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It Takes Two to Tango

Bacterial heterogeneity and host cell features govern Salmonella infection

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Abstract

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Salmonella enterica serovar Typhimurium (*S*.Tm) causes enterocolitis with significant worldwide morbidity and mortality. The general aim of this thesis is to investigate variation in host cell invasion mechanisms used by *S*.Tm across different host cell contexts, as well as the influence of bacterial cell-cell heterogeneity on invasion-relevant *S*.Tm behaviours. The thesis is divided into four sub-projects, each a section in the presented work.

First, a genetic barcoding technique for tracking bacteria in mixed consortium infections was developed and applied to evaluate the dependency on the type three secretion system 1 (T3SS-1) and its effectors for host cell entry. It was found that *S*.Tm invasion of cultured epithelial cells and monocytes is mainly mediated by T3SS-1, or by cooperative uptake of bystander bacteria. T3SS-1-independent entry was possible in cultured macrophages, although T3SS-1-dependent entry was predominant also there. In fact, active invasion was promoted by the same T3SS-1 effectors in all three cell types.

Second, an in-depth comparison of *S*.Tm infections in cell line cultures and in the mouse gut mucosa *in vivo* highlighted a "discreet-invasion" modality *in vivo*, in sharp contrast to the prevailing "ruffle" model for host cell invasion. While ruffle-mediated entry into epithelial cell lines was driven by the T3SS-1 effectors SopBEE2, discreet-invasion into the murine gut absorptive epithelium is driven predominantly by the SipA effector, as well as the SiiE adhesin. Furthermore, discreet-invasion targeted apicolateral "hot spots" near cell-cell junctions, dependent on the local cell neighbourhood, which was further charted in the final two sub-projects.

Third, single-bacterium characteristics among *S*.Tm populations were studied using time-lapse microscopy. The indistinct nature of the shift from growth to virulence induction spawned a transient subpopulation of *S*.Tm "doublets", cell division intermediates also exhibiting pronounced swimming and host cell invasion aptitude. The longer doublets also displayed a different search pattern during near-surface swimming, highlighting bacterial cell length heterogeneity as a key determinant of target search atop epithelia.

Fourth, the morphogenic impact of clinically relevant antibiotics were explored, in context of the previous data. Even *S*.Tm bacteria with the most extreme morphological abnormalities (e.g. highly filamentous or coccoid individuals), induced by chloramphenicol, ciprofloxacin, nitrofurantoin, and meropenem, could robustly swim and invade epithelial host cells. While high concentrations of these antibiotics were effective at suppressing growth and virulence, a range of low, sub-inhibitory concentrations even enhanced host cell invasion capacity and affected the near-surface swimming behaviour among surviving bacteria.

In summary, the present investigation highlights the pivotal importance of taking both host cell features and bacterial heterogeneity into account when studying infection processes.

Keywords: Salmonella, swimming, swim pattern, targetting, virulence, invasion, host-pathogen interactions, epithelium, epithelial cells, macrophages, monocytes, monolayer, flagella, genome, genetic, barcoding, discreet-invasion, shape, morphology, antibiotics, persistence

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Dedicated to my mother Katja,
and to the many who prayed and hoped

Papers

This thesis is based on the following papers and manuscript, referenced throughout by their Roman numerals:

I. Di Martino, M.L., **Ek, V**., Hardt, W.D., Eriksson, J., Sellin, M.E., 2019. Barcoded Consortium Infections Resolve Cell Type-dependent *Salmonella enterica* serovar Typhimurium Entry Mechanisms. MBio 10, e00603-19.

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II. Fattinger, S.A., Böck, D., Di Martino, M.L., Deuring, S., Samperio Ventayol, P., **Ek, V.**, Furter, M., Kreibich, S., Bosia, F., Müller-Hauser, A.A., Nguyen, B.D., Rohde, M., Pilhofer, M., Hardt, W.D., Sellin, M.E., 2020. *Salmonella* Typhimurium Discreet-Invasion of the Murine Gut Absorptive Epithelium. PLoS Pathog. 16, e1008503.

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III. **Ek, V.**, Fattinger, S.A., Florbrant, A., Hardt, W.-D., Di Martino, M.L., Eriksson, J., Sellin, M.E., 2022. A motile doublet form of *Salmonella* Typhimurium diversifies target search behavior at the epithelial surface. Mol. Microbiol. 117, 1156–1172.

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IV. **Ek, V.**, Florbrant, A., Eriksson, J., Sellin, M.E., *unpublished manuscript*. Sub-MIC concentrations of antibiotics boost virulence in the surviving *Salmonella* Typhimurium population.

Copies of papers and manuscript are included at the back of the thesis.

Thesis defence

This thesis will be presented and publicly defended at 13:15 on the 16th of December 2022, in room A1:111a at the Biomedical Centre (BMC) of Uppsala University, in Uppsala, Sweden.

The examination committee includes:

Faculty examiner (opponent)

- Professor **Francisco García-del Portillo**
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Contents

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
ATP	Adenosine-tri-phosphate
CCW	Counter-clockwise
CHL	Chloramphenicol
CIP	Ciprofloxacin
CW	Clockwise
LPS	Lipopolysaccharide
MER	Meropenem
MIC	Minimum inhibitory concentration
MOI	Multiplicity of infection
MUC1	Mucin 1
NAIP	Neuronal apoptosis inhibitory protein
NIT	Nitrofurantoin
NLRC4	NOD-like receptor (NLR) containing caspase activating and recruitment domain 4
NOD	Nucleotide-binding oligomerisation domain
NTS	Non-typhoidal *Salmonella*
OD_{600}	Optical density at 600nm; absorbance of 600nm light
PBP	Penicillin-binding protein
SCV	*Salmonella*-containing vacuole
T3SS	Type-tree secretion system
TLR	Toll-like receptor
WHO	World Health Organisation

Populärvetenskaplig sammanfattning på svenska

Bakterier finns överallt: på land, i vatten, på höga berg och i djupa dalar. En av de platser där de trivs bäst är dock i vår tarm. I tarmen trängs en miljon miljoner (1000 miljarder) bakterier per kubikcentimeter, och det är därför inte svårt att förstå att de har en enorm påverkan på vår hälsa. Tarmbakterier hjälper oss att bryta ner maten och att hålla en lugn miljö trots trängseln. Även om dessa i grunden är självviska, då de tjänar på att leva i samexistens med människan, kallas de ibland för ”goda” bakterier och ingår i vår normala tarmflora – men alla bakterier är inte lika fogliga.

Salmonella är ett släkte av små, stavformade bakterier som är ungefär två mikrometer (miljondels meter) långa. De består i grunden av en bit DNA som skyddas av ett hölje av långa sockerarter och fetter i flera lager, vilket skapar en stark barriär mot utsidan. Ur höljet sticker långa svans-liknande utskott ut, flageller, som *Salmonella* kan använda för att simma runt i sin närmiljö. *Salmonella* lever normalt sett några dagar till veckor, och deras enda drift är att replikera DNAt så inte släktet dör ut. *Salmonella*, likt många andra bakterier, växer till och delar sig på mitten ungefär en gång i halvtimmen, helt av sig själv, så länge det finns rikligt med näringsämnen. Detta gör att bakteriestammen växer exponentiellt (en blir två, som blir fyra, som blir åtta osv.) och snart börjar konkurrera om näringen som finns. De vill då sprida ut sig för att leta efter mer näring, till exempel med hjälp av flagellerna.

Om man äter eller dricker mat eller vatten förorenad med *Salmonella* kan dessa bakterier komma åt fria näringsämnen i vår tarm. I tarmen kan de växa snabbt, men *Salmonella* kan också invadera tarmväggen för att få tillgång till ytterligare en miljö att kolonisera och gömma sig i. Om kroppen upptäcker *Salmonella* sätter detta igång ett immunförsvar som gör att man får en sjukdom som kallas ”salmonellos” då man blir varm och inflammerad och får magont och diarré. Detta sköljer vanligtvis ut bakterien ur kroppen ”den naturliga vägen”, och så småningom blir de flesta bra igen helt utan läkemedel. Dock överlever *Salmonella* detta och kan sprida sig vidare i mljön, via toaletten eller andra ytor så som dörrhandtag, marken, eller vattnet, och kanske hamna i maten för någon annan. *Salmonella* är en av världens mest vanliga sjukdomsframkallande bakterier och den smittar häpnadsväckande hundratals miljoner människor årligen på det här viset, de allra

flesta i utvecklingsländer med undermålig sanitet. Av dessa dör tyvärr tusentals människor, ofta på grund av uttorkning efter diarré. I Sverige skedde det största utbrottet hittills under en värmebölja år 1953, då dålig hygien i ett slakteri i Alvesta ledde till att nästan 9000 personer insjuknade p.g.a. underarten *Salmonella enterica* serovar Typhimurium (*S.*Tm), varav 2400 lades in på sjukhus och 90 personer avled (Folkhälsomyndigheten).

I den här avhandlingen studeras just *S.*Tm och dess sjukdomsalstrande mekanismer i fyra olika artiklar (varav tre av fyra är publicerade i vetenskapliga tidskrifter). I **första artikeln** behandlas en metod för att märka bakteriestammar men en ”streckkod”, vilket möjliggör att man kan blanda flera stammar och jämföra dem i samma experiment och veta vilken stam som gjorde vad genom att ”skanna” dem. Man kan också använda streckkoderna för interna tekniska kontroller av sitt infektionsexperiment. Vi använder denna metod för att kartlägga hur olika uppsättningar av faktorer behövs för att *S.*Tm ska lyckas ta sig in i olika typer av värdceller. I **andra artikeln** bemöts den traditionella modellen för just värdcellsinvasion, där *S.*Tm frambringar enorma vågor på cellytan vilket leder till att bakterien slukas och på så vis tar sig in i cellen. I vår undersökning hittar vi att den traditionella förklaringsmodellen, som härstammar från tidiga undersökningar i cellinjer, inte stämmer i den komplexa tarmen på däggdjur där invasionen är betydligt mer diskret. Dessutom finner vi att *S.*Tm tenderar att söka efter vissa cellgrannskap i tarmväggen för att invadera där (riktat mot skyddande epitelceller nära de slemutsöndrande bägarcellerna). I **tredje artikeln** fokuserar vi mer på bakterien, och finner att bakterier i ett naturligt steg i celldelningen simmar rakare. I detta steg har *S.*Tm en dubbelt så lång form som normalt, och vi kallar bakterierna i detta steg för ”dubbletter”. Dubbletternas simning gör att de snabbare simmar fram till värdcellerna i våra experiment, och att de är överlägsna på att breda ut sig i sidled vilket bidrar till spridningen av hela populationen. I **fjärde artikeln** (opublicerad) fortsätter studierna av vikten av formen på *S.*Tm, där vi visar att olika antibiotika (vår enda klass av läkemedel mot bakterier) i låga men under en behandling förekommande doser tvingar bakterien till många olika former. I våra experiment blev *S.*Tm allt från helt runda sfärer till långa spaghetti-liknande former, och vi såg att detta också påverkade simningen och invasionen av värdceller, i linje med kapitel tre. Detta är viktigt då både användandet av antibiotika samt framförallt bakteriernas resistens mot dessa läkemedel ökat lavinartat.

Den forskning som ingår i avhandlingen bidrar till att vi ska förstå de enklaste byggstenarna kring *Salmonellas* invasion av värdceller och simningsmönster. Båda vetenskapliga fynd och nya tekniker bidrar till att den vetenskapliga horisonten trycks utåt, och kan medverka till att kuva *Salmonella* och till ett samhälle som berörs mindre av dessa och närbesläktade sjukdomsbringande bakterier.

Nedan följer avhandlingen på engelska.

Introduction

One of the many great achievements of humankind is the domestication of light. First came the taming of fire. Sitting close to the flickering light of the fireplace, the early humans could now see enough to repair tools and cook also when waning daylight no longer allowed it. At the fireside they also socialised, dissipated information, and learnt new things from each other through – imaginably vibrant – storytelling. Wielding torches, they ventured deep into caves and wandered out into the dark night, and there found answers previously out of reach. By controlling light, the early humans could discover and explore much more of our world. Over time, light sources improved as the domestication of light continued, and in the 17th century, by shepherding light through a series of polished glass lenses, yet another part of our world revealed itself with the introduction of light microscopy.

The early microscopes opened the door to a colossal amount of scientific exploration. The Italian scientist Marcello Malpighi studied the tissues of anatomical samples through his microscope, and his detailed descriptions founded the field of medical histology (Fughelli, Stella and Sterpetti, 2019). The microscope also allowed the Englishman Robert Hooke to observe rectangular patterns in cork trees and coin the term 'cell' to describe them, as they were reminiscent of the 'cellulae' (small rooms for study and prayer) of period-typical Christian monasteries (Gest, 2004; Sepel, Loreto and Rocha, 2009). The Dutchman Antoni van Leeuwenhoek applied his version of the microscope to investigate water samples, wherein he discovered a plethora of little particles just small enough to be hidden from the naked eye. He observed that the particles moved around in the water and correctly deduced that he was observing tiny lifeforms, composed of single, independent entities of Hooke's cells rather than Malpighi's tissues. van Leeuwenhoek referred to the creatures as 'animalcules', and these studies of what we today refer to as microbes are by many considered the birth of microbiology (Gest, 2004). Similar to the early humans' ventures into the dark, light allowed new ventures into histology, cell biology, and microbiology, which were soon followed by many other fields.

Microbiology taught us that microorganisms display a near-infinite diversity, and occupy all habitats on Earth: in the boiling waters of arctic geysers, at punishing pressures in the deep sea, and encased in temperatures far below freezing (Thakur, Singh and Zhang, 2022). Some of these have adapted to the

human body where they live peacefully in concert with the body, as part of our natural flora. Such symbioses help us metabolise dietary and pharmaceutical compounds, and enhance our resistance to pathogens (Man, de Steenhuijsen Piters and Bogaert, 2017; Adak and Khan, 2018; Erin Chen, Fischbach and Belkaid, 2018). The integration of microbes into human life is mathematically evident as the human body forms a habitat that comprises more microbial cells than human cells (Sender, Fuchs and Milo, 2016). The intestinal flora is particularly interesting, as it contains the highest microbial density of any such niche on the planet (Sender, Fuchs and Milo, 2016). However, some bacteria found a more vicious interaction as they adapted to circumvent or attack the human defences using specialised virulence weaponry to break through the noise of trillions of bacteria and cause disease. Indeed, the intestinal lumen contains up to 100 trillion microbes per cubic centimetre (Berg, 1996; Sender, Fuchs and Milo, 2016), but as few as ten pathogenic bacterial cells can be enough to evoke destructive illness (Kothary and Babu, 2001).

This thesis covers four chapters of research involving the enteric *Salmonella*, focusing on the central *Salmonella enterica* serovar Typhimurium with significant impact on human health worldwide. Below follows an introduction to scientific themes that are relevant to the thesis, including an overview of the *Salmonella* and their virulence, bacterial heterogeneity, and different host-pathogen interaction models, followed by a summary of the four papers included in the present investigation and future perspectives.

The *Salmonellae*

The *Salmonella* (or *Salmonellae*) is a group of common gastrointestinal bacteria. They enter humans and numerous other organisms through contaminated food and drink, then propagate in the intestine, and are excreted intermittently with the faeces, creating their basic infectious cycle loop (i.e. the faecal-oral route; Gerba, 2009). The traditional description of these bacteria is that they are rod-shaped, generally 2-3μm long, and stain Gram-negative. In a nutshell, their structural organisation is as follows: *Salmonella* are delineated by twin lipid bilayers interlaid with a thin cell wall consisting of long peptidoglycans (sometimes called the sacculus) which protects against extracellular dangers and gives shape and structure (Vollmer, Blanot and de Pedro, 2008a). Lining the outside of the outer membrane is a dense jungle of lipopolysaccharides (LPS), giving further rigidity, through which are protruding appendages used for adhesion to surfaces as well as several flagella for swimming and exploration of the immediate surroundings (Moens and Vanderleyden, 1996; Nakamura and Minamino, 2019). However, despite fitting within the constraints of such a basic description, there is astonishing complexity between species and serovars of the *Salmonella* genus. Indeed, only a few but

fundamental properties between *Salmonella* species makes the difference between those that are merely opportunistic and those that are gravely dangerous to human health.

Salmonella cause enterocolitis with significant world-wide morbidity. Human-adapted *Salmonella* can penetrate deeper and also cause typhoid fever (or typhoid), which is commonplace in history books and has been connected to several catastrophic disease outbreaks. Before modern hygiene standards and the breakthrough of antibiotics, typhoid killed up to one in five of the infected and made the rest very ill (Mercer, 2021). This is most often exemplified through the unfortunate story of "Typhoid Mary", a New York cook who carried the bacteria asymptomatically and single-handedly spread the disease via her food to hundreds of people at the dawn of the 20th century (Marineli *et al.*, 2013). Further back in history, in the early days of the USA, *Salmonella* has been implicated to have caused the downfall of the entire 17th-century colony of Jamestown and was a major cause of death during the 18th-century civil war (Pavli and Maltezou, 2022). Even further back, typhoid is a likely candidate behind *cocoliztli*, a catastrophic epidemic outbreak that contributed to the downfall of the Aztec society (Vågene *et al.*, 2018) and is even a contender for what finally felled Alexander the Great (Oldach *et al.*, 2003; Mishra, Mengestab and Khosa, 2022). *Salmonella* outbreaks today are both less frequent and much more contained, although confined outbursts affecting hundreds of people are still recorded, borne by e.g. chocolate, ham, poultry, milk, vegetables and sesame products, as well as via irrigation and drinking water (Kapperud *et al.*, 1990; Kozlica *et al.*, 2010; Martínez *et al.*, 2017; Brandwagt *et al.*, 2018; Jourdan-da Silva *et al.*, 2018; Meinen *et al.*, 2019; Nichols *et al.*, 2021; Samarasekera, 2022). Truly, the *Salmonella* are relentless adversaries of human society and have been for millennia (Zhou *et al.*, 2018).

Genetic organisation

The *Salmonella* machinery to grow, replicate, survive, and instigate disease is encoded in its multi-faceted genome (fig. 1). A dominant part of the genome is present on a circular double-stranded DNA chromosome. The chromosome (sometimes referred to as the nucleoid in its packed 3D form) is a discrete, well-defined physical object stored in the cytoplasm of the bacterium (Kleckner *et al.*, 2014). It encodes the basal functions of the bacterium which are highly conserved amongst *Salmonella* strains, such as the machinery for replication, transcription and translation, stress response, motility, and similar (Kothapalli *et al.*, 2005). Apart from these conserved functions, the chromosome harbours several discrete gene clusters responsible for the assembly of many key virulence factors, e.g. the type 3 secretion systems (T3SSs, discussed in detail later; Lou *et al.*, 2019). These clusters are referred to as the *Salmonella* pathogenicity islands (SPIs), and were acquired through lateral genetic interchange with other pathogens (Haneda *et al.*, 2009; Nieto *et al.*,

2016). The SPIs are essential for within-host virulence but not for general survival, as they are spontaneously lost upon prolonged culture in a lab environment (Jacobsen *et al.*, 2011).

Besides the chromosome, *Salmonella* host different plasmids of sizes ranging between 2-200 kilobases (Rotger and Casadesús, 1999; Rychlik, Gregorova and Hradecka, 2006). The best described are the different so-called "virulence plasmids", which encode functions conferring intra-macrophage survival (*spvRABCD*) and host cell adhesion (*pef*, a plasmid-encoded fimbriae) although these contribute only modestly to *Salmonella* pathogenesis (Barrow and Lovell, 1989; Bäumler *et al.*, 1996; Bäumler, Tsolis and Heffron, 1996; Rotger and Casadesús, 1999; Rychlik, Gregorova and Hradecka, 2006). In fact, these can be experimentally exchanged between different species without affecting virulence inside their host (Barrow and Lovell, 1989). *Salmonella* can also carry other plasmids that produce more evident properties. These include large, conjugative plasmids which are generally utilised by the bacteria to spread antibiotic resistance genes, and small plasmids encoding restriction enzyme systems giving phage infection resistance (Rychlik, Gregorova and Hradecka, 2006).

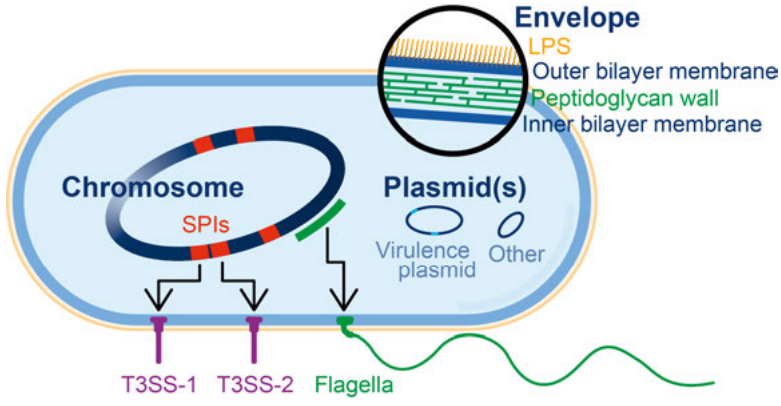

Figure 1. The *Salmonella* cell and genetic organisation. The bacterial envelope is made up of twin flexible bilayer membranes on either side of its rigid peptidoglycan wall. The outer membrane is fortified with LPS. Inside the bacterium, most genes are hosted on the DNA chromosome, such as the flagella genes and the SPIs that encode the T3SSs. Additionally, the bacterium carries one to several plasmids (depending on isolate), e.g. a virulence plasmid encoding functions for survival or host cell adhesion.

The mobile plasmids exemplify the plasticity of the genetic schema underlying the varying abilities of singular *Salmonella* serovars (some of which are discussed in the next section). Additionally, as in most prokaryotes, the *Salmonella* chromosome itself is not static (Jacobsen *et al.*, 2011). On the contrary, duplications and inversions of chromosomal genes are key regulatory mechanisms of *Salmonella* gene expression, with downstream effects on gene

dosage (Malhotra and Seshasayee, 2022) and causing phase variation in the expression of some genes (e.g. the *fliC*/*fljB* genes for the flagella, discussed later). Furthermore, despite a relatively high DNA-mismatch-repair proficiency and replication-machinery fidelity, *Salmonella* has an average mutation rate of 500 mutated genomes per million bacteria in each generation (corresponding to ~0.0005 per genome per generation; Pan *et al.*, 2022). As an effect, there is substantial bacterium-to-bacterium variation within isogenic *Salmonella* populations. Genetic variations may prove disadvantageous, at which point they are quickly cleared from the culture due to selective pressures (Pan *et al.*, 2022). In contrast, a variation may prove beneficial, causing it to become fixed in part of the population, perhaps warranting its own serovar classification and thus forming a new branch on the family tree. Bacterial species on such trees are conventionally categorised by comparison of their genes, and within the *Salmonella* genus by comparison of their surface antigens (many of which are virulence factors; Tindall *et al.*, 2005). Through genetic comparison and temporal modelling, it is possible to approximate likely points in time when genes were acquired and generate a taxonomy of the species. The first draft of the *Salmonella* modern taxonomy using the current convention (based on the Kauffmann-White-Le Minor scheme) was done already in 1987 (Tindall *et al.*, 2005) and is elaborated on below.

A brief taxonomy

It is clear that *Salmonella* is an expansive group of closely related but dissimilar bacteria, and how to classify and order them is a long-debated affair. According to the latest agreed-on standard (Tindall *et al.*, 2005), *Salmonella* is part of the Enterobacteriales family, and diverged along with *Escherichia* from a common ancestor approximately 100-150 million years ago (Doolittle *et al.*, 1996). *Salmonella* is classified into two species, *bongori* and *enterica*, both almost exclusively consisting of commensals of cold-blooded animals (Desai *et al.*, 2013). However, *S. enterica* consists of six subspecies, one of which is also found in warm-blooded animals, somewhat confusingly also named *enterica* (i.e. *Salmonella enterica* subsp. *enterica*). For clarity, it is herein simply referred to as *Salmonella*. This subspecies is then further categorised either by genetics (classically phage typing but now whole-genome sequencing; Anderson *et al.*, 1977; Baggesen *et al.*, 2010) or based on the composition of surface-available antigens (Popoff and Minor, 1997); traditionally into a serotype based on the outer portion of LPS (somatic antigen, or O-antigen) and a serovar based on the flagella (H-antigen), although today the word "serovar" is used synonymously for both. To date, over 2,600 serovars of *Salmonella* have been identified (Desai *et al.*, 2013).

The *Salmonella* serovars are distinctively adapted to thrive in a specific environment and host range, and are grouped thereafter into "generalists" or "specialists" (Baumler and Fang, 2013a). The generalists are found in many,

diverse host species, and typically cause enteric infections that are cleared by the host immune defence without intervention. Specialists, in contrast, are highly adapted to a specific host and cause severe disease. These bacteria are capable of sticking to and penetrating the host defences and disseminating to systemic sites after the initial invasion of the gut. They are also better adapted to survive and replicate within the host (or rather, within a distinct set of host cells). Such specialists are responsible for causing the already-mentioned typhoid fever, and are therefore referred to as typhoid serovars and include the human-specific serovars *Salmonella* Typhi (hereafter *S.*Typhi) and Paratyphi. Generalists, on the other hand, comprise non-typhoid serovars such as *Salmonella* Typhimurium (hereafter *S.*Tm) and Enteritidis.

Much work has been focused on the human-critical *S.*Tm, as a representative member of the non-typhoidal *Salmonella* (NTS; Baumler and Fang, 2013a). *S.*Tm exists in the world's research laboratories in the form of multiple reference strains isolated from clinics or outbreaks in animals, e.g. SR-11, D23580, LT2, 14028, and SL1344 (Curtiss *et al.*, 1988; McClelland *et al.*, 2001; Kröger *et al.*, 2012, 2013; Canals *et al.*, 2019). *S.*Tm SL1344 is a highly virulent, well-characterised strain isolated from English cattle (Kröger *et al.*, 2013). It features a few, key traits. Firstly, although most *S.*Tm strains carry a virulence plasmid which is self-transmissible (i.e. encodes the conjugation machinery), the virulence plasmid of *S.*Tm SL1344 lacks these genes and is thus immobilised (Ahmer, Tran and Heffron, 1999). Secondly, *S.*Tm SL1344 carries a significant virulence gene, encoding the effector protein SopE (discussed later in this introduction), located on an ancient phage insertion on its chromosome (Hardt *et al.*, 1998). These two properties make this strain a good choice for studying properties of *S.*Tm virulence. In this thesis work, *S.*Tm SL1344 is the main subject and from here on the focus, unless otherwise specified.

Key differences between *S.*Typhi and *S.*Tm

Members of the same genus are by definition genetically similar and commonly have comparable lifestyles but are separated by functional differences. Such dissimilarities take shape in many ways, e.g. in disease burden, as is often the case when comparing host-adapted to generalist members. The human-adapted *S.*Typhi and the generalist *S.*Tm are one such pair, which share ~90% of their genetic code (Parkhill *et al.*, 2001). Interestingly, although *S.*Tm diverged from a common ancestor millions of years ago (Doolittle *et al.*, 1996), the human-adapted *S.*Typhi is a mere 50,000 years old and co-evolved with the first hunter-gatherers (Kidgell *et al.*, 2002). Importantly, *S.*Typhi and *S.*Tm differ in their virulence programs (reviewed by Johnson, Mylona and Frankel, 2018), which here merit contrasting.

The most straightforward difference is that *S.*Typhi carries the cytolethal typhoid toxin (Spanò, Ugalde and Galán, 2008), which can be found in some other *Salmonella* serovars but not in *S.*Tm (Suez *et al.*, 2013). The typhoid

toxin is expressed and released into the extracellular space after the bacterium has been internalised into SCVs (Hodak and Galán, 2013; Chang, Song and Galán, 2016). From there, it can intoxicate multiple cells through autocrine or paracrine pathways mediated by its cell surface glycoprotein receptor podocalyxin 1 (on epithelial cells) and CD45 (on blood cells; Spanò, Ugalde and Galán, 2008; Song, Gao and Galán, 2013; Deng *et al.*, 2014). After internalisation, the toxin exhibits DNase I-like activity which prompts DNA-damage responses including cell cycle arrest (Hodak and Galán, 2013). Since the host cell receptors are specific to humans, this demonstrates *S.*Typhi host adaption not present in *S.*Tm (Deng *et al.*, 2014).

*S.*Typhi and *S.*Tm also differ in their opposing interaction with intestinal inflammation. *S.*Typhi carries the Vi antigen, a polysaccharide capsule shielding LPS from immune recognition and thereby prevents phagocytosis and confers serum resistance (Hart *et al.*, 2016). The Vi antigen also binds cell surface prohibitin, which dampens inflammation and neutrophil influx (Sharma and Qadri, 2004; Raffatellu, Chessa, *et al.*, 2005; Winter *et al.*, 2008). Thus, the human-specific pathogen *S.*Typhi has evolved to evade immune detection as part of its pathogenesis, allowing it to infiltrate the body undetected. However, the Vi antigen is absent in *S.*Tm (Hart *et al.*, 2016). In the healthy gut, the endogenous microbiota in the luminal niche competes for nutrients such as short-chain fatty acids as well as host-derived resources such as oxygen, lactate, nitrate, and tetrathionate (Rogers, Tsolis and Bäumler, 2021). As a side effect, the fierce competition for these nutrients confers colonisation resistance towards pathogens. However, *S.*Tm have evolved to not evade but to exploit the host immune defence to overcome this competition. By actively triggering strong inflammation, much of the gut-residing microbiota is decimated, and the fragile gut symbiosis turns into dysbiosis (Weiss and Hennet, 2017). While the *S.*Tm population is also severely reduced (Maier *et al.*, 2014), surviving *S.*Tm can exploit the emptied growth niche and as such overcome colonisation resistance (Stecher *et al.*, 2007; Winter *et al.*, 2010). As such, *S.*Typhi stealthily infiltrates the body while *S.*Tm instead triggers and exploits the immune defence.

*S.*Typhi and *S.*Tm also differ in their ability for *rnn* homologous recombination. Such events are guided by *rnn* operons, which are hosted in several copies by many enteric pathogens, including *Salmonella* (Sanderson and Liu, 1998). *S.*Typhi and *S.*Tm both carry seven *rnn* operons, and will stochastically generate gene inversions and translocations via recombination between them in lab conditions (Kothapalli *et al.*, 2005). However, *S.*Tm rarely endures such rearrangements in nature, suggesting a strong selection for the typical configuration, while *S.*Typhi tolerates comprehensive recombination, producing high variability between isolates (Kothapalli *et al.*, 2005).

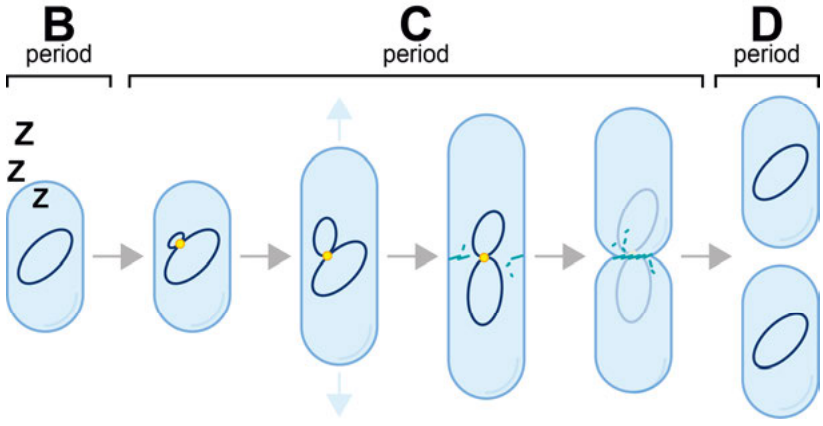

Figure 2. The *Salmonella* cell cycle, consisting of the B/C/D phases. The initial B period is a resting phase from the previous cycle. The C period is where the bacterial genome (here represented by the chromosome; dark blue circle) is replicated by the replisome (yellow), and the original and the nascent copy are simultaneously segregated towards different cell poles. At the same time, the bacterium grows in the longitudinal direction and reaches twice its regular size. Towards the end of the C period, FtsZ peptides (teal) form long filaments that restricts the cell envelope, forming a visible "waist" that eventually transforms into the division septum. The D period is the final period, where constriction is completed, resulting in two separate daughter cells.

Growth

The rudimentary cell cycle of carbon-based life includes four consecutive steps: (1) replication and organisation of the genetic material, (2) biomass production and growth, (3) generation of new progeny by division or budding, and, sometimes, (4) rest in between cycles. Our understanding of the cell cycle specific to bacteria is founded on early experiments in *E. coli* (Cooper and Helmstetter, 1968), and although the model established back then has been iteratively improved upon for decades, the definition of the bacterial cell cycle largely remains the same.

The *S.*Tm cell cycle is comprised of three steps that are very similar to the rudimentary cycle, with slightly overlapping but separate stages referred to as the B, C, and D periods (fig. 2; Wang and Levin, 2009). A new cycle starts in the B period, which is the resting phase between the previous reproduction cycle and the next. The length of the B period varies, as the transition into the next phase is dictated by e.g. starvation signals (Flint, 1987; Wang and Levin, 2009; Gray *et al.*, 2019). The duration of the C and D periods are largely constant, thus the length of the dynamic B period determines the growth speed (Cooper and Helmstetter, 1968). The B period ends if the cell determines it passes all checkpoints, upon which replication is initiated. During the next phase, the C period, the genome is replicated by the bacterial DNA polymerase

(Wang and Levin, 2009). As it is copied, the bacterium simultaneously segregates the copy and the original DNA to opposite poles of the cell (Wang and Levin, 2009). Thus, as DNA replication and DNA segregation occur simultaneously, the B/C/D cell cycle model is not equivalent to the standard G1/S/G2/M model in eukaryotes where these steps are separated into the S and M phases (Meunier, Cornet and Campos, 2021). Next, the bacterium grows in length along the longitudinal axis (during the C period) and the nanoscale machinery to divide the cell forms and division starts (during the D period), which is elaborated on below. Lastly, the mother cell splits into two identical daughter cells that immediately enter the rest phase (the B period), completing the cycle.

Cell envelope

The outmost majority of bacteria are equipped with at least one phospholipid bilayer membrane and a peptidoglycan cell wall of varying thickness (Silhavy, Kahne and Walker, 2010). This barrier is fitting to protect bacteria from the unpredictable and often hostile environment but makes growth fairly complex as it requires the biosynthesis of both cell wall and membrane(s) (Silhavy, Kahne and Walker, 2010). During this balancing act, bacteria also need to heed their internal turgor pressure in order not to simply burst during cell division. Interestingly, this pressure can reach about a third of the pressure in the tires of a racing bicycle (Osawa and Erickson, 2018).

Membrane biogenesis components are produced intracellularly and incorporated either in the inner membrane or transported to the periplasm for use in the outer (in Gram-negatives; Silhavy, Kahne and Walker, 2010). The transport system depends on the nature of the component. Proteins targeted to the inner membrane are transported using the Tat system, while those targeting the outer membrane are transported by the Bam system. Protein transport over the internal membrane happens post-translationally in an unfolded state by the ATPase SecA (Zimmer, Nam and Rapoport, 2008) assisted by the SecB chaperone (Randall and Hardy, 2002), and secreted proteins are accepted by new chaperones to protect them in the periplasm, e.g. by SurA (Bitto and McKay, 2003). The more stable lipids are instead transported solely by the Lol system (Narita and Tokuda, 2006). Lipoproteins are generally transported to the outer membrane but can carry “Lol avoidance signals” to remain in the cytoplasm (Narita and Tokuda, 2006). Finally, as these constituents arrive at their respective assembly site, they are incorporated into the membrane by special incorporation complexes.

Elongation of the peptidoglycan wall differs between Gram positives and negatives (Billaudeau *et al.*, 2017) but is known to be the driving force of cell division in both (Coltharp *et al.*, 2016; Daley, Skoglund and Söderström, 2016). Peptidoglycan biosynthesis has been thoroughly characterised (Goffin and Ghuysen, 1998; Höltje, 1998). The peptidoglycan layer is made up of a dense mesh of long filaments of alternating units of *N*-acetylglucosamine

(Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac), which are cross-linked via peptides attaching to Mur*N*Ac as the layer maturates (Vollmer, Blanot and de Pedro, 2008b). Glc*N*Ac and Mur*N*Ac are both produced from a disaccharide precursor that is synthesised intracellularly and then transferred to the outside of the cytoplasmic membrane. There, the precursors are incorporated into the existing peptidoglycan meshwork through enzymatic reactions catalysed by penicillin-binding proteins (PBPs). Aptly named as they are targeted by beta-lactam antibiotics such as penicillin, the main function of PBPs is to mediate the synthesis, maturation and recycling of the peptidoglycan layer (Sauvage *et al.*, 2008). The PBPs, together with a few other constituents, form the peptidoglycan elongation machinery. In fact, they form either one out of two different machines. Peptidoglycan biosynthesis utilises two spatially separated, mutually exclusive pathways (Satta, Fontana and Canepari, 1994), which are guided by different targeting components and comprise different sets of PBPs. These have important functions in cell elongation and cell division, referred to below as the primary (elongation) and secondary (division).

Cell elongation

The primary peptidoglycan synthesis machinery is responsible for elongating the lateral walls in the longitudinal direction (during the C period) and is referred to as the "elongasome" (Domínguez-Escobar *et al.*, 2011). It uses the actin homologue MreB as a guide, cytosolic polypeptides which forms filaments parallel to the peptidoglycan layer but along the inside of the inner cell membrane. These filaments act as a scaffold for the biosynthesis machinery and are also understood to organise the movement of the whole complex (Domínguez-Escobar *et al.*, 2011). MreB filaments form processions in spiralling patterns along the inside of the cell wall, driven by the ongoing cell wall synthesis (Domínguez-Escobar *et al.*, 2011; van Teeffelen *et al.*, 2011; Hussain *et al.*, 2018). Such spirals are aligned with the long axis of the cell, directing peptidoglycan biosynthesis along the length of the bacterium (Hussain *et al.*, 2018). This contributes to upholding the elongated shape and strengthens the poles, preserving the rod morphology (Hussain *et al.*, 2018; Rohs and Bernhardt, 2021).

Cell division

To comprehend the secondary peptidoglycan synthesis machinery requires a little bit more background. Bacteria commonly divide through binary fission, and a fundamental component of such cell division is the highly conserved protein FtsZ (fig. 2; reviewed in detail by Mahone and Goley, 2020). FtsZ is a cytoskeletal component homologous to tubulin and forms a circular structure along the rim of the bacterial body at the central plane during division. This structure is referred to as the "FtsZ ring" (or Z ring) and is constituted by multiple layers of overlapping, shorter protofilaments of ~30 FtsZ subunits tethered to the inside of the inner cell membrane (Chen and Erickson, 2005). The

Z ring is tightly regulated both spatially and temporally, and is only ever located in the middle of the cell and only present during cell division (Vicente and Rico, 2006; Dajkovic *et al.*, 2008). The ring is strong, but also highly dynamic, and is constantly rearranging itself, powered by the GTP hydrolyse activity of FtsZ (Mukherjee and Lutkenhaus, 1998). Signifying this Z ring dynamicity, protofilament bundles have been observed to travel in streams along the inner lining of the ring, similar to trains on a railroad, completing about two full laps per minute at maximum speed (Loose and Mitchison, 2014). These bundles dynamically reconfigure the ring to increasingly smaller configurations only limited by the rigidity of the envelope, a process which visibly bends the lipid membrane and forms an invagination (observable with a microscope; Erickson and Osawa, 2017). However, FtsZ alone is not powerful enough to constrict the septum fully, in order to complete fission and split the cell (Daley, Skoglund and Söderström, 2016; Erickson and Osawa, 2017).

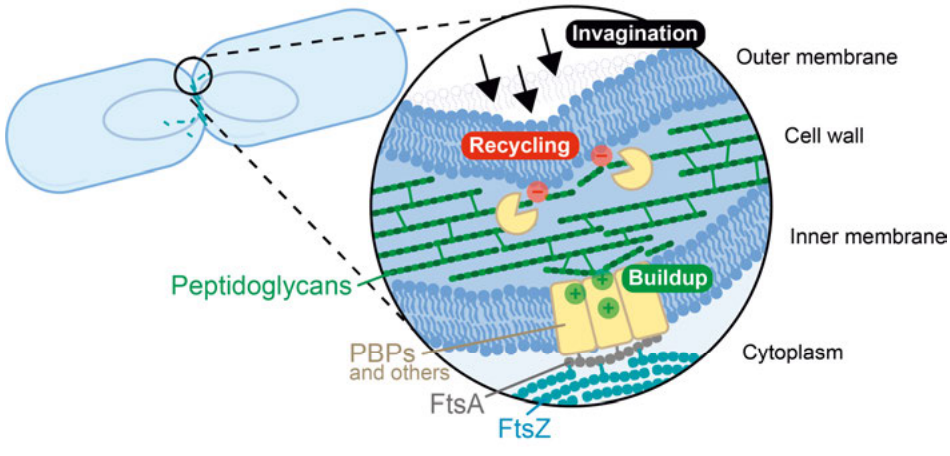

Figure 3. *Salmonella* cell division. The cell division is started by FtsZ (teal), forming a membrane-associated ring along the internal circumference of the bacterium, creating an indentation in the bacterial envelope, which is visible by microscopy. The inwards-directional build-up and breakdown of the peptidoglycan cell wall (green) by the divisome (yellow) along the FtsZ ring stabilises the indentation, and allows the constantly reorganised ring to shrink closer to the cell centre. This constrictive mechanism is repeated until the forming septum is completed, upon which the divisome complex dissociates.

This is where the peptidoglycan layer comes into play, and the secondary peptidoglycan synthesis machinery. This intricate machinery is constituted of over 30 proteins, including division-specific PBPs (normally PBP2 and PBP3; Castanheira and García-del Portillo, 2017), the PBP recruiter ZipA (Hale and de Boer, 1997), DNA translocases (Liu, Draper and Donachie, 1998), the AmiABC amidases (Priyadarshini, de Pedro and Young, 2007; Nakamura *et*

al., 2020), several linker proteins, and an ATP hydrolysis sub-complex to power it all (Schmidt *et al.*, 2004), forming a complex reminiscent of the previously mentioned elongasome referred to as the "divisome" (reviewed by Du and Lutkenhaus, 2017). Interestingly, *S.*Tm also carry alternative PBPs. As intracellular *S.*Tm sense the acidic environment inside phagosomes, they acidify also their cytoplasm mediated by the transcription-regulating two-component system OmpR/EnvZ (also regulating SPI-2; Lee, Detweiler and Falkow, 2000; Chakraborty, Mizusaki and Kenney, 2015) and PBP3 activity is downregulated (via downregulation of the periplasmic protease Prc; López-Escarpa, Castanheira and García-del Portillo, 2022). Instead, specifically for *S.*Tm, there is a switch to the PBP2/3 paralogues $PBP2_{SAL}$ and $PBP3_{SAL}$ (Castanheira and García-del Portillo, 2017; Castanheira *et al.*, 2017, 2020), mediated by the effects of OmpR and Prc (López-Escarpa, Castanheira and García-del Portillo, 2022).

The divisome also includes FtsZ itself along with the FtsZ membrane anchor FtsA (Addinall and Lutkenhaus, 1996) and associates with the moving FtsZ filament bundles (Bisson-Filho *et al.*, 2017). As the moving divisome travels along the FtsZ track it will build up and link together the nearby peptidoglycan layer along the innermost leading edge of the nascent septum while e.g. amidases cleave the outer layers in the trailing edge (Priyadarshini, de Pedro and Young, 2007; Nakamura *et al.*, 2020), thereby creating a peptidoglycan cell wall extrusion along the Z ring. This stabilises the Z ring and allows new filaments to reposition themselves further inwards, approaching the central axis of the cell; as peptidoglycan polymerisation continues, the Z ring guiding the divisome shrinks. This forms a steadily-closing loop, leading to a build-up of peptidoglycans that over time fully closes the septum, thus completing cell division (fig. 3). FtsZ can also regulate cell division directly in some species (via modulation of the peptidoglycan synthesis machinery itself; Mahone and Goley, 2020), fortifying its role as a central regulator of bacterial cell division. As such, the driving force of cell division is the synthesis of the cell wall rather than that of the cell membrane (Coltharp *et al.*, 2016; Daley, Skoglund and Söderström, 2016).

Growth speed

Talking about driving forces, bacteria generally have a short lifespan so replication is inherently required to be a rapid affair, ensuring stable growth of the bacterial population. *S.*Tm replicates asexually through symmetric cell division, with a doubling time of merely 20-30 minutes or so, under rich conditions. A key phenomenon to reaching such fast division lies in that bacterial cells can start new cell cycles during ongoing cycles, resulting in several cell cycles underway concurrently (Micali *et al.*, 2018). This is possible due to the uncoupling of the two steps of growth: replication of the chromosome and the elongation and division of the cell. Consequently, the rate-limiting mechanism of bacterial cell division becomes two (or several), independently progressing

cycles syncing to form an AND gate; in other words, the growing cell is continuously preparing to divide and its chromosome is constantly replicating, and when all required parts are completed the cell division event takes place (Wallden *et al.*, 2016; Micali *et al.*, 2018). This sanctions very short doubling times.

The growth speed of bacteria is however anything but constant. Conversely, an *S.*Tm culture starting from a single bacterium with a doubling time of 30 minutes would otherwise exponentially multiply to almost 100 trillion (10^{14}) individuals within the first 24h, i.e. more cells than the total colon microbiota, the densest habitat on Earth (Sender, Fuchs and Milo, 2016), if left unhindered. The growth speed is governed by multiple factors such as access to nutrients, e.g. amino acids and electron acceptors (Ashino *et al.*, 2019). As bacteria recognise the dwindling supply of such nutrients, they active growth-limiting programs in order not to starve (Gray *et al.*, 2019). *Bacillus subtilis* exemplifies the extreme plasticity of such growth regulation, and has been demonstrated to survive and actively grow at concentrations representing 10,000-fold diluted lysogeny broth (LB) for several months, by slowing the doubling time from ~40 min to almost 4 days (Gray *et al.*, 2019). Similarly, *Escherichia coli* can completely shut off their replication to survive without carbon for over 260 days (Flint, 1987). A final growth-limiting factor is that the increasing number of bacteria is counteracted by a steadily increasing frequency of cell death. The relation between the effect of these signals is best seen in a classical growth curve, typically generated by inoculating a single batch of a liquid medium with a few bacteria and measuring their growth over time (usually via optical absorbance of light at 600nm; OD_{600}). In such experiments, the number of bacteria in a growing culture follows a sigmoidal curve with several phases: an (1) initial slow growth (referred to as the lag phase), followed by a spurt of fast growth in the (2) exponential phase, eventually settling into (3) a stationary phase and a cell number plateau as cell death and cell generation approach an equilibrium (such "invisible" growth referred to as cryptic growth; Gray *et al.*, 2019). Some also include a fourth stage, the (4) decline phase, where the number of bacteria decreases over time as cell death overtakes growth mainly due to declining access to free nutrients (Ashino *et al.*, 2019), and long-term experiments *in vitro* can also include a final (5) static phase (also referred to as the extended stationary phase) when growth and death balance out, supporting a low but sustainable number of bacteria (Finkel, 2006). Thus, the growth speed roughly follows the bacterial access to nutrients.

The number of bacteria that are maintained in the stationary phase of planktonic growth depends on the local environment, and *S.*Tm has several options to spread to other environments for further expansion. *S.*Tm can use their flagella to swim very fast (~10x its body length per second; Macnab and Koshland, 1972; Misselwitz *et al.*, 2012) and as such can spread through liquids in the environment. In the intestine, they also spread via swimming and by the

movement of peristalsis. *S.*Tm, as an invasive bacterium, can also access the intracellular niche, in intracellular vacuoles (*Salmonella*-containing vacuoles; SCVs) and free in the cytosol (García-Del Portillo, 2001; Malik-Kale, Winfree and Steele-Mortimer, 2012; Pucciarelli and García-Del Portillo, 2017). In fact, it can also utilise the invasive capacity to cycle back and forth between the intestinal lumen and the epithelial layer of the intestine, utilising the available nutrients in both niches (Knodler *et al.*, 2010; Laughlin *et al.*, 2014; Geiser *et al.*, 2021). Thus, *S.*Tm is equipped with the tools to spread out and access multiple growth niches, and cycle in between them, to grow and multiply.

Infectious life cycle

Salmonella are ubiquitous worldwide and can persist on many surfaces and for years in soil (Davies and Wray, 1996). Apart from infecting hominids, they survive and replicate in many wild creatures including most birds, reptiles, most wild game such as boar, deer, and racoons, numerous insects, and rodents (Davies and Wray, 1996; Baumler and Fang, 2013b). The spread of *Salmonella* from wildlife to animals in food production is common, and most cases of human salmonellosis are caused by contaminations in food production chains involving poultry, pigs, and cattle (Hoelzer, Switt and Wiedmann, 2011; Hilbert *et al.*, 2012), not least since *Salmonella* also persist in animal housing and feed for years (Davies and Wray, 1996).

Salmonella are transmitted between animals or humans via the faecal-oral route through food, water, or direct contact with animals (Hilbert *et al.*, 2012). A primary colonisation may then happen anywhere along the length of the intestinal tract but is generally localised to specific areas due to host adaption (Bäumler *et al.*, 1998). Human-adapted *Salmonella* species generally infect the human ileum, the distal part of the small intestine, although also targeting the jejunum and colon, while in mice *S.*Tm also generally infect the enlarged cecum portion and the proximal colon. There, *Salmonella* localise along the intestinal wall and replicate in the luminal niche, but as facultative intracellular bacteria are also able to exploit the intracellular niche within host cells (Bäumler *et al.*, 1998). To access this niche, *Salmonella* can cross the physical boundary of the intestinal wall in multiple ways, including M cell uptake and dendritic cell lumen sampling (Jones, Ghori and Falkow, 1994; Leoni Swart and Hensel, 2012). They can also actively penetrate the protective mucus barrier, bind to the intestinal mucosal surface, and invade into absorptive epithelial cells or the mucus-producing goblet cells (Fattinger, Sellin and Hardt, 2021). *Salmonella* can also internalise into or be taken up by mucosa-residing macrophages, wherein they can silently lodge and persist (Helaine *et al.*, 2014; Stapels *et al.*, 2018). Host-adapted specialist species, e.g. *S.*Typhi for humans, can also penetrate even further and make it into the bloodstream or lymph, and spread to systemic sites (Mastroeni and Grant, 2013). Commonly, however, *Salmonella* are detected by the immune defence and cleared from the tissue,

through a protective program causing diarrhoea (more about the human disease in the next section). Lack of adequate sanitation and efficient water treatment cause re-seeding of *Salmonella* into the environment (Kirk *et al.*, 2015), completing the life cycle.

Salmonella in human disease

The ubiquitous *Salmonella* are also one of the most common sources of food poisoning worldwide and the cause of significant morbidity. The majority of medical cases occur in South-East Asia and sub-Saharan Africa, largely due to insufficient sanitation and lack of access to clean water (Mogasale *et al.*, 2014; Kirk *et al.*, 2015; Balasubramanian *et al.*, 2019; Stanaway *et al.*, 2019). The increased ambient temperature also contributes to enhanced survival in the environment and food (Lake *et al.*, 2009; Akil, Anwar Ahmad and Reddy, 2014). However, *Salmonella* is common also in colder climates. Estimates show that around 10% of Europeans come into contact with *Salmonella* species annually, even reaching up to 60% in some European countries, although this may not always lead to disease (Mølbak *et al.*, 2014). Salmonellosis is also the cause of considerable mortality. The Global Burden of Diseases, Injuries, and Risk Factors Study of 2017 estimated that almost 100 million people (95.1) are infected by non-typhoidal strains every year globally, leading to over 50 000 deaths (50 771; Stanaway *et al.*, 2019). Similar estimates of typhoid strains reach just over 20 million (20.6) but cause 223 000 deaths (Mogasale *et al.*, 2014). As such, *Salmonella* are problematic bacteria that cause over 270 000 deaths annually and are regarded as a high-priority pathogen in the fight against antimicrobial resistance by the World Health Organisation (WHO; World Health Organization, 2017).

Salmonella cause a range of diseases referred to jointly as *salmonellosis*, but which is commonly divided into enterocolitis, sepsis, and enteric/typhoid fever (or simply typhoid; Coburn, Grassl and Finlay, 2007; Woc-Colburn and Bobak, 2009). For the purpose of this thesis, these will be grouped into two groups, by severity: (1) enterocolitis, caused by non-typhoidal *Salmonella* such as *S.*Tm (although also typhoidal strain can cause enterocolitis), and (2) sepsis and enteric fever, typically caused by typhoidal *Salmonella* such as *S.*Typhi.

Enterocolitis

Salmonella-induced enterocolitis is an inclusive term for inflammations of the intestinal tract triggered via *Salmonella* recognition by the host immune defence (Kurtz, Goggins and McLachlan, 2017). Inflammation can be instigated in multiple ways, involving either the innate or adaptive immune system.

Innate responses to *Salmonella* begin to be well-characterised. As part of the innate immune system, a local response is instigated upon recognition of the T3SSs or flagella of *Salmonella* intracellularly by epithelial cells and

phagocytes (by the NAIP/NLRC4 inflammasome; Zhao *et al.,* 2011). Such recognition cause death signals in the cell, which activates caspases causing gasdermin D-mediated pore formation and lysis (sometimes referred to as pyroptosis; Shi *et al.*, 2015; Fattinger, Sellin and Hardt, 2021). In the intestinal epithelium, this is joined by a focal contraction of numerous neighbouring cells, pushing out the infected cell from the intact protective epithelial layer (Knodler *et al.*, 2010; Sellin *et al.*, 2014; Rauch *et al.*, 2017; Samperio Ventayol *et al.*, 2021). Cells in the intestinal epithelium as well as macrophages are also equipped with toll-like receptors (TLRs) and nucleotide-binding oligomerisation domains (NODs), which are specific pattern recognition receptors that identify conserved *Salmonella*-specific pathogen-associated molecular patterns such as LPS (TLR4; Hoshino *et al.*, 2016), flagella (TLR5; Hayashi *et al.*, 2001), or peptidoglycans (NOD1 and NOD2; Girardin, Boneca, Carneiro, *et al.*, 2003; Girardin, Boneca, Viala, *et al.*, 2003). In response, a cloud of interleukins and inflammatory lipids are secreted from the cell, which hyperactivates and recruits phagocytes in the local area (Kaiser and Hardt, 2011; Zanoni *et al.*, 2017; Wu *et al.*, 2019). The first response is inflammation, which involves neutrophils and inflammatory monocytes in the local cell neighbourhood and prevents bacterial dissemination. Clinical symptoms of enterocolitis manifest later, within 6 to 48 hours, in the form of nausea, vomiting, diarrhoea, abdominal pain, along with fever and chills which usually last 2 to 7 days after *Salmonella* ingestion (Giannella, 1996).

The adaptive immune response is the second line of defence, generally triggered by the recognition of *Salmonella* LPS and flagellin by dendritic cells residing in the intestinal mucosa (Kurtz, Goggins and McLachlan, 2017). This induces their maturation and antigen presentation (Kurtz, Goggins and McLachlan, 2017), and may also trigger their migration to lymphoid tissue to involve lymphocytes and prompt the generation of *Salmonella*-specific T and B cells (Sierro *et al.*, 2001). This generally puts a stop to the infection and yields partial protective immunity to future infections (Kurtz, Goggins and McLachlan, 2017). However, as mentioned, *S.*Tm is known to exploit the dysbiosis in the gut lumen as a result of the activated immune defence to boost its own growth and overcome colonisation resistance (Stecher *et al.*, 2007; Winter *et al.*, 2010).

Enteric fever

Enteric fever is caused by *Salmonella* which penetrate far into the intestinal mucosa and the bloodstream (sepsis). By getting access to the bloodstream or lymph, *Salmonella* can spread to cause secondary infections at a multitude of systemic sites (Mastroeni and Grant, 2013). Specifically, *Salmonella* in the deep mucosa or submucosa can invade inflammation-borne phagocytic leukocytes, such as macrophages, monocytes, dendritic cells, or neutrophils, wherein they can survive and replicate without eliciting a further immune response (Kurtz, Goggins and McLachlan, 2017; McLaughlin *et al.*, 2019). As

these cells migrate away, the bacteria can translocate to systemic sites and cause secondary infections in e.g. the liver, spleen, gallbladder, or bone marrow (Mastroeni and Grant, 2013).

Due to its unspecific nature, the fever can produce many, non-specific symptoms and is often clinically indistinguishable between infectious and non-infectious diseases, further increasing the challenge to identify and treat the disease (reviewed by Waddington, Darton and Pollard, 2014). Symptoms are equally variant and include abdominal pain, nausea, constipation, diarrhoea, headache, and cough, but also myalgia, arthralgia, chills, anorexia, weight loss, and rash, all of which also vary greatly in severity between patients (Waddington, Darton and Pollard, 2014).

Enteric fever is generally caused by host-adapted specialist species, e.g. *S.*Typhi for humans. However, also a specific invasive NTS (iNTS) serovar, *S.*Tm ST313, has emerged as a ruthless pathogen by causing multiple epidemics in several countries in Africa (Feasey *et al.*, 2012; Murray *et al.*, 2022). The pathogen targets the immune deficient, with the most important risk factor being HIV infection in adults, and malaria, HIV, and malnutrition in children, and has devastating mortality of up to 25% of those afflicted that do not receive appropriate antibiotics (Feasey *et al.*, 2012). This underlines the importance of effective treatments for *Salmonella* pathogens.

Treatment

The gastrointestinal tract is a challenging bottleneck for *Salmonella*, necessitating an infectious dose (i.e. the number of bacteria required to be ingested to prompt disease) of >1000 bacteria for enterocolitis and >100 000 (typhoidal) bacteria for sepsis and enteric fever (Kothary and Babu, 2001). Therefore, most infections cause self-clearing enterocolitis that does not require medical therapy and consequently are likely to go undiagnosed or unreported. However, for individuals at risk, such as the immunodeficient, the very young and the elderly (Pham and McSorley, 2015), there are treatment options.

Since the discovery of penicillin, antibiotics have been the only real cure against bacterial pathogens in a physician's arsenal. Generally, antibiotics target key aspects of the central dogma (i.e. replication, transcription, and translation; Crick, 1970), leading to the inhibition of growth or outright death of a bacterium. However, as bacteria are quickly adapting to thwart the effects of antibiotics, patient treatment requires specific knowledge of the resistance phenotype of the in-patient-specific strain. Multiple antibiotics are still efficient against the *S.*Tm SL1344 reference strain at least *in vitro* (as explored in **Paper IV**). Relevant to the thesis are for example ciprofloxacin (CIP; a fluoroquinolone), chloramphenicol (CHL; an amphenicol), meropenem (MER; a beta-lactam) and nitrofurantoin (NIT; a nitrofuran). CIP impedes DNA replication and transcription by trapping DNA-topoisomerases at their DNA cleavage site, thereby causing tension in the DNA double helix which generates DNA double-strand breaks, activating the stringent response (Drlica *et al.*,

2008). CHL blocks protein synthesis by obstructing the bacterial 50S ribosomal subunit, inhibiting the elongation of the nascent peptide chain, and limiting growth (Marks *et al.*, 2016). Beta-lactams such as MER stops the extension of the peptidoglycans chain during cell well biosynthesis, thereby also hindering cell growth (Lima *et al.*, 2020). Finally, NIT breaks up into several, highly reactive forms inside the bacterium, collectively inhibiting replication, translation, cell wall assembly, and protein synthesis, blocking growth and triggering the bacterial stress response (Aedo, Tang and Brynildsen, 2021).

Evidence shows that antibiotic treatment may not be beneficial at all, and would rather increase the recovery time of patients compared to no treatment (Stapels *et al.*, 2018). Furthermore, careless use of antimicrobials has borne rampant multidrug-resistant strains (Parkhill *et al.*, 2001; Mather *et al.*, 2013; Wiesner *et al.*, 2016; Martínez *et al.*, 2017; Klemm *et al.*, 2018), necessitating the development of new antimicrobials for treatment (World Health Organization, 2017), as well as the deployment of preventative treatment options such as vaccines. Today, there are multiple licenced vaccines against *S.*Typhi: a killed whole-cell vaccine, a live attenuated vaccine, a Vi capsular polysaccharide vaccine, and novel derivations thereof (Waddington, Darton and Pollard, 2014; Kurtz, Goggins and McLachlan, 2017). However, they have low efficiency (protective in 50-60% of cases) and have several other limitations, for example that they are restricted to adults, require specialised training to administer, and the protection only lasts for 2-3 years (Kurtz, Goggins and McLachlan, 2017). Thus, no highly efficient vaccine that can be easily deployed in poor countries exists today. The lack of vaccines and antibiotics poses a problem that necessitates action before we completely run out of options (World Health Organization, 2017).

Host invasion

To exploit intracellular niches, and to penetrate further to systemic sites, *Salmonella* utilise an arsenal of specialised proteins and molecular machines to move around, attach to and penetrate defensive barriers such as the intestinal epithelium, invade and linger within host cells, evade immune cells, and ultimately survive (Ibarra and Steele-Mortimer, 2009a; Jajere, 2019). From the point of view of the host, this bacterial arsenal is responsible for the severity of the pathogen-borne disease and its symptoms, which is referred to as virulence. There are both direct and indirect features of virulence, e.g. the "classic" virulence factors such as the flagella, type 3-secretion systems, and a host of both adhesins (e.g. the giant SiiE adhesin) and toxins (e.g. endotoxin; LPS), but also indirect concepts such as diversity, cell shape, and swim modality, which also play important roles in virulence (as discussed in detail in **Papers III-IV**).

A central concept is that the energy cost of expressing the virulence machinery is not insignificant, and is as such tightly regulated depending on environmental cues connected to their path through the host gastrointestinal tract and beyond. Studies have put such cues to significant scrutiny (Kröger *et al.*, 2013; Srikumar *et al.*, 2015; Canals *et al.*, 2019; Avican *et al.*, 2021). For example, some components are activated by elevated temperature (Mo *et al.*, 2006), while others are activated by acid stress (Muller *et al.*, 2009; Karash, Jiang and Kwon, 2022). Similarly, flagellar swimming (a vital virulence determinant) is activated by starvation signals such as ATP depletion and the lack of certain amino acids (e.g. histidine; Galloway and Taylor, 1980). The most common functions to move and invade are therefore generally inactive in growth-permitting, rich conditions, while starvation and stress cue the expression of new virulence machinery. In this chapter, the virulence factors driving host cell invasion are detailed.

The *S.*Tm type-3 secretion systems

Bacteria are often equipped with several large molecular complexes that facilitate the secretion of select content (Green and Mecsas, 2016). These "secretion systems" are used by many bacteria for many different purposes, and can be specific to a single protein or secrete a broad repertoire. Further, the secretion systems have different configurations to discharge cargo over a varying number of phospholipid membranes either to the immediate extracellular space or into the cytoplasm of e.g. host cells (Green and Mecsas, 2016). Twelve secretion systems have been identified so far (Green and Mecsas, 2016), of which two are generally present in most bacteria, specifically the so-named general secretion system (Sec; Driessen, Manting and van der Does, 2001) and the twin-arginine translocation system (Tat; Berks, Palmer and Sargent, 2005). Gram-positive bacteria can also produce the SecA2, sortase, type 7, and other secretion systems, while gram-negative bacteria carry a range of classes simply referred to as the type 1-6 secretion systems.

In the context of *Salmonella* host cell invasion, the type 3 secretion systems (T3SS) are of crucial importance. *S.*Tm carries two T3SSs, referred to as T3SS-1 and -2, which allow *Salmonella* to invade non-phagocytic epithelial cells, and create and maintain an intracellular replication niche (McGhie *et al.*, 2009), that warrant further description below.

Common structure

The T3SSs are dedicated structures with the explicit function to deliver effector proteins into the cytoplasm of a target host cell (reviewed by Galán *et al.*, 2014). Fundamentally, the T3SS can be regarded as a stack of ring-like protein complexes, forming a long injector system. Its base spans the bacterial envelope and connects the long, extracellular "needle" filament to the cytoplasmic sorting platform. The envelope-embedded base consists of a ~25nm wide tube

that spans both cell membranes (Galán *et al.*, 2014), while the hollow needle protrudes ~21nm from the base into the extracellular space (Wee and Hughes, 2015) and carries a special complex formed by lipophilic proteins at the distal tip. The tip is inserted into the lipid bilayer of a target cell, connecting the bacterial cytoplasm to the target's cytoplasm through the hollow T3SS (Galán *et al.*, 2014).

However, effectors do not simply diffuse from the bacterium into the host cell. Firstly, the channel is not wide enough for passive transfer. Indeed, the needle is a very thin structure of only ~2.5nm in inner diameter and thus can only support the active translocation of unfolded proteins (Loquet *et al.*, 2012). Secondly, effector secretion happens in a specific, sequential manner controlled by the T3SS sorting platform in the bacterial cytoplasm. The sorting platform is a cage-like mesh that consists of the protein SaO supported by OrgA and OrgB, powered by the hexameric adenosine triphosphatase InvC (Lara-Tejero *et al.*, 2011; Cheng *et al.*, 2017). InvC is connected to the export apparatus protein InvA through the small coupler protein InvR, showing the intimate link between the sorting platform and the export apparatus. Apart from InvA, the export apparatus consists of SpaP, SpaQ, SpaR, SpaS (Wagner *et al.*, 2010). The Spa proteins are structural proteins that form an entrance into the secretion apparatus from the bacterial cytoplasm, as well as a rigid chute into which effector proteins are fed by the sorting platform (Wagner *et al.*, 2010). The SaO-OrgA-OrgB sorting platform forms a chamber-like structure wherein the effectors are uncoupled from their cognate chaperones and are unfolded (Puhar and Sansonetti, 2014; Hu *et al.*, 2017), sequentially loading the secretion system with unfolded effectors (Lara-Tejero *et al.*, 2011). These are then transported through the T3SS with the help of specific translocases and are secreted into the host cell cytoplasm where the effectors promptly fold into their active form (Lara-Tejero *et al.*, 2011). As such, the sorting platform together with the export apparatus hosted in the base complex, carries out organised, sequential secretion of the effectors (Kubori *et al.*, 1998; Wagner *et al.*, 2010; Lara-Tejero *et al.*, 2011; Hu *et al.*, 2017). Secretion happens at an impressive speed of around 7-60 molecules per second, and secretion is normally completed within a few minutes of contact with the target cell (Puhar and Sansonetti, 2014).

T3SS-1

The T3SS-1 is critical for host cell invasion. This secretion system mediates a robust, irreversible binding between bacterium and host cell as *S.*Tm inserts its T3SS-1 translocon tip into the host cell membrane, referred to as "docking" (detailed in the section on adhesion). The T3SS-1 then facilitates the injection of effector proteins from the docked *Salmonella* into the host cell. The T3SS-1 itself (and some of its effectors) is encoded on an SPI (specifically SPI-1). Its effectors are involved in host cell invasion (Lou *et al.*, 2019), detailed below (and further explored in **Papers I-II**).

The effectors SopB, SopE, and SopE2 (SopBEE2) are linked to host cell invasion in many models, and their effects have been extensively studied in epithelial and phagocytic cell lines (Bakshi *et al.*, 2000; Hapfelmeier *et al.*, 2004; Raffatellu, Wilson, *et al.*, 2005). The effectors SopE and SopE2 are G-nucleotide exchange factors (GEFs) that activate host Rho GTPases such as Rac-1, Cdc42, and RhoG, culminating in Arp2/3 activation, prompting explosive actin nucleation and reorganisation (Hardt *et al.*, 1998; Bakshi *et al.*, 2000; Friebel *et al.*, 2001; Hapfelmeier *et al.*, 2004). SopB is an immunogenic inositol phosphatase phosphatase, which activates e.g. endogenous GEFs and downstream Cdc42 and RhoG, to promote cytoskeletal reorganization in concert with SopE and SopE2 (Zhou *et al.*, 2001; Patel and Galán, 2006). In epithelial and phagocytic cell lines, SopBEE2 generate massive entry structures referred to as "ruffles", which mediate *Salmonella* internalisation via macropinocytosis (Francis *et al.*, 1993). Ruffles can even be so large that also bystander bacteria are taken up into the cell (Lorkowski *et al.*, 2014), referred to as cooperative invasion.

Other T3SS-1 effectors are SipA, which has multiple functions but importantly stabilises actin filaments during ruffle formation (Zhou, Mooseker and Galán, 1999b, 1999a; McGhie, Hayward and Koronakis, 2004), and SptP, which is responsible for reversing the changes on the host cell actin network after internalisation by acting as a GTPase-activating protein (GAP) for the Rho GTPases (Johnson *et al.*, 2017). SipA, and in come contexts SptP, contribute to efficient internalisation. As such, many effector proteins are secreted and are involved in shaping the internalisation mechanism. However, the T3SS-1 also has alternative functions, such as regulating the host cell immune response. AvrA is an effector responsible for immune response inhibition *in vivo* (Hardt and Galán, 1997; Wu, Jones and Neish, 2012), although many T3SS-1 effectors mould the local milieu by regulating immune signalling (Lou *et al.*, 2019). This is important, as innate immunity NODs are sensitive to T3SS-1 effectors and can also sense cytoskeleton rearrangements via the Rho GTPases (Keestra-Gounder and Tsolis, 2017). Indeed, the T3SS-1 has many critical functions during host invasion.

T3SS-2

The T3SS-2 is important for intracellular survival but is not involved in the invasion step itself (Jennings, Thurston and Holden, 2017). Similar to the T3SS-1, this system is also hosted on a SPI (here SPI-2). It is activated in SCV-lodged *S.*Tm post-internalisation, where it will inject a subset of at least 28 possible effectors past the vacuolar membrane and into the host cell cytosol. These effectors have varied functions (Jennings, Thurston and Holden, 2017), ranging from detoxifying nearby lysosomes and maintaining vacuole stability, to keeping the immune response at bay. Others are involved in trafficking, tethering the SCV to microtubules, recruiting late endosomes and motor proteins like kinesin-1, or inhibiting F-actin formation around the SCV

(Jennings, Thurston and Holden, 2017). These functions stabilise the intravacuolar niche and promote *S.*Tm survival and proliferation (which is part of **Paper I-II**).

Adhesion to host cells

Preceding T3SS-1-mediated internalisation, *Salmonella* need to closely adhere to the host cell surface. Bacteria attach to such surfaces by van der Waals and electrostatic forces, and cell shape and surface-facing area will affect such interactions (van Loosdrecht *et al.*, 1990; Kendall and Roberts, 2015). However, adhesion selectivity is essential for appropriate target cell selection, so bacteria have evolved specific adhesion proteins – adhesins – that determine the host range, virulence, disease progression, and transmission efficiency of the bacterium, so also in *Salmonella* (Yue *et al.*, 2012). These include the giant *Salmonella* adhesin SiiE, a host of fimbriae and pili, and some miscellaneous adhesins (fig. 4). In the context of virulence, and specifically, for the host cell adhesion preceding invasion, the determining factors of the interaction between host and pathogen are thus four-fold: (1) cell shape and size, (2) expressed set of adhesins, (3) distribution of said adhesins on the bacterial surface (depending on cell shape etc., an important point in **Papers III-IV**), and (4) the presence of accessible adhesin target structures on the host cell surface. As such, for *Salmonella* species, the set of expressed adhesins hints at their host adaption; *S.*Typhi, a human pathogen, carries a separate set of adhesins to the generalist pathogen *S.*Tm. Below follows a detailed of known *S.*Tm adhesion factors.

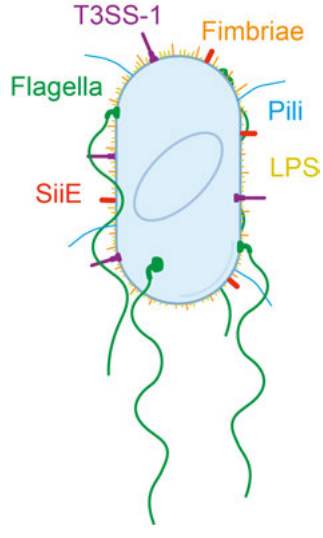

Figure 4. The *Salmonella* surface. Surface-available adhesins produced by *Salmonella*. Also LPS, which is recognised by host immunity, is shown. Not to scale in prevalence or size.

The giant adhesin

The giant *Salmonella* adhesin SiiE is a very large adhesin located on the outer membrane, consisting of 53 repetitions of a bacterial immunoglobulin domain enabling it to reach beyond the height of the sprawling *S.*Tm LPS (Peters *et al.*, 2017). It is carried on SPI-4, along with the SiiABCDF genes encoding a SiiE-specific type-1 export system which seats SiiE in the membrane. SiiE is activated extracellularly, as host cells commonly restrict access to the calcium required for both SiiE secretion and function (Peters *et al.*, 2017). The giant adhesin binds in a lectin-like manner, targeting host cell membrane glycans

(specifically containing GlcNAc and α2–3-linked sialic acid; Barlag and Hensel, 2015). SiiE is dispensable for both invasion of non-polarised epithelial cells (Gerlach *et al.*, 2007) and for intracellular functions (Li *et al.*, 2019), but is important for targeting these glycans on cells in the brush border of polarised epithelia. In the intestinal lumen, SiiE is therefore the initial facilitator between *S.*Tm and the epithelium (confirmed in **Paper II**). SiiE targets the apical cell surface-bound mucin 1 (MUC1) in a receptor-like manner, which is critical for apical invasion into epithelial cells in the intestinal mucosa (Li *et al.*, 2019). The efficient binding of MUC1 is dependent on also binding glycans on the host cell surface (Li *et al.*, 2019). Furthermore, SiiE-mediated binding to the brush border microvilli may be required to properly position the T3SS-1 on the surface of enterocytes, in order to facilitate effector translocation (Wagner *et al.*, 2014). Thus, SiiE is a crucial adhesion factor for invasion into the host gut mucosa *in vivo*.

Fimbriae

The *Salmonella* fimbriae are a broad group of adhesins that include an array of 35 general operons, including Agf, BcF, Csg, Fim, Lpf, Pef, and Saf, as well as *Salmonella*-specific operons named Stb, Stc, Std, Stf, Sth, Sti, and Stj. However, only a discrete set of 5-14 different adhesins is carried by any specific strain, an example of differential host adaptation within the genus (Yue *et al.*, 2012). A notable difference to *Escherichia coli* is that *Salmonella* do not carry curli fimbriae (*csg* operon), but nonetheless Agf fimbriae (*agf* operon) can aggregate to form filaments with highly similar functions (Collinson *et al.*, 1996; Römling *et al.*, 1998). Most of these are differently expressed depending on the context, and some are highly specific to a certain environment, e.g. Fim, Std, and Stj fimbriae are important specifically for host gut colonisation in chickens and mice, and long-term intestinal persistence (Boddicker *et al.*, 2002a; Ledeboer and Jones, 2005; Akkoç *et al.*, 2009; Chessa *et al.*, 2009). Similarly, Pef and Lpf contribute to adhesion to invasion targets in the mouse intestine (Bäumler *et al.*, 1996; Bäumler, Tsolis and Heffron, 1996) and, together with Bcf and Csg fimbriae, to forming biofilms and binding extracellular matrix (Ledeboer *et al.*, 2006). As such, the *Salmonella* fimbriae consist of varied adhesins important for adhesion in different contexts.

Pili

For the purpose of host cell adhesion, bacteria have evolved long, filamentous pili with a tip distal enough to lodge adhesion factors outside any repulsive electrostatic interactions between bacterium and host cell (Proft and Baker, 2008). While *S.*Tm hosts many and diverse fimbriae, it is not equipped with many pili, and is for example missing the type IV pili required for twitching motility (a topic of the section on motility below). However, it does carry a few pili that mediate part of its host-specificity, e.g. the Fim and Saf pili.

Fim is a type I pilus and a factor in long-term, but reversible, binding of mammalian epithelial cells (Misselwitz *et al.*, 2011). The Fim pilus has a cumulative adhesion effect with fimbriae of the Lpf, Pef and Agf loci (van der Velden *et al.*, 1998), but is negatively correlated with Stb (Wu *et al.*, 2012). Interestingly, these pili are specific for planktonic and swimming bacteria, as they are only expressed in liquid media (Wu *et al.*, 2012). Furthermore, the *S.*Tm-specific variant of the tip protein of the Fim pilus, FimH, comes in two allelic variations, one highly adhesive to human epithelial cells and the other adhesive to those of other hosts (Boddicker *et al.*, 2002b; Misselwitz *et al.*, 2011), showing minor host adaptions to humans for *S.*Tm. The Saf pilus is important for host recognition and biofilm formation. Its tip consists of chains of two tip proteins, both of which are required for efficient host recognition: SafD, representing initial host cell recognition and adhesion, and SafA, forming poly-adhesive chains (Zeng *et al.*, 2017). The Saf tip is rather long, formed by >100 SafD-(SafA) elements (Zeng *et al.*, 2017), making them highly important for virulence in pigs but not in mice (Zeng *et al.*, 2017).

Flagella during adhesion

The flagella, generally used for swimming (expanded on further below), are also an important adhesion factor (Horstmann *et al.*, 2020). Cholesterol is prevalent in all animal cell membranes and intensifies the tight membrane packing, thus enhancing membrane rigidity and integrity (Mouritsen and Zuckermann, 2004; Crawford, Reeve and Gunn, 2010). The FliC flagellin, a structural component of *S.*Tm flagella, binds cholesterol on host cells, while an alternate flagellin FljB is not necessary for cholesterol binding (Crawford, Reeve and Gunn, 2010). Methylation of the flagella can increase these hydrophobic interactions with the cell membrane further, while flagella lacking methylation are outcompeted in the mouse gut *in vivo* (Horstmann *et al.*, 2020).

Docking

The T3SS-1 mediates second-wave adhesion through host cell "docking", which is a strong, irreversible binding to the cell subsequent to the initial, transient interaction. Docking needs to be facilitated by the reversible binding to the cell by other adhesins and involves the insertion of a pore-forming protein complex into the host cell membrane (Misselwitz *et al.*, 2011). The pore-forming complex consists of the SipB and SipC proteins, which in turn are associated with the T3SS-1 tip protein SipD, effectively anchoring the bacterium to the host cell. All three proteins are essential for host cell invasion (Misselwitz *et al.*, 2011). SipB is known to bind cholesterol (Hayward *et al.*, 2005) while SipC is known to bind and nucleate/rearrange actin (Chang, Chen and Zhou, 2005). The SipC (*S.*Tm) homologue IpaC (of *Shigella flexneri*) interacts with

intermediate filaments of the host cell, which stabilises docking and is required for the formation of the pore; intermediate filaments might hence be a target also for SipC (Russo *et al.*, 2016).

Swimming and movement

A crucial factor for the survival and propagation of many bacteria is movement. It is also important in the context of disease, and the ability to move is a key virulence factor for many pathogenic species, including *Salmonella* (Das *et al.*, 2018). Movement can be achieved through both passive diffusion and self-propelled motility. However, it is important to first understand microscopic physics and why small things such as bacteria abide by seemingly different physical rules than we do.

Microbial life at low Reynold's numbers

After extensive studies in the field of biophysics, we understand a great deal of the microscopic and macroscopic laws that govern movement throughout the field of biology, reviewed in great detail by the late Howard C. Berg (Berg, 2018). These laws are vital for understanding life at microscopic dimensions (Purcell, 1998; Brewer, Peltzer and Lage, 2022). For example, small things moving through fluids are governed by viscous forces (drag) but not by inertia (Stokes' law; Stokes, 2010). This can be quantified by calculating the Reynolds number, which is simply the relative size of inertia over viscosity (Sommerfeld, 1908; Stokes, 2010). At low Reynolds numbers, flow tends to be laminar and movement depends on viscosity, while at high Reynolds numbers flow is turbulent and movement depends largely on inertia. For example, if we would compare a fish to a bacterium swimming in identical beakers, we could observe their swimming modalities and see that the fish propels itself by accelerating water (pushing water away), while the bacterium propels itself by viscous shear (pushing/pulling itself away). As such, one knows the Reynolds number is large for the fish and low for the bacterium; fish and bacteria depend on different physical properties to propel themselves (Berg, 2018). Indeed, the lack of inertia on the bacterium means that a bacterium that stops swimming does not coast longer than a fraction of the diameter of a hydrogen atom (0.04 Ångström), or, as Howard C. Berg put it, "the fish knows a great deal about inertia, the bacterium knows nothing" (Berg, 2018).

Diffusion

Passive diffusion involves concepts such as sliding (spreading by latitudinal growth of underlying host cell layer), but also diffusion via Brownian motion, the random movement of particles arising from shifts in thermal energy (ten Hagen,

van Teeffelen and Löwen, 2011). Brownian motion thus depends on entropy and describes that particles in solution will bump into other molecules (e.g. water molecules) in the media in which they are suspended, causing them to move around each other and resulting in a so-called random walk (Einstein, 1956; Berg, 2018).

Motility

Motility, in contrast to diffusion, is directional and requires robust force generated by the individual cell, and includes several types of movement, e.g. twitching motility, gliding, and swimming (Henrichsen, 1972; Spormann and Kaiser, 1995; Burrows, 2012; Grognot and Taute, 2021). Twitching is a well-defined form of motility using a grapple hook-like "catch and pull" mechanism to generate momentum, typically dependent on type IV pili that extend, attach, and contract to pull a bacterial cell forward (e.g. in *Pseudomonas aeruginosa*; Mattick, 2002). Gliding, on the other hand, incorporates several poorly-understood mechanisms of directional motility, proposed to drive the bacterium via machinery analogous to caterpillar treads (e.g. in *Flavobacterium*; Braun *et al.*, 2005) and "jet engines" spewing out polysaccharides to push the bacterium forward (e.g. in cyanobacteria; Khayatan, Meeks and Risser, 2015). However, *Salmonella* do not have the type IV pili required for twitching motility (Mattick, 2003; Park, Pontes and Groisman, 2015) and have not been found to employ gliding. They are, however, equipped with an elegant apparatus required for swimming. Swimming is the fastest known form of self-propelled movement in bacteria by far, at around 25µm/s for enterobacteria such as *Salmonella* (Macnab and Koshland, 1972; Misselwitz *et al.*, 2012) both in contrast to gliding at <5µm/s (varies between mechanisms; Spormann and Kaiser, 1995; Hoiczyk and Baumeister, 1998; Nan and Zusman, 2016) and twitching at <0.5µm/s (Burrows, 2012). It forms the prevailing method of movement in *Salmonella* and is also a core focus of this thesis (especially in **Papers II-IV**).

Swimming

The laws of hydrodynamics state that a polarised cell shape is necessary for efficient swimming through liquids, or that effective bacterial swimmers must have a "fore" and an "aft" (Young, 2006). As such, *Salmonella* form rod-shaped vessels that in liquid swim straight forwards along their internal longitudinal axis (or *foreward*, in keeping with the nautical analogy; Berg and Turner, 1990). However, near a surface, *Salmonella* and similar bacteria swim in roughly circular, overlapping loops (Lauga *et al.*, 2006; Ledesma-Aguilar, Löwen and Yeomans, 2012; Park, Kim and Lim, 2019). In fact, in simplistic experiments where e.g. *Salmonella* are put on glass, this is commonly observed also in reality (presented in **Papers III-IV**). To understand why one has to understand how the bacterium swims, and the forces that are generated by swimming.

Bacterial swimming is driven by one to several extracellular flagella, long hair-like filaments that protrude from the bacterium. Different bacteria have different numbers of flagella differentially distributed on the cell body, which determines the swimming locomotion characteristics of the species. *S.*Tm carries ~2-8 randomly distributed (peritrichous) flagella (Bonifield and Hughes, 2003; Misselwitz *et al.*, 2012; Furter *et al.*, 2019). The flagella are self-assembling nanomachines that consist of ~20,000 subunits of a single protein, flagellin (Renault *et al.*, 2017). As briefly mentioned in the chapter on host cell adhesion, *S.*Tm has two genes for flagellin, *fljB* and *fliC*, which autonomously switch their expression at a frequency of 10^{-3}–10^{-4} per cell per generation (Yamaguchi *et al.*, 2020). Thus, *S.*Tm flagella are either made from FljB or FliC, an example of phase variation (explained further below) thought to shroud the flagella from host immune recognition (Yamaguchi *et al.*, 2020). The two types of *S.*Tm flagella are structurally nearly identical, except for the outermost domain of FljB flagella which is somewhat more flexible and mobile (Yamaguchi *et al.*, 2020). This has the effect that FljB flagella are better at facilitating swimming under high-viscosity conditions (Yamaguchi *et al.*, 2020), albeit FliC remains a requisite for adhesion to host cell cholesterols (Crawford, Reeve and Gunn, 2010).

Structurally, the long, extracellular flagella filaments form a hollow, helical structure several times longer (>7µm) than the bacterial cell body (~2µm), that protrudes out from the bacterium (Erhardt *et al.*, 2011). The flagellar filament is linked to the bacterial membrane via a 0.055µm (55nm) long extracellular curved filament referred to as the "hook", which in turn is connected to the flagellar basal body implanted in the membrane (Erhardt *et al.*, 2011). The base of the flagellum is a T3SS, and during flagellum assembly, it pumps flagellin from the bacterial cytoplasm through the 2nm diameter hollow tube of the flagellum to its distal end, where flagellin self-assembles at the tip (Renault *et al.*, 2017). The flagellum grows at an impressive speed of ~100nm (~213 flagellin subunits) per second, albeit it slows down rapidly as the flagellum grows in length, at least in laboratory settings (Renault *et al.*, 2017). Consequently, the assembly of the expansive flagella requires significant energy, and it has been proposed to be a multi-generation process in nature where flagella are inherited for several generations (Aizawa and Kubori, 1998).

When assembled, the flagellum, naturally conforming to the shape of a left-handed helix turning counter-clockwise (CCW) along its length, is rotated to generate forward momentum by viscous shear (Berg and Anderson, 1973). The force for the rotation is generated in the base, consisting of a series of ring complexes which host a static motor (stator proteins), as well as a rotating multi-unit rod through their centre (rotor proteins) attaching to the hook filament extracellularly. The *S.*Tm stators consist of the transmembrane MotA, located in the inner membrane, and MotB, tethering MotA to the peptidoglycan cell wall, both of which are required for locomotion (Muramoto and Macnab, 1998). Powered by the proton motive force, several MotA jointly rotates

the rotor rod CCW, under the control of a set of proteins referred to as the "switch complex" (FliG, FliM, and FliN). This can switch the rotor to clockwise (CW) rotation in response to certain chemotactic signals (further detailed later; Paul *et al.*, 2011). In multi-flagellated species such as *S.*Tm, several flagella bundle together during swimming, mediated by the matching flow generated by their CCW rotation, forming a uniform forward force (Flores *et al.*, 2005).

Finally we arrive at an explanation as to why bacteria such as *Salmonella* swim in circles along a flat surface (explored by e.g. Lauga *et al.*, 2006). The CCW-spinning flagella bundle naturally generates a counteracting CW rotation of the bacterial body along the axis of swimming (the y-axis), stabilising forward movement similar to an arrow shot from a bow. Bacteria close to surfaces will experience drag forces from said surface, at a 90° angle to the direction of swimming (the x-axis) due to its CW rotation. A similar drag but in the opposite direction is generated on the flagella bundle, rotating CCW (i.e. -90°). Since the body and flagella bundle are spatially separated, the result of these opposite forces is the rotation of the bacterium (along the z-axis; fig. 5A). Viewed from above, the bacterium will turn slightly to its right, eventually looping around to form the aforementioned overlapping circular paths (fig. 5B; Lauga *et al.*, 2006).

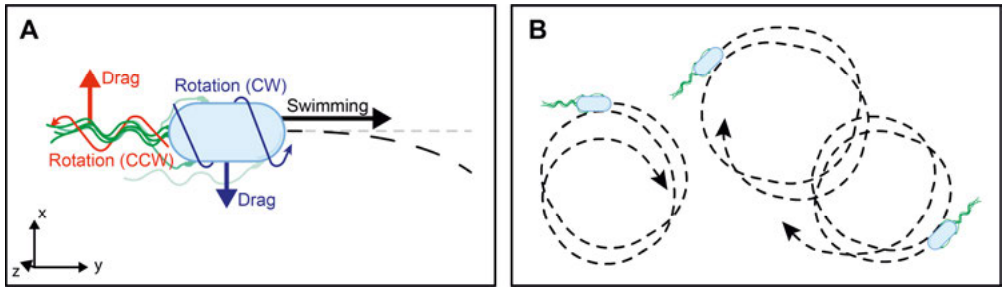

Figure 5. *Salmonella* close to surfaces swim in overlapping circles due to drag. (A) Forces on a bacterium close to a surface, as viewed from above. The CCW torque from the flagella bundle causes counter-rotation on the bacterial body. Both bundle and body close to surfaces experience drag, and in different directions due to their opposite rotations, causing the bacterium to slowly turn clockwise, i.e. slowly turn right in the direction of its movement (if viewed from above). (B) The swimming results in imperfect but circular swim patterns. Not to scale.

Furthermore, while there is no known way to direct steering of the swimming during runs, extracellular signals (mediated by e.g. CheY) can trigger the switch complex to reverse the rotational axis of its flagellum (Paul *et al.*, 2011). In a bundle, a single flagellum rotating CW rather than CCW is enough to generate significant turbulence, leading to the complete unbundling and loss of unison in the driving forces (Flores *et al.*, 2005). This unstructured move-

ment in all directions is referred to as "tumbling", and without collective forward motion, the bacterium grinds to a temporary stop, which is utilised for a form of chemotaxis (described below).

Chemotaxis

In the environment, there are flurries of chemicals that act as attractants (such as nutrients) and repellents (such as toxins), creating landscapes of gradient signals. Such signals are beneficial for finding infection targets (Matilla and Krell, 2018) and also outside the host, for e.g. colony expansion, localization of hosts or symbiotic partners and contribution to microbial diversity by the generation of spatial segregation in bacterial communities (Keegstra, Carrara and Stocker, 2022). The chemical landscapes are navigated by *S.*Tm using a run-and-tumble strategy, in which attractants sensed by the bacterium causes it to frequently tumble (Sourjik and Wingreen, 2012). *S.*Tm (and likely other bacteria) also reorients itself if not swimming along the direction of the gradient, i.e. towards attractants and away from repellents (Nakai, Ando and Goto, 2021). In summary, this leads to a biased random walk, with longer runs and net movement in the preferred direction (Sourjik and Wingreen, 2012; Nakai, Ando and Goto, 2021). Indeed, this forms a careful course correction system that adjusts the course about every second (Berg and Brown, 1972), which trumps swimming randomly (Taktikos, Stark and Zaburdaev, 2013).

Swarming

The traditional view of the lone, swimming bacterium as the communicable agent is challenged by insights into the concept of swarming (reviewed in Kearns, 2010). This special type of motility happens only on surfaces (where swimming is impossible) and is characterised by several bacteria organised side by side in "rafts", conserved in only three classes of bacteria, including *Salmonella* (Kearns, 2010). The benefit of swarming is still not completely elucidated, but it may generate more force than swimming individually and may in many aspects have similar functions to a biofilm (e.g. for protection). Although it is conceptually different from swimming, the formation of swarms requires flagella (even additional flagella synthesis) along with the presence of a surfactant and increased cell-cell interactions (Kearns, 2010). Indeed, the flagella are dominant in motility for many bacteria, and are imperative to pathogens such as *Salmonella*, for both swimming and swarming.

Heterogeneity

*S.*Tm multiply through clonal expansion; a single mother cell copies her DNA, puts one copy in each of the two cell poles, and splits down the middle to create two identical daughter cells. By effect, this means that the two daughter cells will inherit the same genetic code. However, single-cell analyses have

for over a decade highlighted that there is a lot of phenotypic variation in clonal bacterial populations, including their morphology (when inspected through a microscope; Ackermann, 2015; van Teeseling, de Pedro and Cava, 2017), growth (Roostalu *et al.*, 2008; Wallden *et al.*, 2016), nutrient absorption (Ackermann, 2015), and resistance to toxic chemicals such as antibiotics (Balaban *et al.*, 2004; Arnoldini *et al.*, 2014). Thus, a phenotype is not determined by genotype and environment alone.

Causes of heterogeneity

"Adversity has the effect of eliciting talents, which in prosperous circumstances would have lain dormant"—Horace (65BC-6BC)

A certain heterogeneity is universally beneficial, evident by its presence in virtually all organisms that ever existed throughout history, ranging from colossal, intricate dinosaurs to microscopic, simplistic bacteria. The common denominator is that small variation within a population are better for the community than complete homogeny. Indeed, in line with the quote above, also modern game theory suggests two models of microbiological heterogeneity based on adversity: division of labour and bet-hedging (Wolf, Vazirani and Arkin, 2005; Veening, Smits and Kuipers, 2008; Ackermann, 2015; Sánchez-Romero and Casadesús, 2018; García-Pastor, Puerta-Fernández and Casadesús, 2019).

Division of labour

A constant environment permits the formation of specialised, cooperating subpopulations within the main population (García-Pastor, Puerta-Fernández and Casadesús, 2019). This idea is based on natural heterogeneity, i.e. the natural variance between individuals (e.g. gene expression variance, discussed in the next section). Under selective forces, such heterogeneity could select for two phenotypically different subpopulations, which are stronger together (when remaining in the same environment) but that would be weaker apart (explored in **Paper III-IV**).

An example in *S.*Tm is SPI-1, which encodes the T3SS-1 and thus the phenotype for host cell invasion (Sánchez-Romero and Casadesús, 2018). In such *S.*Tm populations, small numbers of SPI-1-expressing cells can be sufficient to endow the population with the capacity to invade and permit host colonisation regardless of the bottlenecks encountered by *Salmonella* populations inside animals. Similarly, it has been shown that a tiny subset of *S.*Tm normally expresses the Std fimbriae, which confer adhesion to the murine cecum as well as differential expression of several key virulence factors (Chessa *et al.*, 2009; García-Pastor *et al.*, 2018; García-Pastor, Puerta-Fernández and Casadesús, 2019). Such adhesion factors can endow the expressing subpopulation with similarly invasion capabilities specifically in the cecum, contributing to local host colonisation.

Bet hedging

Bet hedging is a similar concept to spreading the risk but in a fluctuating environment. Bet hedging entails that maladapted phenotypes develop within isogenic populations so that these subpopulations lose fitness in the existing environment in anticipation of a future environment (Grimbergen *et al.*, 2015). An example of bet hedging is persister bacteria, individuals that stochastically enter a state of slowed growth of dormancy in a protective environment, for example inside its host cell (Lewis, 2010). In dormancy, the reduced cellular activity results in reduced targets for antibiotics, meaning that they have a vastly increased resilience towards antibiotics. After treatment, the persisters can come out of dormancy and produce normally growing offspring. Thus, by gambling that e.g. the chemical challenge of antibiotics therapy may be coming, and going into dormancy, a small bacteria population survives and can repopulate.

Genetic multistability

Gene expression is well-studied, and it is well-established to be heterogeneous. Any certain gene, present in two neighbouring, isogenic bacteria, will have a chance to be naturally expressed at slightly different levels in the two bacteria (Ackermann *et al.*, 2008). Some heterogeneity is simply caused by noise, but several contributing mechanisms have been described (García-Pastor, Puerta-Fernández and Casadesús, 2019), such as bistability and phase variation.

Bistability

In a given environment, some genes are essential to (not) be expressed for the survival of the bacterium. *S.*Typhi, for example, has ~350 essential genes required for cell division, DNA replication, transcription, and translation (in laboratory conditions; Langridge *et al.*, 2009). The expression of non-essential genes can however vary, and can stochastically give rise to subpopulations which stably express the gene and those that stably do not. Hence, the gene is bistable. A cluster of such genes is the aforementioned T3SS-1, which bistability establishes co-existing phenotypes for host cell invasive and non-invasive specialists (Sánchez-Romero and Casadesús, 2018).

Phase variation

Phase variation can be thought of as “reverse bistability”, and describes cells that are far from stable but transition relatively frequently between states (Casadesús and Low, 2013; García-Pastor, Puerta-Fernández and Casadesús, 2019). This can be caused by the expansion and contraction of DNA sequence repeats, through epigenetic control of gene expression by DNA methylation, and via the formation of regulatory feedback loops transmissible to daughter

cells (Casadesús and Low, 2013). Genetic rearrangement is another mechanism, where *S.*Tm phase variation of the genes encoding the two antigenically distinct flagellins FljB and FliC is a common example. Flagellin phase variation is mediated by a post-transcriptional mechanism, worth divulging into in slightly more detail (Bonifield and Hughes, 2003). The flagellin FljB is co-expressed with a protein FljA under the same promoter, which is located on an invertible DNA segment. FljA inhibits the expression of the unlinked fliC gene. However, the *Hin* recombinase can invert the *fljAB* promoter, blocking the expression of FljB flagellin and the FljA inhibitor and thus allowing FliC expression, or invert it back, allowing FljB expression. Consequently, a single bacterial cell will produce FljB or FliC, but never both at the same time, but both can be available in subpopulations within a culture.

Bacterial shape variability

"To be brutally honest, few people care that bacteria have different shape" stated Kevin D. Young in his 2006 review on the impact of shape on the selective value of bacteria, and continued: "which is a shame, because the bacteria seem to care very much" (Young, 2006). However, as Young exemplifies in his review, the shape of bacteria has immense effects on their lifestyle and capabilities, and by studying shape it is possible to predict how proficient a microbe is at different tasks or in overcoming certain obstacles long before experimental confirmation.

However, although microbiologists today acknowledge the importance of the cell shape of their studied bacteria, it is often assumed to be more static than it is, and oversimplified to be merely the format of the small bag containing more exciting genetics and biochemistry (Young, 2006). This does not recognise that, to our current knowledge, shape has bearing on bacterial growth and cell division, differentiation, access to nutrients, motility and dispersal/spread, attachment to different surfaces and host cells, and predation (Young, 2006). One bacterial shape may benefit from an enhanced ability to form clingy biofilms, allowing it to survive on acidic soil or other hostile environments; another is well-adapted to the intestinal lumen, where it is shaped to spread better or produce sturdy colonies impervious to the chaotic nature of peristalsis (Young, 2006; Frirdich *et al.*, 2014; Stahl *et al.*, 2016; Bartlett *et al.*, 2017; Salama, 2020). As such, shape partly defines the specific milieu wherein a bacterium thrives, and, in the context of human health, the medical consequences of pathological bacteria. So, in examining and understanding the factors literally shaping the bacterium and its cellular frame, we can understand much of its behaviour and impact.

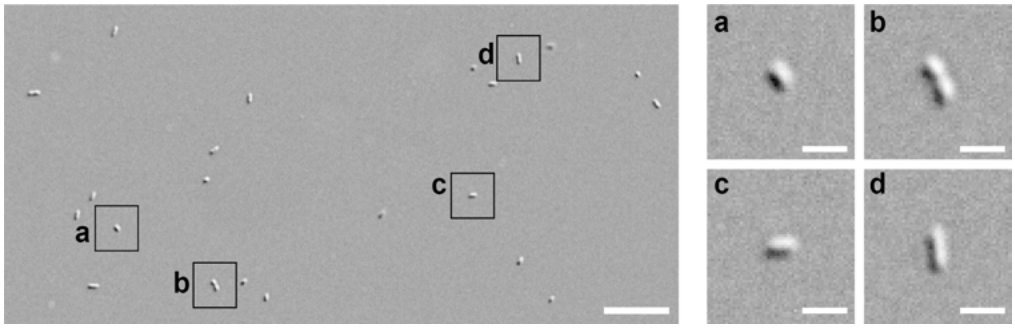

Figure 6. Bacteria in different growth phases cause shape heterogeneity in a typical, isogenic *S.*Tm culture. Untreated *S.*Tm grown for 4h in hypertonic Luria Broth. Four examples of different morphologies are shown (a-d, in no particular order). Scale bar 20µm (large image), 3µm (inserts).

Determinants of shape

As most cells, bacteria are structurally organised around a complex and overlapping network of internal filaments referred to as the cytoskeleton. Such cytoskeletal proteins create the foundation of the cell shape (van Teeseling, de Pedro and Cava, 2017) but are also key to the vast majority of cellular functions, working as scaffolding for cell division, cellular processes, trafficking highways, uptake and secretion, and motility. The prokaryotic cytoskeleton includes proteins such as the previously-mentioned MreB and ParM (Domínguez-Escobar *et al.*, 2011; Brzoska *et al.*, 2016; Hussain *et al.*, 2018), homologues of eukaryotic actin, forming a dense mesh underneath the cell membrane, and important for maintaining the rod shape in e.g. *S.*Tm (Margolin, 2009). It is reinforced with other Par-family proteins, functionally analogous to eukaryotic tubulin (factors of e.g. segregation of genetic material in cell division; Brzoska *et al.*, 2016) and FtsZ, responsible for cell constriction during cell division (as detailed above). The smooth hemispheres forming the caps on the ends of rod-shaped bacteria are shaped by spiral patterns of cytoskeletal proteins, in *E. coli* known as RodZ (Shiomi, Sakai and Niki, 2008), or crescentin, forming the crescent shape of *Caulobacter crescentus* (Ausmees, Kuhn and Jacobs-Wagner, 2003; Charbon, Cabeen and Jacobs-Wagner, 2009). These cytoskeleton components form the basic frame of the cell, which is then reinforced by the rigid peptidoglycan cell wall. Due to this rigidity, supported by the underlying cytoskeleton, the cell wall is a core determinant of bacterial shape (Huang *et al.*, 2008), although the shape varies during growth (fig. 6). However, there are also peptidoglycan-independent factors of shape. *Borrelia burgdorferi* and *Treponema phagedenis* spirochetes depend on periplasmic flagella for twisting the bacteria apart from the motility function (Motaleb *et al.*, 2000). Also, membrane composition is important, as *Rhodobacter sphaeroides* lacking its membrane lipid cardiolipin become spherical (Lin and Thanbichler, 2013), although this effect may be indirect

(e.g. negatively affecting MreB organisation of the cell wall). Similar effects have been seen when chemically inhibiting cell wall in e.g. *Pseudomonas aeruginosa* (Monahan *et al.*, 2014).

Morphology is a phenotypic trait determined by both intrinsic and extrinsic factors. In fact, morphology is often influenced by external stimuli to a degree that is not realised with traditional bulk assays. *Escherichia coli* can adjust its solute content and the amount of intracellular water to form spherical forms up to three times the volume of a regular cell, in order to sustain extreme extracellular declines in osmolality (Sun, Sun and Huang, 2014). Lack of nutrients also causes rod-shaped bacteria to take up coccoid forms, such as *Bacillus subtilis* (Gray *et al.*, 2019), and spore-forming bacteria to go into sporulation, generating smaller, coccoid spore morphologies (van Teeseling, de Pedro and Cava, 2017). Indeed, the shape is plastic to protect against exogenous dangers, often mediated via stress (e.g. antibiotics exposure) discussed below.

Stress

Stress is common in the microbial world. Bacteria are subjected to intense pressures in their environment and depend on the ability to detect and react to numerous extracellular cues, including near-UV radiation, low pH, temperature, osmotic shock, oxidative stress, and nutrient deprivation (reviewed by Trastoy *et al.*, 2018). To defend against such pressures, bacteria are equipped with emergency alert systems, responding to harmful indicators by e.g. activating specific gene expression programs.

In bacteria, the gene-expression RNA polymerase is produced as an inactive protein that together with cofactors forms the active enzyme complex, the "holoenzyme" (Borukhov and Nudler, 2003). One such cofactor is a so-called sigma factor, which determines the promoter specificity of the holoenzyme and as such the discrete set of genes that are transcribed (homologous to TFIIB in eukaryotes; Feklístov *et al.*, 2014). In *E. coli* and *Salmonella*, there are only seven different sigma factors, the relative level of which determines the expression levels of all genes. Three of these are pertinent to the stress responses: σ^E (RpoE) for extracellular stress, σ^H (RpoH) for heat stress, and σ^S (RpoS) for nutrient starvation, although they all have overlapping activating and also can activate each other (Bang *et al.*, 2005). These initiate the production of protective chaperones and enzymes to counter stressful environments before critical damage is done. One such critical threat is damage to the DNA. Sensing of DNA damage induces the so-called SOS system, which consists of over 50 genes encoding elements promoting DNA integrity as well as factors allowing for the survival and continuous replication despite widespread DNA damage, including e.g. RecA (Maslowska, Makiela-Dzbenska and Fijalkowska, 2019). Such functions include excision repair, homologous recombination, translesion DNA replication, and even cell division arrest (Maslowska, Makiela-Dzbenska and Fijalkowska, 2019). The sigma factors and SOS system are as such two key components in the survival of bacteria, including

*S.*Tm, and notably, SOS can also be induced as a response to antibiotics (Miller *et al.*, 2004; Maslowska, Makiela-Dzbenska and Fijalkowska, 2019).

Antibiotics and shape

Many antibiotics influence bacterial cell shape (reviewed by Cushnie, O'Driscoll and Lamb, 2016). Penicillin enters bacteria and directly inhibits PBPs from maintaining the cell wall. In some cases, this can be averted by swapping to an alternative PBP with low affinity to beta-lactams, such as the *S.*Tm $PBP3_{SAL}$ (Castanheira *et al.*, 2020). In other cases, this causes the cell wall to collapse. Consequently, this can cause the bacterium to acquire a characteristic spherical shape referred to as the L-form or spheroplast (Monahan *et al.*, 2014; Cushnie, O'Driscoll and Lamb, 2016). Importantly, this is an avenue of antibiotic tolerance, as the spheroplast outer membrane remains intact and integrity is upheld, and these bacteria often can revert to their original form after antibiotic treatment is relieved (Cushnie, O'Driscoll and Lamb, 2016). Indeed, *Escherichia coli* conforms to a more elastic but smaller cell, as well as going into dormancy, to similar challenges with ampicillin (Uzoechi and Abu-Lail, 2020). *Pseudomonas aeruginosa* also adapts similar spherical forms upon treatment with carbapenems and penicillin antibiotics, facilitating a larger tolerance at the cost of becoming more susceptible to others (Monahan *et al.*, 2014). Antibiotics can also cause cell division failure in bacteria, resulting in extremely elongated morphologies with intense filamentation (Navarro *et al.*, 2022). This can for example be facilitated by SfiA, an SOS mediator activated by the DNA damage induced by some antibiotics (Huisman, D'Ari and Gottesman, 1984). Antibiotics and stress thus affect the bacterial shape in multiple ways, and an altered morphology is also a tool for antibiotic tolerance.

Experimental considerations

The recent decade has spawned a plethora of new options in the ever-expanding toolbox to study bacteria and host-pathogen interactions. Simplistic model systems are fantastic tools for understanding biochemical interactions or investigating certain interactions in isolation, while more complex systems are representative of the clinical infection but often ill-characterised and hard to manipulate and control. Additionally, it is critical to consider if a given phenomenon is better to study in bulk or on the single-cell level. Below, such methodological considerations are discussed.

Models for infection studies

When studying host-pathogen interactions, it is vital to use a fitting host model. The reductionist's approach is to simplify experiments and remove unnecessary elements to be able to focus on the studied phenomenon. In contrast,

more inclusive experimental models incorporate many of the features that are present in real-life situations. In the case of the intestine, such features include multiple cell types in a three-dimensional (3D) architecture, gas exchange, nutrient gradients, blood flow, the immune system, commensal microbiota, and physiologically relevant biomechanical forces such as peristalsis (Barrila *et al.*, 2021). However, complex models have drawbacks as they require more advanced technical solutions (e.g. for culturing, measuring, or microscopy), are often low-throughput, and require specific expertise, all of which makes them leagues more expensive. Thus, different models should not be seen in a one-dimensional space of worse-to-better, but rather be compared on multiple levels such as simplicity, cost, throughput, and representative power. Likewise, comparative studies using multiple models of diverse complexities will always be the best option for multifaceted analyses. Below, these models are compared and contrasted in the example of bacterial invasion of intestinal mucosal host cells, often for the prospect of microscopy, as later also presented in the current investigation (although more concepts can be extrapolated). They are discussed in increasing order of biological complexity and decreasing simplicity of required experimental setup, ranging from *in silico* modelling through *in vitro* models to *in vivo* tissues.

Pure bacterial cultures

Bacterial cultures in different forms and compositions have proved to be powerful tools for genetic and biochemical exploration. For example, pure bacterial cultures have been used to define gene expression and gene essentiality in great detail (Kröger *et al.*, 2013; Canals *et al.*, 2019; Avican *et al.*, 2021). Furthermore, as detailed above, all from the days of the invention of the microscope, microbiology has also had one foot in observational studies, which deserves mentioning although it is not a model per se. During the 20th century, fluorescence microscopy, confocal, and electron microscopy were invented to complement regular transmitted light microscopy, later further complemented by modern light-sheet and super-resolution microscopy techniques (some of which have now been combined to form correlative techniques; Ryter, 1990; Swaim, 2010; Zanacchi, Bianchini and Vicidomini, 2014; Whitehead *et al.*, 2017; Prakash *et al.*, 2022). This toolset has enabled the study of the anatomy of bacteria at high-magnification, build-up and temporal tracking of their intracellular trafficking, and their biophysical measurements in detail, as well as that of structures of specific interest e.g. the T3SS-1 and flagella. Modern developments in imaging techniques and advanced image analyses e.g. using machine learning promise even stronger tools to come. Indeed, a comparative experiment in pure bacteria culture should often be considered, in parallel with more complex infection models.

In silico modelling

Epidemiological computational approaches were already a decade ago successful in assessing epidemics (Siettos and Russo, 2013), and modernised approaches can today fruitfully predict the decision-making of individuals in epidemics, such as if they will accept vaccines or abide by social distancing rules, and what the outcomes of such decisions will have on state economics (Chang *et al.*, 2020; Martcheva, Tuncer and Ngonghala, 2021).

Similar strides have been made also at a higher magnification, as insights into game theory and spatial modelling today allow for powerful simulations of single pathogens interacting with their host (Ewald *et al.*, 2020). One study determined that the establishment of persistent infections requires a bacterial species that can survive both intra- and extracellularly, such as *Salmonella* (Eswarappa, 2009). Another study investigated host-triggered virulence and fitness of such intracellular bacteria, and established a mathematical model determining that an attenuated bacterial strain can be selected for by changing the host's defence system to which the bacterium is confronted (Tago and Meyer, 2016). Yet another study investigated the role of intestinal microbiota in clearing pathogens and proved that increasing the fitness of the commensal microbiota is a method to eradicate pathogens (Wu and Ross, 2016). Such insights are crucial to developing efficient future treatments and vaccines against pathogens such as *Salmonella*. Thus, *in silico* modelling is computationally powerful for predicting biology, but is often based on frequent simplification and assumptions.

Cell line culture models

The traditional and powerful cell line culture models remain one of our most potent tools. Transformed or immortalised cell lines such as HeLa cells poorly recapitulate the *in vivo* intestine, but are in comparison to most other models extremely well-characterised. They are also usually easy and cheap to propagate and grow at scale. Moreover, they are homogenous, as they are all the same cell type, and are easily manipulated, their genomes are commonly known, and gene manipulation is readily available. A key property in host-pathogen interaction studies is that it is also easy to modulate the ratio of bacteria to host cells, referred to as the multiplicity of infection (MOI).

The choice of cell line is important. The HeLa cell is a human cervical carcinoma cell line, known to be the most used and well-characterised cell line in existence (Landry *et al.*, 2013). It is commonly used as a first-line model of human pathogens, due to its widespread availability in research laboratories. Similar but intestine-specific human cell lines include the Caco-2 colorectal adenocarcinoma cells and the Caco-2-derived C2Bbe1 cell line, a hyperpolarising clone that forms extremely tight cell-cell adherence junctions and a defined apical brush border (Gerlach *et al.*, 2008). The effects of polarisation have also traditionally been studied in Madin-Darby canine kidney (MDCK)

cells, which have even been employed in 3D cultures (O'Brien, Zegers and Mostov, 2002). For biochemical interrogation of the host cell, the traditional Cos7 monkey kidney cell line and the HEK293 human embryonic kidney cell line remain popular tools (Nozaki *et al.*, 2018; Chabloz *et al.*, 2020; Bourgeois *et al.*, 2021). The transimmortalised mouse intestinal line m-ICcl2 maintains a crypt phenotype and can be used to study infection of murine intestinal stem cells (Bens *et al.*, 1996). Lastly, myeloid monocyte cell lines such as U937, THP-1, J774A or RAW264.7 can be used to study monocyte behaviour and can be stimulated to differentiate into macrophages for studying phagocyte behaviour (Château and Caravano, 1993; Ekman *et al.*, 1999; Tierrez and Garcáia-del Portillo, 2005; Valle and Guiney, 2005; Hirano *et al.*, 2017). Primary macrophages and other immune cells can also be derived from bone-marrow progenitors, blood, or peritoneal lavage (Vincent *et al.*, 1992; Malbec *et al.*, 2007; Meurer *et al.*, 2016).

Cell line models remain defined and efficient models for studies of biochemistry and selective invasion of specific cell types in isolation, and enable the study of e.g. different levels of polarisation and epithelial integrity, which also can be customised through genetic transformation. In these models, techniques such as gentamicin protection assays allow quantitative comparisons between conditions, and their largely 2D organisation allows top-down imaging experiments. Furthermore, modern methods such as single-cell RNA sequencing and dual-species RNA sequencing promise powerful comparisons of properties even of single invasion events into single host cells (Westermann, Gorski and Vogel, 2012; Avital *et al.*, 2017). However, cell lines are commonly transformed or immortalised, and thus are altered genetically and phenotypically from what they are meant to represent. Furthermore, albeit cheap and simple to propagate, cell line monolayers lack physiological architecture and consist only of a single cell type, and hence do not reflect the complexity of tissues.

Enteroids and enteroid-derived monolayers

In the last decade, the discovery that primary stem cells of the intestinal epithelium can be grown into self-organising "mini guts" has garnered incredible interest as a new model of e.g. intestinal host-pathogen interactions (Sato and Clevers, 2013). Called enteroids (or colonoids when derived from the colon), these three-dimensional structures are capable of partially recapitulating the distinct identity, cell type heterogeneity, and behaviour of the original intestinal tissue in culture (Taelman, Diaz and Guiu, 2022). By either microinjecting bacteria into the enteroids lumen (Geiser *et al.*, 2021) or switching the polarisation of the enteroid to create an "inside-out" enteroid (Co *et al.*, 2019, 2021; Nash *et al.*, 2021), it is possible to study bacterial infection in an almost natural environment. It is also possible to break up the enteroids and establish a 2D monolayer with an apical and basal side, akin to cell line models, albeit with the identity and cell type heterogeneity of the parental enteroid maintained

(exploited in **Paper III**). This further simplifies infection protocols, and most traditional procedures (described in the previous section) can be adapted for such models. However, enteroid cultures require expertise and are expensive to handle. They also lack features such as blood flow and immune cells, although development towards such advanced features is in progress in many types of organoids (the general mini-organ), by organoid vascularisation (Yu, 2021) and organoid co-cultures with immune cells during infection (de Waal *et al.*, 2022; Han *et al.*, 2022). Furthermore, enteroid cultures require special equipment, as well as an adapted imaging setup due to their sheer size and culture conditions.

Nevertheless, to date, enteroids and enteroid-derived monolayers are, next to tissue explants, likely the most physiological models for quantitative studies of infection of the diverse and dynamic intestinal epithelium outside of the body (Aguilar *et al.*, 2021). It is also possible that such models can be applied clinically for personalised medicine using patient-derived organoid panel tests, although this is yet underdeveloped and cost-ineffective (Li *et al.*, 2020).

Animal models

Despite strides in modern method development, research is still dependent on animal models for systemic interactions. Such analyses can be employed in multiple ways, using mucosal tissue explants (Russo *et al.*, 2016), ligated loops (recently used to investigate *S.*Tm motility; Westerman, McClelland and Elfenbein, 2021), or in the intact animal *in vivo* (Tsolis *et al.*, 2011). For example, the now considered classic streptomycin pre-treated colitis mouse model (used in **Paper II**; Barthel *et al.*, 2003) allows interrogation of *S.*Tm-inflicted gut disease on a systematic level of both the bacterium in its natural context, as well as of the host. However, animal models are costly, necessitate special housing and licenced handlers, and are unfit for exploratory studies due to ethical concerns. Therefore, it may prove that enteroid-based models, which have fewer such restraints, eventually will replace animal models in the future, as those models mature. Nevertheless, animal models allow challenging the complete biological host, with the full range of possible symptoms on the tissue, immune system, and microbiota, as well as assaying systematic spread and disease severity on observational behaviour, at the cost of ethical and experimental considerations.

Bulk assays vs. single-cell studies

Bulk studies are commonly employed to study infectious bacteria. Studies in bulk effectively interrogate average behaviours and general properties of the population, but cannot recognise subpopulations or unique behaviours of single microbes or host cells. This is rapidly changing as e.g. single-cell analysis enters the combinatory multi-omics age (Perkel, 2021) and machine learning allows high-throughput single-cell segmentation in image data (Cutler *et al.*,

2022). Further, we can compare this to single-cell mathematical models (Ewald *et al.*, 2020). The main upside with assays in bulk is the low learning threshold, but as single-cell studies also can describe bulk data (e.g. population averages), single-cell methods are likely to become more and more common as they grow more manageable and affordable.

Single-cell analyses can today be employed in imaging, to interrogate behaviours on even a single-molecule level using light microscopy, fluorescence microscopy, atomic force microscopy, and cryo-electron tomography (a major theme of **Paper II-IV;** Hodzic, 2016; Watson, Taherian Fard and Mar, 2022). Technologies such as single-cell proteomics, single-cell mass-spectrometry, and single-cell RNA sequencing open up further avenues for comparisons (Watson, Taherian Fard and Mar, 2022). Data from such techniques will likely be deployed to dissect cell-cell heterogeneity in the future. The present investigation contains multiple examples of single-cell data, specifically using single-cell light microscopy and advanced image analysis to illuminate *S.*Tm host-cell invasion, as detailed in the next chapter.

Present investigation

Overall aim

Salmonella are dangerous pathogens, and non-typhoidal serovars such as *S.*Tm are key adversaries of human health. Early recognised for their pathological potential, *S.*Tm has been the target of great scientific effort over the past few decades, leading to a wealth of information about the *S.*Tm biochemical inner workings. Studies of *S.*Tm host cell colonisation were traditionally done in bulk and in separated systems, e.g. by parallel infections in isolated wells in a plate. By comparing different wells it is possible to compare properties between populations (e.g. between a wild-type and a mutant), scored as the average of each population. However, most variation within these populations is lost in such data, as are colourful subgroups or unique phenomena.

Recent technological advances such as flow cytometry and single-cell transcriptomics enabled analyses on the level of individual cells, and comparison of properties not only between, but within, populations. Similarly, modern high-resolution microscopy can be utilised to analyse bacterial cultures and differentiate fluorescence levels, shapes, and behaviours between individual cells. Such techniques have uncovered a rich variation in gene expression within individuals of bacterial populations (e.g. bistable expression, as discussed previously), also in strongly conserved functions such as the production of the invasion-critical T3SS-1 (Sánchez-Romero and Casadesús, 2021), and in morphology within populations (Ackermann, 2015). In an *S.*Tm culture, a bacterium may thus be conventionally rod-shaped and express both the T3SS-1 and flagella while an adjacent cell may be round and express neither; neighbouring cells differ strongly in virulence potential. As such, insights gained from traditional, bulk biochemical studies can benefit from complementation with single-cell analyses of within-population variation, using methods such as high-resolution microscopy.

The general aim of the thesis work was to investigate the mechanisms for *S.*Tm invasion of the mammalian gut epithelium at scale, and using modern single-cell methodologies. To this end, the project was divided into several subprojects. We investigated effector dependence for *S.*Tm invasion into different cultured host cells (**Paper I**), mechanisms for host cell entry *in vivo* (**Paper II**), growth and virulence interdependencies and their effects on swim-

ming (**Paper III**), assayed also under sub-MIC antibiotic therapy stress (**Paper IV**). These subprojects are all described below and appended at the end of the thesis.

Paper I: *S.*Tm depends on distinct invasion modalities for different host cell types, as assayed using an internally controlled, scalable method based on genetic barcoding

Background

Host cell invasion is driven by multiple virulence factors, such as the T3SSs, distinct T3SS effectors, and flagella. These factors can be immunogenic but are generally required for efficient internalisation. However, depending on the context, certain functions may be dispensable. Conversely, when epithelium scanning is completed and a host cell has been approached and adhered to, the expression of new flagella may be less critical. Similarly, invasion into phagocytes, which actively sample the extracellular volume as part of their immunological activities, is not strictly dependent on active internalisation via T3SS-1 (Virella, 2007), but invading bacteria instead depend on factors to survive within the hostile macrophage (Drecktrah *et al.*, 2006). Such compelling differences between possible natural host cell types remain incompletely charted, in part because of a lack of quantifiable methods.

In this paper, we developed a method to study the differences in *S.*Tm host cell invasion modes in three models of cell types *S.*Tm might encounter during its infectious cycle: epithelial cells, phagocytic macrophages, and non-phagocytic monocytes.

Summary

To study these differences, we in this paper developed a scalable method to study invasion depending on the bacterial composition and host cell context. The method is based on the construction of consortia with chromosomally tagged ("barcoded") *S.*Tm strains. These strains were sourced from our local strain collection of wild-type and gene-deleted *S.*Tm SL1344 mutants, which were transformed to carry a short (40bp) and unique barcode (Grant *et al.*, 2008). Seven defined strains, e.g. three wild-type and four single-deletion mutants, all with different barcodes, were mixed 1:1:1:1:1:1:1 to create a consortium. Consortia could then be used as individual inocula in classical gentamicin protection assays, where internalised bacteria remain detectable, but extracellular bacteria are killed by the antibiotic. The relative abundance of the

seven strains could then be compared by qPCR or amplicon sequencing. This version of the assay is internally controlled due to the multiple isogenic (apart from the barcode) strains in each well, and is scalable due to the reliance on consortia rather than single mutants, as well as the genome-based detection method instead of classic plating.

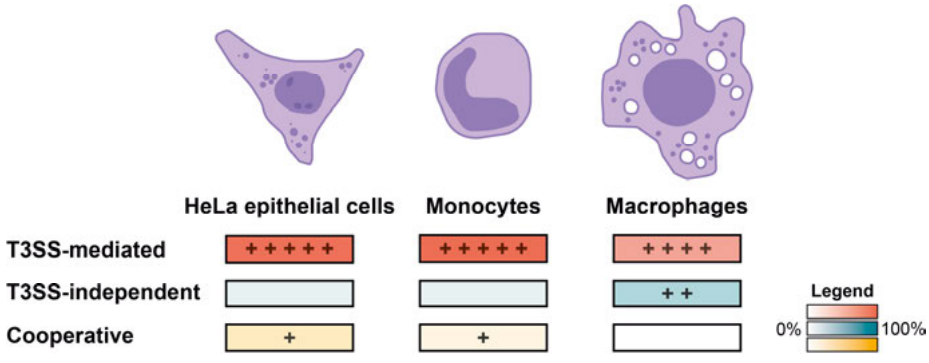

Figure 7. Relative number of T3SS-1-mediated, T3SS-1-independent, and cooperative invasions observed in the three cell types. Values from short-term invasion experiments using barcoded consortia as described in paper I.

We applied the barcoded consortia to study differences in the invasion of the mentioned host cell types. In HeLa epithelial cells and U937 monocytes, we confirmed that invasion is T3SS-1-dependent (<1% of events are T3SS-1-independent). Cooperative invasion, i.e. when the local entry structure triggered by invading bacteria is large enough to take up bystander, possibly non-invasive bacteria, represented the entry mechanism for 4-7% of bacteria at high MOIs in these cells. Somewhat surprisingly, invasion into the phagocytic U937-derived macrophages was also generally T3SS-1-mediated, although the T3SS-1-independent entry increased to ~25% of total events. Also unexpected was that no cooperative entry could be detected in the macrophages. Lastly, we concluded that the combined effects of the T3SS-1 effectors SopB, SopE, and SopE2 (the "ruffle inducers") were sufficient for the dependence on T3SS-1 across all three cultured cell types.

From these data, we conclude that the host cell context is important for the mode of *S.*Tm invasion, e.g. for the relative contribution of T3SS-1-dependent, cooperative, or T3SS-1-independent entry (fig. 7; explored further in **Paper II**).

Paper II:
*S.*Tm invasion of the murine gut absorptive epithelium is highly different to transformed cell line infection models, producing discreet invasion structures at cell-cell junctions

Background

The "ruffle" invasion model is characteristic of *S.*Tm. Ruffles are the effect of an explosive uptake mechanism where *S.*Tm injects its "ruffle inducers" (primarily the SopB, SopE, and SopE2 effectors, collectively SopBEE2) through the T3SS-1 to trigger the formation of lamellipodia and filopodia around itself, leading to uptake via induced macropinocytosis. Another associated effector, SipA, is important for stabilising the actin filaments of these structures, but is not involved in inducing the ruffles themselves. The characterisation of this mechanism has come from traditional experiments using transformed cell line models, which are heavily used still today since they are well-characterised, amenable to high-throughput analysis, and allow a standardised and consistent testing ground. However, gathering evidence shows that these models poorly recapitulate the architecture, behaviour, and complexity of the host gut mucosal epithelium.

We showed previously that invasion depends on the host cell context (in **Paper I**), and we here considered challenging the classical ruffle invasion model in a variety of transformed cell lines as well as in the gut absorptive epithelium *in vivo* in mice. In cell lines, ruffles are known to encompass large protrusions around the triggering *S.*Tm. However, preliminary data showed that such extensive ruffles are rarely observed in the gut, or are much more discreet than those in cell lines. We here explored this notion in-depth, using a combination of bacterial genetics and high-resolution microscopy.

Summary

We first confirmed the dependence of the ruffle inducers for cell line entry, as SopBEE2 triple deletion mutants did not successfully invade any out of three non-polarised, transformed epithelial cell lines. Also aligning with previous data, strains producing SopBEE2 do not require SipA for invasion into these cell lines. Remarkably, however, we found that SipA expression is critical specifically for the invasion of the mouse gut mucosa epithelium. This also depends on the large SiiE adhesin, which is dispensable in most cell line infections. Furthermore, strains lacking SopBEE2, which had close to zero invasion events in our experiments with cell lines, were still able to invade the

gut mucosa *in vivo* to some capacity. As such, we concluded that *S.*Tm invasion into epithelial cell lines is driven by SopBEE2, while invasion into the murine gut absorptive epithelium is driven predominantly by SipA and the SiiE adhesin.

Since invasion into the gut epithelium was shown to be possible without the traditional drivers of large entry structures (SopBEE2), we next employed light microscopy and scanning electron microscopy to investigate the size and morphology of the entry structures in both cell lines and the mouse gut. In the cell line experiments, *S.*Tm indeed induced expansive, SopBEE2-dependent ruffles. Strikingly, these were lacking in the mouse mucosa. Only marginal structural changes could be observed in the form of elongated, bent, and deformed microvilli around the bacterium as it seemed to "sink" into the cell. Indeed, this gentle invasion did not dramatically perturb the actin cytoskeleton (normally underlying the production of lamellipodia and filopodia) and lead to a tight and smooth vacuolar compartment. Indeed, due to the lack of expansive ruffles, this invasion mode also did not allow for cooperative invasion. We termed this gentler, SipA-dependent mode of *S.*Tm entry into the murine gut epithelium "discreet-invasion".

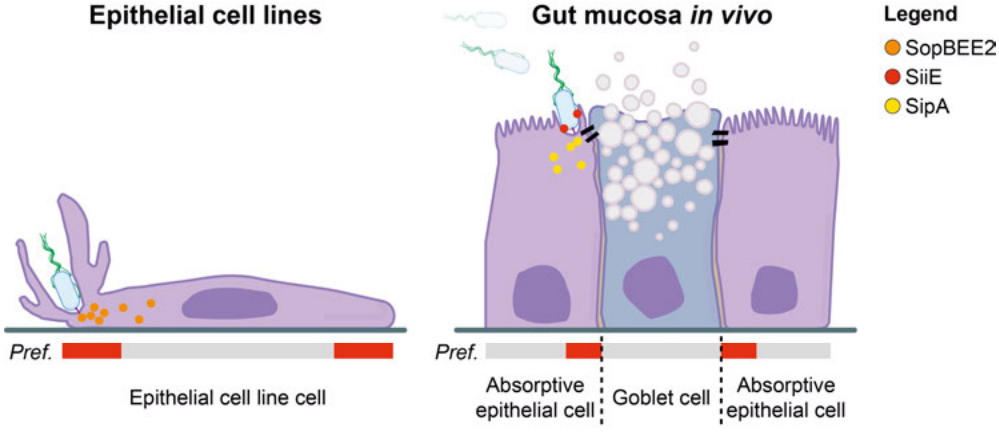

Figure 8. *S.*Tm invasion into epithelial cell lines in culture and gut mucosa absorptive epithelial cells *in vivo* display different adhesion and internalisation features. *S.*Tm preferentially adheres to cell edges along the surface while *in vivo* it selectively targets cell-cell junctions between absorptive epithelial cells and goblet cells. Invasion into epithelial cell lines displayed classic ruffle-mediated internalisation dependent on the T3SS-1 effectors SopEE2. In contrast, the "discreet- invasion" into the gut mucosal epithelium did not require SopBEE2, but was dependent on SipA, as well as preceded by adhesion via the SPI-4 giant adhesin SiiE.

Additionally, in our microscopy analyses, we noted that *S.*Tm frequently localised to the cell-cell junctional zones separating individual epithelial cells in the gut. Indeed, we found that ~80% of the adhered bacteria localised within 2µm from these junctions. We also found that especially the cell-cell junction

between an absorptive epithelial cell and a neighbouring goblet cell was targeted. In fact, *S.*Tm was five times as likely to localise to the cell-cell junctions of the goblet cell-adjacent absorptive epithelial cells. In full agreement with these data, invasion events were similarly localised to the outer rim of the cells, suggesting that this may be an actively targeted site for *S.*Tm.

In conclusion, this paper explores the vital differences between host cell contexts for studying bacterial invasions (fig. 8). Specifically, findings from established cell line models cannot always be extrapolated to the context of the intact gut mucosa. Furthermore, it found that specific cell-cell junctional zones are explicit targets for the invasive *S.*Tm, underlining the potential importance of near-surface swimming for epithelial cell colonisation patterns (further explored in **Paper III** and **Paper IV**).

Paper III: *Salmonella* "doublets" swim straighter and diversify the search for invasion target sites at the epithelial surface

Background

The *S.*Tm life cycle consists of two basic, opposing stages: growth and virulence. When nutrients become scarce (or by environmental cues), the virulence machinery is activated, e.g. by inducing the expression of the T3SS-1, and activation of the flagella so the bacterium starts swimming. Simultaneously, as virulence is costly, growth decelerates (Sturm *et al.*, 2011). As such, in the most simplistic explanatory model, *S.*Tm should either grow or express virulence, and there should be no bacteria that are simultaneously growing and e.g. actively swimming or invading host cells. *S.*Tm is commonly studied in bulk, however, where such cell-cell variation is occluded.

However, in qualitative microscopy data of *S.*Tm host cell infections, we observed that some invading *S.*Tm seemingly were in the middle of their division cycle, carrying a septum (the characteristic "waist" in the middle of a dividing cell). Indeed, a preliminary single-cell study of an *S.*Tm liquid culture found many such mid-division bacteria that were also swimming. If some *S.*Tm cells are capable of simultaneous growth and virulence expression, this suggests substantial overlap between the two states and that they are not exclusive of each other. In this paper, we decided to look into these simultaneously dividing and virulent individuals and quantify and compare their swimming and host cell invasion propensity to that of their brethren.

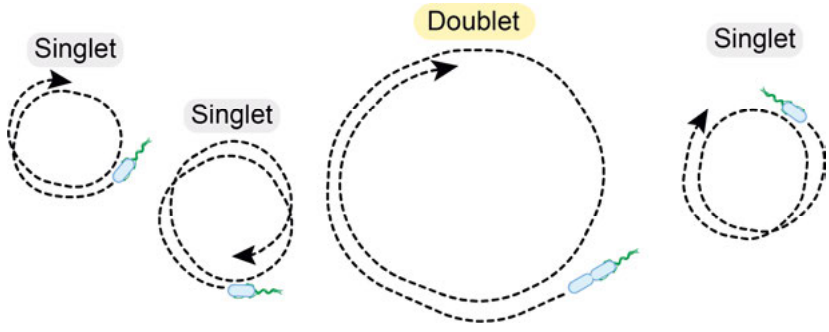

Figure 9. *S.*Tm "doublets" swim at equal speed, and much straighter, than the archetype "singlets". Individual *S.*Tm tracked over time show characteristic circular patterns, here as a representation. Not to scale.

Summary

Throughout the study, we contrasted the ~2µm long paradigmatic *S.*Tm bacteria, referred to as "singlets", to the waist-bearing, typically 3-4µm long subpopulation referred to as "doublets". Doublets were as expected most prevalent when the *S.*Tm growth rate is the highest (late exponential growth phase) and was observed to commonly split during host cell entry, confirming that they are late-stage cell division intermediates. However, we found that doublets were also present in cultures at all stages of the *S.*Tm growth curve, also prevalent at the late exponential growth phase where nutrients are scarce. *S.*Tm in this phase express virulence and is the most invasive (Ibarra and Steele-Mortimer, 2009b), and such cultures are routinely used for creating inocula in infection experiments. Also in such inocula, doublets were prevalent and were observed to swim, confirming a substantial overlap between growth and virulence expression. The consequences for host cell invasion were however not obvious, as doublets (at ~15-20% of the population) were in minority to the singlets, as anticipated.

However, despite their fractional prevalence in the inocula, single-cell analyses of cell line culture infections revealed that this *S.*Tm subpopulation is highly prone to invasion, as up to >70% of invasions into host cells were performed by doublets. This represents a 2.6-fold increase in prevalence after transitioning from the environment to the intracellular population, which we found to not rely on either increased T3SS-1 expression or a bigger ruffle size, and only marginally on increased adhesion capacity. As doublets were especially enriched inside host cells when using short infection times, we hypothesised that their approach to the host cell is more efficient and that doublets benefit from e.g. (1) a faster swim speed, or (2) a more efficient target search behaviour. By single-cell particle tracking in movies of swimming *S.*Tm, however, we determined that singlets and doublets had similar swim speeds, hence refuting (1) while (2) remained unexplored.

Peritrichously flagellated, rod-shaped bacteria tend to scout in large circles when they swim along solid boundaries (Lauga *et al.*, 2006; Park, Kim and Lim, 2019). In the motility data, however, doublets were determined to swim much straighter than singlets (i.e. in larger circles; fig. 9). By further analysing the *S.*Tm swim patterns, we noted that the straighter swim route caused doublets to survey a much higher number of unique host cells during their pre-invasion near-surface swimming. This is likely important for finding fitting invasion sites (as outlined in **Paper II**), or perhaps in the search for appropriate host cell types (as touched upon in **Paper I** and **Paper II**). Surprisingly, doublets were also more prone to swim than singlets, implying that these dividing cells also diverted energy to simultaneously execute the virulence program. These two properties, straighter swimming and a higher fraction of motile individuals, in combination produce a more mobile subpopulation that explores larger epithelial surface areas, which simultaneously decreases competition in the local, initial area. Arguably, this contributes to a higher virulence also of the whole *S.*Tm population.

The shape of the bacterial cell thus has a major role in *S.*Tm virulence, and size heterogeneity in the population can enhance the host cell invasiveness of the population. Morphology is as such a key determinant of host cell invasion capacity (which is further explored in **Paper IV**).

Paper IV: Low concentrations of antibiotics induce *Salmonella* morphology changes, alter host cell search behaviour, and boost host cell invasion

Background

The very shape of bacteria directly influences most aspects of their life (Young, 2006), in agreement with our data that *S.*Tm morphology is a key determinant in its virulence (discussed in **Paper III**). The bacterial morphology can also be influenced chemically, which is notable for many antibiotic therapies. For example, the archetypical beta-lactams antibiotics target peptidoglycan wall biosynthesis, with downstream effects such as elongation, filamentation, or loss of rod shape. This is observed at low concentrations of antibiotics, far below their therapeutic range (i.e. below the MIC; Uzoechi and Abu-Lail, 2020; Ponmalar, Swain and Basu, 2022). Interestingly, periods of such concentrations occur intermittently during antibiotics therapy (Craig, 2001), however, effects on the morphology, swimming or host cell invasion of host-residing *S.*Tm during such treatment gaps have not yet been comprehensively elucidated.

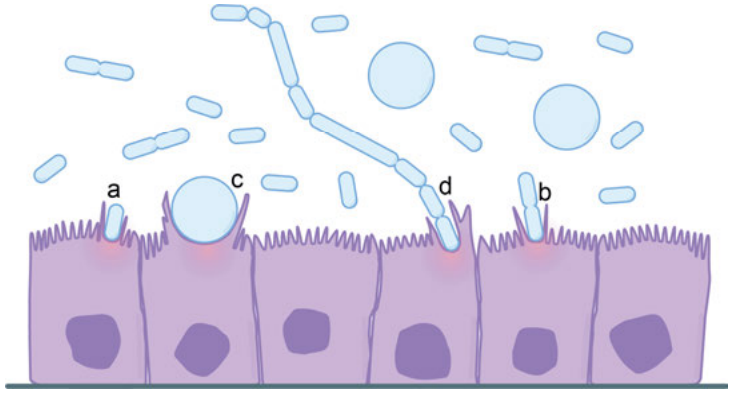

Figure 10. *S*.Tm host cell invasion is a remarkably resilient trait, upheld despite grossly abnormal morphology caused by antibiotic-inflicted stress. Host cell invasion of epithelial cells by representatives of different *S*.Tm morphologies inducible with antibiotics, including CHL and NIT (larger number of replication intermediates/doublets), and the morphology extremes induced by CIP (long, filamentous/annelid-like), and MER (large coccoid spheres). Morphotypes are here represented by (a) singlets, (b) doublets, (c) coccoid, and (d) filamentous annelid-like *S*.Tm. Not to scale.

This opens up an intriguing avenue of experimentation, where the morphology of *S*.Tm may be purposely altered by low-dose antibiotics, after which their virulence potential can be evaluated to investigate the role of morphology. Here, we utilised our established toolset of single-cell assays to study such effects of treatment with sub-MIC concentrations of four antibiotics (CHL, CIP, NIT, and MER).

Summary

Directly in our first screens, we observed that antibiotics induce vast morphological changes in the normally rod-shaped *S*.Tm, e.g. cell division slowdown/failure, increased cell length, loss of rod shape, and swelling. The most extreme effects depended on the molecular target of the antibiotic and included exceptionally long filamentous forms (~50x their normal length upon CIP treatment) and large, spherical cells (upon MER treatment), both in line with previous literature (Lederberg and st. Clair, 1958; Mason *et al.*, 1995). The morphological effects were dose-dependent, as very high concentrations were effective in killing or blocking the growth of the bacteria and very low concentrations did not produce any effects at all, and intermediate effects in between. However, in a broad span below the MIC, the morphological abnormalities consistently appeared during treatments.

To test the effects of these morphological changes on virulence, we employed single-cell tracking to measure swim dynamics along with well-established techniques to assay T3SS-1 activity (similar to **Paper III**). We found that sub-MIC concentrations of the tested antibiotic allowed swimming,

T3SS-1 expression, and host cell invasion (fig. 10). Remarkably, *S.*Tm treated with 24% down to 3% of the MIC of either CHL, CIP, or NIT even showed increased invasion in multiple experiments. Further, although it cannot fully account for the increased virulence, we found that longer individuals in the antibiotics-treated populations swam straighter, similar to the longer, hyperinvasive doublets in naturally-occurring *S.*Tm populations (discussed in **Paper III**). From these combined data, we conclude that while antibiotic therapy is efficient ≥MIC, sub-MIC levels of antibiotics may boost the host cell invasion capacity among surviving bacteria.

Lastly, we made multiple qualitative observations during our exploration. First, filamentous *S.*Tm continued to grow even at the host cell interface, budding off single bacteria from its ends. This indicates that the filamentous bacteria were capable of growth and generally, but not completely, deficient in cell division. This agrees with numerous bulk studies showing growth (i.e. increasing biomass) below MIC, although such bulk assays do not consider the gross effects on morphology and cell-cell variation. Second, filamentous *S.*Tm could stretch >100μm in length, thus spanning multiple epithelial cells along its length. These bacteria were also observed to in a short temporal window initiate invasion into several separate epithelial cells, specifically at both of its poles. This could indicate a tendency for instigating ruffle-mediated entry at the poles in these antibiotics-treated bacteria, an interesting avenue of future research. Third, the spherical *S.*Tm lacking a functional cell wall after MER treatment were observed to both swim (albeit slower) and invade (albeit rarely), pushing the envelope of what is possible to rescue after a chemical challenge that induces extreme cell wall defects. Although not quantified, preliminary observations also suggested that the effects on morphology are reversible by simply diluting the antibiotic in the culture medium further, in line with similar studies in other bacteria (Monahan *et al.*, 2014). These observations hint at a wealth of potential discoveries in the field of sub-MIC antibiotic therapy, in the context of host cell invasion for *S.*Tm, but also other pathogenic bacterial species.

Conclusion and future perspectives

Salmonella are dynamic, resilient adversaries of human health. Despite immense efforts over the last century, much remains unknown in our basic understanding of these pathogens, and the genus remains on the WHO high priority list in the light of AMR (World Health Organization, 2017). In the present investigation, several contributions to our understanding of *S.*Tm pathogenesis are presented. Specifically, we have mapped the distinct invasion modalities of *S.*Tm in different host cell contexts, first by developing a genetic barcoding technique applied to multiple host cell types and then by in-depth comparison of cell line cultures to the mouse gut mucosa *in vivo*. We also investigated *S.*Tm invasion target search during near-surface swimming in the mouse gut mucosa, in tissue culture, and on glass, identifying preferred invasion targets and exploring the swim patterns that probe for such targets during the initial phase of infection. The morphology of *S.*Tm was also thoroughly documented as a key virulence determinant, also in the context of low concentrations of antibiotics. With these findings as a basis, there are multiple avenues for further investigation.

Primarily, enticing questions about the *S.*Tm search for invasion targets and host cell selection remain unexplored. Outlined above are findings that *S.*Tm targets the neighbouring area of cell-cell junctions between absorptive epithelial cells and goblet cells in the host gut mucosa, and that swim patterns are conceivably critical in the search for such targets. We predict that such infection hotspots stem from unevenness in the otherwise homogenous surfaces along which *S.*Tm swims, and although multiple findings hint this may be the case (Misselwitz *et al.*, 2012; Furter *et al.*, 2019; Cooper *et al.*, 2021) this has not been formally proven. It would likely be fruitful to compare how such hotspots are found on more host cell models of varying cell polarisation and cell layer tightness, as we found that such properties impact invasion. The gut absorptive epithelium is confluent, and its cells are columnar and polarised, features that many common cell lines lack, which as such can likely be used for rewarding phenotypical comparison to *in vivo* data. In particular, it would be interesting to see how swimming *S.*Tm behaves atop varying confluent Caco-2 cultures, also in comparison with the strongly polarising Caco-2-derived C2Bbe1 cell line.

Furthermore, a longer bacterial morphology correlated with straighter swimming. As such, this would favour the discovery of physically distant infection niches, but this connection was not formally tested, although the ratio

of length to swim straightness generally remained constant throughout these experiments. Indeed, it would seem that *S.*Tm growth and cell division (and thereof morphology) and its virulence are more integrated than previously assumed, which would be interesting to scrutinise further. Similarly, the extreme *S.*Tm morphologies caused by sub-MIC antibiotics were shown to still be compatible with the ability to invade cell line models, and in a specific concentration range even showed increased host cell invasion propensity in our data. Future experiments could also explore how this translates to the host gut *in vivo* where sub-MIC antibiotics could lead to similar changes in gut-resident *S.*Tm. Such studies could lead to clinically relevant insights for the treatment dosing in the context of this and similar pathogens.

*S.*Tm dependence on host cell adhesion and the T3SS-1 is an integrated concept in the present investigation. We have already outlined several findings in this area, for example, that the individual T3SS-1 effectors have altered prominence depending on host cell context, and that these can be rescued to a certain degree due to bistable expression in the population. Notably, we found that *S.*Tm invasion into the *in vivo* gut mucosa differed greatly from traditional cell line cultures, and that cooperative invasion in the mucosa was limited by the small size of the *S.*Tm entry structures. Conceivably, such virulence features can in the future be explored also in other models, using the stringent, scalable, and cost-effective barcoded consortium infection method. This technique fills a methodological gap in the analysis of competitiveness for internalisation within bacterial populations, and is powerful enough to detect genetic bottlenecks. Barcoded consortium infections could be employed to compare cell line models and the *in vivo* mouse gut mucosa to e.g. modern mouse enteroid models or enteroid-derived monolayers. For example, systems can be compared on the relative *S.*Tm T3SS-1 effector dependence for invasion into these systems, or on the SiiE adhesin dependency (vital in the *in vivo* host gut mucosa) to that of other adhesins. Indeed, comparison of such virulence components between model systems is critical to determine if a model is powerful enough to discriminate studied behaviours (e.g. if *S.*Tm cooperative invasion occurs, ruffles may be a valid indicator of host cell invasion, while in other systems it might not). We already have published such data on cell line models of the epithelium, monocytes, and macrophages, as well as the *in vivo* mouse gut mucosa, to serve as a framework for such comparisons. Indeed, we envisage the barcoded consortium infections as a powerful tool for these applications. Additionally, it could be used to assay invasion properties, e.g. T3SS-1 effector dependence, along the gut of a single mouse, which could be a powerful method to discover tissue-specific and bacterial load-specific effects for the invasion along the gut mucosa *in vivo*.

Thus, the above-presented investigation serves as a methodological and conceptual base for future exploration of the diverse invasion mechanisms employed by *S.*Tm.

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“Variety's the very spice of life,
That gives it all its flavour.”

— William Cowper,
The Task, 1785

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