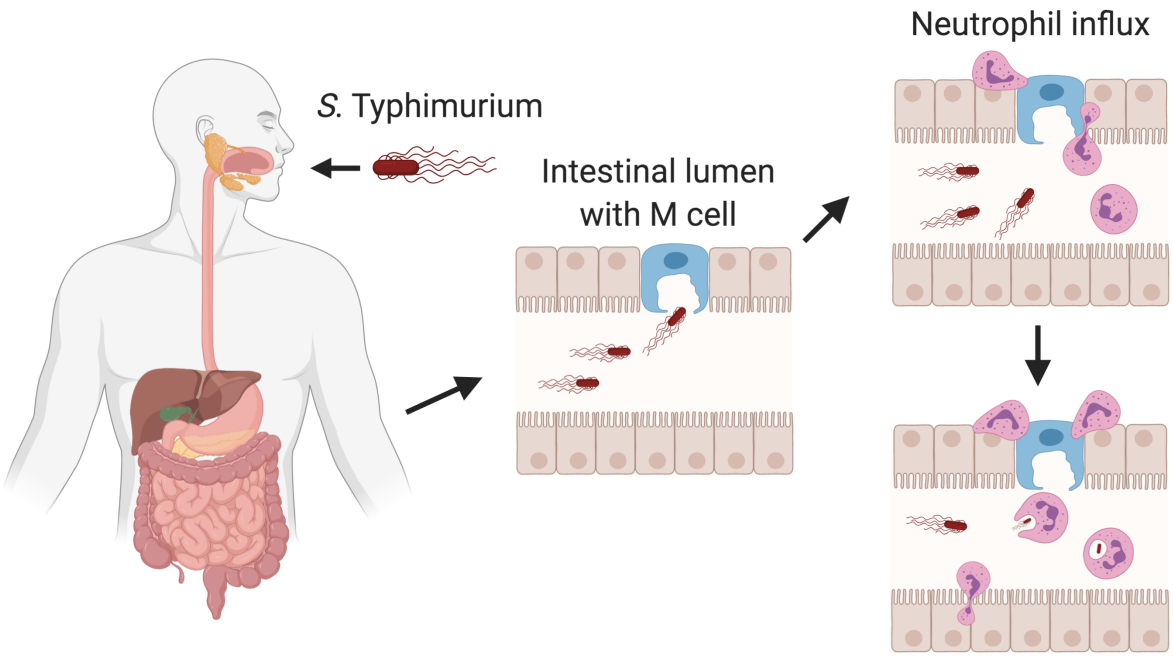

FIGURE 3 Illustration of the pathogenesis of non-typhoidal salmonellosis. Following ingestion of *S.* Typhimurium the bacteria will travel to the intestine where it will induce inflammation. This inflammation manifests as an influx of neutrophils and profuse diarrhea.

2.5 Mouse models of salmonellosis

As briefly remarked in the beginning of this literature review, most details regarding the pathogenesis of the three types of diseases have been mainly studied using mouse models. For example with *S.* Typhi being solely a human-specific pathogen studies to molecular detail are especially cumbersome. In order to understand the pathogenesis of typhoid fever better there is an appropriate match-pair in *S.* Typhimurium and susceptible mice as the pair recapitulating the systemic dissemination seen in human typhoid fever fairly well (110–112). However, prominent mouse models used to study aspects of the systemic dissemination of *S.*

Typhimurium are not wild-type in that the mouse lineages, as for example the BALB/c, are mutants for a protein called Nramp1 (also known as slc11a1) (113).

The importance of Nramp1, a membrane transport protein, comes from it limiting the ability of intracellular pathogens to proliferate within cells (114). The cells where Nramp1 is expressed are the same ones that *S.* Typhi first comes in contact with during typhoidal pathogenesis, in dendritic cells (115), macrophages (116), and neutrophils (117). The function of Nramp1 is to import metal ions into intracellular compartments of these cells (118,119) and in this way possibly affect the oxidative status (120–122) or the expression of other antimicrobial proteins (123) resulting in inhibition of growth of internalized organisms. Hence when this protein is non-functional, it allows for systematic dissemination of *S.* Typhimurium in mice resulting in a typhoid fever-like disease.

Mouse models with non-functional variant of Nramp1 do not however recapitulate carrier-states of typhoid fever due to the mice succumbing to the infection before carriage is established (110). Instead, to study carriage one infects mice with functional Nramp1, such as 129/Sv, with *S.* Typhimurium resulting in the bacterium causing a chronic infection (124,125). Both mouse model using Nramp1-negative mice and Nramp1-positive mice give insights into the pathogenesis of typhoidal fever. Yet neither of these models result in the mice having gastroenteritis, which is characteristic of non-typhoidal salmonellosis. To model this one can also use mouse models, such as C57BL/6, but with the mice being pre-treated with streptomycin. Originally the streptomycin pre-treated mouse model was discovered in the 1950's by showing that pre-treating mice with the antibiotic allowed for lower infectious dose of *S.* Enteritidis (126). The model was systematically launched as a model for colitis caused by non-typhoidal salmonellae a half a decade later (127). As such this model recapitulates one of the hallmarks of pathogenesis of non-typhoidal salmonellosis in humans by showing a massive infiltration of immune cells into the intestine and similar histopathology (127–129). All in all these mouse models have given researchers good tools to understand the pathogenesis of different *Salmonella* on a molecular level with important contributions on identifying important genes for virulence.

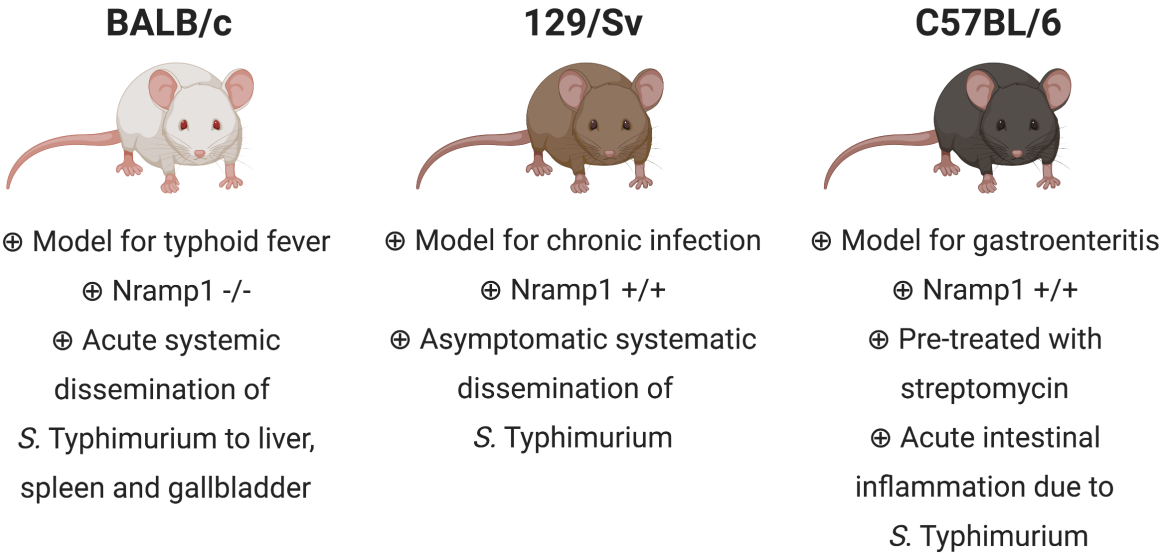

FIGURE 4 Characteristics of some mouse models for research into *Salmonella* infections.

2.6 Macrophage models of salmonellosis

As has been highlighted many times in the text, an important cell type for the pathogenesis associated with *Salmonella* is the macrophage. This detail is specifically pertinent to the systemic dissemination of *S.* Typhi during typhoid fever, and *S.* Typhimurium during typhoid fever-like illness in susceptible mice, as the bacteria can be found within splenic (36,38,41,42) and liver macrophages (37,38,44) during an infection. As such, a lot of research have been performed using macrophage cell lines in order to determine genes that are needed for the ability of *Salmonella* to cause disease. But what is so special and interesting about the fact that *Salmonella* can be found within macrophages? The detail that is the most striking in this relationship is the fact that even though macrophages have numerous functions ranging from bone remodeling to iron recycling, macrophages are mainly known for ingesting foreign agents, such as bacteria, via phagocytosis and then degrading them in intracellular compartments (130).

Currently macrophages are seen as two distinct populations based on their origins, both of which possibly are targeted by *Salmonella.* Previously it was thought that all macrophages in the body would be seeded from the bone marrow continuously by the production of motile monocytes that would then develop into specific macrophages for each tissue. Instead tissue resident macrophages, such as the Kupffer cells of the liver and marginal zone and red pulp macrophages of the spleen, are actually embryonically seeded to each organ and renew there during the lifetime

of the host while monocyte-derived macrophages mainly arrive during inflammation and are distinct from resident macrophages (130–132). Hence even thought it has been postulated that typhoid fever involves *S.* Typhi entering macrophages near the M cells and then disseminating throughout the body within them (as presented in (133)) the reality is probably slightly more complex since tissue resident macrophages are non-motile. Regardless, *Salmonella* is able to grow within these cells evolved to ingest and degrade, making the use of macrophages in cell culture prominent.

One of the most commonly used macrophage cell line in *Salmonella* research are the RAW264.7 cells. This cell line couples well with mouse models used to study the systemic spread of *S.* Typhimurium as the RAW264.7 cells were originally isolated from BAB/14 mice following isolation of tumorous tissue after infecting the mice with Abelson Leukemia Virus (134). This ties the cells very well to the mice used for studying typhoidal pathogenesis, BAB/14 mice are actually BALB/c mice with the single difference of them carrying structural genes for immunoglobulin from another mouse strain (135). Hence, RAW264.7 cells do not produce functional Nramp1, as neither does the BALB/c mice. As such RAW264.7 cells have been used to show the importance of Nramp1 by showing that the functional expression of the protein inhibits the intracellular replication of *S.* Typhimurium in the RAW264.7 that otherwise allow for replication of the bacterium (136).

2.7 Uptake/Invasion into macrophages

The importance of the relationship between *Salmonella* and macrophages for pathogenesis becomes evident by the fact that mutants of *Salmonella* that do not have the ability to survive within macrophages are not able to cause disease (137). But what actually happens when a macrophage ingests a *Salmonella* bacterium?

A first step for the macrophage is to catch and ingest its prey, which most often happens via phagocytosis. Phagocytosis can proceed in a few different ways by for example antibodies or complement factors binding to the bacterium, aiding the macrophage to phagocytize the bacterium (138). However, already at this stage it is not clear if phagocytosis is the only means for *Salmonella* to enter macrophage. This due to *Salmonella* having the ability to actively invade cells, albeit a detail best characterized in non-phagocytic cells, by translocating proteins into host cells using

the needle-like type-three secretion system-1 (T3SS-1) encoded on the *Salmonella* pathogenicity island-1 (SPI-1) (133,139). Instead it has been suggested that *Salmonella* enters macrophages not due to phagocytosis, but actually by the bacteria inducing membrane ruffles in the macrophage resulting in ingestion via macropinocytosis (140). The reason such membrane ruffles are induced is in part due to the proteins *Salmonella* translocate having actin-polymerizing activities that results in changes of the cytoskeleton of the macrophage (141–143). For example proteins SipC and SipA, translocated via T3SS-1, have been shown to actively initialize nucleation of actin polymers and stabilizing the subsequent actin leading to manipulation of the host cytoskeleton (144–147).

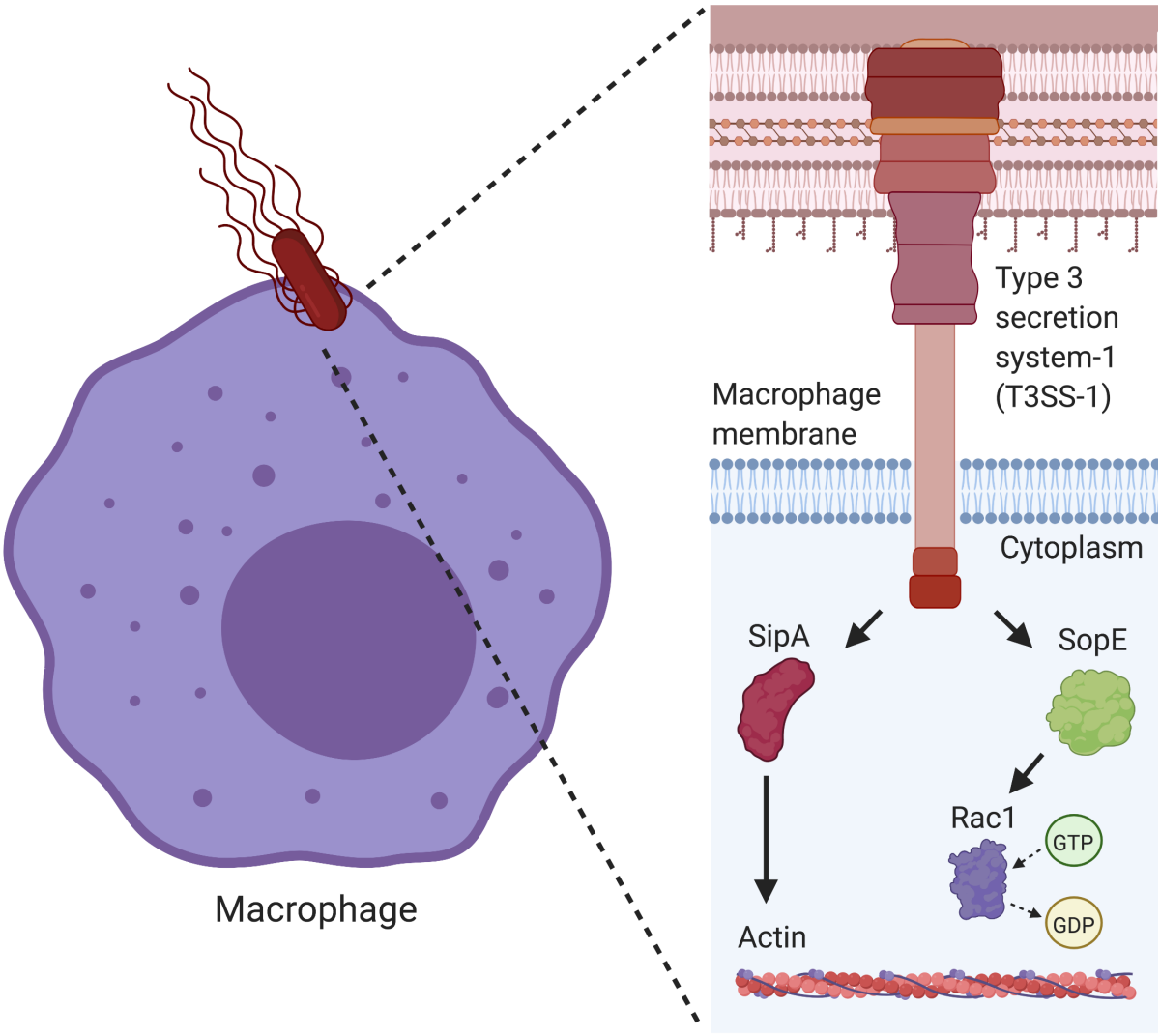

FIGURE 5 Illustration of the injection apparatus used by *Salmonella* when invading host cells. The type 3 secretion system-1 translocates effectors exemplified by SipA and SopE into the host cell resulting in modulation of the actin cytoskeleton and activation of small GTPases.

The translocated SipC and SipA are not enough by themselves to induce invasion of host cells. Examples of other proteins being translocated into the host cell by the T3SS-1 are SopE, SopE2, and SopB. These proteins have in common that they modulate signaling pathways in the cell in order to aid the uptake of *Salmonella*. SopE and SopE2 are both guanine nucleotide exchange factors that work by converting inactivated forms of the host Cdc42 and Rac1 into active forms by exchanging their bound guanine nucleotide from GDP to GTP (148–150). This activation of Cdc42 and Rac1 will lead to downstream effects on the actin cytoskeleton resulting in ruffling of the membrane of the host cell (151–153) resulting in uptake of *Salmonella* (139). SopB on the other hand is not a guanine nucleotide exchange factor, but instead possesses phosphatase activity towards phosphoinositide (154). This enzymatic activity causes accumulation of the signaling molecule phosphatidylinositol-3-phosphate in the host cell leading to formation of the phagosomes wherein *Salmonella* will initially reside within the cell (155–157).

2.8 First phase within the macrophage

Whether the majority of *Salmonella* enter macrophages by actively invading or through phagocytosis it seems that the subsequent steps of the pathogenesis of *Salmonella* within the macrophage are independent of means of entry (158). When inside the cell *Salmonella* finds itself in what looks like a normal phagosome (159) used to ingest and degrade any bacteria. However, this phagosome will instead quickly develop into a spacious phagosome (140) that will eventually end up becoming a *Salmonella*-containing vacuole (SCV) (160–162). What distinguishes these SCVs from a normal phagosome, or the subsequent phagolysosome, is the fact that the intracellular compartment is under the control of *Salmonella* resulting in the SCV having a modified profile of host proteins associated with it (133,160). This control is mediated by *Salmonella* using an other secretion system T3SS-2 encoded on the SPI-2 genetic region that is induced in *Salmonella* during intracellular growth (163).

While the T3SS-2 is similar to T3SS-1 in structure, the proteins being translocated into the host cell via T3SS-2 *Salmonella* have different functions. Many of these proteins have postulated to have specific tasks in trying to inhibit the mechanisms that the macrophage employs in trying to kill *Salmonella*. The importance of the

T3SS-2 translocated proteins is very clear from the fact that *Salmonella* lacking the capacity to produce these components is not able to proliferate in mice, nor within macrophages in cell culture (164–167). Following the formation of the early SCV the process of acidification of the SCV starts from the neutral pH of the contents arriving from outside the cell and eventually reaching pH 4.5 as the SCV progresses towards the phagolysosomal stage (168). Even though this acidification is a normal part of the degradative pathway for the macrophage *Salmonella* does not inhibit it from taking place, but rather slows the process down (169). In contrast, the acidification of the SCV is required for *Salmonella*s ability to grow within macrophages (170) due to acidity being a signal for secreting some of the proteins modulating the SCV (169,171).

2.9 Avoiding killing by the macrophage

In general the stage when most organism internalized by macrophages get killed is when the phagosome that the organism resides in fuses with lysosomes. Lysosomes are intracellular vesicles containing degradative enzymes that when fused with the phagosome create a phagolysosome (168). For *Salmonella* to be able to survive within macrophages it has to avoid this fusion of the early SCV and the lysosomes (172,173). It is thought that SPI-2 is important for this avoidance, however, there are no studies showing a direct mechanism of how *Salmonella* would inhibit this lysosome-to-phagosome fusion. Instead there are refuting data indicating that *Salmonella* actually does not inhibit fusion of the SCV with lysosomes, but instead simply resists killing by the lysosomal content (174–176). A similar finding has also been observed in an other study, albeit not with macrophages, but instead with epithelial cells (177). There might be somewhat of a middle way between these contradicting studies in that a study proposes that *Salmonella* manages to do avoids being killed by lysosomes by segregating its population into several SCVs. In this way there simply aren't enough lysosomes to fuse with the various SCV and hence the lysosomal content are depleted by *Salmonella* letting part of the population be targeted while the rest continue proliferating within SCVs (178).

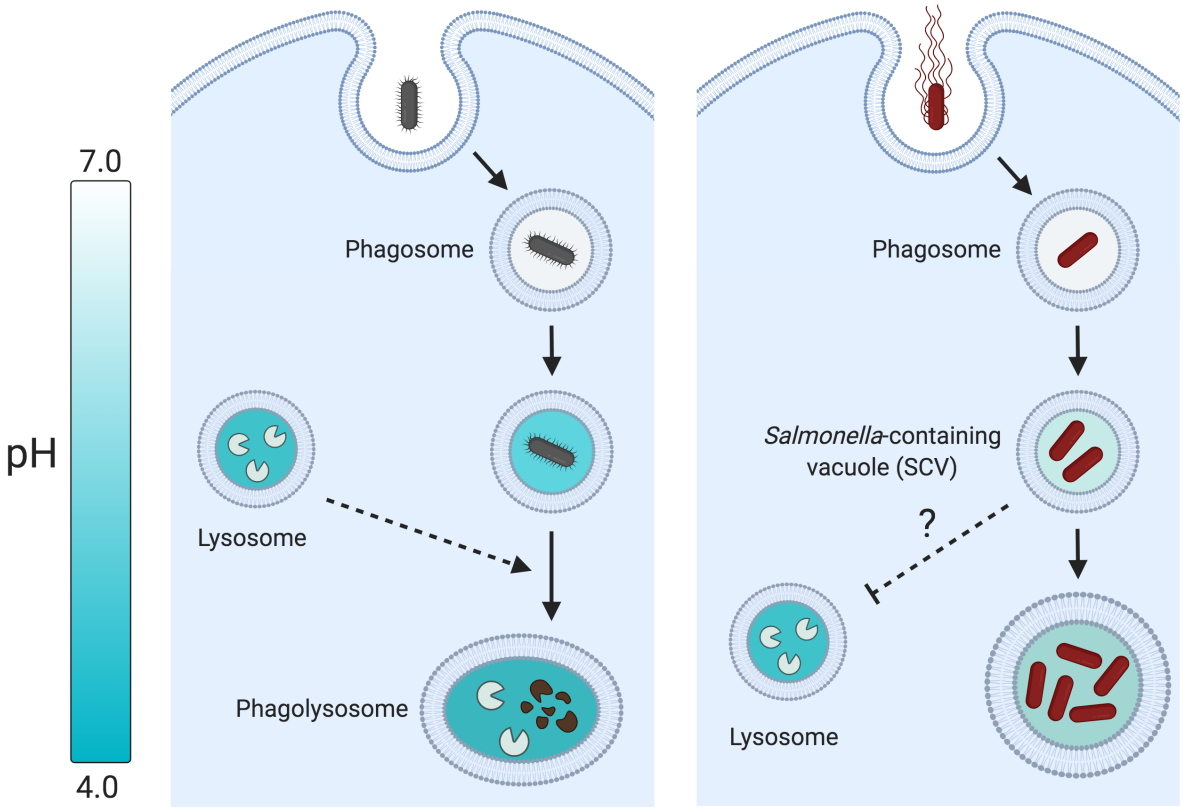

FIGURE 6 Illustration of the endocytic process of a macrophage when ingesting a non-pathogenic bacteria (left) compared to *Salmonella* (right). pH of the phagosomes containing the non-pathogenic bacteria acidifies faster compared to the ones containing *Salmonella*. *Salmonella* also possibly is able to inhibit lysosomal fusion with the phagosome.

Whatever steers the relation between lysosomes, lysosomal fusion and the SCV, this form of killing is not the only way for a macrophage trying to combat intracellular *Salmonella.* A very important additional aspect is the ability of macrophages to utilize redox chemistry. This includes production of ROS and reactive nitrogen species (RNS) that target *Salmonella* within the compartment it resides in. One very prominent enzyme complex in this ROS-mediated killing is the NADPH oxidase. NADPH oxidase uses oxygen and NADPH to produce superoxide and hydrogen peroxide in an event known as the respiratory burst (179–181). The importance of NADPH oxidase in controlling an infection with *Salmonella* has been shown experimentally in mice and macrophages in cell culture where the lack of NADPH oxidase impairs the hosts ability to resist a *Salmonella* infection (182–184). As a matter of fact the importance of NADPH oxidase is not only seen in experimental settings but also in humans in the disease called chronic granulomatous disease, a disease involving mutation in genes encoding for components of NADPH oxidase,

resulting in increased susceptibility towards intracellular pathogens such as *Salmonella* (185).

But how come *Salmonella* is still able to cause infections even in macrophages and mice with functional NADPH oxidase? Once again it seems that the answer can be found within the genetic region of SPI-2 which has been shown to allow for *Salmonella* to evade the NADPH oxidase by somehow blocking the enzyme complexes association with the membrane of the SCV (186–188). Yet, as with lysosomal fusion to the SCVs, similar contradictions exist for the NADPH oxidase in that the effect of the enzyme is more pronounced in the very beginning of the macrophage ingesting *Salmonella*, a time-frame where SPI-2 is proposed not to be active. This points to a temporal discrepancy regarding on how SPI-2 could block NADPH oxidase (189). Additionally, the actual importance of the NADPH oxidase in macrophages during a *Salmonella* infection in mouse might be confused with that of the effect of NADPH oxidase of neutrophils and monocytes, as is proposed in a study showing that macrophage NADPH oxidase does not kill *Salmonella* efficiently during infection, but that of the neutrophils and monocytes do (190).

Still, SPI-2 is not the only defense *Salmonella* uses towards the effects of the NADPH oxidase. As nothing is black or white in biology, but much of it is grey, *Salmonella* still gets exposed to ROS that have the ability to damage bacterial periplasmic proteins (191). For this *Salmonella* has enzymes that detoxify such ROS one of which is called superoxide dismutase (192,193). Superoxide dismutase works by converting superoxide radicals into less reactive hydrogen peroxide. This has been shown to be important for *Salmonella* during growth within macrophages and in mice (191,193,194) by a proposed mechanism that superoxide dismutase does not let superoxide reach targets in cytosol of the bacterium (195). The issue is not solved by superoxide dismutase alone as the by-product of its enzymatic activity is creating hydrogen peroxide. But *Salmonella*s defense extends beyond superoxide in that it also possesses catalases and alkyl hydroxyperoxide reductases, enzymes that detoxify hydrogen peroxide into water and oxygen (192), while also having the ability to regulate the permeability of the outer membrane to block entry of hydrogen peroxide (196). As superoxide dismutase, the hydrogen peroxide quenching enzymes catalase and alkyl hydroxyperoxide reductase have also been shown to be important for the bacterium's ability to grow within macrophages (197–199).

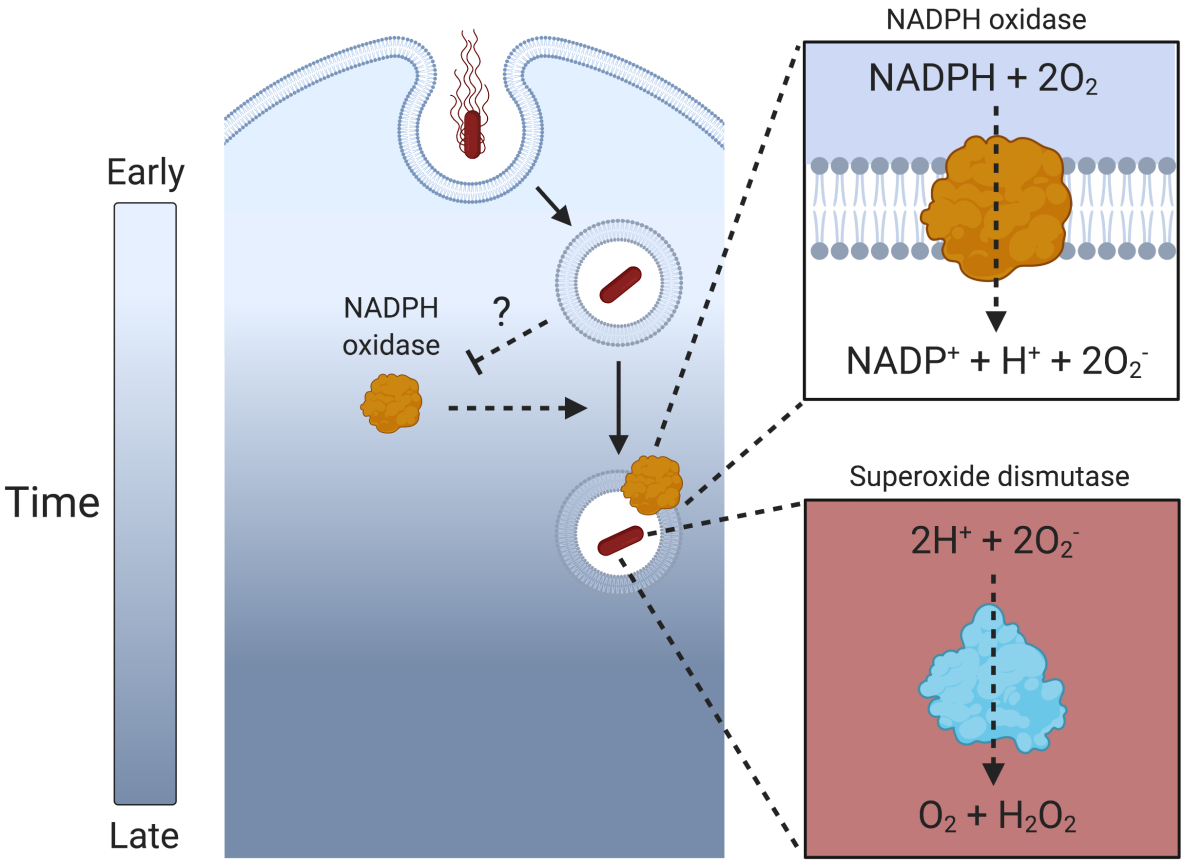

FIGURE 7 Illustration of the innate immunity effector NADPH oxidase and its production of superoxide and the superoxide detoxifying enzyme of *Salmonella* in superoxide dismutase. Chemical equations simplified.

Yet, with the lysosomes and ROS one would think that this would be enough of an arsenal for the macrophage to fight invaders, but still the macrophage has additional mechanisms of killing. One of these additional mechanisms are very analogous to the ROS-mediated killing in the form of RNS (200–202). These RNS are produced as by-products following production of nitric oxide by the inducible nitric oxide synthetase (iNOS), an enzyme mainly found active in the cytosol of macrophages (203–206). The produced nitric oxide can then further react with for example proteins involved in metabolism that contain cysteines (207) or with ROS produced by the NADPH oxidase resulting in new toxic compounds (203,208). This synergy between the NADPH oxidase and iNOS allows for the creation of peroxynitrate, a very strong oxidant with vast biocidal activity (209–211). All of these actions contribute to the importance of iNOS in defense against pathogens as shown by iNOS being needed for efficient killing of *Salmonella* in mice and within macrophages in cell culture (182,183,212–215).

However, as with NADPH oxidase *Salmonella* has also defense mechanism against iNOS similar to the ones used for counteracting NADPH oxidase in the form that the SPI-2 genetic locus is needed for *Salmonella* to survive in macrophages having iNOS activity (216). How this SPI-2 mediated defense against iNOS works is not known, but curiously enough it seems that it's an arms race in the fact that nitric oxide itself generated by macrophages is able to repress the transcription of SPI-2 (217). Other than SPI-2 the main protein in defense against nitric oxide is the flavohemoglobin Hmp, which is under regulatory control of the NsrR regulon (218), whose enzymatic function involves detoxifying nitric oxide by converting it to nitrate. The ability of Hmp to do this has been shown important for growth in macrophages in culture and in mice, hence most likely relating it to the defense against iNOS (219,220). In somewhat of a similar fashion it has been proposed that the nitrate transporter NirC is also involved in protection against iNOS in mice and macrophages by the way of reducing the amount of nitric oxide in the bacteria (221). While an other mechanism of defense against nitric oxide is simply to absorb the damage by quenching it via antioxidants such as glutathione (222).

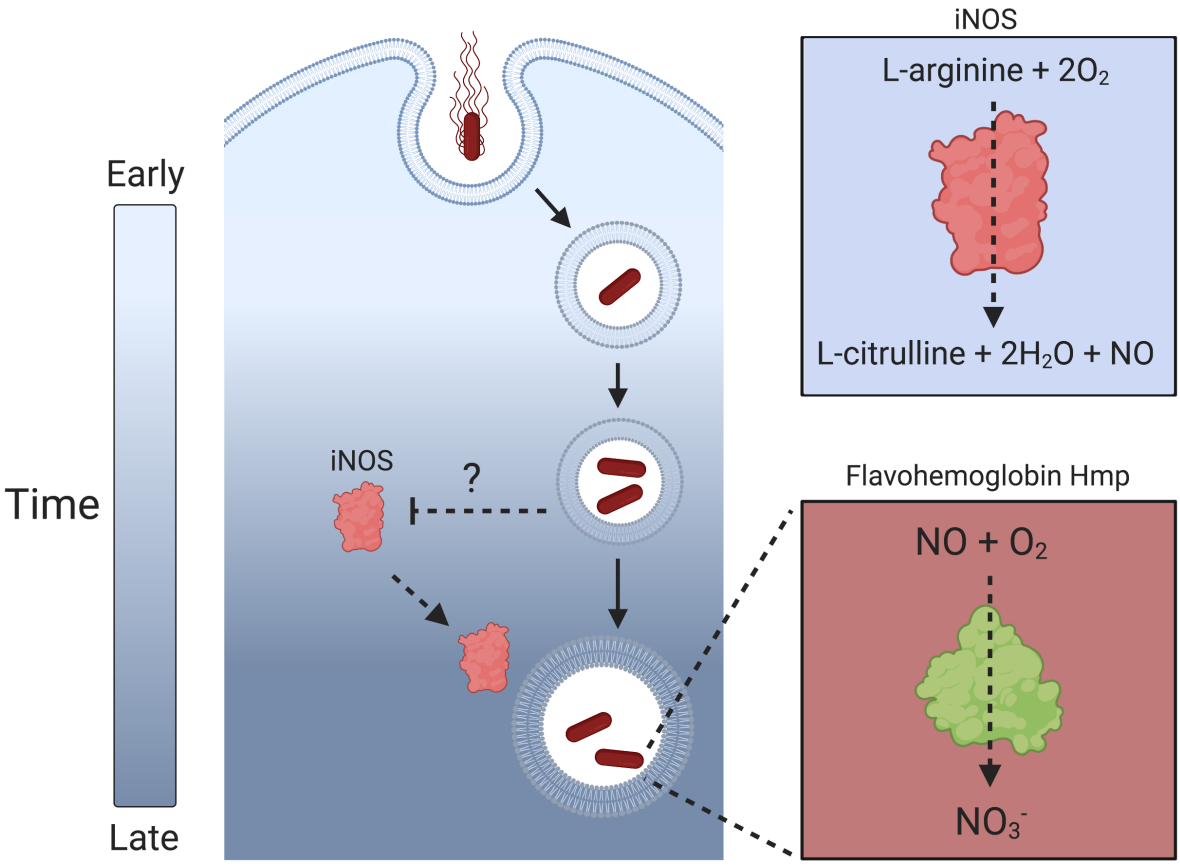

FIGURE 8 Illustration of the innate immunity effector iNOS and its production of nitric oxide and the nitric oxide detoxifying enzyme of *Salmonella* in flavohemoglobin Hmp. Chemical equations simplified.

2.10 Creating conditions for proliferation within the macrophage

In the event that *Salmonella* manages to survive all of the assaults from the macrophage it will find itself creating a network of intracellular compartments that allows for proliferation. One of the effector proteins encoded by SPI-2 that is needed to establish this is SifA. Although most studies performed on the role of SifA in forming the network of intracellular compartments called *Salmonella*-induced filaments (SIF) has been performed in epithelial cells (reviewed in 145,203–205) the importance of SifA in macrophages is unquestionable, albeit it is unclear if actual SIFs form in them (226). First, if *Salmonella* is lacking SifA it will be more likely found in the cytosol of the macrophage, instead of in the vacuolar compartment of the SCV, indicating that the protein is important for for the integrity of the SCV in macrophages (36,227,228). How SifA helps in the maintenance of integrity of the SCV in macrophages is not fully understood, but data from mainly epithelial cells infected with *Salmonella* indicates that SifA blocks the accumulation of motor proteins, such as kinesin, to the SCV and this way possibly decreases the membrane turnover of the SCV leading to higher stability of the compartment (229,230).

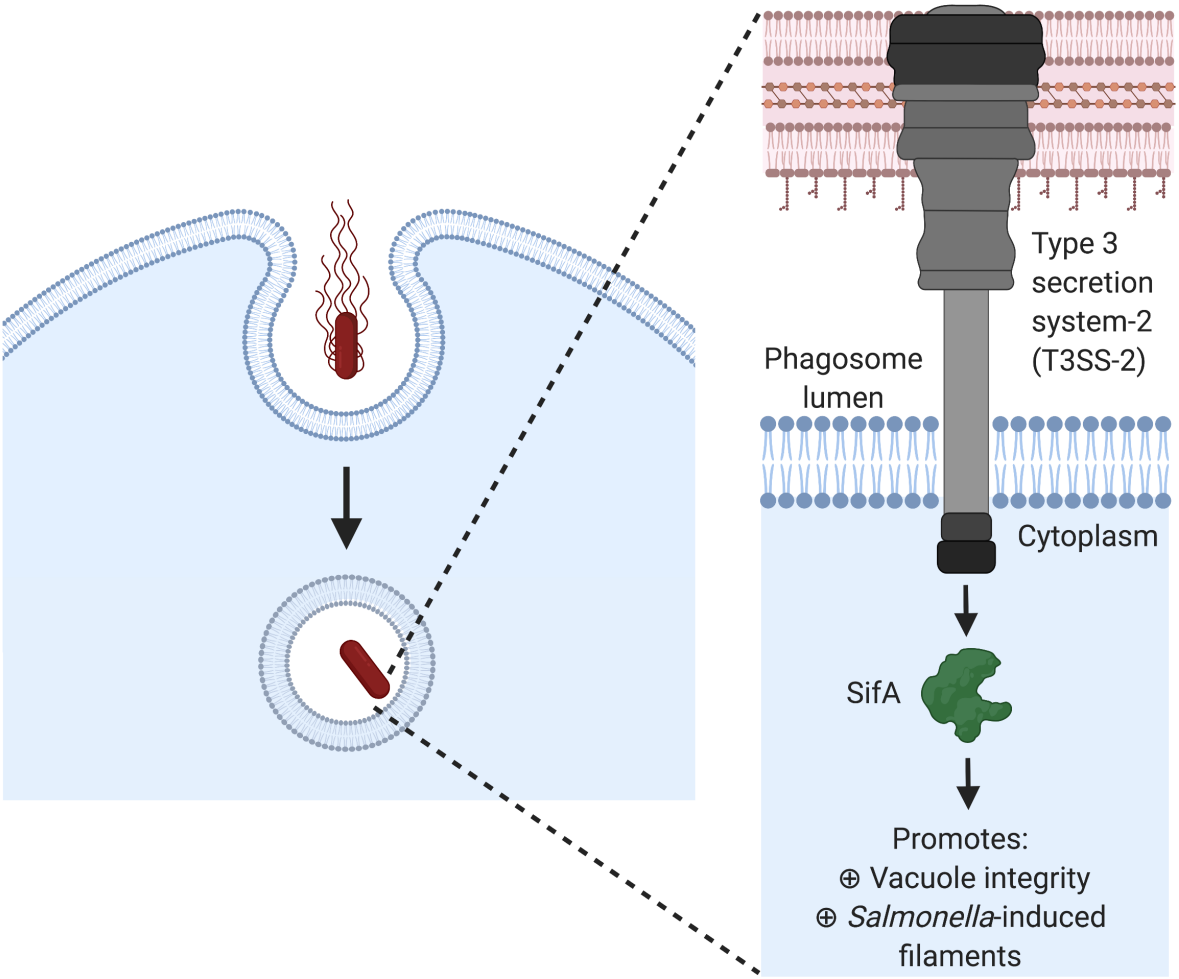

FIGURE 9 Illustration of the injection apparatus used by *Salmonella* when within the host cell. The T3SS-2 translocates effectors exemplified by SifA into the host cell resulting in stability of the vacuole within which *Salmonella* resides.

The establishment of these stable intracellular compartments in the form of SCVs is a prerequisite for *Salmonella* to be able to proliferate within the cell. This due to the fact that the structures formed with the help of SifA promote the nutritional acquisition from other intracellular compartments into the SCV (231,232), an important aspect of intracellular pathogenesis (233–237). However, *Salmonella* seemingly is able to also acquire nutrients into the host cell where it resides by somehow stimulating the cell. This is the case for iron, an important metal for many organisms, through the fact that *Salmonella* is able to steer macrophages to phagocytize erythrocytes and in this way gaining access to increasing amounts of iron while within the macrophage (38,238–240).

Even though the majority of the aspects regarding the intracellular life of *Salmonella* in the macrophage that have been highlighted in the text above have mainly been

determined by experimentation in mice and cell culture using *S.* Typhimurium the results of such studies most likely highlight details also pertaining to human typhoid fever. For example the intracellular life of *Salmonella* in humans has also been established during diagnosis of typhoid fever via blood cultures and bone marrow biopsies as *S.* Typhi is mainly found within cells in the samples (93–95), hence corroborating the relevance of studies on intracellular *S.* Typhimurium in various infection models.

3 RESEARCH AIMS

Antibiotic resistance and intracellular pathogenesis were the main focus of this thesis with both being important aspects of the biology of *Salmonella*. Studies on these issues were undertaken to decipher new information on genetic and molecular determinants needed by *Salmonella* to resist an antibiotic, as well as to proliferate within host cells.

As such one aim of Paper I was to study the importance of muramyl endopeptidases in the ability of *S*. Typhimurium to resist antibiotics. For Paper II the aim was to characterize the importance of a regulator of said muramyl endopeptidases in the form of how periplasmic protease Prc affected the fitness of *S*. Typhimurium during intracellular pathogenesis in macrophages and during infection in mouse. For Paper III the aim was to characterize the response of innate immunity effector iNOS in macrophages on a single cell level during infection with *S*. Typhimurium.

4 MATERIALS AND METHODS

4.1 Bacteria

The bacteria used in this thesis can be divided into two categories. One of the categories includes the *Salmonella* strains under study, *S.* Typhimurium SR-11 and *S.* Typhimurium 14028. Occasional confirmatory experiments were also performed using *S.* Typhimurium SL1344. The parent of strain *S.* Typhimurium SR-11 was originally isolated from infected mouse Peyer's patches (241) and selected further for a more virulent variant able to cause chronic infection resulting in *S.* Typhimurium SR-11 χ4665 used in this thesis (242). *S.* Typhimurium 14028 is a laboratory strain obtained from the American Type Culture Collection and *S.* Typhimurium SL1344 is derived from a study attempting to create a live *Salmonella* vaccine (243).

The other category is bacteria used for genetic manipulation. This category includes *S.* Typhimurium LB5010, a mutant for the DNA restriction modification system (244), which was the main strain used when making mutations into the genome of *Salmonella*. From this strain the mutations were then transferred to either SR-11, 14028 or SL1344 by transduction with the help of phage P22 (245). For cloning intermediary strains of *Escherichia coli* (*E. coli*) Top10, available from Thermofisher, or *E. coli* TG1, available from Nectagen, were used before purification and transformation into *S.* Typhimurium SR-11 or 14028.

4.2 Genetic manipulation of bacteria - Removing genes

Many experiments in this thesis are based on genetic manipulation in the form of removal of genes of *S.* Typhimurium followed by phenotypic observations. This is done by so called recombineering (246). Recombineering is based on homologous recombination where DNA fragments, in the form of a selection marker, are introduced into bacteria via electroporation. This DNA fragment is then subsequently incorporated into the genome, with the help of an exogenous recombineering system, in place of a gene of interest. The bacterium is henceforth described as Δ*gene*-mutant for the gene that was deleted.

More specifically, mutants created in this thesis are done using the pSIM recombineering system (247). To be able to create mutants one has to produce the DNA fragment using PCR. The DNA fragment is to contain an antibiotic resistance

gene, i.e. the selection marker, that is flanked by the upstream and downstream regions of the gene one intends to remove. This can be accomplished by first designing primers, using annotated genome sequences from the NCBI database, by which one part of the primer anneals to the upstream or downstream region of the gene of interest and the other part anneals to a template plasmid containing the selection marker. The designed primers and template plasmid are then mixed and the final DNA fragment can be purified following PCR.

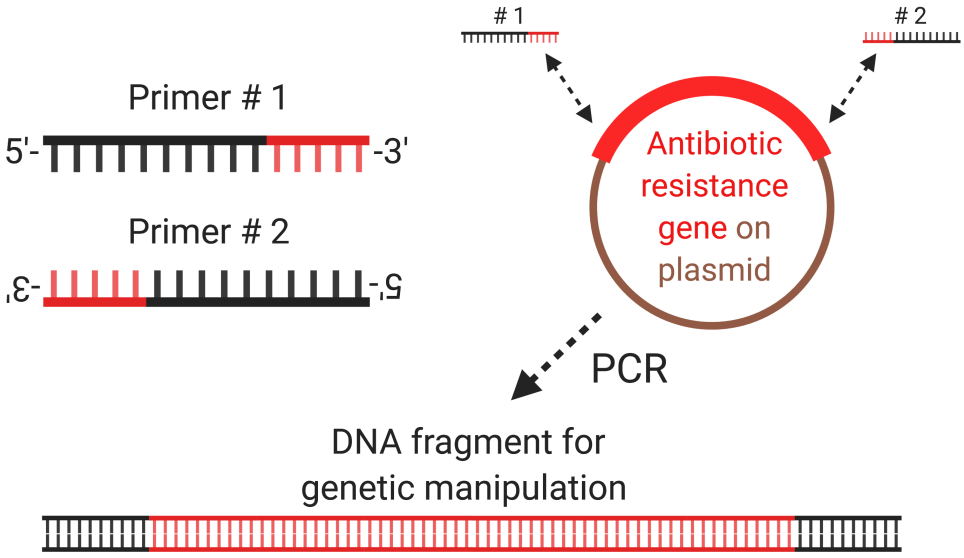

FIGURE 10 Illustration of the principles in creating a DNA fragment used to mutate/remove genes. The black part of the primers corresponds to sequences that are upstream (primer #1) or downstream (primer #2) of the gene of interest. The red part corresponds to sequences of the selection marker on the plasmid and are used in the PCR to amplify the antibiotic resistance gene by using the plasmid as a template. This way the created DNA fragment has an antibiotic resistance gene flanked by sequences matching the bacterial chromosome.

Having produced the DNA fragment bacteria are prepared for recombineering. To start off a pSIM plasmid is introduced into the bacteria one wishes to mutate, i.e. *S.* Typhimurium LB5010, and the bacteria are then grown to the exponential growth phase at 32°C. This temperature is important since the pSIM plasmids contains a temperature sensitive origin of replication. After reaching the exponential growth phase the bacteria are moved to 42°C in order to induce the temperature-dependent expression of the bacteriophage λ recombination system from the plasmid. This recombination system consists of three genes encoding the proteins Exo, Beta and Gam (246,247). Exo exonuclease is thought to trim the incoming DNA fragment to allow for Beta, a single strand DNA binding protein, to protect the newly trimmed

fragment with Gam inhibiting the endogenous bacterial degradation system meant to target incoming foreign DNA (246,247).

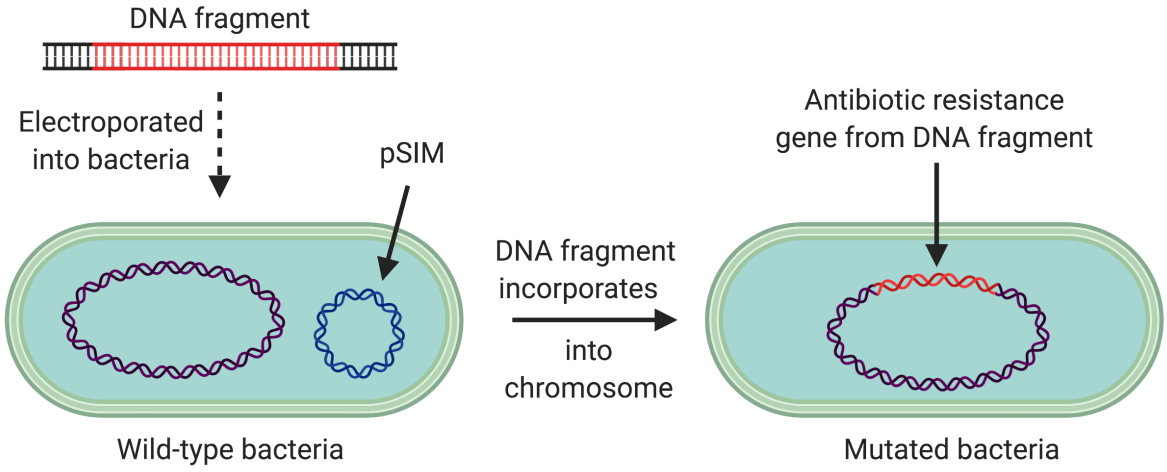

FIGURE 11 Illustration showing DNA fragment created with PCR being electroporated into bacterial cell. The DNA fragment will be incorporated into the chromosome in the place of a gene of interest, based on the homologous sequences on the DNA fragment (in black), with the help of λ recombination system induced from the pSIM plasmid. This will yield a mutated bacteria.

Following induction the bacteria are prepared for electroporation by washing and mixing with the PCR-generated DNA fragment, electroporated, and left to recover in growth media resulting in transformation and recombination of the DNA fragment. After recovery the bacteria are plated onto agar plates containing the antibiotic corresponding to the selection marker designed into the DNA fragment. The colonies yielded the next day are the ones that have successfully incorporated the DNA fragment in the place of the gene of interest and hence a mutant has been created. From hereon the mutation is transferred with the help of phage P22 via transduction into SR-11, 14028 or SL1344.

4.3 Genetic manipulation of bacteria - Complementing genes

Having observed a phenotype in a mutant one can confirm that the gene of interest is responsible for said phenotype by genetic complementation. In this one transforms a plasmid containing the gene of interest back into the mutant to observe whether the phenotype is reverted back to wild-type. In this thesis the plasmids containing genes of interest have been produced by both restriction digestion/ligation cloning method and *in vivo* assembly (IVA) cloning.

Cloning via restriction digestion/ligation is based on designing primers to amplify a gene of interest from a chromosomal template in parallel with designing restriction sites into the primers. The choice of the restriction site has to match the plasmid vector of choice, i.e. the plasmid one wants to clone into, as plasmids have multiple cloning sites incorporated into them consisting of several restriction sites. Once the gene of interest has been amplified by PCR both the DNA fragment and the plasmid vector are digested using restriction enzymes. This results in two linear DNA fragments, i.e. the PCR product and the plasmid, that have corresponding overhangs resulted from the cleavage. These can now be fused together via a ligation reaction where a ligase, an enzyme ligating DNA together, will couple the digested DNA fragment and plasmid together at the cleavage sites. This plasmid will then be transformed into appropriate bacteria for further replicative amplification and purification.

A more recent, and frankly more simple, way to perform cloning is by using IVA cloning (248), a method based on the endogenous homologous recombination system of the bacteria. One designs primers that will amplify the gene of interest from a chromosomal template, but instead of designing restriction sites into the primers one adds sequences that will overlap with the plasmid vector one intends to clone into. Similarly one designs primers that will create a linear version of the plasmid vector when amplified in PCR. This way one has a DNA fragment containing the gene of interest flanked by overlapping regions to the linear plasmid vector. The DNA fragment and linearized vector are then transformed into bacteria whereby they will be fused by the homologous recombination system of the bacteria. The plasmid is then purified from the recipient and transformed into the appropriate strain.

4.4 Antibiotic sensitivity testing

For testing antibiotic sensitivity this thesis employs three different methods in disk diffusion, broth dilution, and drop-on-lawn. In disk diffusion one adds a specific amount of an antibiotic solution to a paper disk. This paper disk is then placed on an agar plate where bacteria shortly before have been evenly spread out on. After allowing for overnight growth one is able to measure an inhibitory zone around the antibiotic disk. The measurements can then be compared between wild-type and mutants. Broth dilution is used to determine the minimum inhibitory concentration

(MIC) by culturing bacteria in growth media containing a scale of concentrations of a specific antibiotic. The MIC is determined as the lowest concentration where no bacterial growth is visibly present in the media following incubation. Drop-on-lawn can be used to visualize antibiotic sensitivity by applying a dilution series of bacteria in droplets onto an agar plate infused with a specific antibiotic. Following incubation one can observe differences between wild-type and mutants by looking at whether there is differences in growth of the droplets of the dilution serie.

4.5 β-galactosidase release assay

β-galactosidase (LacZ) release assay can be used to study autolysis in *Salmonella* as it does not possess the gene for LacZ, unlike *E. coli*. In this thesis the plasmid pKTH3088, which constitutively expresses LacZ, is transformed into *Salmonella*. This results in the cytosol of wild-type and mutants containing LacZ with the enzyme only being present outside the bacterial cell if the cell undergoes lysis. The presence of LacZ can be measured by adding ortho-nitrophenyl-β-galactoside that when metabolized by LacZ yields a colour change. Hence, when bacteria containing pKTH3088 are subjected to a trigger for autolysis, for example a β-lactam antibiotic, one can measure this by collecting the supernatant and determine the amount of extracellular LacZ by inferring from colour intensity and in this way know whether a mutant is more likely to autolyse compared to wild-type.

4.6 Gentamicin protection assay

Gentamicin protection assay can be used to measure various aspects of host-pathogen interactions such as the ability of bacteria to invade cells or to grow within them. This method is not exclusive to *Salmonella* research as it can also be used for other intracellular bacteria with the blueprint for the method already published in the 1970's (249). A few years later it was shown that the antibiotic gentamicin is poor at killing intracellular bacteria, possibly due to it not entering eukaryotic cells, leading to the creation of what now is known as the gentamicin protection assay (250).

In this thesis the gentamicin protection assay is used to determine the ability of *Salmonella* to proliferate within RAW264.7 cells during an overnight infection and as a basis for microscopy in order to study the innate immunity effectors of RAW264.7

cells. For these experiments the RAW264.7 cells were cultured in a 24-well plate and infected with *S.* Typhimurium at a multiplicity of infection (MOI) of 10. Cells were then incubated 37°C and 5% CO_2 for up to 1 hour after which the media was replaced with media containing gentamicin. After this, media containing gentamicin was removed with further experimentation depending on the aim of the study; one can lyse the macrophages and retrieve the bacteria in order to determine the amount of internalized bacteria or leave bacteria to grow overnight with either retrieving the bacteria the to see the proliferation or to fix the sample and stain them for microscopy.

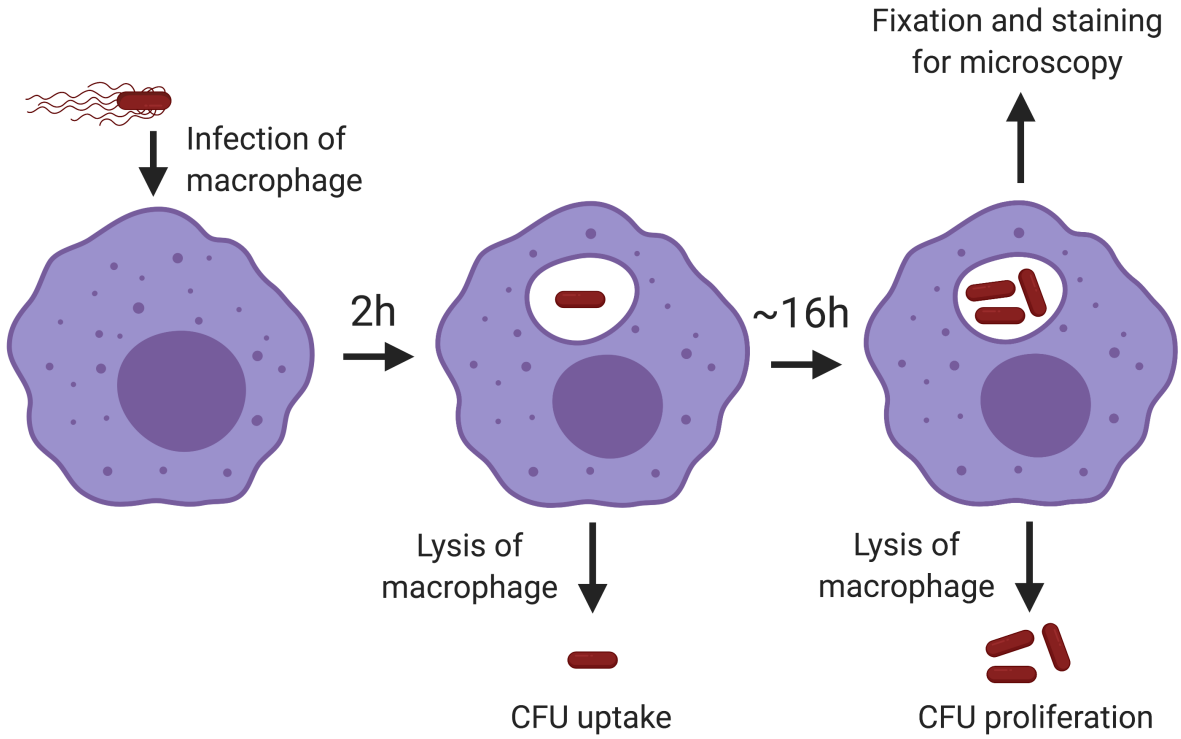

FIGURE 12 Illustration of the gentamicin protection assay and the various uses of it in this thesis. The first two hours post-infection contain killing of extracellular bacteria by gentamicin. CFU stands for colony-forming unit which is determined by calculating number of colonies on agar plates following lysis of the macrophages and retrieval of the internal bacteria.

4.7 Immunofluorescence microscopy

In this thesis immunofluorescence microscopy was used for detection of hypoxia, presence of iNOS, and protein synthesis in RAW264.7 cells. All microscopy starts off with fixation of the samples, often by formaldehyde. Immunofluorescence is based on antibodies conjugated with fluorescent molecules, applied after fixation, from which the signal can then can be detected when imaging using a fluorescence

microscope. These antibodies are often not directly binding to the signal that is being measured, but instead function as signal amplifiers. In the case of hypoxia the primary molecule that the antibody binds to is pimonidazole, while for protein synthesis the antibody binds to is puromycin, and for detecting iNOS the antibody binds to an anti-iNOS antibody that itself is directly binding to iNOS.

4.8 Competitive infection in mouse

To study whether mutations in the genome of *S.* Typhimurium affect the bacterium's fitness in a more complex system compared to a cell culture, competitive infection in mice can be used. The competition between a mutant and wild-type is performed by inoculating the bacteria in a 1:1 mutant/wild-type ratio, determined using optical density and confirmed by enumerating the colony-forming unit (CFU) of each, here applying BALB/c mice. Three days later the mice were sacrificed and liver, spleen and gallbladder harvested. From the organs the ratio of the mutant to wild-type is determined by recovering the bacteria and enumerating the CFU for mutant and wild-type, which can be done due to the mutant having a selection marker.

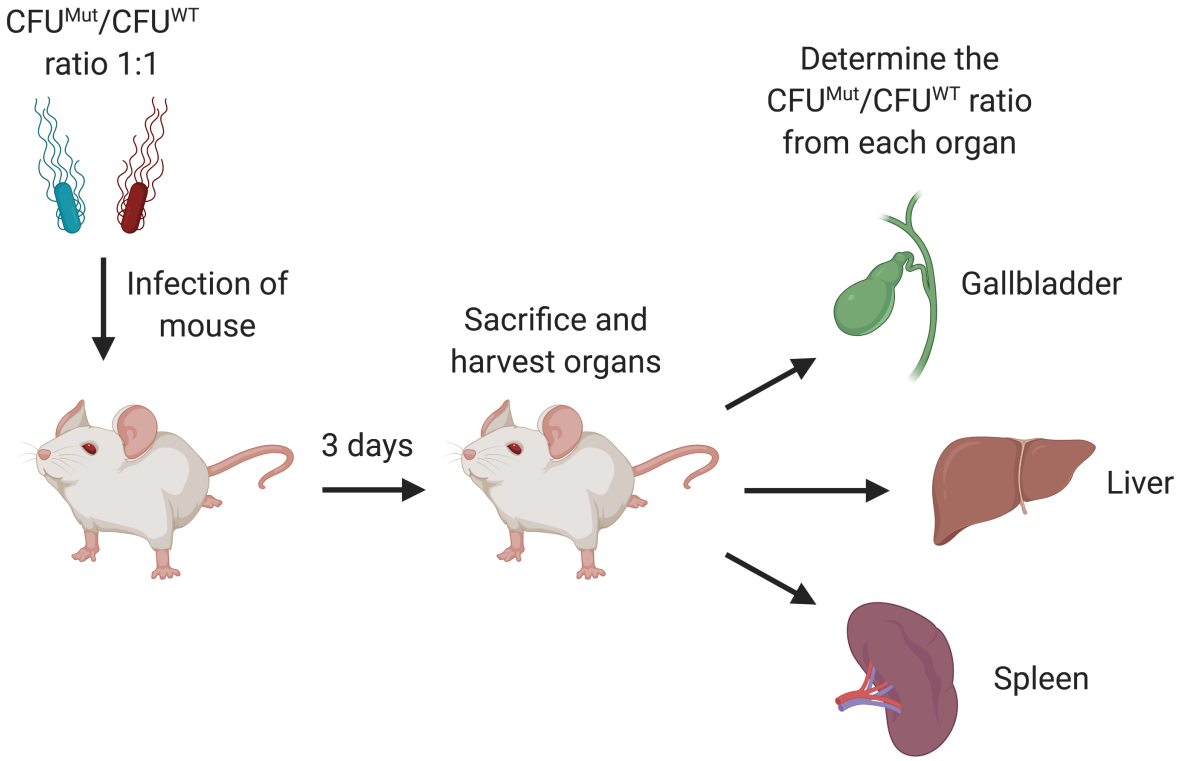

FIGURE 13 Illustration of a competitive infection in mice. CFU^{Mut} stands for the colony-forming unit of the mutant bacteria and CFU^{WT} stands for the colony-forming unit of the wild-type.

4.9 Ethical considerations

As has been described in the literature review section, the human-specific *S.* Typhi causes typhoid fever which is a disease with high mortality. Due to this it is cumbersome to work with *S.* Typhi as the safety protocols involved in working with *S.* Typhi require trained personnel and special laboratories. However, as a complementary way to try to understand the pathogenesis of *S.* Typhi researchers have used the less pathogenic *S.* Typhimurium instead. The advantage with choosing *S.* Typhimurium is that one of its natural hosts is the mouse and in experimental infections the mouse recapitulates various aspects of the human-specific disease typhoid fever. As *S.* Typhimurium requires standard biosafety protocols for a pathogen it makes *Salmonella* research available to more researchers while also enabling faster progress.

The ethical considerations within this thesis mainly in regard to experimentation using animals. However, in order to minimize this the work in this thesis is always first performed in cell culture setting when studying the pathogenesis of intracellular *Salmonella* infection. Only thereafter did we move onto animal models in an effort to try to confirm if our observations made in a cell culture settings holds in a host animal, a much more complex environment for the bacteria, in order to asses whether there is a possibility of our findings to be translated into potential future targets for treatment for example. Hence I believe the ethical aspects of using mouse models to study *Salmonella* pathogenesis are a matter of balancing cost to benefit. I believe that the single animal experiment performed in this thesis outweighs the ethical costs by offering great insight into the role of a specific enzyme in the pathogenesis of *Salmonella* and this knowledge could be used in the future to help design treatments that will eventually help humans.

5 RESULTS AND DISCUSSION

5.1 Paper I

In Paper I we set out to study proteins involved in degradation of peptidoglycan (also known as murein) of the cell wall within *S.* Typhimurium. As a vast majority of antibiotics that have been successful throughout history one way or another target the cell wall such proteins are of interest. However, most such antibiotics have focused on targeting the machinery that synthesizes the cell wall. Yet, less focus has been put on the flipside of this function i.e. enzymes that cut or degrade the cell wall. Such enzymes are collectively referred to as peptidoglycan hydrolases (251,252). Mechanical functions of peptidoglycan hydrolases include creating space in the existing peptidoglycan mesh for new pieces to be incorporated and degradation of old peptidoglycan in a continuous turnover. The enzymes of interest in Paper I belong to peptidoglycan hydrolases, more specifically termed muramyl endopeptidases as they cleave peptidoglycan within the cell wall (251,252).

Paper I studies how the sensitivity of *S.* Typhimurium to various antibiotics is affected when one removes one or several of said muramyl endopeptidases. The main focus was a muramyl endopeptidase named MepS (known as Spr in Paper I). MepS has been characterized *in vitro* to be a DD-endopeptidase that cleaves D-ala-mDAP cross-links in the peptide bridges between the glycan strands of the peptidoglycan. Such enzymatic activity is proposed to be required for incorporation of new peptidoglycan into the existing mesh of the cell wall (253). In this we find that by removing *mepS* from *S.* Typhimurium the bacterium becomes sensitized to vancomycin, an antibiotic that inhibits cell wall synthesis by binding to peptides in peptidoglycan precursors hence sterically blocking penicillin-binding proteins (PBPs) from accessing their substrate (254,255).

This sensitization towards vancomycin is a curiosity since vancomycin is known to not work against Gram-negative enteric bacteria, of which *S.* Typhimurium is one. This resistance relies on the outer membrane that overlies the cell wall resulting in vancomycin not being able to access its target due to the large size of the antibiotic (254). This kind of resistance towards an antibiotic is known as intrinsic antibiotic resistance as simply a feature, i.e. the outer membrane, seemingly is the component needed for resistance, as opposed to spontaneous mutation arising resulting in

antibiotic resistance. As a first step in Paper I we showed the sensitization towards vancomycin after removal of *mepS* from *S.* Typhimurium was not due to a destabilization of the outer membrane allowing for vancomycin to reach its target, but due to some other mechanism.

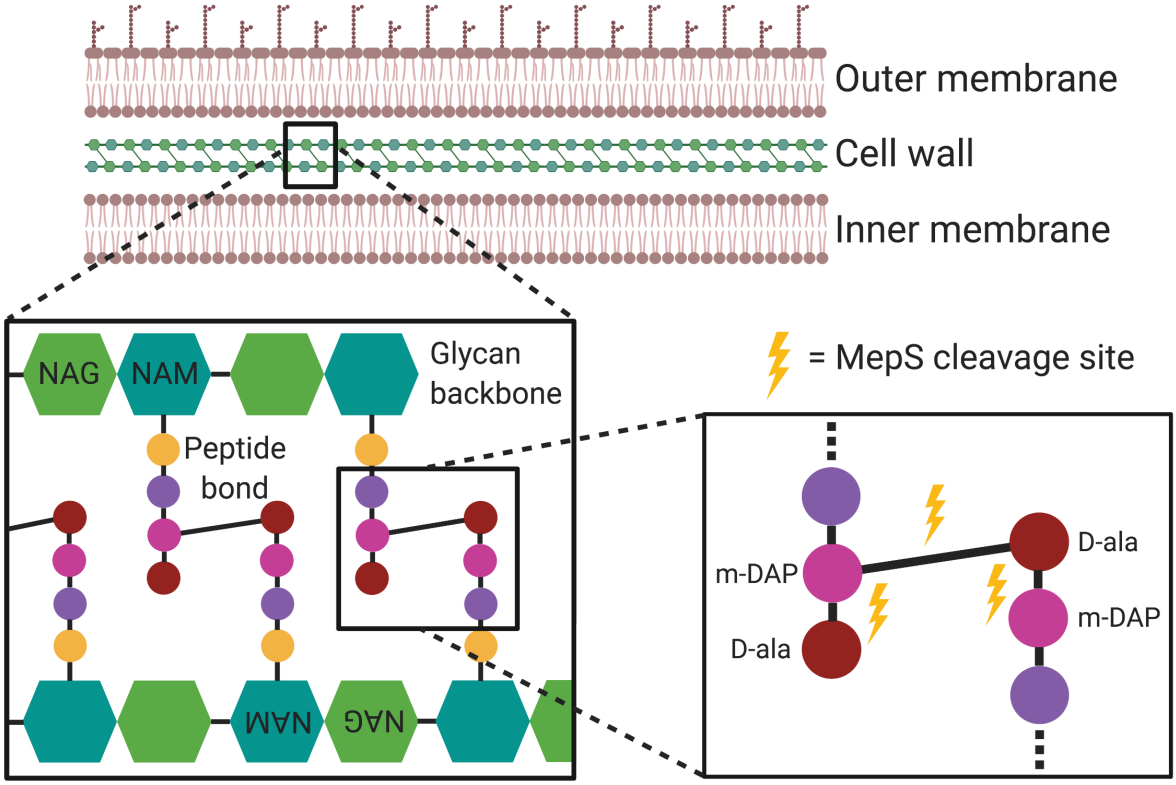

FIGURE 14 Illustration highlighting the cleavage site of muramyl endopeptidase MepS (also known as Spr). The cell envelope of the bacteria consists of the outer membrane, cell wall, and inner membrane. Within the cell wall MepS cleaves the peptide bond between meso-diaminopimelic acid (m-DAP) and D-alanine (D-ala).

We subsequently report in Paper I that the mechanism by which *S.* Typhimurium becomes sensitized towards vancomycin when removing *mepS* is due to the bacteria being more prone to undergo autolysis. Autolysis is a phenomenon where specific cell wall degrading enzymes are activated, following for example stress due to antibiotic exposure, resulting in seemingly uncontrolled cleavage of the cell wall and bacterial lysis (256,257). In Gram-negative bacteria enzymes such as amidases and transglycosylases (258–260), and muremyl endopeptidases (256,261,262) are proposed to be the main drivers in autolysis. One of the main stressors studied that leads to autolysis are β-lactam antibiotics i.e. antibiotics that target enzymes synthesizing the cell wall and their transpeptidase activity (263,264). Yet Paper I is the first report implicating muramyl endopeptidase MepS in autolysis in addition to

showing that vancomycin is able to induce autolysis in a Gram-negative enteric bacterium.

But how does MepS fit into the autolytic system involving amidases, a transglycosase, and other muramyl endopeptidases? Recently some studies have come to shed light on what might be happening during autolysis by using β-lactam antibiotics as the trigger. In this it has been shown that the machinery synthesizing the cell wall at the site of cell division, i.e. the "divisome" of which one component is PBP3 that is targeted by β-lactams, needs to be assembled at the site (260). When this machinery is inhibited the autolytic process is somehow initiated resulting in autolysis. The reason for this has been proposed to be due to autolytic enzymes starting to degrade cell wall while being under the impression that the cell wall synthesizing machinery, which is inhibited by the β-lactam, is synthesizing new cell wall at the same time (265). This imbalance between degradation and synthesis would then result in autolysis.

Interestingly we show that for the intrinsic vancomycin resistance resulting from MepS being present, MepS needs to be catalytically active. However, on the contrary to other muramyl endopeptidases proposed to be autolysins, such as PBP7, MepS seems to act opposite to an autolysin in the sense that the lack of *mepS* results in *S.* Typhimurium being more prone to undergo autolysis and not less. Additionally it has been observed in *E. coli* that overexpression regarding MepS, even a catalytically inactive form in a wild-type background results in the bacteria becoming more resistant to the β-lactam antibiotic mecillinam (266). Hence, the proposed mechanism for this effect of overexpressing MepS resulting in increased mecillinam resistance is far from as simple as MepS cleaving cell wall; the authors show that elevated MepS levels result in increased activity of PBPs that synthesize cell wall leading to the cellular machinery simply outrunning the inhibitory effect of mecillinam.

Whether the proposed mechanistical insights made for MepS and mecillinam in *E. coli* are the same that drives our observations made in Paper I regarding MepS and vancomycin is not clear. To try to understand the underlying mechanism we then selected for spontaneous mutations that would result in vancomycin resistance in bacteria lacking *mepS*. In this we found an additional player in the form of the the

periplasmic protease Prc (known as Tsp in paper I) that when removed reverted the phenotype of the Δ*mepS*-mutant to wild-type, indicating a connection between Prc and MepS. This connection was already previously well established in *E. coli* as MepS has been proposed to be the main substrate for Prc (267). As such, one possible mechanism of how the lack of *mepS* indirectly might affect autolysis is that the function of Prc is instead focused on other targets, such as the cell wall synthesizing enzyme PBP3 (268–270) or muramyl endopeptidase PBP7 (271,272), resulting in dysregulation of the autolytic system due to excessive processing/degradation of its components.

In this way we show in Paper I that the outer membrane is not the only protective factor for a Gram-negative enteric bacterium in its defense towards vancomycin. Instead we suggest that the intrinsic antibiotic resistance towards vancomycin is probably at least twofold in that the outer membrane acts as a protective barrier, but somehow molecules of vancomycin can gain access to the cell wall resulting in a need for a second line of defense in the form of MepS. Hence, Paper I highlights additional features of vancomycin and the resistance determinants in a Gram-negative enteric bacterium. We believe all this increases our understanding of basic biology of *S.* Typhimurium in regards to antibiotic resistance and hopefully will be useful for future studies.

5.2 Paper II

As we observed a phenotype for the bacteria lacking *prc* in Paper I we decided to further probe the Δ*prc*-mutant for additional phenotypes. Prc is a periplasmic protease originally identified as a protease that targets proteins with nonpolar C termini through recognition via its PDZ domain (273–278). The function of Prc is strongly associated with the lipoprotein NlpI to which the protease docks on the inner leaflet of the outer membrane (278–280). This docking to NlpI is needed for full activity of Prc in degrading its substrate MepS (267,278). Yet even though Prc has been previously studied *in vitro* the role of the protease during pathogenesis is less well characterized. Hence in Paper II we found that *S.* Typhimurium lacking *prc* had a significantly reduced ability to proliferate within murine macrophage-like RAW264.7 cells. This ability to proliferate and survive within macrophages is very central to the pathogenesis of typhoid fever as highlighted in the literature review section. First, in

order to verify that the lack of *prc* was the reason for this reduced ability to proliferate we complemented the Δ*prc*-mutant by reintroducing the *prc* gene on a plasmid into the bacteria. This resulted in complementation of the Δ*prc* phenotype thus confirming the genotype-phenotype association for the reduced ability to proliferate within RAW264.7 cells.

Related to Paper I we wanted to know if there were any possible genetic associations to the phenotype of the Δ*prc*-mutant by further removing genes from mutant and observing whether the additional mutation resulted in suppression of the Δ*prc* phenotype. As described in the section for Paper I the main substrate for Prc is thought to be MepS. Thus the obvious starting point in trying to find a suppressive mutation was to remove *mepS* from the Δ*prc*-mutant. Contrary to our expectations, the removal of *mepS* from the Δ*prc*-mutant did not result in the suppression of the reduced ability to proliferate within RAW264.7 cells. As such for gaining tentative suppressor mutations instead of employing a selection-based approach, we opted for a candidate-based approach where known partners of Prc were removed sequentially and possible suppression of the Δ*prc* phenotype was tested. In this we found that by removing the gene for the alternative peptidoglycan synthase $PBP3_{SAL}$ from the Δ*prc*-mutant, the Δ*prc* virulence phenotype was suppressed.

$PBP3_{SAL}$ has not been previously shown to be associated to Prc, however a homologous protein to $PBP3_{SAL}$, PBP3, has been shown to be processed by Prc (268–270). In actuality $PBP3_{SAL}$ itself has only recently been characterized and shown to be important for *S.* Typhimuriums ability to grow within host cells, both in fibroblasts and macrophages (281). The production of $PBP3_{SAL}$ is initiated only at low pH corresponding to the intracellular acidity of vacuoles, for example in macrophages as described in the literature review. Also, $PBP3_{SAL}$ has been suggested to take over from PBP3 in the divisome during these intracellular conditions (281,282). In addition $PBP3_{SAL}$ has been shown to have a lower affinity for β-lactam antibiotics compared to PBP3 resulting in *Salmonella* populations not being able to regrow in β-lactam treated mouse models if the bacterium is lacking *pbp3sal* (283).

To continue studying whether the lack of *prc* had an effect beyond the cell culture experimentation we moved on to a more complex infection model where BALB/c

mice were infected in the form of competitive infections. In this the Δ*prc*-mutant was significantly outcompeted by wild-type when recovering the bacteria from liver, spleen and gall bladder, indicating that Prc is needed during a whole animal infection. As with the cell culture experiments using RAW264.7 cells the additional removal of *pbp3sal* from the Δ*prc*-mutant suppressed this competitive disadvantage resulting from the Δ*prc*-mutation by showing that the Δ*prc*Δ*pbp3sal*-mutant could be recovered from the mice at similar proportions to wild-type.

These results add to the fact established in Paper I where MepS is connected to Prc by now also possibly adding PBP3sal into the repertoire of protein partners to Prc. Interestingly both MepS and PBP3sal are involved in cell wall metabolism, with MepS cleaving the cell wall and PBP3sal synthesizing it. Other proteins involved in cell wall metabolism, such as PBP7 and transglycosylase MltG, have also been established as possible targets for Prc, albeit not shown in *Salmonella* but in *E. coli* (271,272,284). This would indicate that Prc and its proteolytic capability probably has a regulatory function for various aspects of the cell wall synthesis apparatus, now also shown to be involved in infection relevant setting as we show in Paper II.

5.3 Paper III

Having described new genetic determinants for *Salmonella* to be able to proliferate within macrophages, Paper III approaches the question on how *Salmonella* is able to get as far as to start proliferating within macrophages. In this we studied how iNOS, the enzyme tasked with producing nitric oxide employed in bacteria killing, works on the level of individual macrophages during a *Salmonella* infection. The reason to study this aspect of iNOS was due to claims that *Salmonella* inhibits iNOS via a SPI-2 dependent mechanism (216). However, as of today there are no studies showing how *Salmonella* does this happens and no effector proteins being translocated by the T3SS-2 from SPI-2 have been shown mechanistically to inhibit iNOS, as described in the literature review section.

Paper III begins by trying to establish whether, and to what extent, hypoxia would occur in individual RAW264.7 cells infected with *Salmonella*. The interest in studying hypoxia in concert with innate immunity is due to iNOS requiring molecular oxygen to for synthesis of nitric oxide (285,286) and due to hypoxia inducing iNOS expression via HIF-1α (287). In this we noted that infection indeed generated hypoxia, but with

hypoxia not establishing uniformly in the macrophage population. Rather hypoxia correlated with the load of intracellular bacteria and in contrast, and contrary to our expectation, the hypoxic cells containing *Salmonella* appeared negative for presence of iNOS.

This data might strengthen the fact that SPI-2 could be involved in inhibiting iNOS in the infected cell, as we would expect the intracellular *Salmonella* in our studies to express SPI-2 even though we don't present this data in Paper III. However, we saw that iNOS induction in RAW264.7 cells did not require living *Salmonella* to be present, but instead simply that presence of lipopolysaccharide or peptidoglycan was enough to activate iNOS production. Yet, the induction of iNOS in the RAW264.7 cells was concentration dependent only up to a point, with approximately 70% of the RAW264.7 cells not producing iNOS regardless of how much TLR-ligand was provided.

As the presence of *Salmonella* infection in individual RAW264.7 cells correlated with hypoxia, but not with iNOS, the question remained as to what the reason for both of these correlations could be. For this we asked whether the lack of iNOS might actually not be a direct inhibition by *Salmonella* on iNOS or iNOS expression, but instead an indirect effect. This indirect effect could be due to a general effect of *Salmonella* infection on protein synthesis of the macrophage imposed by the immense metabolic burden posed by the proliferating *Salmonella*. This could mean that proliferation of *Salmonella* would indirectly result in the macrophage not being able to defend itself by using iNOS and possibly other means also affected by the general lack of protein synthesis. For this we observed that the presence of large amounts of *Salmonella* correlated with the RAW264.7 cell being less active for protein synthesis. Moreover, later in the infection almost no cells containing *Salmonella* were actively synthesizing proteins, albeit still alive, indicating that the *Salmonella* infection leads to shut-off of protein synthesis in the infected cell.

Although SPI-2 has been suggested to affect the localization of iNOS (216), suggesting a direct mechanism, the details presented in Paper III leads one to think of two possible scenarios for an indirect mechanism. One possible scenario is based on the observation that only part of the RAW264.7 cells could be induced to produce iNOS, which suggest an inherently heterogenic population. Hence, lack of iNOS in

Salmonella infected cells could simply be down to the fact that that specific RAW264.7 cell could not mount an iNOS defense from the beginning. The other possible scenario we term "the chicken race". This scenario assumes that when *Salmonella* infects a RAW264.7 cell it is a question whether *Salmonella* is fast enough to "outrun" the macrophage defenses to the point that hypoxia is induced and hence a broad protein synthesis shut-off that in the end disarms the macrophage. This might well be a possibility since hypoxia in general reduces protein synthesis mainly due to the lack of ATP generation (288–290), but simultaneously confusing due to hypoxia and SPI-1 effectors inducing iNOS expression (287,291).

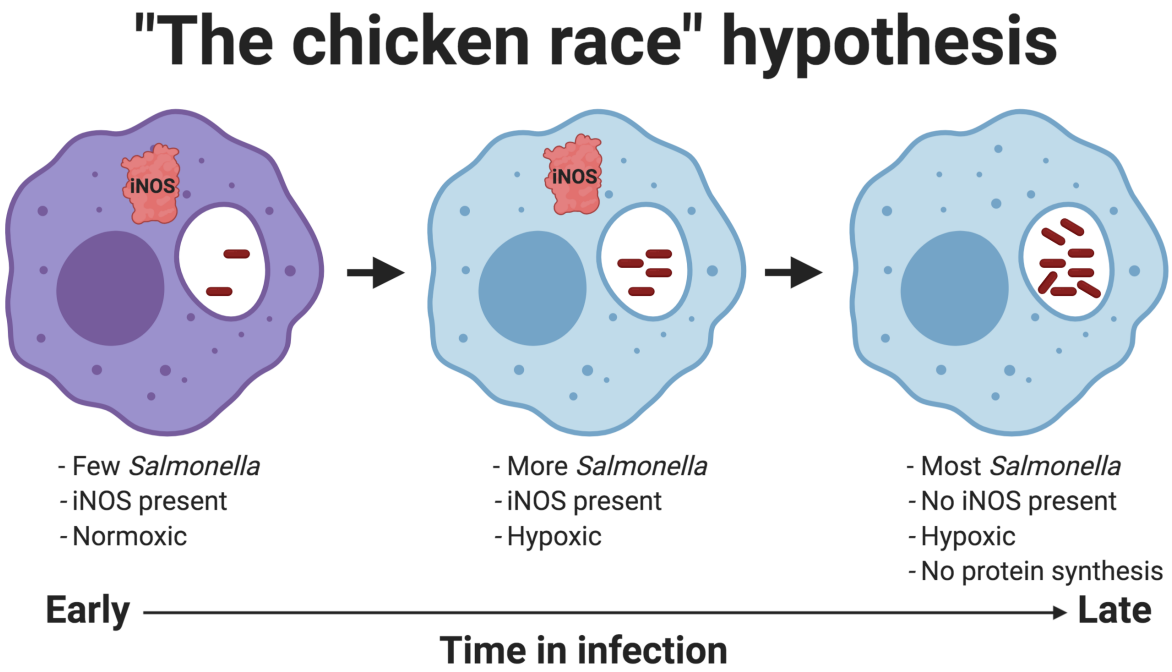

FIGURE 15 A schematic of "the chicken race" hypothesis describing how a *Salmonella* infection possibly proceeds in an individual macrophage in regards to amount of *Salmonella*, presence of iNOS, oxygen status, and level of protein synthesis.

Yet "the chicken race" might not be a race against iNOS itself, but could be seen more as a siege where *Salmonella* has to hold out for long enough for the macrophage to run out of substrate for iNOS in the form of L-arginine, whose availability has been correlated with the microbistatic effect of macrophages (292,293). Once *Salmonella* would get an advantage over the macrophage iNOS would rapidly vanish, due to its short half-life (294), and the bacteria would be free to proliferate. Hence, the response of *Salmonella* against iNOS during a successful infection could be a combination of a SPI-2-mediated direct inhibition of the

localization of iNOS (216), RNS detoxifying enzymes (220), and an indirect shut-off of iNOS production once the macrophage has exhausted its reserves of L-arginine.

6 CONCLUSION

Salmonella enterica is a species of Gram-negative bacteria causing substantial burden on human health worldwide. The diseases *Salmonella enterica* can cause range from gastroenteritis to typhoid fever. Typhoid fever is caused by *S.* Typhi and is the most severe form of the diseases in that it results in the systematic dissemination of the bacterium throughout the body with organs such as liver, spleen, and bone marrow affected. This is accompanied with high mortality, especially for children, and made worse due to the rapidly developing antibiotic resistance. Yet *S.* Typhi does not cause acute gastroenteritis, which instead is a characteristic of human infections due to *S*. Typhimurium. Curiously, when infecting mice with *S*. Typhimurium a typhoid fever-like illness manifests allowing *S*. Typhimurium to be used as a model organism.

By studying the intrinsic antibiotic resistance of *S*. Typhimurium towards the cell wall synthesis inhibitor vancomycin in Paper I we begin to highlight new aspects of this form of antibiotic resistance. Previously vancomycin resistance in Gram-negative enteric bacteria was thought to be due to the antibiotic not being able to pass the outer membrane. However, in Paper I we show that this intrinsic resistance does not solely rely on the outer membrane, but that there also is a contribution from the muramyl endopeptidase MepS, a cell wall cleaving enzyme. We also show that this contribution is most likely due to MepS stabilizing the autolytic system and is dependent on the periplasmic protease Prc (also known as Tsp). By these results we add to the knowledge of determinants for antibiotic resistance towards a specific antibiotic.

In Paper II we move on from antibiotic resistance to intracellular pathogenesis. In this we show that Prc is needed for full fitness of *S*. Typhimurium in both macrophages and mice. This we suggest is due to the *Salmonella*s lesser ability to proliferate within host cells when lacking *prc*. Surprisingly, this attribute of Prc is not dependent on MepS, as could be hypothesized due to literature and observations made in Paper I, but instead is dependent on peptidoglycan synthase $PBP3_{SAL,}$ a protein recently shown to aid *S*. Typhimurium in proliferating within host cells. These results suggest that the pool of targets for the periplasmic protease Prc contains even more

proteins involved in cell wall synthesis than previously shown highlighting the importance of the regulatory potential of this protease.

As for the ability of *S*. Typhimurium to proliferate within macrophages we show in Paper III that the presence of *S*. Typhimurium in said macrophages correlates with presence of hypoxia and absence of the innate immunity effector iNOS, an enzyme producing RNS tasked with killing the bacteria. We hypothesize that the lack of iNOS might be due to an indirect effect by hypoxia leading to shut-off of protein synthesis, thus allowing for *S*. Typhimurium to proliferate within the cells. These results could implicate that some of the effects of SPI-2 could be indirect through the ability of the pathogenicity island to allow intracellular replication which eventually results in hypoxia.

7 POINTS OF PERSPECTIVE

This thesis presents work relying heavily on molecular biology techniques in order to try to understand fundamental biological aspects of *Salmonella*. This lends the research performed in this thesis to the option of further experimentation as there are always ways in which one can go deeper into the study questions by using anything from very sophisticated genetic manipulation to omics-based data collections. One such avenue of future research that can be applied to this thesis is to find out more exact mechanistic details of the biology that has been studied. Even though we haven't managed to give definitive answers on said mechanical details we still believe our contribution is valuable to the field and hope others will build upon our work.

In Paper I specific details that could be answered in the future are for example how MepS actually contributes to the intrinsic vancomycin resistance in *S*. Typhimurium. We suggest that MepS is involved in stabilizing the autolytic system, but are not able to present exactly how this happens. To give specific details of MepS's involvement is of course not a simple task already due to the fact that one in general doesn't know how the autolytic system works. Additionally, it is counterintuitive that a catalytically active MepS is needed for the phenotype as this would mean that the bacteria needs an enzyme that cleaves cell wall in order to stabilize an autolytic process that when initiated results in cleavage of the cell wall.

As for Paper II the question remains in what the mechanistic relationship between Prc and $PBP3_{SAL}$ is. To begin answering this one could by *in vitro* experimentation show whether Prc degrades/processes $PBP3_{SAL}$ in a similar fashion that Prc degrades MepS and processes PBP3. This aspect could then be extended to *in vivo* by attempting to show degradation or processing of $PBP3_{SAL}$ in the absence and presence of Prc in cell culture or mouse infection experiments. However, this would be complicated since the level of expression of $PBP3_{SAL}$ is at best low leading to detection of $PBP3_{SAL}$ being difficult in such experiments.

As for Paper III we show that *S*. Typhimurium infection of individual macrophages correlates with hypoxia and lack of iNOS. However, we do not definitively show is how these are mechanistically couple to each other. One possibly could be due to lack of protein synthesis in the macrophage resulting in the lack of iNOS when *S*.

Typhimurium is present. This would require experimentation establishing many parts of the correlations all from how *Salmonella* infection results in hypoxia to how this hypoxia would lead to shut-off of protein synthesis. Additionally one could consider measuring nitric oxide levels, possibly via indirect means through reporter-fusions, within macrophages as the presence of iNOS in slightly hypoxic *Salmonella* infected cells might not necessary mean that the enzyme is catalytically active due to substrate unavailability. This way the temporal relationship between hypoxia, iNOS, and *Salmonella* within a RAW264.7 cell could be strengthened and possibly additional mechanistic insights could be discovered.

8 ACKNOWLEDGEMENTS

First and foremost all the figures in this thesis were created with BioRender.com.

First, my supervisors:

Thank you to my main supervisor **Mikael Rhen.** I hope you soon meet the day when you don't have to open up your email to find meeting requests and other annoying things, but instead get to be at peace and sneak into the lab to do random experiments. As a tribute I'll bless this page with some of my favourite quotes by Mikael during the years:

"If you really like someone don't buy them a diamond, buy them glycogen, it's more expensive."

"You don't need to be smart to be in science, you just need to be able to hit your head into the wall over and over again."

"Plan every experiment so that you don't need to use statistics."

Thank you to my co-supervisor **Edmund Loh**. Unfortunately my original Ph.D. plan involving RNA helicases derailed very early on which meant we were not able to utilize your expertise fully. Regardless, I wish you luck in your career so that you become so famous that people 200 years from now will be buying paintings of you!

To the students I've had the pleasure to supervise during my Ph.D. studies. You've all been very important, all for different reasons, for my personal development.

Amanda. The first student I had the pleasure to supervise. Bright high school student at the time, now on her way to becoming a medical doctor. Summer of 2017.

Iina. My first medical student. An archetype of a Finnish person, persistent as all hell and great work ethic. A great future medical doctor! Fall of 2018.

Fredrik. I hope you are feeling better and have found your way back. Spring of 2019.

Yas. A true firecracker. The time we had together was definitely educational for both of us. Will never forget the day you were extremely angry at the fact that neither I nor Padryk could answer your very insightful and difficult questions. Students like you are a perfect preparation for a future Ph.D. defense! Summer of 2019.

Nicole. If I was even half as smart as you my Ph.D. studies would've been way easier. One day once the pandemic is over we will celebrate your acceptance into

medical school and you acing your first exam! Remember me when you're famous! Summer of 2019.

Mikael. You survived a project that was way too hard and managed to sort out other things along the way. Once again, a persistent Finn with great work ethic. Hope you get to do what you want in the future! Fall of 2019.

Iiris. Brilliant and easy-going student. I felt like getting to supervise you was some kind of karma being paid back to me for all the tougher times during my Ph.D. studies. I wish you all the luck in finding the medical profession that will not consume your whole life. Fall of 2020.

To Rhen lab members:

Marie. It is truly unfortunate that our time together was so short, I would've needed longer. To manage to finish the project should definitely warrant at least a Nobel prize, simply for the effort. I'm curious to continue following you in seeing how big of a farm one can take care of while simultaneously being a successful scientist. Godspeed!

Rikki. Thank you for the ease of collaborating. It is unfortunate we only got to meet twice and you never arriving at your intended destination in Stockholm. I wish you all the best!

To Henriques-Normark lab, Loh lab, Sotiriou lab members:

Jens. Smålands gift to the academic elite of Stockholm. Has a mysterious essence of seemingly not caring or worrying about anything, while simultaneously doing so. Scientists will study this unique specimen for centuries to come! Also saved me from becoming homeless. A prominent contributor to the non-scientific discussions in the lab and founder of the Wednesday lab group runs. Thanks Jens!

Stephen. A chameleon of a man. Put him wherever you want and he will fit in and do the job required, be it a professional bioinformatician or a hillbilly on the Finnish countryside! Always ready for a good time, even during bad times. "In the land of the blind, the man with one eye is king". Thanks Stephen!

Padryk. The only person I've ever met with no ability to stop working. Was some sort of a machine in his previous life and will eventually be reincarnated as one again. Also great with students, is there anything this man can't do? Thanks Padryk!

John. Keep on fighting the power by listening to Rage Against the Machine on Friday afternoons, we will eventually win. Don't give up on teaching! Thanks John!

Francesco. Ever-present in the Wednesday lab group runs. Thank you for the company!

Jill, Felix, Hannes, Sissi, **Anshika, Elisabeth, Vitor** and **Mario**. Fellow current and past Ph.D. students all adding their own flavour to the work atmosphere. Everyone has their own journey through their Ph.D. studies and it has been an interesting time in being able to share parts of mine with yours!

Vicky, Karina, Jenny, Karin and **Priyanka.** Thank you for showing care for fellow lab members. Much appreciated!

Anuj, Peter, Katrin, Sandra and **Marie-Stephanie.** To the ever-present old guard of the BHN group, I salute you!

Birgitta and Staffan. Thank you for the close association with the Rhen lab. Our lives definitely are much more comfortable being part of the BHN supergroup, than if we were doing everything ourselves. From one ABBA fan to two other ABBA fans; Thank you for the music!

Georgios. Thank you for letting me be a token bacteriologist in your group meetings!

Maria and **Anna.** Thank you to the "lab moms" for all their effort in organizing various aspects about research that is too difficult for a tiny Ph.D. student to understand.

To family:

Madeleine. The one that has gotten to be part of the "behind the scenes". Tack för tålamodet!

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