# The expression of virulence genes increases membrane permeability and sensitivity to envelope stress in *Salmonella* Typhimurium

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# Abbreviations

| | |
|---|---|
| ATR | Acid tolerance response |
| BAM | β-barrel assembly machinery |
| BIg | Bacterial immunoglobulin domains |
| CAMP | Cationic antimicrobial peptide |
| CFU | Colony forming unit |
| CRP | cAMP receptor protein |
| Cryo-TEM | Cryogenic transmission electron microscopy |
| $DiSC_3(5)$ | 3,3'-Dipropylthiadicarbocyanine Iodide |
| EEA1 | Early endosome antigen 1 |
| EPEC | Enteropathogenic *Escherichia coli* |
| EPS | Extracellular polymeric substances |
| ESR | Envelope stress responses |
| FMM | Functional membrane microdomains |
| GEF | Guanine exchange factor |
| GFP | Green fluorescent protein |
| HGT | Horizontal gene transfer |
| hil | Hyper invasive locus |
| H-NS | Histone-like nucleoid structuring protein |
| hrs | Hours |
| IgA | Immunoglobulin A |
| IL-8 | Interleukin-8 |
| LAMP | Lysosomal associated membrane protein |
| LB | Lysogeny Broth |
| LCFA | Long chain fatty acids |
| LCM | Low complexity microbiota |
| LCN2 | Lipocalin-2 |
| LPS | Lipopolysaccharide |
| McpC | Methyl-accepting chemotaxis protein |
| NPN | N-phenyl-1-naphthylamine |
| NTS | Non-typhoidal *Salmonella* |
| OD | Optical density |
| ORF | Open reading frame |
| p. i. | Post infection |

| | |
|---|---|
| PMF | Proton motive force |
| *prg* | PhoP repressed genes |
| Psp | Phage-shock protein |
| ROS | Reactive oxygen species |
| SCV | *Salmonella* containing vacuole |
| SCFA | Short chain fatty acids |
| SIF | *Salmonella* induced filaments |
| SKIP | SifA-and-kinesin-interacting-protein |
| *S*. Tm | *Salmonella enterica* serovar Typhimurium |
| SPI | *Salmonella* Pathogenicity Island |
| SAR | *Salmonella* reference collection |
| T1SS | Type one secretion system |
| T3SS | Type three secretion system |
| T6SS | Type six secretion system |
| TE | Tris-EDTA |
| TNF-α | Tumor Necrosis Factor α |
| WHO | World Health Organization |
| WITS | Wild-type isogenic tagged strains |
| WT | Wild Type |
| VF | Virulence factor |

# Abstract German

*Salmonella enterica* serovar Typhimurium (*S.* Tm) stellt eines der vorherrschenden enterischen Pathogene dar, die Menschen und Tiere infizieren und mit Diarrhöe in Verbindung gebracht werden. Um die Krankheit im Wirt auszulösen, verlässt sich *S.* Tm stark auf die Expression von spezialisierten Virulenzfaktoren. Der Transkriptionsregulator HilD spielt dabei eine zentrale Rolle in der Regulation des Netzwerkes von *S.* Tm, das die Expression der Virulenz antreibt. Bemerkenswert ist, dass das HilD Regulon nur in einem Teil der in vivo und in vitro Population von *S.* Tm exprimiert wird. Die HilD-exprimierenden Zellen können in die Epithelzellen des Wirts eindringen und eine Entzündung des Darms auslösen, die das Wachstum von *S.* Tm im Darmlumen fördert. Die Expression von Virulenzfaktoren, welche die Immunantwort des Wirtes regulieren und dabei eine vorteilhafte Nische erzeugt, ist ein Beispiel für Kooperation. Im Falle von *S.* Tm stellt die Entzündung eine Art Gemeingut dar, welche *S.* Tm dabei hilft mit der Mikrobiota zu konkurrieren und die Wahrscheinlichkeit der Übertragung zum nächsten Wirt zu erhöhen. Dennoch stellt die Expression von Virulenzfaktoren wesentliche Kosten auf der Ebene des einzelnen Bakteriums dar. Die Kosten der Virulenz erhöhen das Aufkommen von weniger virulenten Mutanten – sogenannten Betrügern (engl. Cheaters), die von der Entzündung profitieren können, ohne dabei zur Produktion beizutragen. Ein erweitertes Verständnis der Kosten der Virulenz und deren Regulation würde es möglich machen, die ökologischen Faktoren zu identifizieren und so zu modulieren, dass weniger virulente *S.* Tm entstehen. Bisher wurde nur ein Kostenfaktor zur Virulenzexpression in *S.* Tm beschrieben, nämlich eine Halbierung der Wachstumsrate in Zellen, in denen das HilD Regulon exprimiert wird. Da die meisten Invasionsfaktoren, die von HilD kontrolliert werden, in der Zellmembran eingebettet sind, nehmen wir an, dass die Expression des HilD Regulons *S.* Tm intrinsisch empfindlicher für Membranstress machen könnte. Außerdem ist es bekannt, dass *S.* Tm als Antwort auf Membranstress die Expression von durch HilD regulierten Funktionen verringert. Aus diesem Grund nehmen wir an, dass die Expression von diesen Genen ein wesentlicher Bestandteil der allgemeinen Membranstress-antwort ist. Wir untersuchten die Hypothese durch den Vergleich von Membrandurchlässigkeit, Sterblichkeit und HilD Aktivität in Anwesenheit von Membranstress in Populationen von *S.* Tm Stämmen, in welchen die Expression der HilD abhängigen Regulatoren und Funktionen genetisch modifiziert wurden. Unsere Ergebnisse zeigen einen Kompromiss zwischen Membranintegrität und Virulenzexpression in *S.* Tm, bei welchem ungünstige Umweltbedingungen die Virulenzexpression verringern. Dieser Effekt der Virulenzexpression auf die Membran von *S.* Tm und die Stressresistenz ist unabhängig von

der zuvor beschriebenen Reduktion der Wachstumsrate und stellt eine erhebliche Belastung während der in vivo Infektion in Mäusen dar. Die beschriebenen Fitnesskosten der Virulenzexpression sind ein neuer Faktor, der zur inhärenten Labilität der Virulenz in *S.* Tm und zur Selektion von virulenzverringernden Mutationen beiträgt. Diese genetische Labilität der Virulenz in *S.* Tm könnte dafür genutzt werden, diese zunehmend antibiotikaresistenten Bakterien zu bekämpfen. Da die in der Membran eingebetteten Virulenzfaktoren während des Infektionsprozesses für viele pathogene Bakterien essenziell sind, könnten unsere Ergebnisse dabei helfen, die Entwicklungen neuer Anti-virulenz Strategien voranzutreiben.

## Abstract English

*Salmonella enterica* serovar Typhimurium (*S.* Tm) represents one of the most prevalent enteric pathogens infecting humans and animals, associated with diarrheal disease. To trigger disease in the host, *S.* Tm critically relies on expression of specialized virulence factors. The transcription regulator HilD plays a central role in *S.* Tm regulatory network driving expression of virulence. Remarkably, the HilD regulon is only expressed in a fraction of *S.* Tm population both *in vitro* and *in vivo*. HilD-expressing cells are able to invade host epithelial cells and trigger gut inflammation, which fosters growth of *S.* Tm in the gut lumen. Expression of virulence factors modulates the host immune response therefore creating a favorable niche is an example of cooperative trait. In case of *S.* Tm inflammation represents a public good that helps *S.* Tm to outcompete the microbiota and to maximize the transmission to the next host. However, expression of virulence factors imposes a significant fitness cost to *S.* Tm at the single-cell level. Cost of virulence promotes emergence of virulence attenuated mutants – namely cheaters, which can benefit from inflammation, without contributing to its production. Therefore, understanding the cost of virulence and how it relates to virulence regulation could allow the identification and modulation of ecological factors to drive the evolution of *S.* Tm toward attenuation. Until now, the only described cost of virulence expression in *S.* Tm is a 2-fold reduction of the growth rate in cells expressing HilD regulon. Since most invasion factors controlled by HilD are embedded within envelope, we hypothesized that expression of the HilD regulon could render *S.* Tm intrinsically more sensitive to envelope stress. Moreover, it is known that in response to stress affecting membrane homeostasis, *S.* Tm generally downregulate expression of HilD-regulated functions. Therefore, we speculated that expression of HilD-controlled genes could be an integral part of general envelope stress response specific to *S.* Tm. We addressed this hypothesis, by comparing the membrane permeability, death rate and HilD activity in presence of membrane targeting stress in populations of *S.* Tm strains in which the expression of regulators and functions downstream of HilD was genetically tuned. Our results reveal a trade-off between envelope integrity and virulence expression in *S.* Tm which could explain the downregulation of virulence expression in response to hostile environmental conditions. This effect of virulence expression on *S.* Tm membrane status and stress resistance is independent from previously described growth rate reduction and presents a significant burden during *in vivo* infection in mice. Herein, the described fitness cost of virulence expression is a novel factor contributing to the inherent instability of virulence in *S.* Tm and selection for virulence attenuated mutants. This genetic instability of virulence in *S.* Tm could be exploited to fight against this pathogen, which is

becoming increasingly resistant to antibiotics. Since membrane embedded virulence factors are critical during infection process of many bacterial pathogens, our findings can inspire development of new anti-virulence strategies.

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# CHAPTER I

## The expression of virulence genes increases membrane permeability and sensitivity to envelope stress in *Salmonella* Typhimurium

# Introduction

## *Evolution of virulence*

### Virulence and virulence factors

Virulence understood as the ability of pathogen to inflict harm upon the host requires the presence of virulence factors (VFs). These VFs expressed by the pathogen, acting individually or together at different stages, provide bacteria with traits enabling host exploitation and escaping immunity response. Depending on their role, bacterial VFs can be grouped into five functional categories (1-5) (Finlay & Falkow, 1989). (1) Membrane proteins which play roles in colonization, adhesion, invasion, and confer antibiotic resistance. (2) Polysaccharide capsules which surround the bacterial cell and protect it from the environment. (3) Cell wall and outer membrane components, such as lipopolysaccharides (LPS) or lipoteichoic acids. In the Gram-negative bacteria, LPS – the major outer membrane glycolipid protects pathogen against complement-mediated immunity and is a potent inducer of inflammation. Gram-positive bacteria are naturally surrounded by a thick cell wall protecting the cell from external environment. (4) Secretory proteins which can modify the host cell environment. Pathogenic bacteria use different secretion systems (I-VI) to transport these protein toxins inside the host cell or into the environment. (5) Other VFs such as siderophores- iron scavenging molecules, which can increase bacterial survival under challenging iron depleted conditions (Wu et al., 2008). The amount of harm caused to their host is highly variable between pathogens. Interestingly, the presence of VFs by itself does not guarantee that the bacterium will induce any damage. *Neisseria meningitidis* can remain non-invasive in the human host despite carrying VFs like capsule and type IV pili (Laver et al., 2015). Comparative analysis of genomes of innocuous and pathogenic strains revealed that evolution towards virulence mostly relies on acquisition of novel VFs, rather than loss of specific genes (Maurelli, 2007).

### Direct selection and coincidental evolution of virulence

Acquisition of VFs by either horizontal gene transfer (HGT) or mutation, can be beneficial in specific ecological context. Virulence can allow exploitation of the host and thus successful reproduction of the pathogen. This implies that increased virulence should be evolutionary selected for. If the acquisition or evolution of the VF by the pathogen takes place in the same host where they trigger the disease of interest, the process is termed a direct selection. For example, the lysogenic conversion of *Vibrio* spp. by a cholera-toxin-encoding phage gave rise

to the human pathogenic *Vibrio cholerae* species (Waldor & Mekalanos, 1996). While in the human gut, presence of the cholera toxin leads to increased pathogen loads and improved transmission, thus making it a beneficial trait for the pathogen (William & John, 2014). Another explanation for increased virulence is coincidental evolution. This occurs when the virulence trait has been selected in another ecological context than the host, where it triggers the disease. Many of the virulence determinants might originate from the coevolution of bacteria with unicellular eukaryotes, acquired long before a given bacterium became a pathogen of higher animals. For example, The Type Three Secretion System (T3SS) of *Pseudomonas aeruginosa* is important for the interaction with mammalian host (Hauser et al., 1998), however, it also plays a role in killing the slime mold *Dictyostelium discoideum* (Pukatzki et al., 2002). Among the VFs of Enteropathogenic *Escherichia coli* (EPEC) are the prophage-borne Stx-toxins. Most likely, the acquisition of Stx-prophage has been selected for not in human but bovine host. In the bovine digestive tract, which is the main zoonotic reservoir of EPEC, the presence of Stx-prophage improves EPEC's survival in presence of a protozoan *Tetrahymena pyriformis.* Interestingly, the same prophage coincidently increased EPEC's virulence in the human gut by inhibiting the protein synthesis of the host cells (Steinberg & Levin, 2007).

### Competition driven adaptation into virulence

In the complex environments interspecies competition can be the underlying cause of selection for increased virulence to the host. Such competition-driven evolution of virulence can be advantageous to the pathogen in the long term. For the pathogens residing in the host digestive tract the huge barrier preventing their colonization comes from presence of intestinal microbiota providing colonization resistance to the host. Certain pathogens like *S.* Tm (Stecher et al., 2007; Stecher et al., 2008), *Citrobacter rodentium* (Mullineaux-Sanders et al., 2019) or *V. cholerae* (Ma & Mekalanos, 2010) use dedicated VFs to trigger host inflammatory response. The subsequent gut inflammation leads to clearance of microbiota emptying the niche and providing high energy nutrients for pathogen to thrive. In this light, competition against residual microbiota for the niche and resources could have been the underlying cause for intestinal pathogens to evolve virulence traits harming the host. However, the competition-driven adaptation into virulence is not always beneficial for the pathogen in a long term. Bacterium *Haemophilus influenzae* asymptomatically colonizes the host nasal cavity. Occasionally among the harmless population mutants with increased virulence capable of colonizing the bloodstream emerge. These mutants can multiply in this unoccupied niche

leading to bacteremia in the host. As bacteremia is lethal for the host the advantage for the increased virulence mutant is temporary and limited to population colonizing that affected patient. In a long term, perspective these mutants would not be transmitted to the next host and therefore lost (Margolis & Levin, 2007). These examples show that competition for resources can be important factor triggering evolution of virulence.

### Anthropogenic factors influencing virulence evolution

The medical practices applied in disease prevention and treatment have impact on virulence emergence. The use of antibiotics on one hand revolutionized medicine and saved numerous lives, however, the widespread use of large quantities of antibiotics led to increased number of bacterial strains resistant to treatment (Modi et al., 2014; Blair et al., 2015). In addition to leading to rise of resistant mutants, antibiotics can also impact evolution of virulence. The gut microbiota are not only sensitive to the effect of pathogen-triggered inflammation (Stecher et al., 2007) but also treatment with antibiotics. In case of the gut inflammation triggered by *S.* Tm, the resident commensal *E. coli* strains can also bloom under such circumstances. These mixed blooms favor HGT, therefore contributing to exchange of VFs (Stecher et al., 2012; Diard et al., 2017). Disruption of a protective microflora caused by administration of antibiotics (Modi et al., 2014; Ubeda et al., 2017) can open the niche for bloom of intestinal pathogens (Stecher & Hardt, 2011; Larcombe et al., 2016) creating similar conditions as inflammation. Another milestone in disease prevention – vaccines, can also impact evolution of virulence. During infection of *S.* Tm, or as a result of the oral vaccination with acid inactivated *S*. Tm; the O-antigen specific immunoglobulins A (IgA) are produced. Subsequent IgA-mediated cross-linking enchains dividing pathogen cells preventing separation of the daughter cells after division resulting in so called “enchained growth” (Moor et al., 2017). Pathogens trapped in such way cannot exchange mobile genetic elements via HGT with other bacteria present in the niche thus slowing down evolution of virulence. These examples illustrate that human-derived factors also influence evolution of pathogens.

### Loss of virulence

Virulence traits are not only gained but can also be lost in the evolutionary trajectories. Whereas presence of VFs enables the pathogen to exploit its host to the benefit of the invader, maintenance and expression of VFs might impose a cost on the pathogen as well. Especially under suboptimal conditions, the energy resources necessary for the functioning of VFs can

inflict selective pressures favoring the loss of virulence. Very often VFs are under tight spatiotemporal control and their expression triggered only under specific environmental conditions (Mekalanos, 1992). In *S.* Tm, the expression of VFs is critical for the invasion process and is controlled by a complex regulatory network coupled with environmental sensing (Fàbrega & Vila, 2013). Despite the tight control, the virulence expressing cells of *S.* Tm were shown to have a two-fold reduction in their growth rate (Sturm et al., 2011). The cost is partially mitigated by the bimodal expression where only fraction of population is expressing VFs (Hautefort et al., 2003). The concept that VF expression is costly for the pathogen itself, brings a new perspective into the traditional view of virulence and has only recently begun to be investigated in more details. The emergence of virulence attenuated clones can also occur within the host. During the chronic infection with *P. aeruginosa* avirulent clones are routinely isolated from the patients (Winstanley et al., 2016). Moreover, the relaxation of selective pressure for virulence can also favor VF loss. For instance, in enterohemorrhagic *E. coli* upon sub-cultivation, the loss of genes encoding the Shiga toxin was observed (Bielaszewska et al., 2007). These examples illustrate that virulence is not a fixed trait but can be lost under certain circumstances.

### Virulence as a cooperative trait

Many bacterial traits are cooperative. Some of these traits are associated with virulence (Griffin et al., 2004) whereas other like biofilm formation can lead to increased antibiotic resistance (Rainey & Rainey, 2003). Biofilm formation provides numerous advantages to bacteria. Cells within the biofilm are more tolerant to mechanical removal, presence of antibiotic or disinfectants (Davies, 2003). In *P. aeruginosa* biofilm formation requires production and secretion of the extracellular polymeric substances (EPS) that enclose bacteria within the biofilm. Another important compound is iron which is obtained by production of iron scavenging siderophores (Banin et al., 2005). Both these traits, EPS and siderophore production, can be considered cooperative traits as their benefits are accessible to the entire population. Mathematical modelling predicted that targeting pathogens cooperative traits such as multicellular organization has therapeutical potential with reduced risk of resistance emergence in comparison to antibiotic treatment (André & Godelle, 2005). Ross-Gillespie employed treatment of *P. aeruginosa* biofilms with gallium as it can be uptaken by the siderophores instead of iron. Authors showed that indeed gallium inhibits *P. aeruginosa* growth and resistance to such treatment emerged at much slower rates than resistance to antibiotics (Ross-Gillespie et al., 2014).

## *S. Tm virulence as example of cooperative trait*

### *S.* Tm as a global importance pathogen

Majority of people associate *Salmonella* infection with unpleasant food poisoning originating from poultry and egg consumption. Despite improvements in food preservation and efforts put into the food safety precautions, this pathogen continues to infect people worldwide (Majowicz et al., 2010). *Salmonella* is a Gram negative, rod shaped, facultative anaerobic, facultative intra-cellular member of the family Enterobacteriaceae. *Salmonella* spp. is a broad-host pathogen which colonizes major livestock species like poultry, cattle, and pigs. Humans become infected by ingesting food or water contaminated with animal feces. Poultry and poultry products are recognized as primary source of disease (Saravanan et al., 2015). The genus *Salmonella* is currently classified into two species *Salmonella enterica* and *Salmonella bongori* (Lan et al., 2009; Chattaway et al., 2021). The latter, initially classified as *Salmonella enterica* subspecies V, is rarely associated with human infections. *Salmonella enterica* based on the genomic relatedness and biochemical properties is further divided into five subspecies denoted by the Roman numbers. The subspecies are classified as following: I. *S. enterica* subsp. *enterica*: II. *S. enterica* subsp. *salamae:* III. *S. enterica* subsp. *arizonae;* IV. *S. enterica* subsp. *houtenae*; and V. *S. enterica* subsp. *indica.* The subspecies of *S. enterica* subsp. *enterica* accounts for 99% of *Salmonella* infections in humans and warm-blooded animals. In addition to classification based on phylogeny, the Kauffman and White system classifies *Salmonella* into serotypes based on three major antigenic determinants including somatic (O), capsular (K) and flagellar (H) (Lan et al., 2009). The somatic antigen forms the oligosaccharide component of bacterial LPS and is a heat-stable antigen. Specific serotype can express more than one O antigen. The H antigens are mainly found in bacterial flagella, are heat-sensitive and are involved in activation of host immune responses. The K antigens are heat-sensitive polysaccharides mainly located in the bacterial capsule; these antigens are rarely found among *Salmonella* serotypes. The current classification system contains more than 2500 described serovars (Chattaway et al., 2021). In the nomenclature of the serotype the subspecies is usually omitted. In clinics the most important distinction for *Salmonella* is between typhoidal serovars causing enteric fever and non-typhoidal *Salmonella* (NTS) serovars that most commonly cause gastroenteritis. Enteric fever has been reported endemic in Southeast and Central Asia causing 22 million infections yearly, resulting in 200 000 deaths (Jajere, 2019). Treatment of typhoid fever requires antibiotic therapy. Humans infected with NTS serovars most commonly develop self-limiting gastroenteritis with symptoms such as

fever, stomach crumps, diarrhea, and vomiting. In treatment of NTS specific therapy is not required, however, the outbreak investigation is often carried. The important NTS serovars associated with the foodborne *Salmonella* outbreaks in humans include Typhimurium, Enteritidis, Heidelberg and Newport (Jajere, 2019). The most common form of NTS infection – gastroenteritis, occurs in about 93.8 million cases per year and results in 155 000 deaths (Majowicz et al., 2010).

## Virulence factors of *S.* Tm

The key virulence traits of *S.* Tm are attributed to the timely expression of the VFs such as flagella, adhesin, or two T3SS. Many of important VFs are encoded within horizontally acquired *Salmonella* pathogenicity islands (SPIs) (Hensel, 2004). These genetic elements retained increased proportion of adenine-thymine pairs in comparison to rest of the genome. Both, SPI-1 and SPI-2 encode T3SS apparatus important for the epithelial cells invasion and macrophage survival, respectively. The SPI-3 encoded proteins help *S.* Tm survive within macrophages and in the magnesium depleted environments. (Jajere, 2019) The SPI-4 contains an operon of six open reading frames (ORFs), namely *siiABCDEF,* encoding adhesin which is important for the initial attachment to host epithelial cells (Kiss et al., 2007). The SPI-5 encodes multiple T3SS effector proteins. The SPI-6 encodes Type Six Secretion System (T6SS) which transports bacterial proteins into host cytoplasm or cellular environment in response to external conditions. (Jajere, 2019; Mulder et al., 2012). In addition to VFs encoded within the SPIs, *S.* Tm harbors additional factors contributing to successful infection including chemotaxis apparatus and flagella (Josenhans & Suerbaum, 2002). Described VFs acting in the orchestrated manner enable *S.* Tm to overcome host barriers and complete the infection process.

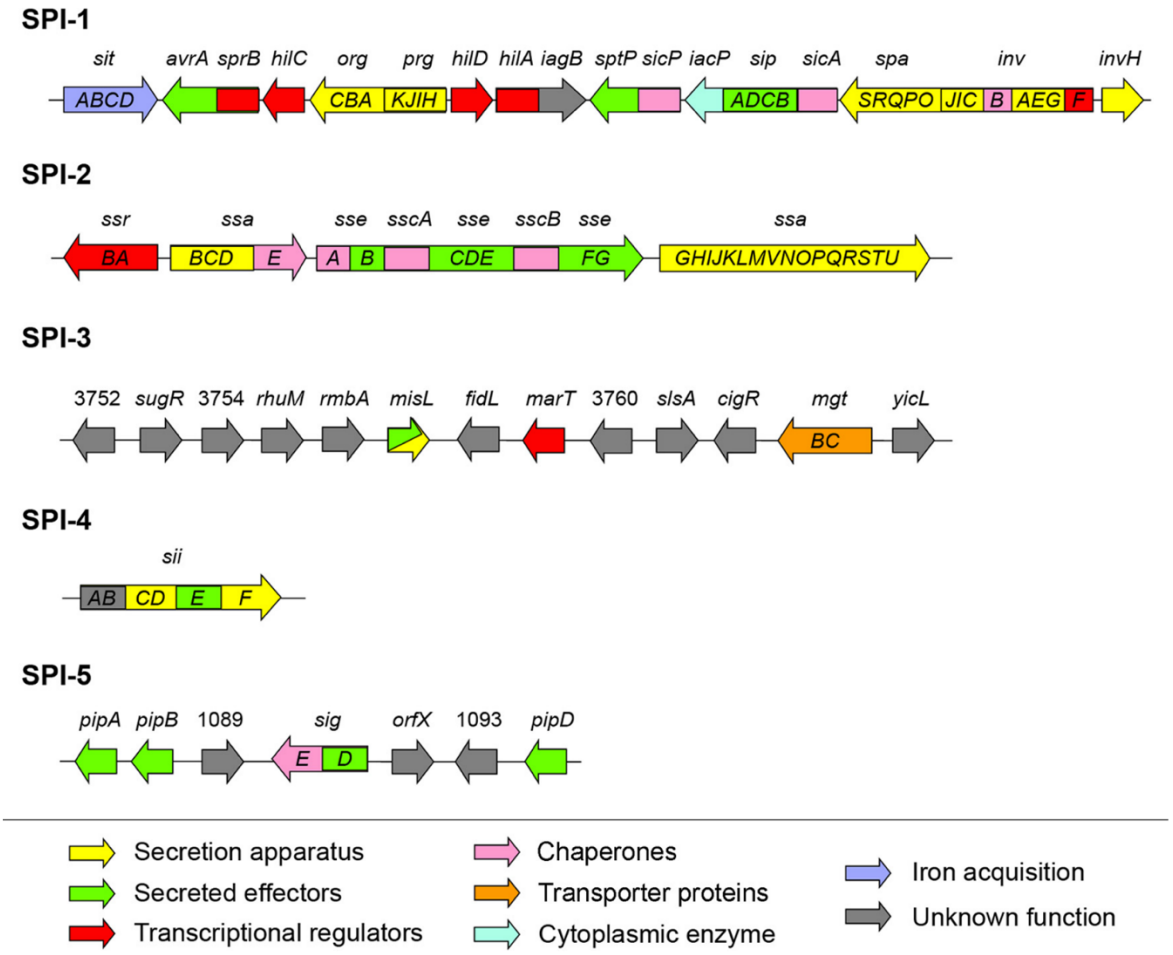


**Fig. 1. Schematic overview of five SPIs of *S.* Tm genome**. Arrows rare representing genes or operons. Colors indicate their putative functions: yellow: secretion apparatus, green: secreted effectors, red: transcriptional regulators, pink: chaperones, orange: transporter proteins, blue: cytoplasmic enzyme, violet: iron acquisition, grey: unknown function (Fàbrega & Vila, 2013)

To approach host epithelial cells *S.* Tm needs to scout the dense intestine environment. Motility is largely increasing pathogen chance to encounter epithelial cells and successfully invade it. The move of *S.* Tm does not occur at random, but it happens along the concentration gradients thanks to the chemotaxis apparatus. The motility is achieved thanks to presence of flagella. *S.* Tm strains lacking functional chemotaxis or flagella are less efficient in reaching the intestinal monolayer in the early stages of infection (Stecher et al., 2004).

Majority of *S.* Tm serovars possess flagella and usually up to 10 flagella are distributed at random on bacterial surface (Van Asten & Van Dijk, 2005). The base of flagella embedded in bacterial envelope is similar to the base of T3SS, however, instead of the needle-like structure through which effectors are translocated, flagella possess a long appendage that rotates counter- or clockwise providing motility (Terashima et al., 2008). The flagellar motor is

composed of three proteins: FliM, FliN and FliG. The motor determines the direction of movement – clockwise or counterclockwise, and its rotation can change upon sensing the chemotactic signal. The energy driving rotation comes from the proton motive force. The flagellin can be composed of either the FliC or the FljB protein and these two proteins are expressed alternately in a phenomenon of phase variation (García-Pastor et al., 2019; Yamamoto & Kutsukake, 2006). In this process the DNA invertase Hin catalyzes a site-specific recombination that turns promoters on or off. The type of flagellin impacts the virulence of S. Tm in mice. The locked ON FljB flagellin mutants are attenuated in mice, whereas the FliC locked on mutants display wild type (WT) virulence (Ikeda et al., 2001). The assembly of flagella is a very complex, hierarchical process which relies on timely expression of flagellar operons divided into three classes: early, middle, and late (Terashima et al., 2008; Chilcott & Hughes, 2000). In general, if strain is defective in expression of early or middle genes, its late genes cannot be expressed, and following the same logic, strains defective for early genes cannot express neither middle nor late genes. The expression of flagellar genes and functioning of flagella are regulated in a complex manner which is sensitive to the environmental cues and physiology of the cell.

*S.* Tm uses the flagellar motility to traverse the gut lumen space towards the epithelial cell layer (Stecher et al., 2004). This directional movement is possible thanks to coupling of flagellar motility with chemotaxis sensing. In response to changes in for example nutrient concentration, the sensory kinase CheA phosphorylates the response regulator CheY. The latter undergoes conformational changes when phosphorylated and binds the flagellar rotor protein FliM, thus changing the direction of flagellar rotation toward clockwise (Sarkar et al., 2010; Welch et al., 1993). The rotation change of one or more flagella disrupts the bacterial movement and causes cells to tumble. During this event bacteria reorients itself at random and changes movement direction. In the presence of attractant such as nutrients, flagellar motor rotates in the counterclockwise direction. A sequence of move- tumble events leads bacteria in direction of increasing concentration of the attractant. The phosphorylation of the CheY protein provides a fast regulation in response to environmental changes. Another level of regulation is provided by acetylation of the CheY protein. Binding of the acetylated CheY to both, FliM and CheA is repressed. Regulation based on acetylation is slower than phosphorylation and reflects changes in the metabolic state of the cell (Barak & Eisenbach, 2004; Liarzi et al., 2010). Due to coupling of chemotaxis sensing with flagellar motor *S.* Tm can adjust directionality of its movement based on external and internal inputs.

After approaching host epithelial cell layer *S.* Tm begins invasion process of the host cells to initiate the systemic infection. Within the intestinal mucosa epithelial cells have a polarized organization with an apical side facing towards the intestinal lumen and a basolateral side (Gerlach et al., 2008). To establish contact and invade polarized epithelial cells, pathogen relies on its VFs: the T3SS-1 and SPI-4 encoded Type One Secretion System (T1SS). The main component of the T1SS is the non-fimbrial giant adhesin SiiE, which with the molecular weight of 595 kDa is the largest protein of *S.* Tm proteome. This protein is secreted by T1SS, and its C-terminal part establishes contact with the host cell surface. Subsequently the T3SS-1 needle can be positioned against the host cell surface. Structurally the SiiE protein is composed of 53 BIg -bacterial immunoglobulin domains. The T1SS is composed of three proteins: SiiC, D and F, where the latter is an ATPase subunit, SiiD – the periplasmic protein and SiiC the outer membrane pore. In addition, SiiA and SiiB perform regulatory functions (Wille et al., 2014) controlling the SPI-4 dependent adhesion. During the infection, genes responsible for the adhesion are co-regulated with genes encoding the SPI-1 invasion machinery, thus providing high efficiency of breaching the epithelial cell barrier (Barlag & Hensel, 2015).

When correctly attached, *S.* Tm needs to translocate bacterial effectors inside the host cytoplasm to trigger cytoskeletal rearrangements subsequently leading to bacterial engulfment. This process depends on the SPI-1 encoded genes. *S.* Tm SPI-1 spans over 40kb and contains 39 genes encoding T3SS-1, chaperones and effector proteins as well as transcriptional regulators which control many virulence genes within and outside of SPI-1 (Song et al., 2017). The role of T3SS-1 is to deliver bacterial effector proteins into the host cell cytoplasm. The T3SS-1 is a complex multi-protein machinery comprising the three key elements: envelope embedded needle complex, an inner membrane export apparatus, and cytoplasmic sorting platform (Hu et al., 2017). A sorting platform, consisting of five proteins: SpaO, OrgA, OrgB, InvI and InvC; determines the order of protein secretion and energizes the secretion process. Additional chaperones are required for the appropriate loading of effectors and translocases into the sorting platform. The needle complex base is embedded within the export apparatus composed of InvA, SpaP, SpaQ, SpaR and SpaS proteins. The export apparatus plays a role in the assembly and stabilization of the needle complex. The base of a needle complex consists of several rings anchored in the bacterial envelope. The InvG protein is a structural component of the outer ring, PrgH and PrgK proteins constitute the inner rings of the needle complex base. This cylindrical base of the needle complex spans ~26 nm in diameter. The PrgI protein arranged in a helical manner, is the main component of the needle-

like structure stretching to ~60 nm from the bacterial envelope. The InvJ protein controls the length of the needle segment. The inner rod of the needle complex is made up of the PrgJ protein. In the center of this structure is a channel ~20 Å in diameter through which the cargo can traverse towards the host cell cytoplasm. The tip of a needle complex is capped with a SipD protein which upon contact with the host cell surface forms a platform for the translocon proteins SipB and SipC. The SipB protein is inserted into the host cell membranes and forms a channel through which T3SS-1 effectors are transported into host cell cytoplasm. (Lou et al., 2019; Hu et al., 2017). The SipC translocon protein is targeting the F-actin promoting pathogen internalization. (Kaniga et al., 1995; Hayward & Koronakis, 1999). Study by Ellermeier and Slauch established that the T3SS-1 requires the DsbA protein for its full activation (Ellermeier & Slauch, 2004). Once the connection spanning through bacterial envelope and host cell membrane is successfully established, pathogen can translocate its effector proteins toward the cytoplasm of the host cell.

*S.* Tm relies on a subset of effector proteins to initiate the cytoskeletal remodeling of the host cell and induction of the proinflammatory responses. Many of the effectors affect the polymerization of the actin cytoskeleton on the site of bacterial entry to stimulate creation of the membrane ruffles enabling engulfment of the bacteria. Proteins encoded within SPI-5 and transported via T3SS-1, namely SopE, SopE2 and SopB are crucial during this stage of invasion. The SopE functions as a guanine exchange factor (GEF) and activates Cdc42 and Rac-1. The two latter are host Rho GTPases involved in cell signaling governing cytoskeletal rearrangements (Fàbrega & Vila, 2013). The SopE triggered signaling cascade leads to induction of the proinflammatory cytokines such as interleukin-8 (IL-8) or tumor necrosis factor α (TNF-α) causing mucosal inflammation. Whereas the SopE protein is not present in all *Salmonella* species, the highly homologous SopE2 – another GEF protein, is present in all *Salmonella* strains (Bakshi et al., 2000). To ensure that induced cytoskeletal rearrangements localize near the bacterium-host contact, the SipA effector directly binding actin filaments is involved. SipA inhibits actin filaments depolymerization leading to their accumulation at the site of infection promoting creation of the ruffles in the host membrane. (Hapfelmeier et al., 2004; Higashide et al., 2002; Zhou et al., 1999). The SopB protein, initially reported in *S.* Tm Dublin, interferes with phosphatidylinositol signaling pathway. This signaling disturbance leads to increased secretion of chloride and subsequently onset of diarrhea. (Norris et al., 1998; Zhang et al., 2002). Additional effectors, namely SopD, SopA and IacP also partake in this stage of enteropathogenesis. The SopD protein acts together with SopB promoting fluid secretion and inflammatory response leading to onset of enteritis (Jones et al., 1998). The

SopA protein is an ubuquitin ligase resembling the mammalian HECT E3 protein (Zhang et al., 2006) which ubiquitinates proteins involved in the inflammation onset. The cytoplasmic enzyme IacP presumably plays a role in posttranslational modifications of SopB, SopD and SopA ensuring proper secretion of these effectors (Kim et al., 2011). Some of the SPI-1 effectors can also interact with the SPI-2 effectors. The actin-binding SipA, together with SifA participates in correct positioning of the *Salmonella* containing vacuole (SCV) (Brawn et al., 2007). On the other hand, some effectors can antagonize the effect of others, for instance SptP can disrupt the actin cytoskeleton (Fu & Galán, 1998). Therefore, it is crucial that secretion of effectors happens in a controlled and timely manner according to their role in the infection process.

The host cell cytoskeletal rearrangements triggered by the bacterial effectors leads to *S.* Tm engulfment by the epithelial cells. Once this step is completed, *S.* Tm is localized inside the SCVs (Haraga et al., 2008). These membrane enclosed compartments create environmental niche for the pathogen to replicate. To create and maintain the SCVs, *S.* Tm employs at least 43 effectors secreted by two T3SS encoded within SPI-1 and SPI-2 (Figueira & Holden, 2012; Galan, 2001; LaRock, 2015). The effectors secreted by SPI-2 T3SS-2 contribute to SCV maturation and biogenesis of *Salmonella* induced filaments (SIFs) (Garcia-del Portillo et al., 1993; Knuff & Finlay, 2017).

In the early stages, SCVs in HeLa cells resemble the early endosomes and are decorated with markers for endocytic sorting and recycling pathways. Bacterial effectors are partially responsible for maturation of the SCVs. For instance, the SopB effector engages the Rab5 GTPase which in turn recruits the early endosome antigen 1 (EEA1) to the vacuole membrane (Knuff & Finlay, 2017). Additionally, the early SCVs are also characterized by markers like Rab4, Rab11 and endocytic markers transferrin receptor. Along with the maturation of SCVs is associated with loss of the early markers and acquisition of late endosomal markers like Rab7, lysosomal associated membrane proteins (LAMPs) 1, 2 and 3; and ATPase. Unlike late endosomes, SCVs are not enriched for markers like cathepsin D, lysobiphosphatidic acid and mannose-6-phosphate receptor. The final stage of late SCV is a unique compartment enabling the replication of *S.* Tm (McGhie et al., 2009; Knuff & Finlay, 2017).

The bacterial effectors translocated using T3SS-2 play important role in the SCV maturation process. The SifA effector, forms complexes with host factor SifA-and-kinesin-interacting-protein (SKIP). Thus created SifA-SKIP complexes bind to Rab9, preventing Rab9-dependent

M6PR recruitment to the SCV membrane. These markers recruit lysosomal enzymes, therefore their absence, protects SCVs from the host defenses. The SopD2 effector interferes with the Rab7-dependent recruitment of the microtubule-based trafficking thus preventing delivery of SCVs to lysosomes. The SseJ has two enzymatic activities: glycerophospholipid:cholesterol acyltransferase and phospholipase A activity, which reshape the lipid composition of SCV membranes. These alterations contribute to formation of the unique compartment enabling replication of *S.* tm in the late SCV (Knuff & Finlay, 2017).

In parallel with *S.* tm replication within SCV, the formation of SIFs take place. SIFs are the lysosomal glycoprotein-containing tubules extending outside the SCV (Figueira & Holden, 2012). SIFs biogenesis is associated with T3SS-2 secreted effectors: SifA, SseJ, SopD, PipB2, SseF, SseG, SpvB and SteA (Knuff & Finlay, 2017). Collectively these effectors participate in processes like: SIF biogenesis, maintenance, and modifications of SCV membranes, SCV positioning, recruiting microtubule motor activity enabling SIFs extension along the microtubules. It is hypothesized that SIFs modify the host vesicular trafficking to supply *S.* Tm with nutrients and membrane components thus promoting bacterial replication.

Interestingly, findings by Knodler et al. suggest that not entire *S.* Tm population replicates within the SCVs once inside the host epithelial cell. Authors showed that pathogen forms two sub-populations with distinct doubling rates. Interestingly, a portion of fast replicating cells was localized inside the cytosol; and these cells were expressing SPI-1 encoded genes, whereas the slower replicating sub-population was localized inside the SCVs and expressing SPI-2 genes. (Knodler et al., 2010; Knodler, 2015; Hallstrom & McCormick, 2011).

A fraction of the *S*. Tm cells surviving and replicating within epithelial cells or macrophages can cross the basolateral membrane of epithelium, where they utilize dendritic cells as vehicles for systemic dissemination (Voedisch et al., 2009; Swart et al., 2016). Findings by Bravo-Blas et al. surprisingly revealed that *S.* tm can also travel toward the lymph nodes without any carrier (Bravo-Blas et al., 2019). In the streptomycin treated mouse model, the arrival in the lymph nodes was found to impose a bottleneck, as less than 300 *S.* Tm per day could reach this site (Kaiser et al., 2013). Moreover, studies using isogenic tagged strains indicated further bottlenecks during the systemic infection (Mastroeni & Grant, 2013). Further studies revealed that *S*. Tm displays a high diversity on the single cell level within infected organs. Studies in spleen showed *S*. Tm sub-populations characterized by distinct growth rates and thus presumably different metabolism (Claudi et al., 2014).

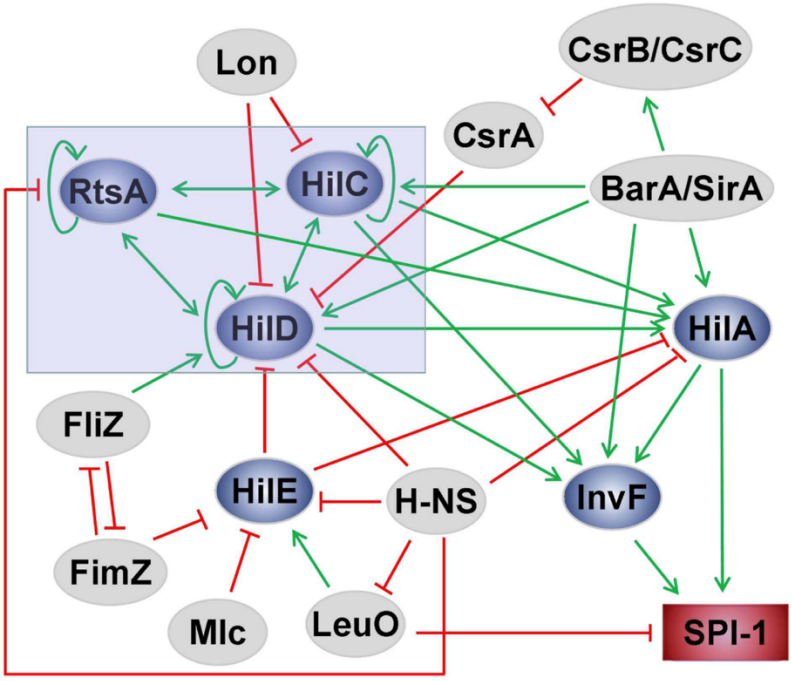


**Fig. 2. Schematic overview of SPI-1 regulatory network.** Blue ovals represent regulators more directly involved in regulation of SPI-1, with HilD-HilC-RtsA amplification loop framed in a blue square. Grey ovals indicate other regulatory proteins indirectly affecting SPI-1 expression as a part of HilD regulon. Green arrows represent activating effect, and red lines with flat ends inhibitory effect. (Lou et al., 2019)

The infection process by *S.* Tm relies on the complex, highly organized series of steps involving activity of dedicated VFs. Up- or downregulation of these VFs needs to be coordinated with signals from both, external environment, and internal cues reflecting physiological state of the pathogen itself. Some secreted effectors antagonize activity of the others, making it crucial for the infection steps to occur in the correct order. Timely initiated virulence program in accordance with environmental signals increases *S.* Tm chance of a successful infection (Golubeva et al., 2012).

The regulatory network governing the virulence program is of astonishing complexity. Since the SPIs carry the genes which play crucial roles during infection, their regulation is of critical importance. It is especially important for the regulation of SPI-1, as expression of genes encoded within this pathogenicity island are initiating the virulence program of *S.* Tm. The SPI-1 expression depends on regulators encoded within the island itself, as well as outside the

SPI-1. In conditions mimicking the host intestinal lumen with low oxygen and high osmolarity, the SPI-1 is being maximally transcribed (Fàbrega & Vila, 2013). The SPI-1 is a genetic element originally introduced into *S*. Tm genome via horizontal gene transfer, and over the course of evolution maintained the GC content lower than the rest of the genome. To mitigate the cost of uncontrolled expression originating from the newly acquired genetic elements *S.* tm employs the histone-like nucleoid structuring protein (H-NS) which selectively silences regions with a low GC content (Rosen et al., 2006). H-NS-mediated repression of transcription can be alleviated by actions of various DNA-binding proteins, such as HilD or LeuO (Kalafatis & Slauch, 2021).

The SPI-1 itself, crucial for invasion, contains genes encoding four important virulence regulators: HilA, HilD. HilC and InvF. The research from 1992 mentioned for the first time the hyper invasive locus (*hil)* in the *S*. Tm genome (Lee et al., 1992). Deletion of the *hil* locus led to drastic decrease in bacterial entry into Hep-2 cells, showing its essential role during bacterial invasion. Another study from 1995 identified the HilA as a novel OmpR/ToxR family protein. The promoter of HilA is a target for H-NS silencing which prevents its transcription (Olekhnovich & Kadner, 2006), presence of the binding of HilC and HilD to their respective binging boxes can de-repress this effect and activate HilA transcription (Bajaj et al., 1995). This transcriptional activator drives the expression of the SPI-1 genes apart from *hilC* and *hilD*. Within the SPI-1 HilA binds to the *invF* and *prgH* promoters, activating T3SS-1 genes expression. Moreover, HilA regulatory function spans beyond the SPI-1 encoded genes. Under invasion-inducing conditions, HilA activates expression of the *sii* operon encoded within SPI-4 by binding the promoter of the first gene in this operon *siiA* (Thijs et al., 2007). The co-regulation of SPI-1 and SPI-4 genes by HilA is consistent with importance of these loci for intestinal colonization. In addition, promoter of the SPI-5 encoded SopB (SigD) effector, is also activated by HilA binding (Thijs et al., 2007). In contrast, HilA can also act as a repressor under the invasion-inducing conditions silencing the SPI-2 encoded genes i.e., *ssaH* and *sseL*. Moreover, HilA was found to negatively regulate expression of the *flhD* gene, thus downregulating the motility (Thijs et al., 2007).

HilD is an AraC-like global transcription regulator encoded within the SPI-1 which regulon encompasses about 250 genes (Petrone et al., 2014; Martínez-Flores et al., 2016). HilD positively regulates expression of the SPI-1 genes, as well as many other genes involved in *S*. tm virulence. HilD directly controls expression of HilA through a feed forward loop which it forms with HilC and RtsA. The HilC and RtsA are also AraC-like regulators which recognize

the same DNA motif as HilD. Each of these three regulators can induce transcription of *hilC, hilD* and *rtsA* genes (Ellermeier et al., 2005). This feed forward loop is fueling virulence expression. Narm et al., showed that HilC, HilD and RtsA can form homodimers as well as heterodimers while in the solution (Narm et al., 2020). The HilD protein has a special place in the *S.* Tm virulence regulation because the majority of external regulatory inputs are integrated through HilD (Golubeva et al., 2012; Baxter et al., 2003; Kim et al., 2019; Golubeva et al., 2016). For this reason, the regulation of HilD expression is of crucial importance.

The *hilD* promoter region is characterized by the low GC content and is silenced by the H-NS. HilD can overcome this H-NS silencing to activate its own promoter (Kalafatis & Slauch, 2021). The *hilD* mRNA stability provides another level of controlling virulence expression. The 5' end of *hilD* mRNA contains two mutually exclusive stem loop structures. First of these stem loops contains the ribosome binding site and the *hilD* start codon. This stem-loop is stabilized by the global regulator CsrA. Factors reducing stability of the first stem-loop, enhance *hilD* translation and thus virulence expression. Formation of the second stem-loop, liberates ribosome binding site and start codon, promoting *hilD* translation and subsequent activation of the virulence program. The second stem loop is energetically favorable and in the absence of additional regulatory components should predominate increasing virulence expression. However, CsrA binding to the first stem-loop, stabilizes this structure and shifts the balance towards repression of *hilD* (Hung et al., 2019). Post-translational modifications can also impact HilD stability and its DNA-binding properties. Acetylation of the lysine K297 by the protein acetyltransferase increases HilD stability but reduces its ability to bind DNA (Sang et al., 2017). The HilD affinity to DNA is also regulated by HilE on the protein-protein level. Encoded outside of the SPI-1, HilE is negatively regulating HilD. HilE specifically binds to HilD, but not HilC or RtsA. This protein-protein interaction prevents HilD from binding to its target DNA (Grenz et al., 2018).

### Cross-regulation between SPI-1 and other virulence functions

During infection, *S.* Tm relies on the direct motility using chemotaxis to optimally colonize the host gut. To achieve that, chemoreceptors detect presence of attractants or repellents in the environment and trigger signaling cascade controlling direction of the flagellar motor. Although flagellar motility exists in both, HilD-expressing and HilD-non-expressing cells, Cooper et al. observed that these two subpopulations display a different swimming behavior (Cooper et al., 2021). The authors showed that in the SPI-1 inducing conditions, HilD derepresses the methyl-

accepting chemotaxis protein (McpC) expression by removing H-NS silencer from the *mcpC* promoter region. Presence of the McpC protein results in a counterclockwise movement of flagellar motor and a smooth swimming phenotype. Smooth swimming increases the net movement of *S.* Tm toward epithelial cell layer (Cooper et al., 2021). Cells which do not express McpC protein, retain their chemotactic functions, display tumbling behavior, and change direction more often. Interestingly, Hoffmann et al., showed that certain non-chemotactic mutants showing smooth swimming behavior can be more invasive (Hoffmann et al., 2017).

Regarding importance of motility during *S.* Tm gut infection, pathogen developed several levels of cross-regulation between the SPI-1 and flagella. Hautefort et al. showed that during the early infection of epithelial cells (2 hours post infection) flagellar gene expression is repressed. However, during the late infection (4 and 6 hours post infection) flagellar biosynthesis genes were upregulated (Hautefort et al., 2008). These data demonstrated that flagellar expression is changing over time during the infection process. Similar to the SPI-1 genes, flagellar operons also require a precise spatiotemporal expression coordinated with environmental sensing. Expression of flagellar genes is under the spatiotemporal control depending on transcriptional hierarchy of three promoter classes. The flagellar master operon *flhDC* is under control of $\sigma^{70}$-dependent flagellar class 1 promoter. A functional complex $FlhD_4C_2$ is required for transcription of flagellar genes under class 2 promoters. One class 2 gene – *fliA* encodes for a transcription factor $\sigma^{28}$ enables transcription of the class 3 promoters (Chilcott & Hughes, 2000). Expression of flagellar master regulator – *flhDC* is positively or negatively influenced by other factors. The regulator RtsB functions as a repressor of flagellar class 1 genes. RtsB is encoded together with RtsA in an operon *rtsAB* which is transcriptionally activated by HilD, HilC and RtsA. (Ellermeier & Slauch, 2003). Interestingly, Singer et al. showed that HilD can directly activate *flhDC* via activation of the P5 transcriptional start site (Singer et al., 2014). Yet another level of crosstalk between regulatory networks of flagella and SPI-1 is based on FlhDC-dependent expression of *fliZ.* The *fliZ* expressed form flagellar promoters of class 2 and 3 functions as a regulator of HilD activity acting post-translationally (Iyoda et al., 2001; Kage et al., 2008; Chubiz et al., 2010). The regulatory cross talk between flagellar and SPI-1 regulons encompasses several feedback loops implemented on various levels supporting multi-process interplay between these regulons. Depending on the environmental niche and stage of infection, HilD could activate *flhDC* directly binding to its promoter or alternatively repress its transcription via RtsB. Singer et al. hypothesize that in the early stages of infection, SPI-1 genes are induced, and motility is downregulated, however,

flagellar genes remain in a state enabling immediate activation via HilD at the later point of infection (Singer et al., 2014). These regulatory intersections suggest that concerted spatiotemporal expression of flagellar genes are important for successful infection.

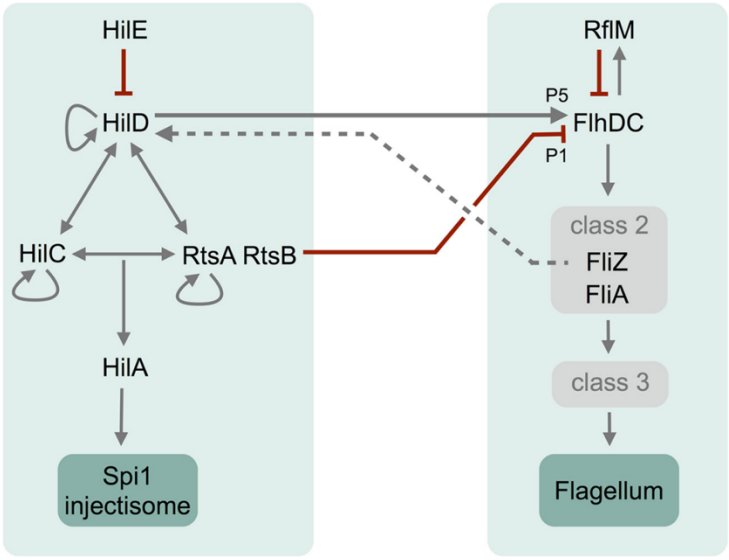


**Fig 3. Schematic overview of the crosstalk between the SPI-1 and flagellar regulons.** For the simplification, only protein names are depicted. Horizontal arrows connecting two green blocks represent the crosstalk interactions between SPI-1 and flagellar regulons. Grey arrows indicate activating effects and red blunt lines inhibitory effects. The dashed line between class 2 product FliZ and HilD represents posttranscriptional activation (Singer et al., 2014).

Coordinated action of SPI-4 encoded giant adhesin and T3SS-1 increases the invasion of polarized epithelial cell. The adhesin is attaching *S*. Tm to epithelial cell and positions bacteria in orientation enabling penetration of T3SS-1 injectosome and delivery of effectors. This functional cooperation is reflected in direct co-regulation of these VFs (Keersmaecker et al., 2005; Thijs et al., 2007; Petrone et al., 2014; Thijs et al., 2007; Gerlach et al., 2008; Main-Hester et al., 2008). The HilA, SPI-1 encoded virulence regulator, has been shown to bind in the promoter region of *siiA* gene (Thijs et al., 2007) and within *siiE* coding region (Keersmaecker et al., 2005). Results of Main-Hester et al. showed that HilA is de-repressing expression of SPI-4 genes silenced by H-NS (Main-Hester et al., 2008). In addition, Petrone et al. observed direct regulation of *siiA* by the HilD – SPI-1 encoded master regulator of virulence (Petrone et al., 2014). Binding of the SPI-4 locus by both HilA and HilD suggest importance of coordinated expression of both SPI-1 and SPI-4 for ensuring successful invasion of polarized epithelial cells. Interestingly, in the ΔSPI-1 background, expression of

HilA or HilD had little or no effect on *siiE* transcript levels, suggesting that these regulators act in concert with additional SPI-1 encoded function to activate SPI-4 (Main-Hester et al., 2008).

In the host gut *S.* Tm expresses SPI-1 genes which mediate the invasion of the epithelium. When internalized, bacteria downregulate SPI-1 genes and upregulates expression of SPI-2 genes which support its intracellular replication. During the late stationary phase HilD induces expression of SPI-2 encoded genes by binding to the promoter of *ssrAB* (Martínez et al., 2014). Subsequently the SPI-2 encoded protein SsrB which is a positive regulator of SPI-2, can act as a repressor of SPI-1 genes during intracellular stage of infection (Pérez-Morales et al., 2017). Such regulatory crosstalk between SPI-1 and SPI-2 allows *S.* Tm to express appropriate VFs depending on the environmental niche.

### Cooperative virulence of *S.* Tm

*S.* Tm virulence process is an example of cooperative trait. Infection with *S.* Tm begins by ingestion of contaminated food or water. Pathogen being transmitted via the oral route begins its journey within the digestive system of the host. In the initial stage of infection bacteria encounter the first environmental barrier as it passes through the stomach. There, *S.* Tm is exposed to the acidic pH, and as a response activates the acid tolerance response (ATR) which enables bacteria to maintain its intracellular pH value higher than extracellular (Foster & Hall, 1991). The surviving *S.* Tm arrives in the small intestine where it needs to cross the mucus to access the intestinal epithelial cells. To further colonize the host *S.* Tm needs to cross the intestinal epithelium. To achieve this, the pathogen can exploit phagocytic intestinal cells, such as antigen sampling M-cells or dendritic cells. However, *S.* Tm is also able to force its own uptake into non-phagocytic epithelial cells in a process called invasion (Hume et al., 2017). This process relies on delivery of bacterial effector proteins inside the host cells via T3SS-1 encoded within SPI-1 (Song et al., 2017). As a result, the host cells undergo extensive cytoskeletal rearrangement leading to formation of the membrane ruffles that engulf bacteria into the SCVs. These intracellular vesicles provide a compartment where *S.* Tm can survive and replicate (Kuhle & Hensel, 2004). The bacterial entry is detected by the host immune system triggered by the presence of pathogen VFs or bacterial recognition by pathogen-associated molecular pattern receptors (Tükel et al., 2005). These immune responses lead to cytokines and chemokines release driving subsequent inflammatory response which drastically changes the environment in the gut lumen (Sellin et al., 2015).

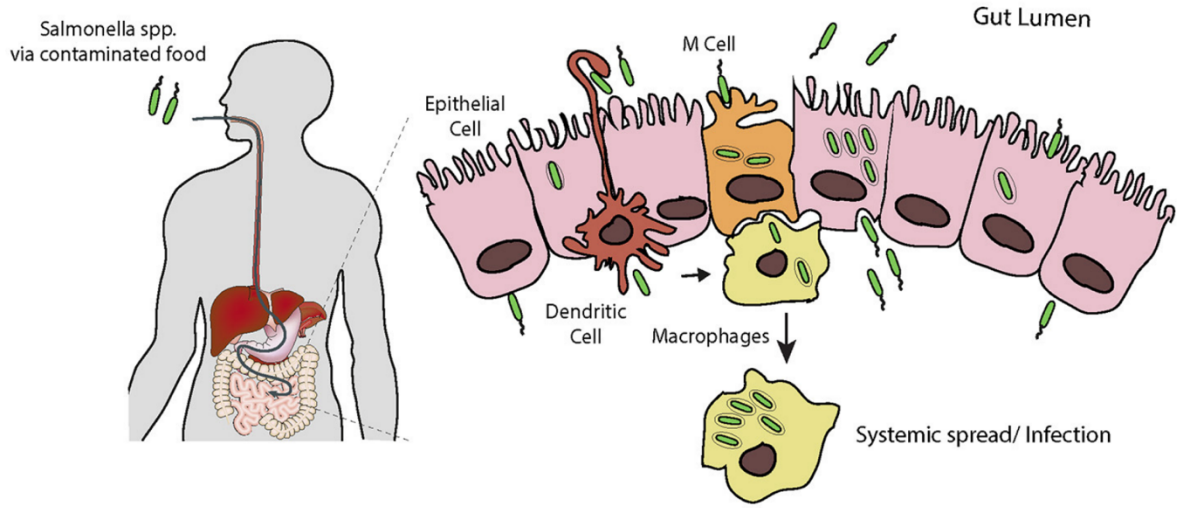


**Fig 4. Schematic overview of *S.* Tm crossing the epithelial layer.** Following ingestion and passage through the stomach, *S.* Tm encounters the intestinal epithelial layer. Bacteria can cross epithelial barrier using phagocytic intestinal cells like antigen sampling M-cells or dendritic cells. Moreover *S.* Tm can force its own uptake by invading non-phagocytic epithelial cells from the apical side, i.e., the intestinal lumen. Following the uptake, bacteria can replicate intracellularly, or be transcytosed and released on the basolateral side of epithelial layer, from here *S.* Tm can further infect epithelial cells (Hume et al., 2017).

This host inflammatory response is important for *S.* Tm as it helps pathogen to outcompete commensal bacteria inhabiting the host gut. The human gut is colonized by remarkable number of commensal bacteria, majority of these bacteria assists in the food digestion and thus the uptake of nutrients (Riccio & Rossano, 2020). In addition, this commensal consortium provides protection against gut infection creating colonization resistance barrier (Ducarmon et al., 2019). This protective barrier decreases the chances of *S.* Tm to successfully approach and attach to the host epithelial cells. However, several outcomes of the host inflammatory response shift the balance between the protective microbiota and *S.* Tm in favor of the pathogen by boosting its growth (Stecher et al., 2007). Mucosal inflammation provides access to high energy nutrients which *S.* Tm can access using its motility and chemotaxis (Stecher et al., 2008). This nutrient accessibility promotes *S.* Tm replication, whereas commensals growth is impeded in conditions of gut inflammation. The lipocalin-2 secreted by epithelial cells during inflammatory response interferes with the iron uptake by commensal bacteria, but not by *S.* Tm (Raffatellu et al., 2009). Another advantage for *S.* Tm is conferred by its ability to utilize tetrathionate, which is a respiratory electron acceptor generated during gut inflammation, whereas majority of commensals fail to utilize it (Winter et al., 2010). Additionally, *S.* Tm effector SopE initiates generation of nitric oxide by stimulating expression of nitric oxide synthase in activated macrophages. In reaction with reactive oxygen species (ROS), nitric

oxide leads to nitrate, which is preferentially used as an anaerobic electron acceptor by *S.* Tm (Lopez et al., 2012). Along with the pathogen, certain commensal Enterobacteriaceae can also benefit from inflammation in the gut lumen (Stecher et al., 2012). Through the lens of the cooperative trait the gut inflammation can be seen as the "common good".

In case of *S.* Tm infection, these benefits of inflammation are brought by a fraction of cells expressing VFs in the gut. In WT *S.* Tm only a sub-population of cells is expressing T3SS-1 *in vitro* and *in vivo* (Hautefort et al., 2003; Bumann, 2002). The SPI-1 ON phenotype is heritable and maintained for several generations if bacteria are transferred into the SPI-1 non-inducing conditions (Sturm et al., 2011). While the mechanism controlling the SPI-1 bistability in *S.* Tm remains unclear, the core architecture of regulatory circuit driving SPI-1 expression was described to function as a signal amplifier with an activation threshold (Saini et al., 2010). In this system activation of the promoter of a master regulator of virulence – HilD, is a critical step for the induction of the SPI-1 genes, while HilC and RtsA function as transcription amplifiers. Moreover, the SPI-1 regulatory circuit is known to accommodate signals from various global regulators which are fed into the circuit via HilD (Golubeva et al., 2012). This complex regulatory structure assures both, expression of SPI-1 genes under favorable conditions, and in the fraction of the population.

The study by Sturm et al., using fluorescent reporter on a single cells level, showed that cells expressing T3SS-1 display retarded growth in comparison to T3SS-1-negative cells (Sturm et al., 2011). This suggested that VFs expression imposes a cost for the pathogen, and the payoff for the sub-population having invasion capacity is growth retardation. In such conditions the "cheater" mutants can emerge, which would benefit from the inflammation as a public good and avoid paying cost of its production (West et al., 2006). Indeed, the fast-growing virulence attenuated mutants were observed during within host evolution (Diard et al., 2013). The emergence of avirulent mutants which can outcompete WT *S.* Tm, threatens transmission of the pathogen into secondary host. However, in this case the cooperative virulence is stabilized by the presence of the fast-growing WT subpopulation which does not produce VFs. These cells can slow down the rise of cheaters and thus ensure transmission of the virulent phenotype. The mathematical modeling performed by Diard et al. showed that it is crucial for WT *S.* Tm to sustain a substantial fraction of phenotypically avirulent cells to prevent cheaters overcoming the population. The modelled scenario of WT *S.* Tm being outcompeted by avirulent mutants is predicted to involve decrease of inflammation, regrowth of microbiota and clearance of *S.* Tm in the gut. Therefore, both SPI-1 expressing, and non-expressing sub-

populations are needed for the successful infection, as they both have different and complementary roles in this process (Diard et al., 2013; Sánchez-Romero & Casadesús, 2018). The tight regulatory network driving bimodal expression of virulence ensures stabilization of cooperative virulence in *S*. Tm.

Although, the SPI-1 expression is tightly controlled in a spatiotemporal manner depending on the presence of environmental cues, not all *S*. Tm cells express these VFs in a given niche. In their study, Hautefort et al., using fluorescent reporter fusion, showed that *prgI* (encoding T3SS-1 needle) is expressed in about 50% of *S.* Tm cells under the in vitro experimental conditions (LB and cell culture) (Hautefort et al., 2003). Nowadays, the notion that genetically isogenic bacterial populations can contain cells displaying variety of different phenotypes is broadly accepted in microbiology. However, in the past, as majority of experiments were performed on the bulk of cells and obtained results reflected the averaged phenotype, making this important feature to be long overlooked (Kreibich & Hardt, 2015). A certain cell-to-cell variation is a consequence of the noisy gene expression. However, presence of features amplifying these stochastic differences, like regulatory feedback loops, allows emergence of stable subpopulations with distinct gene expression patterns (Ackermann, 2015). The phenotypic heterogeneity can be beneficial for bacteria allowing it to divide a labor or engage in the bet hedging.

In the conventional view of how bacteria adapt to the fluctuating environment, the central notion is that individual bacteria sense the environmental cues and respond to them by signal transduction and subsequent changes in the gene expression patterns. However, in some cases, such as when environment changes are very rapid, this strategy might not be sufficient to ensure bacterial survival (Ackermann, 2015). A possible solution is that a small number of the population can express features which are not necessarily the most optimal for the environment organism is currently in but would be highly advantageous and allow survival in another environment. This strategy where a small number of individuals shows a certain phenotype independently of the environmental cues is called bet hedging and it allows organisms to persist in a fluctuating environment.

The division of labor ensures a payoff in a constant environment where each subpopulation has a specific phenotype. The benefits for a given cell depend on the phenotypes of the other cells sharing the same microenvironment (West & Cooper, 2016). In case of *S*. tm infection, phenotypically virulent subpopulation triggers the host inflammatory response that helps

eliminate bacteria from different species as described in section "*S.* Tm virulence is a cooperative trait". Phenotypically avirulent subpopulation mostly remain in the host gut lumen where they can reproduce successfully. The cooperation of these two subpopulations is an example of division of labor.

*Environment sensing as a crucial factor in virulence regulation in S. Tm*

Two-component regulatory systems of *S.* Tm

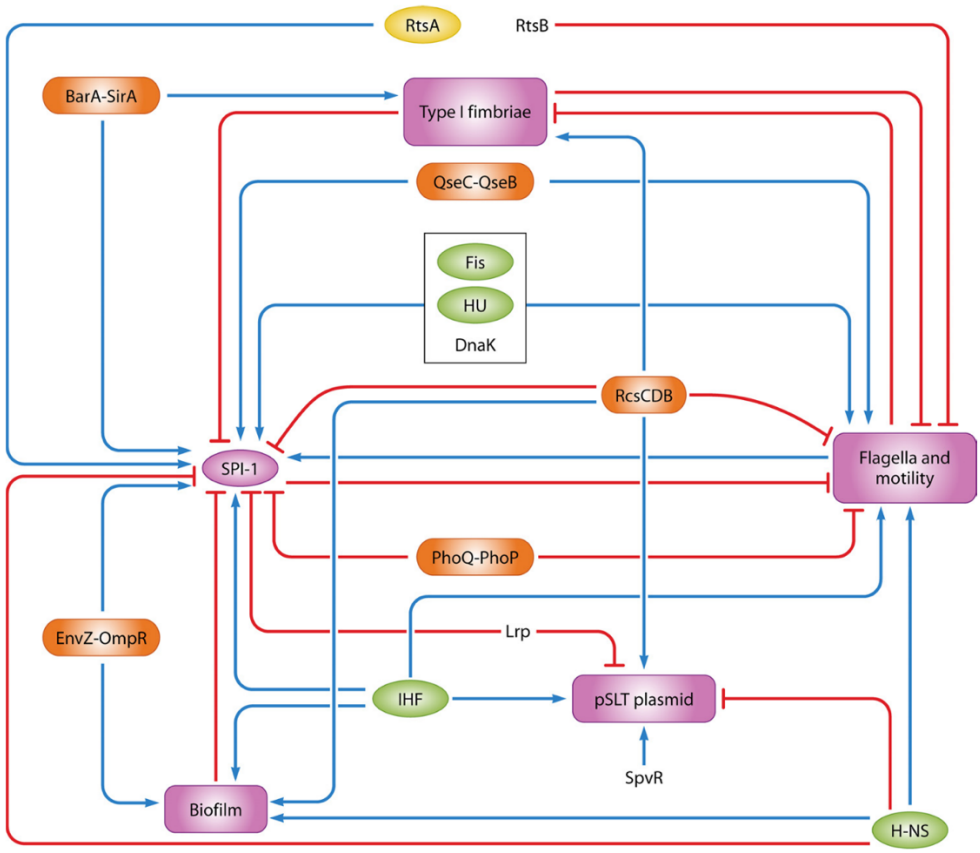


**Fig 5. Schematic overview of crosstalk between virulence elements of *S.* Tm and two-component systems enabling sensing of the environmental cues.** Red lines indicate inhibitory effects and blue arrows indicate activating effects (Fàbrega & Vila, 2013).

To ensure timely virulence expression, environmental cues are sensed by bacterial two component systems and converted into cellular response and as such integrated by the virulence regulators. Among the most relevant for virulence expression two component systems are: PhoP/PhoQ, EnvZ/OmpR, BarA/SirA. (Groisman et al., 2021; Kenney & Anand, 2020; Zere et al., 2015). In principle these two component systems consist of the membrane embedded sensor kinases (PhoQ, EnvZ and BarA) which in response to environmental stimuli

phosphorylates the cytoplasmic response regulator (PhoP, OmpR, SirA). These systems allow *S.* Tm sensing of the environmental conditions and adjust the expression of specific genes to adapt to these conditions (de Pina et al., 2021).

One of the most important two component systems used by *S.* Tm is the PhoP/PhoQ system. PhoQ is activated by several signals such as: low $Mg^{2+}$ concentration, antimicrobial peptides, long chain unsaturated fatty acids, mildly acidic pH and increased osmolarity (Groisman et al., 2021). In response to these stimuli PhoQ phosphorylates PhoP and such modified PhoP can bind to its target DNA. Phosphorylated PhoP controls genes involved in virulence, cell surface modifications, $Mg^{2+}$ homeostasis within the cytoplasm, resistance to antimicrobial agents. Conditions activating the PhoP/PhoQ system can be found inside macrophages. The PhoP/PhoQ system activation triggered by certain stimuli can counteract the effect of this stimuli. PhoP/Q is sensitive to concentration of $Mg^{2+}$ and as its concentration decreases, the level of phosphorylated PhoP increases. Presence of divalent cations, like $Mg^{2+}$, neutralizes the negative charge of the phosphate residues residing in LPS, thus stabilizing bacterial envelope. When bacteria experience environment depleted in $Mg^{2+}$, PhoP-activated genes covalently modify the LPS in a way which helps to compensate for the lack of these cations. These covalent modifications help to avoid electrostatic repulsion between phosphate residues within LPS (Groisman et al., 2021). $Ca^{2+}$ and $Mn^{2+}$ also interact with PhoQ resulting in PhoP inactivation (Vescovi et al., 1996). By contrast $Ni^{2+}$, $Cu^{2+}$, and $Ba^{2+}$ had no effect on PhoP/Q in concentrations up to 300 $\mu$M. PhoQ is activated by different cationic antimicrobial peptides (CAMPs) in sublethal concentrations (Bader et al., 2003). These peptides with their ability to penetrate the *S.* Tm outer membrane can reach PhoP's periplasmic domain. Interestingly the strength of activity of antimicrobial peptide and of PhoQ activation are not correlated (Shprung et al., 2012). Instead PhoPQ activation is correlated with hydrophobicity and amphipathicity of the given peptide. PhoQ was found to respond most efficiently to peptides with high hydrophobicity and high positive charge. The mildly acidic pH also activates the PhoP/Q system. *S.* Tm encounters such pH inside the macrophage phagosomes (Rathman et al., 1996). The PhoQ's response to acidic pH is mediated by PhoQ's cytoplasmic domain, unlike sensing $Mg^{2+}$ and antimicrobial peptides which are sensed by the PhoQ's periplasmic domain (Choi & Groisman, 2016). *E. coli* carrying either its own *phoPQ* genes or the one from *S.* Tm, was able to sense hyperosmotic stress from 300mM NaCl (Yuan et al., 2017). This PhoP/Q activation is transient with recovery to the pre-stimuli state within 15 min. PhoQ senses the osmolarity perturbed membrane via its transmembrane domain, which can respond to both ionic and nonionic osmolytes, such as sucrose or sorbitol (Yuan et al., 2017).

Moreover, several long-chain unsaturated fatty acids, including linoleic, linolenic, and palmitoleic acid, added to the LB were found to downregulate transcription of PhoP-activated genes 2- to 4-fold (Viarengo et al., 2013). Majority of PhoP-regulated genes are PhoP activated (Groisman et al., 2021). However, regarding the SPI-1 expression, PhoP is repressing the *prg* genes (PhoP repressed genes) and *hilA.* (Groisman et al., 2021). PhoP binds directly to the *hilA* promoter (Palmer & Kim, 2019). As the conditions activating PhoP/Q can be found in the macrophages, downregulation of the invasion genes makes sense at this stage of infection as invasion factors have already fulfilled their role.

The sensor kinase EnvZ responds to cytoplasmic signals that arise from changes in extracellular environment. The OmpR can act canonically (phosphorylation dependent) to regulate porin genes *ompF* and *ompC,* and non-canonically (phosphorylation independent) to activate the acid stress response (Kenney & Anand, 2020). Several factors influence porin levels, such as: temperature, pH, osmolarity and growth phase. Depending on the condition the relative abundance of the porins changes, whereas their total amount remains relatively the same. In low osmolarity conditions, the major porin of the outer membrane is OmpF exhibiting the larger pore and faster flux. In high osmolarity conditions, ompF transcription is repressed and OmpC becomes dominant porin in the outer membrane, exhibiting a smaller pore and slower flux. The virulence of *S*. Tm mutants lacking *envZ* and *ompR* is reduced. The OmpR activates expression of the SPI-2 encoded two-component regulatory system SsrAB. In turn, the SsrAB regulates the T3SS-2 required for replication inside macrophages. In response to acid pH, the EnvZ/OmpR system can work non-canonically. In these conditions, the OmpR phosphorylation is very low, but the OmpR is still binding to EnvZ. This interaction of EnvZ with OmpR can lead to OmpR dimerization. The OmpR in a dimer form can bind DNA and regulate transcription in the absence of phosphorylation (Desai & Kenney, 2017; Kenney & Anand, 2020).

The sensor kinase BarA and its cognate regulator SirA are involved in carbohydrate metabolism, motility, biofilm formation and invasion (Fàbrega & Vila, 2013). The SirA activates the expression of two small RNAs, *csrB* and *csrC,* which inhibit production of CsrA protein. CsrA is an RNA binding protein altering stability of its target mRNA, regulating virulence and central carbon metabolism, CsrA protein binds the 5’ end of *hilD* mRNA thus preventing translation of HilD protein – the central positive regulator of virulence in *S*. Tm (Hung et al., 2019). In this regulatory network, SirA, by activating small RNAs which titrate CsrA protein, preserves HilD activity. Lawhon et al. described a model where SirA can be induced by acetate

in the concentration found in the distal ileum without involvement of BarA (Lawhon et al., 2002). In the distal ileum characterized by pH=6.7, because of the difference of pH between the ileum and bacterial content, acetate ions accumulate within bacteria. Within bacteria acetate kinase converts acetate into acetyl phosphate. Additionally, acetyl phosphate can be also produced from endogenous acetyl-CoA if it exceeds the metabolic needs. The acetyl phosphate might phosphorylate BarA, SirA or both, thus triggering expression of invasion genes (Lawhon et al., 2002).

### Impact of metabolism on the regulation of SPI-1

To ensure successful infection *S.* Tm needs the ability to grow on different substrates present in the infection site. This versatile bacterium can metabolize different carbon sources including short chain fatty acids (SCFA) such as acetate, succinate and several glucose derivatives (Bohnhoff et al., 1964; Cummings et al., 1987). Glucose was shown to be an important carbon source for *S.* Tm *in vitro* (Bowden et al., 2014). Presence of glucose determines the cAMP levels within the cell. Decreasing glucose corresponds with increasing concentrations of intracellular cAMP. This molecule is a cis-activator of the cAMP receptor protein (CRP). Thus, decreased levels of glucose lead to transcription of the CRP controlled genes (Fandl et al., 1990). Addition of glucose to the M9 media was found to repress expression of SPI-1 genes (Sridhar & Steele-Mortimer, 2016). Study by El Mouali demonstrated that CRP-cAMP indirectly affects the level of HilD. The CRP-cAMP transcriptionally inhibits the Hfq-dependent small RNA Spot 42. When the CRP-cAMP repression is relieved, the Spot 42 positively affects expression of *hilD* mRNA interacting with its 3' UTR (El Mouali et al., 2018).

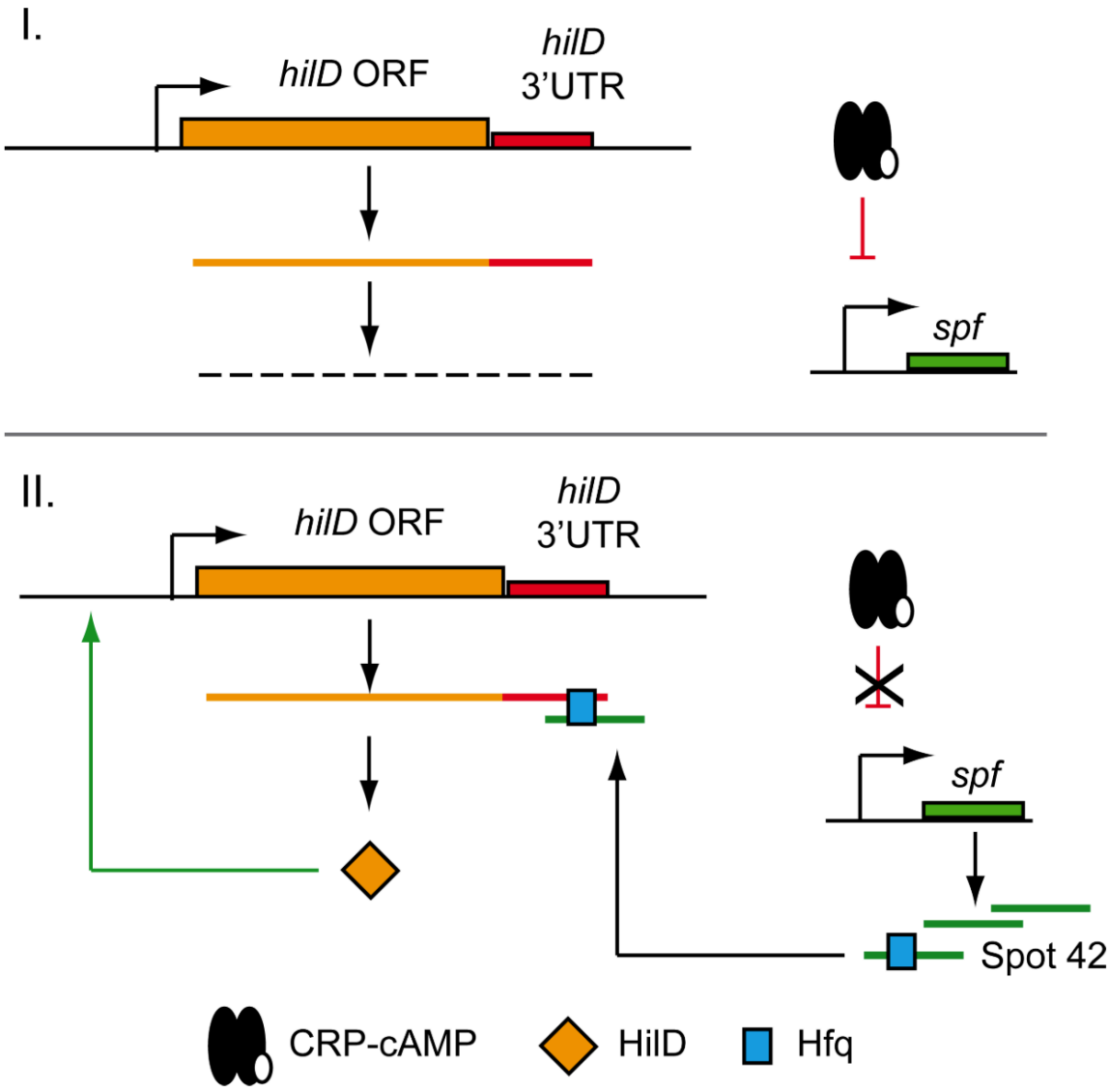


**Fig 6. Proposed model of activation of *hilD* expression via the Spot 42 small RNA**. I. When Spot 42 small RNA is inhibited by the CRP-cAMP complex (e.g., low glucose) expression of SPI-1 genes is repressed. II. When the Spot 42 small RNA is expressed, it promotes the SPI-1 genes expression. In the scheme genes are represented as rectangles, yellow – *hilD*, red – 3' UTR of *hilD*, green – *spf*; horizontal lines in the same colors represent RNA of these genes; and blue, yellow and black shapes indicate proteins: Hfq, HilD and CRP-cAMP (El Mouali et al., 2018).

Next to the acetate inducing expression of *S.* Tm invasion genes, two other SCFA, namely propionate and butyrate were found to have the opposite effect (Gantois et al., 2006). The recent findings by Hockenberry et al. suggest that SCFA do not impact the subpopulation of *S.* Tm cells expressing invasion genes by transcriptional downregulation but rather by decreasing the growth rate of T3SS-1 expressing cells (Hockenberry et al., 2021). It is likely that *S.* Tm senses the concentration of SCFA of the mammalian intestinal tract as an input to regulate virulence expression. Also, long chain fatty acids (LCFA) can impact virulence

expression in the intestine. Study by Golubeva et al. suggested that LCFA can bind directly to HilD protein and block its ability to activate transcription of the invasion genes (Golubeva et al., 2016). Authors postulate that concentration of LCFA should decrease along the intestinal tract as these molecules get absorbed in the small intestine. Therefore, in the distal ileum, the LCFA concentration would be low. These studies suggest that both SCFA and LCFA can serve as environmental signals modulating *S.* Tm virulence expression.

### Environmental signals leading to SPI-1 downregulation

To design successful anti-virulence strategies, we need to better understand the cost of virulence traits for the individual cells. So far for *S.* Tm the only described cost of virulence is a two-fold reduction in growth rate in VFs expressing cells (Sturm et al., 2011). It remains unclear whether the growth retardation is the only difficulty which virulent *S.* Tm cells face during infection. Interestingly, it was shown that under stressful environmental conditions *S.* Tm tends to downregulate T3SS-1. This could suggest that virulent cells might be particularly vulnerable to external stress conditions. There are many components of the external environment which can lead to downregulation of the SPI-1 encoded genes. One of such elements is increased temperature. As a mesophile *S.* Tm can survive and replicate in temperature range of 15-45°C (Montville & Matthews, 2001). Often infection caused by the pathogen leads to increased body temperature of the host, potentially affecting virulence expression patterns of the pathogen. Exposure to sub-lethal heat stress of 42°C caused down-regulation of the SPI-1 encoded genes (*invA, invB, invG, invC, prgH* and *prgK*) (Sirsat et al., 2011). Another important compound present within the host are the CAMPs. CAMPs are important components of innate immunity in animals (Hancock & Scott, 2000). They act within minutes after infection and are effective against broad range of pathogens including bacteria and fungi. As positively charged molecules CAMPs interact with the anionic bacterial surface, predominantly binding to the lipid A moiety of lipopolysaccharide. Binding to lipid A subsequently leads to increased permeability of the outer membrane and progression to the periplasm where CAMPs can access inner membrane. The loss of integrity of the inner membrane is thought to have the bactericidal effect (Daugelavicius et al., 2000). In *S.* Tm the PhoP/Q two component system is crucial in resistance to CAMPs (Groisman et al., 2021). Many PhoP/Q regulated genes are associated with remodeling of the bacterial envelope. Moreover, the flagellar and SPI-1 genes were found to be downregulated (Bader et al., 2003). PhoP negatively regulates *hilA* expression by binding to its promoter thus preventing binding of activators HilD, HilC and RtsA (Palmer & Kim, 2019). Another example of stress condition

threatening *S*. Tm survival in the host is presence of bile. Bile, synthesized by hepatocytes in the liver, is a fluid composed of bile salts, cholesterol, various electrolytes and proteins (Hofmann, 1984). In the intestine bile promotes digestion of fats and facilitates absorption of fat-soluble vitamins. However, it also has antibacterial activity, as the bile salts disrupt the membranes, denature proteins and cause DNA damage in bacteria (Begley et al., 2005) (Prieto et al., 2004). The ability of *S*. Tm to grow in the presence of bile can be modulated as bacterium can adapt to by the growth in sublethal concentration of bile salts. This adaptation involves many changes in the gene expression, including upregulation of DNA damage and stress response, but also repression of invasion genes (Hernández et al., 2012). All the mentioned stress conditions can be associated with the membrane damage suggesting that invasion genes regulation is sensitive to the envelope homeostasis. Not only the external conditions, but also disruptions in the folding of outer membrane proteins affect bacterial membrane homeostasis. The β-barrel assembly machinery (BAM) complex is required for correct assembly of the β-barrel proteins. When the system is malfunctioning the unfolded outer membrane proteins accumulate in the cytoplasm inducing outer membrane stress (Walsh et al., 2003). Interestingly, deletion of *bamB* encoding an outer membrane lipoprotein led to downregulation of SPI-1 and flagellar genes (Fardini et al., 2007). Palmer and Slauch showed that *S.* Tm can sense the outer membrane β-barrel protein assembly through the outer membrane lipoprotein sensor RcsF (Palmer & Slauch, 2020). The stimulated RcsF activates the RcsCDB system which is silencing the *flhDC* the master flagellar regulator and *hilD, hilC and rtsA.* Taken together, these examples indicate that virulence regulation in *S*. Tm is sensitive to changes in the membrane homeostasis.

The envelope is the external barrier protecting bacteria from many antimicrobials including antibiotics, bacteriophages, or microbial warfare agents. Maintenance of integrity of this essential barrier is crucial for bacterial survival. State of the membrane is constantly monitored and if the membrane integrity is compromised the envelope stress responses (ESR) are activated (Saha et al., 2021). The Gram-negative bacteria possess several components of ESR, being among others: $\sigma^E$ response, Cpx, Rcs (Guest & Raivio, 2016).

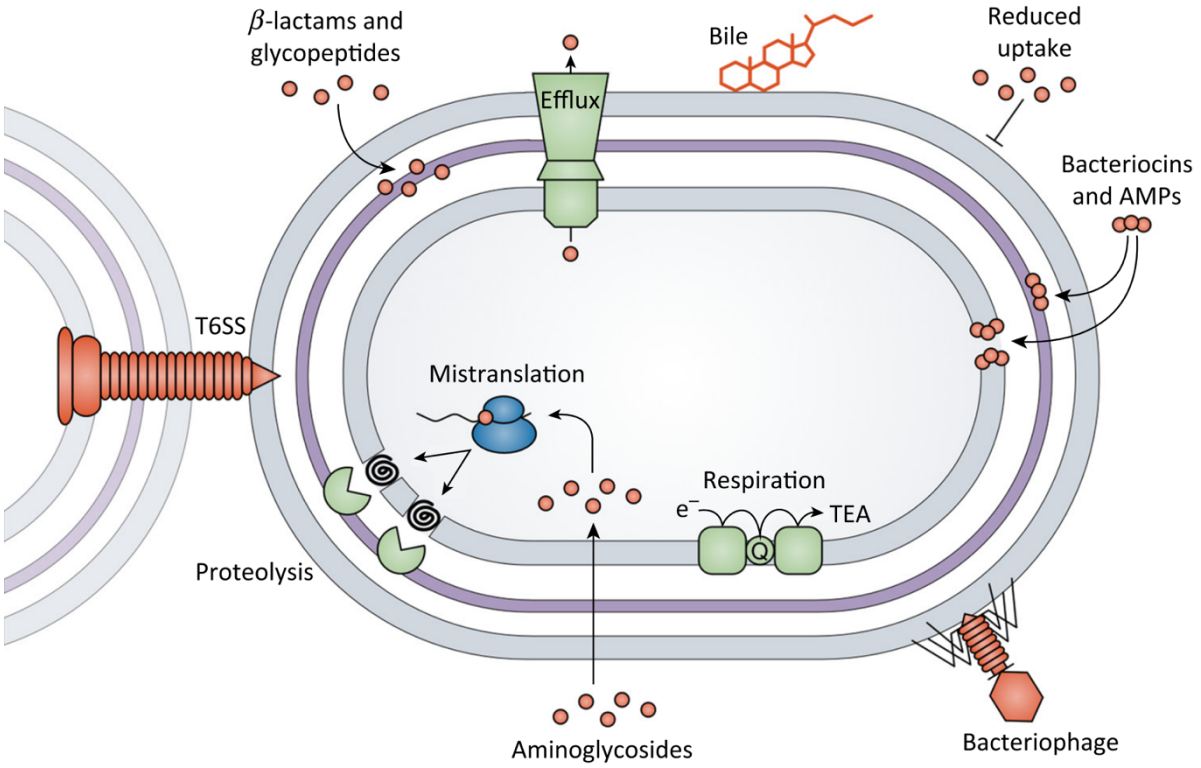


**Fig. 7. Schematic overview of factors targeting bacterial membrane**. Antimicrobial agents that interact with the envelope (shown in red) exert toxicities at the cell wall, through protein misfolding, and by disrupting membranes. Envelope stress responses (ESRs) are activated by these threats and induce a variety of protective mechanisms (shown in green) that act mainly to decrease the intracellular concentration of antimicrobial substances, prevent their uptake or action, and/or alleviate the resulting toxicities (Guest & Raivio, 2016)

The Cpx system is specifically dedicated to disruptions occurring at the inner membrane and it involves a two-component system, where CpxA is a sensor kinase and CpxR is response regulator. The Cpx response is preventing intoxication by misfolded proteins, mostly via upregulation of the chaperone protein DegP (Cosma et al., 1995; Hung et al., 2001). Studies in *E. coli* and other enteric bacteria showed that CpxA senses various of envelope stress conditions and as a result leading to phosphorylation of the CpxR. The phosphorylated CpxR changes the expression of many genes, mostly linked to the inner membrane (Raivio, 2014). Moreover, it is known that the Cpx system is involved in resistance mechanisms against a wide range of antimicrobials including among other aminoglycosides. The Rcs response involves another two-component system consisting of the outer membrane lipoprotein sensor RcsF and the response regulator RcsB. The Rcs system is activated in response to β-lactam antibiotics (Laubacher & Ades, 2008). Another ESR component - $\sigma^E$ response, was shown to be activated in the presence of CAMPs. When CAMPs bind to the outer membrane, they are

thought to induce conformational changes in the outer membrane proteins, and subsequently to induction of the $\sigma^E$ ESR (Mathur et al., 2007). Presence of several systems ensuring adequate response during exposure to membrane targeting stress, further emphasizes importance of the membrane

Taking the high sensitivity of *S.* Tm to the membrane homeostasis, and subsequent downregulation of virulence expression we hypothesized that perhaps cells expressing VFs have decreased membrane integrity. As many VFs are large membrane embedded structures it cannot be excluded that their presence affects membrane integrity. This could be of important consequences for the design of future therapies.

## Aims of the project

Most studies of infectious bacteria focus on how the virulence of a pathogen impacts its host, leaving a gap in our understanding of how expression of virulence affects the pathogen itself. A few studies addressing this question suggest that expression of virulence is not neutral for the pathogen but can impose a fitness cost (Sturm et al., 2011; Vasanthakrishnan et al., 2015). Until now, the only described fitness cost in *S.* Tm is the 2-fold reduction of growth rate in subpopulation expressing HilD regulon (Sturm et al., 2011). HilD is a master regulator of virulence in *S.* Tm known to regulate important virulence functions such as T3SS-1 or flagella (Fàbrega & Vila, 2013; Martínez-Flores et al., 2016). Since most invasion factors controlled by HilD are embedded within bacterial envelope, we hypothesized that the expression of the HilD regulon could render *S.* Tm intrinsically more sensitive to stressors targeting the envelope. To investigate this question, we set a few specific goals.

Firstly, we wanted to compare the outer membrane permeability in *S.* Tm strains. Using N-phenyl-1-naphthylamine (NPN) uptake assay we aimed to determine if the membrane of HilD expressing cells is more permeable in comparison to cells which do not express HilD. Secondly, to further understand if virulence expression can impact *S.* Tm ability to withstand envelope targeting stress, we designed experiment involving treatments disrupting the bacterial outer membrane. By exposing *S.* Tm cells to envelope stress (heat shock, Tris-EDTA) we wanted to determine if HilD expressing cells are more susceptible to the stress exposure in comparison to their phenotypically avirulent counterparts. By testing a library of *S.* Tm strains in which the expression of functions downstream of HilD was genetically tuned, we aimed to dissect which HilD-regulated functions contribute to membrane permeability and sensitivity to envelope targeting stress. Furthermore, we assessed if *S.* Tm can downregulate virulence expression when exposed to sub-lethal stress dose, and subsequently improve its ability to withstand lethal stress exposure. Lastly, we wanted to understand if increased stress sensitivity is costly during *in vivo* infection in a mouse model.

Via these experimental approaches we aimed to deepen the understanding of cost associated with virulence expression in *S.* Tm.

The doctoral thesis is based on a peer-reviewed publication

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SHORT REPORTS

# The expression of virulence genes increases membrane permeability and sensitivity to envelope stress in *Salmonella* Typhimurium

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## Abstract

Virulence gene expression can represent a substantial fitness cost to pathogenic bacteria. In the model entero-pathogen *Salmonella* Typhimurium (*S*.Tm), such cost favors emergence of attenuated variants during infections that harbor mutations in transcriptional activators of virulence genes (e.g., *hilD* and *hilC*). Therefore, understanding the cost of virulence and how it relates to virulence regulation could allow the identification and modulation of ecological factors to drive the evolution of *S*.Tm toward attenuation. In this study, investigations of membrane status and stress resistance demonstrate that the wild-type (WT) expression level of virulence factors embedded in the envelope increases membrane permeability and sensitizes *S*.Tm to membrane stress. This is independent from a previously described growth defect associated with virulence gene expression in *S*.Tm. Pretreating the bacteria with sublethal stress inhibited virulence expression and increased stress resistance. This trade-off between virulence and stress resistance could explain the repression of virulence expression in response to harsh environments in *S*.Tm. Moreover, we show that virulence-associated stress sensitivity is a burden during infection in mice, contributing to the inherent instability of *S*.Tm virulence. As most bacterial pathogens critically rely on deploying virulence factors in their membrane, our findings could have a broad impact toward the development of antivirulence strategies.

## Introduction

Bacteria constantly sense their environment and adapt to changes by modulating gene expression accordingly. In entero-pathogenic bacteria, physiological and environmental stimuli drive the expression of virulence genes and the outcome of the interaction with the host [1–3]. Virulence can maximize the fitness of pathogens via host exploitation [4,5]. On the other hand, virulence factors can be costly to produce, therefore requiring fine-tuned regulation to ensure balanced and timely expression [6]. However, how regulatory pathways have been

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**Abbreviations:** cat, chloramphenicol acetyltrasferase; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; CFU, colony-forming unit; DiOC2(3), 3,3′-diethyloxa-carbocyanine iodide; DiSC3(5), 3,3′-dipropylthiadicarbocyanine iodide; FDR, false discovery rate; HCD, higher-energy collisional dissociation; HS, heat shock; LB, Lysogeny broth; LCN2, Lipocalin-2; MIPS, monoisotopic precursor selection; NPN, N-phenyl-1-naphthylamine; p.i, postinfection; PI, propidium iodide; SDC, sodium deoxycholate; SOPF, specified opportunistic pathogen free; S.Tm, *Salmonella* Typhimurium; T3SS-1, Type 3 secretion system 1; TCEP, Tris(2-carboxyethyl)phosphin; TE, Tris-EDTA; WT, wild-type; GFP, Green Fluorescent Protein; OD, Optical Density; SCV, Salmonella Containing Vacuole.

selected for in order to tune virulence expression based on environmental stimuli remains to be understood. Here, we address this point in the notorious pathogen *Salmonella* Typhimurium (*S*.Tm).

*S*.Tm is a facultative intracellular entero-pathogen able to prosper in the intestinal lumen of a broad range of hosts [7]. The AraC-like transcription factor HilD is at the center of *Salmonella*'s regulatory network of virulence expression. HilD controls about 250 genes constituting the HilD regulon [8–10]. Expression of this regulon depends on various environmental parameters [2]. Upon entry into stationary phase in rich medium, HilD mediates the OFF to ON virulence switch in a subset of cells, which is reflected by the bimodal expression of reporter genes [11,12]. In the gut, the HilD regulon primes this subset of cells to swim toward, attach to and invade enterocytes [7,13,14]. The resulting intestinal innate immune response favors the growth and the transmission of *S*.Tm [5,15], thus improving *S*.Tm fitness at the population level [16].

However, the fitness cost of virulence expression at the single-cell level and the facultative intracellular lifestyle of *S*.Tm both strongly constrain the expression of the HilD regulon. The production of invasion factors controlled by HilD correlates with a 2-fold reduction of the growth rate [12]. Although such a substantial fitness cost could threaten the evolutionary stability of virulence in *S*.Tm [17], the heterogeneous "all or nothing" (ON/OFF) bimodal expression pattern [11] mitigates the cost by allowing only a fraction of *Salmonella* cells to engage in the costly virulence program [17]. Furthermore, independently from the fitness cost, once inside the *Salmonella*-containing vacuole (SCV), two-component systems silence invasion genes like the Type 3 secretion system 1 (T3SS-1), whereas the expression of a specific set of genes is stimulated to ensure the intracellular survival of *S*.Tm [18,19]. For instance, low pH and osmolarity are sensed by EnvZ/OmpR, which triggers T3SS-2 production via SsrA/B [20]. Moreover, PhoP/PhoQ represses invasion genes upon sensing low $Mg^{2+}$, low pH, and cationic antimicrobial peptides [21–23], which further ensures survival within host cells.

Nevertheless, a number of other signals modulate virulence expression in *S*.Tm outside host cells. Availability of carbon sources, amino acids, divalent cations, phosphate, and oxygen tilt the balance toward inhibition or activation of the HilD regulon (reviewed in [2,3]). Envelope stress, e.g., bile and heat, as well as misassembled outer membrane proteins, generally inhibits T3SS-1 production [24–27]. It is not clear why sensing environmental cues unrelated to the intracellular niche such as bile or excessive heat [26,27] should *per se* inhibit the expression of invasion genes. As such, the evolutionary causes of the repression of invasion factors outside of the SCVs remain to be determined. For this, we must understand what conditions the fact that some *S*.Tm cells should express invasion factors or not, how virulence gene expression intertwines with the general physiology of the bacteria, and to what extent this has shaped regulatory networks in *S*.Tm.

Since most invasion factors controlled by HilD are embedded within the envelope (i.e., T3SS-1, SPI-4 T1SS, flagella, and chemotactic receptors), we hypothesized that the expression of the HilD regulon could render *S*.Tm intrinsically more sensitive to envelope stress and that inhibiting the expression of HilD-controlled genes could correspondingly be an integral part of a general envelope stress response specific to *S*.Tm. This hypothesis was addressed by comparing outer membrane permeability, death rate, and HilD activity in response to envelope stresses in populations of *S*.Tm strains in which the expression of regulators and functions downstream of HilD was genetically tuned. This revealed a clear trade-off between membrane robustness and virulence expression in *S*.Tm. We show that this trade-off influences the selection for avirulent mutants within host, which could therefore inspire the design of novel anti-virulence strategies against *Salmonella*.

## Results

### Bimodal expression of the HilD regulon and proteomic analysis on sorted *S*.Tm cells

In this study, we used the chromosomal P*prgH*::*gfp* reporter (in which *gfp* expression is controlled by a copy of the promoter of the T3SS-1 *prg* operon) inserted in the locus *putPA* [11] as a proxy for the expression of the HilD regulon in *S*.Tm SL1344 (further referred to as wild type or WT). More specifically, the P*prgH*::*gfp* reporter is activated by HilA [28], itself tightly controlled by HilD [29], and does not interfere with T3SS-1 expression. The distribution of the *gfp* expression at late exponential phase in Lysogeny broth (LB) was clearly bimodal with approximately two-third of the population in the OFF state and one-third in the ON state (S1 Fig). As previously reported, the ON/OFF ratio results from the production of HilD, whose activity is controlled by the negative regulator HilE at the posttranslational level [12,17,30]. Accordingly, the proportion of ON cells was increased to about half of the population in the Δ*hilE* mutant. Note that the ON/OFF ratio in WT and its Δ*hilE* derivative varied between experiments, but the latter consistently yielded more ON cells than the WT (**Figs 1A, 2D, and 3A**). Deletion of *hilD*, on the other hand, locked the cells in the OFF state (S1 Fig), validating the P*prgH*::*gfp* reporter as a proper proxy for HilD activity.

To further confirm that the P*prgH*::*gfp* reporter is coexpressed with HilD-regulated genes, we compared the proteomic profile of WT *S*.Tm cells sorted according to the bimodal distribution of the green fluorescence signal (S1 Fig, S1 Table). The translation of SPI-1 T3SS-1 components and effectors, the SPI-4 T1SS, flagella, and chemotaxis systems was increased in GFP positive cells (Table 1), which was consistent with previously published transcriptomic data describing the HilD regulon [8–10]. This analysis validated the use of the P*prgH*::*gfp* fusion as reporter for HilD regulon expression at the single-cell level throughout this study.

### The expression of *hilD* increases the permeability of the outer membrane to a lipophilic compound

Several functions controlled by HilD are large protein complexes embedded in the envelope of *S*.Tm (T3SS-1, SPI-4 T1SS, flagella, and chemotactic receptor clusters (S1 Fig, Table 1, S1 Table), potentially affecting envelope integrity. In order to assess permeability of the outer membrane, we used N-phenyl-1-naphthylamine (NPN), a lipophilic dye that is weakly fluorescent in aqueous environments but becomes highly fluorescent in hydrophobic environments such as the inner leaflet of the outer membrane and the inner membrane [31]. In growth conditions triggering expression of the HilD regulon (i.e., late exponential phase in LB) (Fig 1A), the WT and the Δ*hilE* mutant accumulated significantly more NPN than the Δ*hilD* mutant (Fig 1B). In contrast, adding glucose to the media drastically reduced the expression of the HilD regulon [32] (Fig 1A) and NPN uptake (Fig 1B). As a control, polymyxin B, acting as a detergent, increased NPN uptake independently of glucose presence (Fig 1C). We tested a possible effect of *gfp* expression (used to monitor the HilD regulon induction) by constitutively expressing *gfp* from a plasmid (transcriptional fusion P*rpsM*::*gfp* in pM965) (Fig 1A, S2 Fig), which demonstrated that the presence of GFP did not affect NPN uptake or the measurement of the NPN fluorescent signal (Fig 1B).

We then evaluated the relative contribution of T3SS-1, SPI-4 T1SS, and flagella to the increased membrane permeability in *S*.Tm (Fig 1D). The full SPI-1 deletion (including *hilD*) phenocopied the Δ*hilD* mutant ($p = 0.880$), validating our previous observation. However, the deletion of the *iagB-invG* locus in SPI-1 (i.e., removing operons *iag*, *spt*, *sic*, *iac*, *sip*, *sic*, *spa*, and *inv*, but keeping transcriptional regulators *hilD*, *hilC*, *hilA*, and *invR* intact) or SPI-4

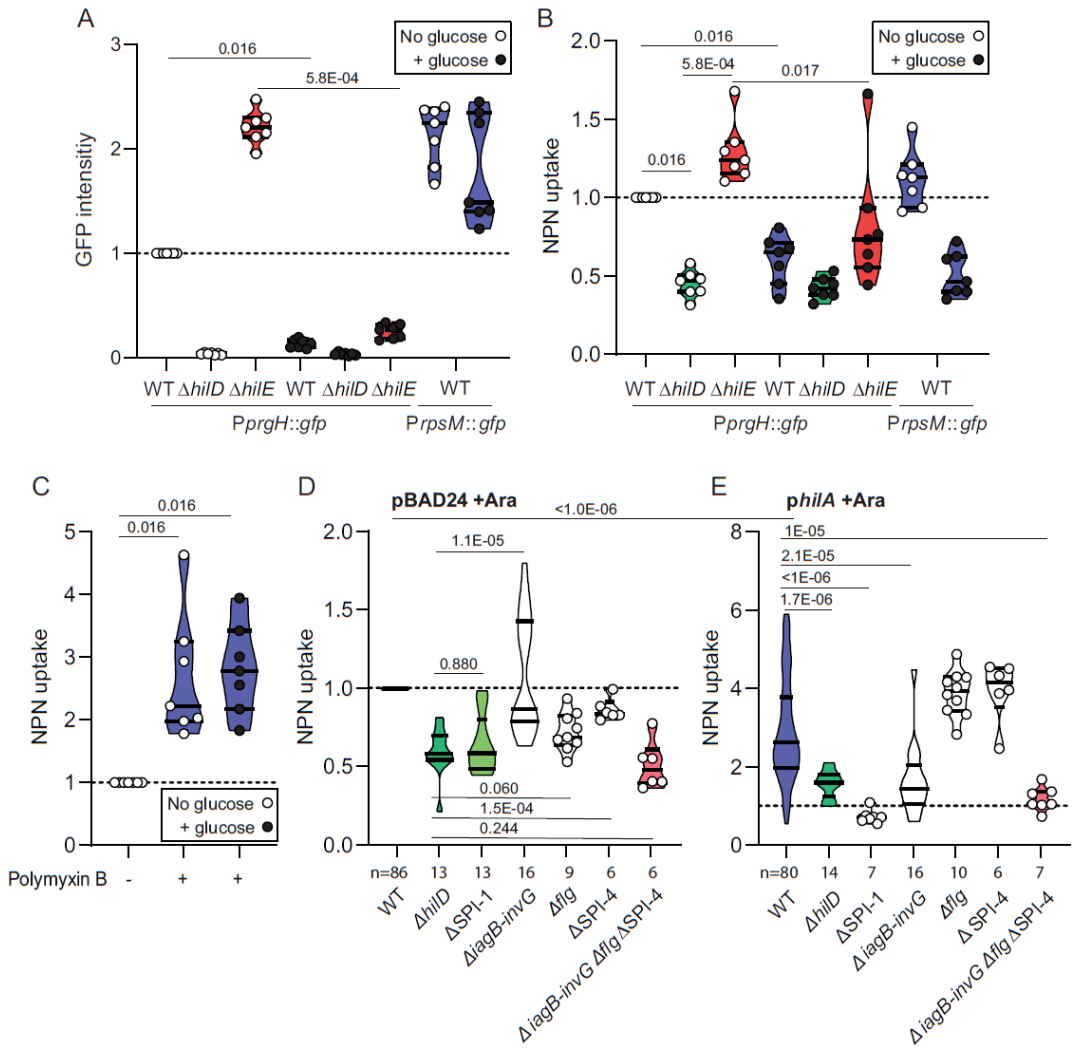


**Fig 1. Expression of the HilD regulon increases the permeability of the outer membrane to NPN.** GFP (from P*prgH::gfp*) (A) and NPN fluorescence (B–E) were measured from cells treated with 10 μM NPN and were divided by the optical density at 600 nm. Values of each repetition are normalized using a parallel experiment on the WT. When indicated, 0.1% of glucose was added to the broth in order to repress virulence expression (+ glucose, black dots). A control strain constitutively expressing GFP from pM965 carrying P*rpsM::gfp* (fluorescence distribution shown in S2 Fig) was used to control for the effect of GFP on the fluorescence readout from NPN uptake. A 2.5-μg/mL Polymyxin B treatment permeabilizing the membrane was used as positive control (C). (D, E) NPN uptake in WT, *ΔhilD*, ΔSPI-1, *ΔiagB-invG*, *Δflg*, ΔSPI-4, and triple mutant *ΔiagB-invG Δflg* ΔSPI-4 carrying either pBAD24 (D) or p*hilA* (E) and treated with 10 μM NPN. Fluorescence values were normalized to the reference WT pBAD24 grown in presence of 1 mM arabinose. Values obtained in the absence of arabinose are shown in S2 Fig. For comparisons against the WT, *p*-values were calculated using the raw data in paired Wilcoxon tests. For comparisons between mutants or conditions, *p*-values were calculated using the normalized dataset in unpaired Mann–Whitney tests. *p*-Values for comparisons discussed in the main text are indicated within the panels with bars marking the compared conditions. S2 Table shows *p*-values for comparisons between groups from panels D and E. Numbers below the x-axis ($n = x$) indicate the number of replicates when nonequal between conditions in a given experiment. $n = 7$ in panels A–C. Source data are provided as a source data file (S1 Data). NPN, N-phenyl-1-naphthylamine; WT, wild-type.

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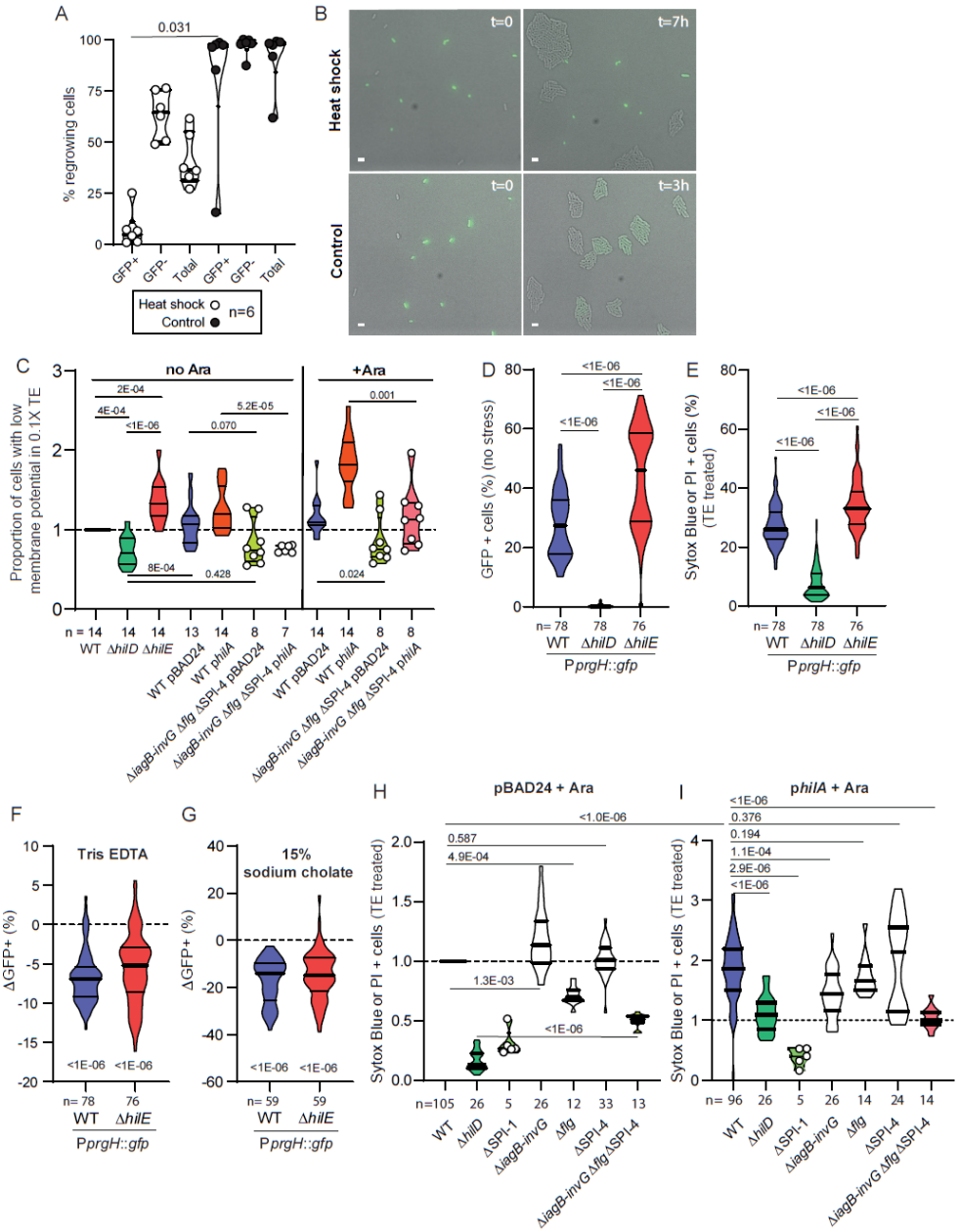


**Fig 2. Expression of the HilD regulon increases sensitivity to membrane stress.** (**A**) Time-lapse microscopy analysis of WT reporter strain (P*prgH*::*gfp*, GFP) after HS (51˚C, 15 minutes) (white dots) and untreated control (black dots). Violin plots represent the fraction of cells able to form microcolonies among cells expressing the HilD regulon (GFP+), not expressing the HilD regulon (GFP–) and the total population. The *p*-value was calculated using a paired Wilcoxon test. (**B**) Representative pictures from time-lapse microscopy experiments. Cells in the upper panel were heat treated. Left picture shows cells at t = 0, and right picture shows cells after 7 hours. Cells in the lower panel are

untreated control. Left picture shows cells at t = 0, and right picture shows cells after 3 hours. Scale bar: 2 μm. (**C**) Cells grown in LB supplemented (+ Ara) or not (no Ara) with 1 mM L-arabinose to induce the overexpression of *hilA* from p*hilA* (derivative of pBAD24) were stained using 30 μm $DiOC_2(3)$ in the presence of 10 mM Tris-1 mM EDTA (0.1X TE) and analyzed by flow cytometry. Unstained WT control and WT cells treated with 100 μm CCCP and 30 μm $DiOC_2(3)$ were used to define the population of cells with low membrane potential in each sample (S4 Fig). The proportion of cells with low membrane potential was then normalized according to values obtained in parallel experiments using the WT. For comparisons against the WT, *p*-values were calculated on the raw data using paired Wilcoxon tests. For comparisons between mutants or conditions, *p*-values were calculated using the normalized dataset and unpaired Mann–Whitney tests. (**D**) Proportion of cells producing GFP (P*prgH::gfp*) from WT, *ΔhilD*, and *ΔhilE* strains, not stained by Sytox blue or PI (i.e., alive) in distilled water, measured by flow cytometry. (**E**) Frequency of cells stained with either Sytox blue or PI (i.e., dead) after treatment with 100 mM Tris-10 mM EDTA (TE treated) from WT, *ΔhilD*, and *ΔhilE* strains measured by flow cytometry. (**F, G**) Reduction of the GFP positive fraction (ΔGFP+) among WT or *ΔhilE* cells alive after TE treatment compared to distilled water control (F) or 15% sodium cholate compared to PBS control (G). Significance of the deviation of the median from 0 (dashed lines) estimated by Wilcoxon signed rank test ($p < 1E-06$). (**H, I**) Normalized frequency of cells stained with either Sytox blue or PI (i.e., dead) after TE treatment from *ΔhilD*, ΔSPI1, *ΔiagB-invG*, *Δflg*, ΔSPI-4, and triple mutant *ΔiagB-invG Δflg* ΔSPI-4 strains harboring pBAD24 (**H**) or p*hilA* (**I**). Data normalized to parallel WT pBAD24 controls. The cultures were supplemented with 1 mM arabinose (+ Ara) to induce *hilA* expression in the strains carrying p*hilA*. For comparisons against the WT, *p*-values were calculated using the raw data in paired Wilcoxon tests. For comparisons between mutants or conditions, *p*-values were calculated using the normalized dataset in unpaired Mann–Whitney tests. *p*-Values for comparisons discussed in the main text are indicated within the panels with bars marking the compared conditions. The S5 Table contains *p*-values for comparisons of data from panels **H** and **I**. Numbers below the x-axis indicate the number of replicates. Source data are provided as a source data file (S1 Data). DiOC2(3), 3,3′-diethyloxa-carbocyanine iodide; HS, heat shock; LB, Lysogeny broth; PI, propidium iodide; TE, Tris-EDTA; WT, wild-type.

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individually had significantly less effect than the Δ*hilD* mutation (Fig 1D). Interestingly, deleting the *flgBCDEFGHIJ* operon (thereafter shortened *flg*) or combining deletions of *iagB-invG*, *flg* and SPI-4 phenocopied the Δ*hilD* mutant ($p > 0.05$) (Fig 1D).

Overproducing HilA, a transcriptional regulator of virulence (including T3SS-1 and SPI-4) controlled by HilD [33], led to a drastic increase in NPN uptake in the WT ($p < 1E-06$). In these experiments, strains carrying the empty vector pBAD24 (Fig 1D) were compared with strains overexpressing *hilA* from the pBAD24 derivative p*hilA*, both growing in the presence of the arabinose inducer (Fig 1E).

Upon *hilA* overexpression, compared to WT, the *iagB-invG* deletion reduced membrane permeability ($p = 2.1E-05$) as well as the triple deletion *iagB-invG flg* SPI-4 (p = 1E-05). However, deleting the *flg* operon (potentially repressed by HilA via inhibition of *flhD* [33]) or SPI-4 did not reduce membrane permeability.

A reproducible pattern of NPN uptake was observed when comparing various mutants in which *hilA* was not overexpressed (pBAD24 carrying strains or in absence of arabinose), confirming that the flagella were the most important contributors to membrane permeability (Fig 1D, S2 Fig). Unexpectedly, the impact of the T3SS-1 was marginal at WT expression level and production of the SPI-4 T1SS remained relatively neutral in all tested conditions. S2 Table gathers statistical analysis results from this dataset.

## The expression of *hilD* reduces resistance to outer membrane disrupting treatments

In general, permeability to NPN is increased by treatments that disrupt the membrane of gram-negative bacteria like polymyxin B (Fig 1C) [34], indolicidin [35], and aminoglycosides acting as divalent cation binding sites on the outer membrane [36]. Increased NPN uptake in *hilD* expressing populations of *S*.Tm (Fig 1B) suggested that the membrane of HilD ON cells could be inherently disrupted, thus making these cells more sensitive to stress targeting the membrane.

Since heat provokes disruption of the outer membrane [37], we first measured survival of *S*. Tm exposed to a mild heat shock (HS, 51˚C, 15 minutes) at the single-cell level with time-lapse fluorescence microscopy. The cells were observed for 16 hours posttreatment. The vast majority of cells expressing the HilD regulon (i.e., GFP positive cells) was unable to resume growth

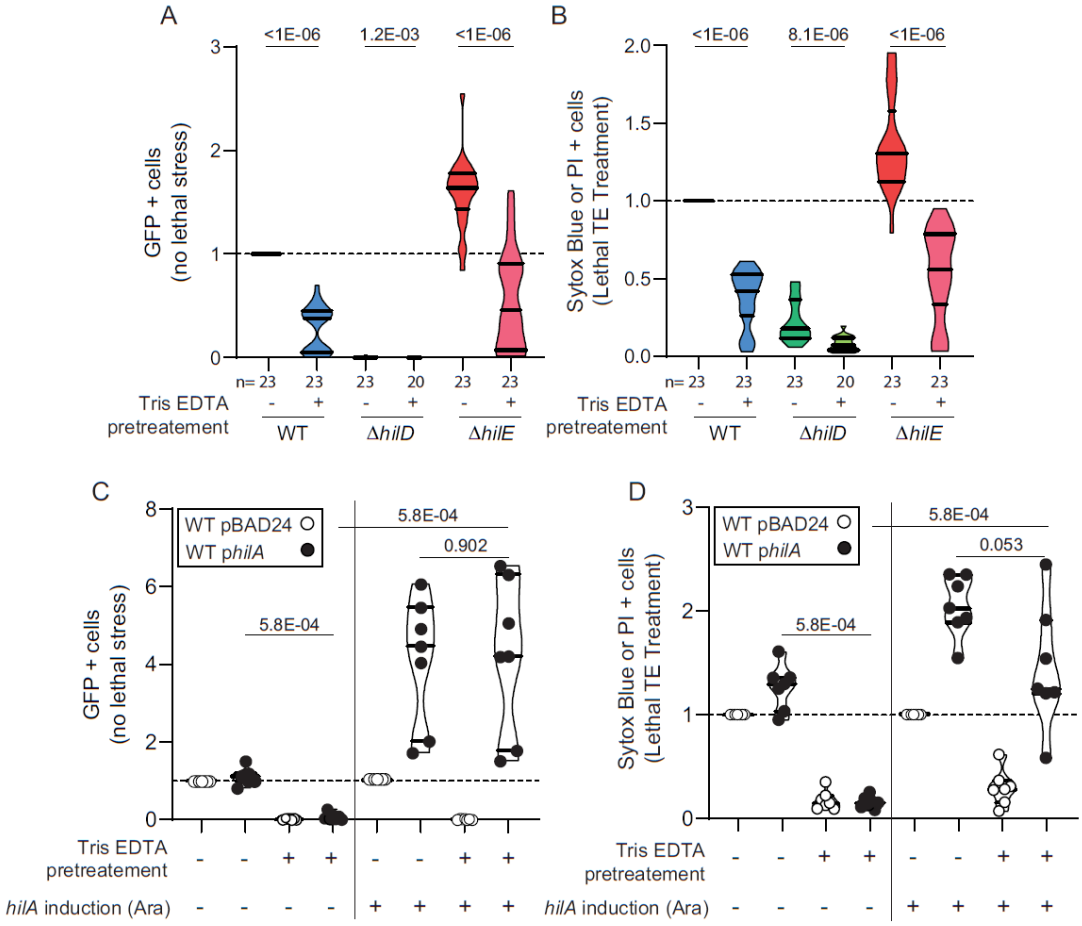


**Fig 3. Sublethal stress inhibits expression of virulence and increases resistance against lethal TE exposure.** Flow cytometry analysis. (A) Proportion of GFP expressing cells (P*prgH*::*gfp*) from WT, *ΔhilD*, and *ΔhilE* strains not stained by Sytox blue or PI (i.e., alive) in absence of lethal treatment (distilled water control). Data normalized to parallel experiments with the WT strain. When indicated, 4 mM Tris–0.4 mM EDTA was added to the broth as sublethal pretreatment. (B) Frequency of cells stained with either Sytox blue or PI (i.e., dead) after treatment with 100 mM Tris-10 mM EDTA (lethal TE treatment) in WT, *ΔhilD*, and *ΔhilE* strains. Data normalized to parallel experiments with the WT strain. When indicated, 4 mM Tris–0.4 mM EDTA was added to the broth. (C) Proportion of GFP expressing cells, unstained by Sytox blue or PI, in distilled water. WT strain carrying the empty vector pBAD24 or the p*hilA* plasmid allowing for overexpression of *hilA*. When indicated, 1 mM arabinose (Ara) and/or 4 mM Tris 0.4 mM EDTA were added to the broth. (D) Frequency of cells stained with either Sytox blue or PI after treatment with 100 mM Tris-10 mM EDTA. When indicated, 1 mM arabinose and/or 4 mM Tris–0.4mM EDTA were supplemented in the medium. (C, D) Data normalized using WT pBAD24 (−Ara or +Ara) as reference, $n = 7$ replicates. For comparisons against the WT, *p*-values were calculated using the raw data in paired Wilcoxon tests. For comparisons between mutants or conditions, *p*-values were calculated using data normalized using corresponding WT or WT pBAD24 as reference in unpaired Mann–Whitney tests. Numbers below the x-axis indicate the number of replicates. Numbers within the graphs are *p*-values for comparisons discussed in the main text and bars below these numbers indicate the compared groups. Source data are provided as a source data file (S1 Data). PI, propidium iodide; TE, Tris-EDTA; WT, wild-type.

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(Fig 2A and 2B HS upper panels, and S3 Fig), while the rest of the population (i.e., GFP negative cells) regrew after a lag period. Untreated cells were able to grow normally regardless of their *hilD* expression state, with ON cells switching OFF and diluting the GFP (Fig 2B control lower panels, S3 Fig).

**Table 1. Membrane-embedded multiprotein complexes coproduced with GFP from the P*prgH*::*gfp* fusion.**

| Functions | Proteins | log2 fold increases ranging from |
|---|---|---|
| Flagellum and chemotaxis system | FliZ, FliB, FliC, FliS, FliL, FljB, MotA, MotB, Trg, CheB, CheM, CheW, CheA, SL1344_3112, SL1344_3189, Aer, and Tcp | 0.63 to 2.20 |
| T3SS-1 components, regulators, and effectors (SPI-1) | AvrA, OrgB, OrgA, PrgK, PrgJ, PrgI, PrgH, HilD, HilA, SptP, IacP, SipA, SipD, SipC, SipB, SicA, SpaS, SpaP, SpaO, InvJ, InvC, InvB, InvA, InvE, InvG, InvF, and InvH | 1.60 to 5.47 |
| SPI-4 T1SS | SiiA, SiiB, SiiC, SiiD, SiiE, and SiiF | 2.29 to 6.22 |

*S*.Tm cells expressing *gfp* controlled by P*prgH* produce significantly more flagella, T3SS-1, and SPI-4 T1SS proteins. These membrane-embedded multiprotein complexes are coproduced with additional proteins listed in S1 Table constituting the HilD regulon.

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To determine if membrane permeability correlated with higher sensitivity to stress in the ON cells, we analyzed HS sensitivity of the triple Δ*iagB-invG* Δ*flg* ΔSPI-4 mutant, which has already proved less permeable to NPN (Fig 1). This mutant produced an amount of *hilD* expressing cells comparable to the WT (S3 Fig), with similarly reduced growth rate compared to OFF cells (S3 Fig). However, a higher proportion of these ON cells was able to resume growth after HS than in WT or the Δ*hilE* mutant (S3 Fig), suggesting that membrane-localized virulence factors increase both membrane permeability and stress sensitivity.

We then measured membrane potential in cells exposed to 10 mM Tris-1 mM EDTA (0.1X Tris-EDTA [TE]), which destabilizes the lipopolysaccharide of gram-negative bacteria [38] and allows entry of the dye 3,3′-diethyloxa-carbocyanine iodide ($DiOC_2(3)$) (Fig 2C) [39]. In the presence of 0.1X TE, membrane potential leads to accumulation of $DiOC_2(3)$ to the point of dye aggregation shifting its fluorescence from green to red. Here, we used flow cytometry because it measures fluorescence in a high number of cells and allows testing multiple conditions in parallel. We followed the gating strategy described in S4 Fig to estimate the proportion of cells with low membrane potential (green only) among the population of stained cells (red and green) in WT and mutant strains. Exposure to the proton ionophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as control condition in which the membrane potential is abolished and most $DiOC_2(3)$ stained cells remained green (depolarized membrane). Unstained cells and cells exposed to CCCP and $DiOC_2(3)$ served as references (S4 Fig). Representative images are provided in S4 Fig.

Again, a pattern consistent with NPN uptake emerged from these experiments. The WT strain and the Δ*hilE* mutant showed significantly higher proportion of cells with low membrane potential compared to the Δ*hilD* mutant. Overexpression of *hilA* in the presence of arabinose increased the proportion of cells with low membrane potential. This was rescued in the Δ*iagB-invG* Δ*flg* ΔSPI-4 triple mutant (Fig 2C). In the context of endogenous *hilA* expression, the triple mutant Δ*iagB-invG* Δ*flg* ΔSPI-4 phenocopied the Δ*hilD* mutant ($p = 0.428$).

Membrane potential in absence of stress was measured in control experiments using 3,3′-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$) (S5 Fig), a red fluorescent hydrophobic probe accumulates in the polarized membrane of cells [40]. This assay was compatible with the readout for expression of the HilD regulon with the reporter P*prgH*::*gfp*. We observed no difference in $DiSC_3(5)$ staining in *hilD* ON (GFP+) versus OFF cells (GFP−) (S5 Fig), suggesting that the membrane of *hilD* expressing cells were not inherently depolarized. Reduction of membrane potential observed with $DiOC_2(3)$ was therefore due to exposure to 10 mM Tris-1 mM EDTA

further destabilizing the outer-membrane, especially when *S*.Tm expressed the HilD regulon (Fig 2C).

This observation led us to evaluate the susceptibility of the ON cells to a more severe 100 mM Tris-10 mM EDTA treatment (TE treatment). We used two complementary approaches, flow cytometry and microscopy, to quantify the proportion of dead cells detectable after TE treatment and the fraction of cells expressing the HilD regulon (GFP+ cells) among the survivors. We used two dyes to stain the dead cells: propidium iodide (PI) or Sytox blue. Fig 2D shows the proportion of GFP+ cells exposed to distilled water used as solvent for TE. After TE treatment, we observed a clear increase in the proportion of dead cells in WT and the Δ*hilE* mutant compared to the Δ*hilD* mutant with both dyes (Fig 2E). Although Sytox blue had the tendency to stain slightly more cells than PI (S6 Fig), we judged the overlap sufficient to pool both staining results in every dataset. The proportion of ON cells decreased in the surviving populations compared to control (Fig 2F). The timing of the experiment (30' of treatment before cytometry analysis) was too short to allow ON cells to switch OFF and to dilute the GFP by cell division. The constitutive expression of *gfp* from the promoter P*rpsM* did not alter the overall pattern of stress sensitivity when comparing WT, *ΔhilD*, and *ΔhilE* strains (S6 Fig). For unclear reasons, WT and *ΔhilD* with P*rpsM*::*gfp* were slightly less sensitive than their P*prgH*::*gfp* counterparts. This nevertheless suggested that expressing *gfp* was not increasing stress sensitivity *per se*. We also ruled out the contribution of nonfluorescent and unstained debris formed during treatment by quantifying the fraction of nonfluorescent events from stressed cells constitutively expressing *gfp* (S6 Fig). Moreover, a treatment with 15% sodium cholate, a natural detergent present in bile, was even more potent at reducing the proportion of ON cells in WT (−14% versus −6.8% with TE) and the Δ*hilE* mutant (−14.7% versus −5.2% with TE) (Fig 2G). The effect of lethal TE treatment was further confirmed by live monitoring of cells in a microfluidic device (S1 Movie). In fact, mainly the ON cells from the WT reporter strain exposed to TE died and became stained by PI (red) concomitantly with losing their GFP, while most OFF cells remained apparently intact (quantification in S7 Fig). Imaging of PI and Sytox blue staining upon TE treatment is presented in S6 Fig.

Additional microscopic analysis of WT reporter bacteria after TE treatment confirmed that, although ca. 70% of the cells were able to resume growth (S8 Fig), the fraction of GFP + cells among the regrowing cells was significantly reduced (17% less GFP+ cells compared to untreated control (S8 Fig)). This indicated that the TE treatment killed a significant amount of bacteria, with a higher probability for the ON cells to die. Flow cytometry and microscopy showed comparable results with 30% of WT cells stained by PI or Sytox blue (Fig 2E) or not regrowing on the agar pad after TE treatment (S8 Fig). Based on this overall death rate and parameters extracted from cytometry experiments (S3 Table), we estimated a 44% death rate for the ON cells, about 2.3 times higher than the death rate of the OFF cells (19%) from WT *S*.Tm (Formula: Death rate = 100 −((% final × % total survivors)/% initial); median values, S3 Table). These values were comparable in the Δ*hilE* mutant (41% and 28% respective death rate for ON and OFF cells). However, the death rate of the locked OFF Δ*hilD* mutant (6.2%) was strikingly lower than for WT or Δ*hilE* mutant OFF cells. This could be attributable to the method used to discriminate between the ON and OFF cells from WT and Δ*hilE* mutant strains based on a fluorescent intensity cutoff when using flow cytometry or microscopy. Meaning that, below this detection cutoff, cells in which the HilD regulon is not fully repressed could bias the overall death rate of the so-called "OFF" subpopulations in WT and Δ*hilE*. Counting colony-forming units (CFUs) posttreatment confirmed the overall death rate being higher in WT and the Δ*hilE* mutant compared to the Δ*hilD* mutant (S4 Table). These data also suggested that Sytox blue or PI staining and counting the regrowing cells under the microscope could underestimate the fraction of cells affected by TE treatment and potentially lysing

(S4 Table). Alternatively, plating might be an extra stress that kills cells after TE treatment which would otherwise form microcolonies under the microscope or would not be stained by PI or Sytox blue.

As observed for NPN uptake, the deletion of *iagB-invG* or SPI-4 alone did not change sensitivity to TE. However, the individual deletion of the *flg* operon significantly increased resistance to TE compared to WT ($p = 4.9E-04$) (Fig 2H).

Although the cumulative deletions of *iagB-invG*, *flg* and SPI-4 increased resistance to TE treatment, they did not fully phenocopy the resistance of the Δ*hilD* mutant (Fig 2H) ($p < 1E-06$). This could be due to the higher sensitivity of this assay compared to NPN uptake measurements in batch cultures. It also suggests that other functions controlled by HilD could play a role in increasing stress sensitivity in the ON cells.

The overexpression of *hilA* (p*hilA* + arabinose) drastically enhanced sensitivity to TE ($p < 1E-06$) (Fig 2I). Under these conditions, the full SPI-1 deletion ($p = 2.9E-06$) and, to a lesser extent, the deletion of *hilD* alone ($p < 1E-06$), of *iagB-invG* ($p = 1.1E-04$), and of *iagB-invG flg* SPI-4 ($p < 1E-06$) restored some resistance. Individual deletions of *flg* or SPI-4 had no effect ($p > 0.05$). Controls in the absence of arabinose reproduce the pattern observed for strains carrying pBAD24 in the presence of arabinose (S9 Fig). S5 Table gathers statistical analysis results from this dataset.

## Exposure to sublethal Tris–EDTA concentration inhibits expression of virulence and increases resistance to lethal stress

Given that the expression of the HilD regulon sensitizes *S*.Tm to heat, sodium cholate and TE exposure, and previous observations that bile or HS inhibit the expression of T3SS-1 [24–27], we hypothesized that exposure to sublethal stress might result in shutting down the HilD regulon, which should protect *S*.Tm against lethal stress.

To address this, we grew the bacteria in LB medium containing TE at sublethal concentration (4 mM Tris–0.4 mM EDTA, 0.04X TE) before exposure to a lethal dose (100 mM Tris-10 mM EDTA). As expected, the proportion of ON cells was strikingly reduced when *S*.Tm grew in the presence of TE at sublethal concentration (Fig 3A), and survival was increased when the bacteria were then exposed to lethal dose (Fig 3B). Overexpression of *hilA* restored P*prgH*::*gfp* expression (Fig 3C, S10 Fig) and, with it, the high death rate in sublethal TE pretreated cells exposed to lethal TE concentration (Fig 3D). This suggested that the expression of genes downstream of *hilA* increased stress sensitivity even if a protective stress response is triggered by sublethal stress. Shutting down the expression of the HilD regulon upon sublethal stress increases the chance of surviving harsher environments that would otherwise be lethal if cells would remain ON.

## HilD-mediated stress sensitivity is costly during symptomatic infections

In contrast to a Δ*hilD* mutant, the *iagB-invG flg* SPI-4 triple deletion restored stress resistance but did not prevent the growth defect associated with HilD expression in the ON cells (S3 Fig). This allowed us to determine whether HilD-mediated stress sensitivity was in itself a significant burden during infection in mice (Fig 4, S12 **Fig**). For this, we compared the outcome of competitions between the WT (yielding ON cells displaying both slow growth and stress sensitivity) and the Δ*hilD* mutant (not yielding ON cells) (competition 1), with competitions between the triple mutant (yielding ON cells displaying slow growth) and the Δ*hilD* triple mutant (not yielding ON cells) (competition 2). To ensure comparable conditions during infections, a fully virulent helper strain (untagged WT) was added to the tagged competitors (Fig 4A). The role of the helper strain was to trigger inflammation that would otherwise not be

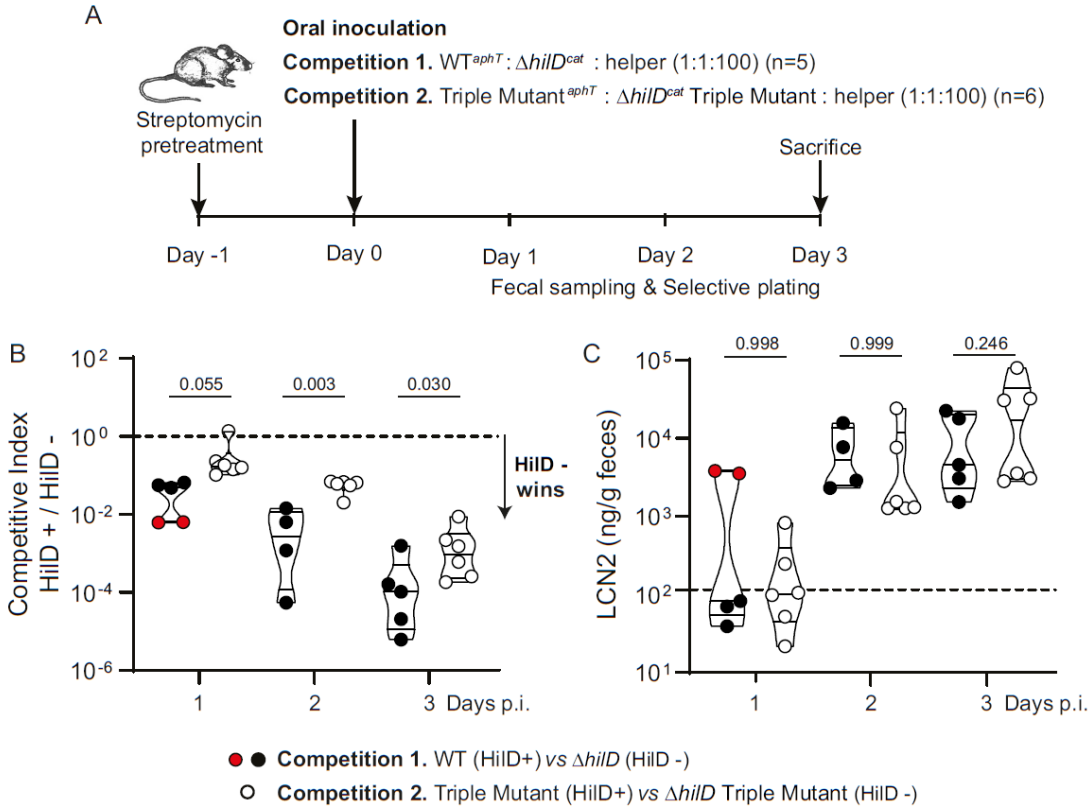


**Fig 4. Virulence-associated stress sensitivity is costly during infection.** (A) Schematic diagram of the experimental infection setup. Two competitions were performed in the presence of a virulent helper strain triggering inflammation in C57BL/6J mice pretreated with streptomycin. Competition 1 featured *S*.Tm WT (yielding ON cells displaying both slow growth and stress sensitivity) and the Δ*hilD* mutant (not yielding ON cells). Competition 2 featured the Δ*iagB-invG* Δ*flg* ΔSPI-4 triple mutant (yielding ON cells displaying slow growth) and the Δ*hilD* triple mutant (not yielding ON cells). (B) Competitive indices calculated from the relative proportions of each competitor in fecal samples. Dots correspond to individual mice at a given time point. Filled dots (black or red depending on the inflammation at day 1 p.i.): competition 1 (WT versus Δ*hilD*); empty dots: competition 2 (Triple mutant versus Δ*hilD* triple mutant). The dashed line represents a 1:1 ratio between HilD+ and HilD− competitors. Values below this line indicate that the HilD− strain outgrew the HilD+ strain. (C) Intestinal inflammation estimated by measuring LCN2 concentration in the feces. Concentrations above $10^2$ ng/g are usually detected in feces from inflamed gut (dashed line). Red dots indicate mice particularly inflamed during competition 1 at day 1 p.i. Two independent inocula per condition. The *p*-values indicated within graphs were generated with one-way ANOVA and corrected by a Sidak multiple comparisons test; bars below *p*-values mark the compared groups. Source data are provided as a source data file (S1 Data). LCN2, Lipocalin 2; *S*.Tm, *Salmonella* Typhimurium; WT, wild-type.

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triggered by strains harboring deletions inactivating T3SS-1 and motility. The helper was added at a 100-fold excess in the inocula to prevent the Δ*hilD* mutants from outgrowing it.

The triple mutant was outgrown by its Δ*hilD* derivative, but at a significantly slower pace than the single Δ*hilD* mutant outgrowing the WT (Fig 4B, S11 Fig). This suggested that, in addition to the growth defect, increased stress sensitivity counts as a substantial burden in WT *S*.Tm cells expressing virulence *in vivo*. Differences between competitive indexes were significant at day 2 and 3 post-infection (p.i.), correlating with the onset of inflammation quantified by measuring the amount of Lipocalin 2 (LCN2) in the feces (Fig 4C) [41]. Along those lines,

the Δ*hilD* mutant outgrew the WT faster at day 1 p.i. in 2 mice particularly inflamed at this time point (marked with red dots in Fig 4B and 4C).

To further address the role of inflammation, we suppressed it by performing competitions between HilD+ versus HilD− strains using an avirulent genetic background unable to produce functional T3SS-1 (Δ*invG*) and T3SS-2 (Δ*ssaV*). Because deleting *iagB-invG* (Fig 2) or *invG* alone (S12 Fig) was not sufficient to reduce stress sensitivity in the WT, and because the T3SS-2 is mainly produced in the SCV, we were able to use Δ*invG* Δ*ssaV* avirulent mutant backgrounds without interfering significantly with stress resistance (experimental setting S12 Fig). In the absence of inflammation, the fitness disadvantage of HilD+ strains relative to Δ*hilD* mutants was not affected by the ability to express or not the full HilD regulon (competitions 3 and 4, S12 Fig). The pace at which HilD− outgrew HilD+ strains was rather slow compared to experiments performed with virulent strains (Fig 4). This may be because the inflammation kills part of the *Salmonella* population [42], therefore accelerating outgrowth of HilD− strains during regrowth. The whole population size of *Salmonella* was decreasing by day 3 p.i. because of competitive exclusion by the regrowing intestinal flora in the absence of inflammation [5]. Competitions 3 and 4 show that only the growth defect influences the outcome of competitions in the absence of inflammation when stress is mild and, with it, the burden of expressing the HilD regulon. Inflammation instead negatively impacts the ON cells weakened by the expression of virulence factors embedded in the membrane. These results demonstrate that stress upon inflammation could contribute to select for avirulent *S*.Tm mutants that naturally emerge during infection [17].

## Discussion

In *S*.Tm, the cost of virulence gene expression drives within-host emergence of attenuated mutants harboring loss-of-function mutations in positive transcriptional regulators of virulence (e.g., *hilD* and *hilC* [17,43]). Such attenuated mutants consistently emerge during chronic infections in mice and loss-of-function mutations in *hilD* were detected in a large collection of sequenced natural isolates at a frequency suggesting positive selection [44]. The genetic instability of virulence in *S*.Tm could be exploited to fight against this pathogen, which is becoming increasingly resistant to antibiotics [45]. However, the tight regulation of bimodal expression of virulence impairs fixation of attenuated mutants during within-host growth, ensuring transmission of the virulent genotype [17]. Understanding the cost of virulence, and how it relates to expression regulation, could allow the identification and the modulation of ecological factors in order to drive the evolution of *S*.Tm toward attenuation.

Until now, the only identified cost of virulence in *S*.Tm was a 2-fold reduction of the growth rate in cells expressing the HilD regulon [12]. The molecular mechanism underlying this growth defect is still unclear. Here, we discovered that the expression of *hilD* increased membrane permeability to the hydrophobic compound NPN (Fig 1) and sensitized *S*.Tm to stresses that disrupt the envelope (i.e., short exposure to mild heat, TE, and sodium cholate (Fig 2)). To identify the functions involved, we compared mutants lacking different operons upregulated by HilD coding for the T3SS-1, the flagella, and the SPI-4 T1SS. At WT expression level, the deletion of several T3SS-1 components (*iagB* to *invG*) did not significantly change membrane permeability or stress resistance (**Figs** 1D **and** 2H). Overexpressing *hilA*, which directly controls T3SS-1 gene expression, increased membrane permeability and sensitivity to stress. In this case, the deletion of *iagB-invG* did reduce membrane permeability to NPN and stress sensitivity (**Figs** 1E **and** 2I). This might relate to the fact that mislocalized T3SS components, like overexpressed secretin, can disrupt the membrane, as reported in *Yesinia enterocolitica* and *Escherichia coli* mutants that lack functional phage-shock proteins [46,47], as well as in

*Pseudomonas aeruginosa* [48]. However, we found that the flagella played a significant role, increasing stress sensitivity in the WT, in accordance with the observation that producing flagella increases the intrinsic death rate in *E. coli* MG1655, a strain that does not possess a *bona fide* T3SS [49]. Cumulating deletions showed that multiple functions controlled by HilD act synergistically to increase sensitivity to membrane stress.

Surprisingly, the cumulated deletions of most of the T3SS-1 component coding genes (*iagB-invG*), the *flg* operon and SPI-4, while increasing stress resistance, did not prevent the reduction in growth rate in the *hilD* expressing cells (**S3B Fig**). From this, we conclude that stress sensitivity and growth defect are actually two independent costs of virulence, the second most likely related to the production of T3SS-1 effectors (SopE, SopE2, SopB, SipA, SptP, SopA, SpvB, and SpvC), which, when removed in addition to the T3SS-1 translocon (*SipB*, *SipC*, and *SipD*), partially restore the growth rate of *S*.Tm [12]. Our *in vivo* competition results (**Fig 4**) also highlight that this independent cost of stress sensitivity is physiologically relevant, as it sensitizes cells expressing the HilD virulence program to the harsh environment in the inflamed gut [42].

The relative contribution of envelope destabilization by multiprotein complexes (T3SS-1 and flagella) and of the energetic burden of secretion and motility to the fitness cost of virulence is difficult to assess as these processes inevitably intertwine. Nevertheless, the comparative proteomic analysis of untreated ON versus OFF cells did not reveal any significant extracytoplasmic stress response in the ON cells, such as the induction of the RpoE or Cpx regulons (**S1 Table**) [50]. Therefore, the bacteria expressing the HilD regulon did not seem more intrinsically stressed than the OFF cells. As previously shown in *Pseudomonas putida* expressing the flagella [51], an energetic cost of virulence can impair the ability to cope with certain stresses. However, membrane potential was comparable between *hilD* ON and OFF *S*.Tm cells.

The repression of the HilD regulon by sublethal exposure to TE was in accordance with known inhibition of T3SS-1 production in *S*.Tm exposed to envelope stress (e.g., heat, bile, or cationic antimicrobial peptides [25–27]) and deprivation of divalent cations sensed by the two-component system PhoPQ [21]. The artificial overexpression of *hilA* showed that decoupling stress response from inhibition of virulence expression was detrimental to *S*.Tm in harsh environment. To the best of our knowledge, this is the first dataset demonstrating a link between regulation of virulence expression and stress response in *S*.Tm driven by the fitness cost of virulence. We propose that such trade-off between virulence and membrane robustness in *S*.Tm shaped the regulation of virulence expression by integrating it into the general stress response. Moreover, since many virulence determinants are typically deployed at the level of the cell envelope, this trade-off and regulatory integration might constitute a more general paradigm relevant for other bacterial pathogens.

To conclude, we demonstrate that the cost of virulence in *S*.Tm is pleiotropic but strongly envelope-related and that such virulence costs can be mitigated by down-regulation concomitant to stress response. Our work reveals that impaired membrane robustness constitutes in itself a significant selective force that can promote the rise of more resistant avirulent mutants within infected hosts (**Fig 4**) [17,43]. This occurs despite the fact that functions less expressed in these mutants, such as flagella and chemotaxis systems, favor growth in the inflamed gut [52]. Therefore, biological stress acting more specifically on the *hilD* expressing cells with impaired membrane robustness could be identified and harnessed for the design of novel antivirulence strategies. Many pathogens rely on similar costly features to ensure pathogenesis (e.g., entero-pathogenic *E. coli*, and *P. aeruginosa*). Hence, our discovery could have broad impact for the development of strategies aiming at fighting pathogens by interfering with the evolutionary stability of virulence.

## Materials and methods

### Strains and media

All the strains and plasmids used in this study are listed in S6 Table. *Salmonella enterica* serovar Typhimurium SB300 (SL1344) [53] and derivatives were cultivated at 37˚C using LB liquid or solid media (Difco United Kingdom). Antibiotic selection was performed with 100 μg/ml ampicillin (Sigma), 6 μg/ml chloramphenicol (Sigma Saint-Louis, MO), and 50 μg/ml kanamycin (Sigma) when needed. Subcultures in late exponential phase (induction of the HilD regulon) were prepared by diluting overnight cultures 1/100 in 2 mL LB and 4-hour incubation at 37˚C with shaking in absence of antibiotic. When stated, media were supplemented with 0.1% w/v D-glucose (Sigma) to inhibit the expression of the HilD regulon. For induction of *hilA* under the inducible P*ara* promoter in p*hilA* [54], a plasmid deriving from pBAD24 [55], media were supplemented with 1 mM L-arabinose (Sigma). Constitutive *gfp* expression was obtained from P*rpsM*::*gfp* cloned in pM965 [13]. Sublethal pretreatment of cells was performed using a mixture of 4 mM Tris (Sigma) and 0.4 mM EDTA (Sigma) added to the subculture broth. Spent LB medium used to trigger expression of the HilD regulon in microfluidic settings was prepared by sterile filtration of the supernatant from centrifuged late exponential phase WT cultures ($OD_{600}$ = 0.8).

### Mutant constructions

*Salmonella* mutants used in this study were constructed by homologous recombination using the λ-Red gene replacement system as described in [56]. To select for recombinants, the chloramphenicol acetyltrasferase (*cat*) gene was amplified from the pKD3 template plasmid using primers containing 40-bp region homologous to flanking regions of the target gene in the chromosome of *Salmonella*. The PCR product was transformed by electroporation into the recipient strain harboring pKD46 helper plasmid encoding λ phage *red*, *gam*, and *exo* genes controlled by an arabinose inducible promoter. Recombinant bacteria were selected on LB plates containing chloramphenicol. Following recombination, the chloramphenicol resistance cassette was removed using the flippase encoded on the pCP20 helper plasmid. Correct gene replacement and resistance cassette deletions were confirmed by PCR. Kanamycin resistance was obtained by integrating the *aphT* resistance cassette together with a neutral barcode (WITS13) in *malX* near *ygdA* as described in [57]. Bacteriophage P22 HT/int-mediated transduction was used to transfer mutations into the desired genetic background [58]. All primers used in this study are listed in S7 Table.

### Stress resistance analysis by flow cytometry

A total of 10 μl of late exponential phase subcultures were diluted in 90 μl of stress media or vehicle combined with dead staining. Stress media contained either 15% (w/v) sodium cholate (Sigma) in PBS or 100 mM Tris (Sigma) −10 mM EDTA (Sigma) in distilled water. Dead staining was either PI (Invitrogen Waltham, MA) at a final concentration of 30 μg/ml or Sytox blue (Invitrogen) at a final concentration of 10 μM. The mixtures were incubated in 96-well plates for 30 minutes at 37˚C. After incubation, cells were diluted 1/10 in filtered PBS and analyzed by flow cytometry using LSR Fortessa (BD Biosciences Franklin Lakes, NJ) operated with the FACS Diva software (BD Biosciences). Data acquisition was performed until 50,000 events of unstained cells were recorded using excitation with 561-nm laser and band-pass filter 610/20 nm for PI and excitation with 405 nm laser and band-pass filter 450/50 nm for Sytox Blue. The GFP signal was recorded using excitation with 488 nm laser and band-pass filter 512/25 nm and 505LP. Data were processed using FlowJo V10 software (FlowJo Ashland, ORE). Events

were first gated for unstained cells (PI or Sytox blue negative) and further for GFP positive cells when reporter strains were used.

## Membrane potential analysis by flow cytometry and fluorescence microscopy

Late exponential phase subcultures (1 ml) were centrifuged 3 minutes at 9,500 rpm. Supernatants were discarded, and cell pellets were resuspended in 1 ml 10 mM Tris-1 mM EDTA before staining by $DiOC_2(3)$ or PBS before staining by $DiSC_3(5)$. Staining were carried out in 96-well plates in a total volume of 50 μL. In the control wells, CCCP was added at a final concentration of 100 μM in DMSO (Sigma). Cells were incubated for 30 minutes at 37˚C. After incubation, $DiOC_2(3)$ (Invitrogen) was added to a final concentration of 30 μM or $DiSC_3(5)$ (Invitrogen) was added to the final concentration of 2 μM. Both dyes were prepared in DMSO. The cells were incubated for 5 minutes at room temperature. Moreover, 20 μL were transferred into 180 μL of 10 mM Tris-1 mM EDTA (for $DiOC_2(3)$ staining) or PBS (for $DiSC_3(5)$ staining) and analyzed by flow cytometry using LSR Fortessa (BD Biosciences) operated by FACS Diva software (BD Biosciences). A total of 50,000 events were recorded using excitation with 488-nm laser and band-pass 542/27 for green fluorescence and excitation with 488-nm laser and band-pass 685/35 for red fluorescence with $DiOC_2(3)$ staining or 640-nm laser and band-pass 670/14 for red fluorescence with $DiSC_3(5)$. Data were processed using FlowJo V10 software (FlowJo).

For fluorescence microscopy imaging, 4 μl of cells were added onto 1% agarose pads directly after the incubation step with $DiOC_2(3)$ or $DiSC_3(5)$. Imaging was performed using an inverted Nikon Ti Eclipse epifluorescence microscope (Nikon, Japan) equipped with a Plan Apo 100× oil immersion objective and a pco.edge 4.2 sCMOS camera. Fluorescence was excited by a SPECTRA X light engine and filtered with a Chroma 84100bs polychroic filter set. FITC setting with cyan laser line (50%, 100 ms exposure), 470/24 nm excitation filter, and 515/30 nm emission filter was used for green fluorescence or with 705/72 nm emission filter for red fluorescence with $DiOC_2(3)$ staining. Cy5 setting with red laser line (50%, 100-ms exposure), 640/30 nm excitation filter and 705/72 nm emission filter was used for red fluorescence with $DiSC_3(5)$.

## Proteomics analysis

**Culture and sorting.** WT SL1344 cells harboring the GFP reporter for HilD regulon expression (fusion P*prgH*::*gfp*) in late exponential phase were collected by centrifugation, resuspended, and diluted in PBS. Samples were sorted according to green fluorescence intensity using a FACS Aria III (BD Biosciences) with scatter and fluorescence channels for GFP, excitation 488 nm, with 514/30nm band-pass with the precision on yield. The flow cytometer was calibrated with CST beads. During the sorting process, the sample and the collection tubes were kept at 4˚C or on ice.

After sorting, cells were collected by centrifugation at 12,000 rpm for 10 minutes at 4˚C. After each centrifugation step, cells resuspended in the remaining supernatant were transferred to smaller tubes: first 5 ml and then 1.5-ml protein low binding microcentrifuge tubes (Eppendorf Germany). After the final centrifugation step, pellets were stored at −80˚C until further processing.

**Sample preparation.** Frozen sorted cell pellets were lysed in 20 μL of lysis buffer (1% sodium deoxycholate (SDC), 10 mM Tris(2-carboxyethyl)phosphin (TCEP), 100 mM Tris, pH = 8.5) using 20 cycles of sonication (30 seconds on, 30 seconds off per cycle) on a Bioruptor (Diagenode SA, Belgium). Following sonication, proteins in the bacterial lysate were heated at

95˚C for 10 minutes. Proteins were then alkylated using 15 mM chloroacetamide at 37˚C for 30 minutes and further digested using sequencing-grade modified trypsin (1/25, w/w, trypsin/protein; Promega, USA) at 37˚C overnight. After digestion, the samples were acidified with TFA to a final concentration of 1%. Peptides desalting was performed using iST cartridges (PreOmics, Germany) following the manufacturer's instructions. After drying the samples under vacuum, peptides were stored at −20˚C and dissolved in 0.1% aqueous formic acid solution at a concentration of 0.25 mg/ml upon use.

**Mass spectrometry analysis.** Peptides were subjected to LC–MS analysis using an Orbitrap Fusion Lumos Mass Spectrometer equipped with a nanoelectrospray ion source (both Thermo Fisher Scientific Waltham, MA). Peptide separation was carried out using an EASY nLC-1200 system (Thermo Fisher Scientific) equipped with a RP-HPLC column (75 μm × 36 cm) packed in-house with C18 resin (ReproSil-Pur C18-AQ, 1.9-μm resin; Dr. Maisch, Germany) and a custom-made column heater (60˚C). Peptides were separated using a step-wise linear gradient from 95% solvent A (0.1% formic acid, 99.9% water) and 5% solvent B (80% acetonitrile, 0.1% formic acid, 19.9% water) to 35% solvent B over 45 minutes, to 50% solvent B over 15 minutes, to 95% solvent B over 2 minutes, and 95% solvent B over 18 minutes at a flow rate of 0.2 μl/min.

The mass spectrometer was operated in DDA mode with a cycle time of 3 seconds between master scans. Each master scan was acquired in the Orbitrap at a resolution of 240,000 FWHM (at 200 m/z) and a scan range from 375 to 1,600 m/z followed by MS2 scans of the most intense precursors in the linear ion trap at "Rapid" scan rate with isolation width of the quadrupole set to 1.4 m/z. Maximum ion injection time was set to 50 ms (MS1) and 35 ms (MS2) with an AGC target set to 250% and "standard," respectively. Only peptides with charge state 2 to 5 were included in the analysis. Monoisotopic precursor selection (MIPS) was set to peptide, and the intensity threshold was set to 5e3. Peptides were fragmented by HCD (higher-energy collisional dissociation) with collision energy set to 35%, and one microscan was acquired for each spectrum. The dynamic exclusion duration was set to 30 seconds.

**Protein identification and label-free quantification.** The acquired raw files were imported into the Progenesis QI software (v2.0, Waters Nonlinear Dynamics United Kingdom), which was used to extract peptide precursor ion intensities across all samples applying the default parameters. The generated mgf files were searched using MASCOT against a decoy database containing normal and reverse sequences of the *Salmonella* Typhimurium (strain SL1344) (UniProt, release date 07.01.2019, in total 10,098 sequences) and commonly observed contaminants generated using the SequenceReverser tool from the MaxQuant software (Version 1.0.13.13). The following search criteria were used: full tryptic specificity was required (cleavage after lysine or arginine residues, unless followed by proline); 3 missed cleavages were allowed; carbamidomethylation (C) was set as fixed modification; oxidation (M) and protein N-terminal acetylation were applied as variable modifications; and mass tolerance of 10 ppm (precursor) and 0.6 Da (fragments). The database search results were filtered using the ion score to set the false discovery rate (FDR) to 1% on the peptide and protein level, respectively, based on the number of reverse protein sequence hits in the datasets.

Quantitative analysis results from label-free quantification were normalized and statistically analyzed using the SafeQuant R package v.2.3.4 (https://github.com/eahrne/SafeQuant (PMID: 27345528)) to obtain protein relative abundances. This analysis included global data normalization by equalizing the total peak/reporter areas across all LC–MS runs, summation of peak areas per protein and LC–MS/MS run, followed by calculation of protein abundance ratios. Only isoform specific peptide ion signals were considered for quantification. The summarized protein expression values were used for statistical testing of between condition differentially abundant proteins. Here, empirical Bayes moderated *t* tests were applied, as

implemented in the R/Bioconductor limma package (http://bioconductor.org/packages/release/bioc/html/limma.html). The resulting per protein and condition comparison *p*-values were adjusted for multiple testing using the Benjamini–Hochberg method.

## Time-lapse microscopy

For HS, 1 mL of the overnight culture was centrifuged and cells resuspended in the same volume of 0.85% KCl (Sigma). Moreover, 70 μl of the suspension was transferred into a 250 μl PCR tube and incubated in a thermocycler (Biometra Germany) 15 minutes at 51+/−0.5˚C. After this, cells were diluted 1/50 in PBS.

For TE treatment, cells in late exponential were diluted 1/10 in 100 mM Tris-10 mM EDTA in $ddH_2O$ (treatment) or $ddH_2O$ (control) prewarmed at 37˚C and incubated at 37˚C for 30 minutes without shaking. After this, the cells were diluted 1/5 in PBS.

Treated and control cells were inoculated onto a thin matrix of LB agarose attached to a microscope slide. These slides were prepared as follows: a sticky frame (Gene Frame AB-0578) was attached to a standard microscope slide. The resulting cavity was filled with heated LB supplemented with 2% agarose (A4718; Sigma) and covered with a standard microscope slide. After cooling and removal of the cover slide, strips of agarose were removed with the use of a surgical scalpel blade, resulting in 2 rectangles of LB agarose (approximately 5 × 7 mm side), one in the center of each half of the frame. Cells (2 μl) were spotted in the center of the pads, and the frame was sealed with a coverslip (24 × 60 mm). Each slide contained one control and one treated sample.

Microscope slides were examined using an inverted microscope (DeltaVision Core, Cytiva Life Sciences Marlborough, MA, mounted on a motorized Olympus IX71 stand) equipped with an environmental chamber (World Precision Instruments Sarasota, FL) set at 37˚C for up to 16 hours. Images were acquired using a 60× 1.42 NA Plan Apo N objective (Olympus Japan), with the matching 1.524 refractive index immersion oil (Cargille-Sacher Laboratories Cedar Grove, NJ) and a solid-state illumination (Spectra X light engine, Lumencore Beaverton, OR).

Images were recorded with a CoolSNAP HQ2 (Photometrics Tuscon, AZ) at each field every 10 minutes using brightfield (filter "POL", transmittance 32% and exposure time 0.015 seconds) and fluorescence once per hour to prevent phototoxicity (single band-pass emission filter 525/48 nm, transmittance 32%, and exposure time 0.025 seconds). Up to 12 fields were selected per condition and acquired using the microscope control software (DeltaVision SoftWoRx Suite 7.1.0, Cytiva Life Sciences). Images have been analyzed by manual counting using Fiji (ImageJ 1.53c). GFP+ and GFP− cells were identified according to a unique threshold based on fluorescence intensity that was applied on the first picture of every series. On average (+/− standard deviation), 565+/−224, 636+/−248, 493+/−117, and 436+/−118 cells were analyzed per experiment in HS, HS control, TE and TE control conditions, respectively.

## Microfluidic time-lapse microscopy

B04A-03 microfluidic plates for bacteria were used with the CellASIC ONIX2 Microfluidic System to observe the effect of TE treatment in real time at single cell level. Cells in late exponential phase were diluted 1/2 in spent LB medium and loaded into a CellASIC plate using the standard loading sequence protocol. With a constant pressure of 15 kPa, the cells were exposed to spent LB medium for 1 hour before switching to the TE treatment supplemented with PI (30 μg/ml) or Sytox blue (10 μg/ml). Cells trapped in the 0.7-μm height-restricted compartments were imaged with a 2-minute acquisition frame rate on an inverted Nikon Ti Eclipse epifluorescence microscope equipped with a Plan Apo 100x oil immersion objective, a

motorized stage and perfect focus system for multiposition time-lapse imaging, and a pco.edge 4.2 sCMOS camera. Fluorescence was excited by a SPECTRA X light engine and filtered with a Chroma 84100bs polychroic filter set. For GFP fluorescence, FITC settings were used (cyan laser line, 470/24 nm excitation filter, and 515/30 nm emission filter). Cy5 settings (red laser line, 640/30 nm excitation filter, and 705/72 nm emission filter) were used for PI fluorescence, while the CFP settings were used for Sytox blue imaging (blue laser line, 440/20 nm excitation filter, and 475/20 nm emission filter). Postprocessing was done with ImageJ. Fluorescence intensities of individual cells at time points 0, 30, and 60 minutes after start of TE treatment were measured with the point tool set to auto-measure and auto-next slice. Intensity cutoffs were set guided by distribution analysis to 180 for PI and 1,600 for GFP.

## NPN uptake assay

Cells in late exponential phase were centrifuged 3 minutes at 9,500 rpm, and the pellet was washed twice in PBS. NPN uptake was measured in a flat bottom 96-well plate (Corning 3904 Corning, NY) with a final NPN (Sigma) concentration of 10 μM. 2.5 μg/mL Polymyxin B (Sigma) was used as permeabilizing agent in the positive controls. In order to achieve an OD ranging from 0.4 to 0.6, 120 μl of the cell suspension was added to each well on top of 80 μl of PBS (with polymyxin B for the positive controls). NPN was added last, and the measurements were performed immediately.

The fluorescence readout was recorded using a plate reader Synergy H4 Hybrid Reader (BioTek Instruments Winooski, VT) controlled with the Gen5 software. The following excitation/emission wavelengths (in nm) were used: 350/420 for the NPN signal and 491/512 for GFP signal; the bandwidth was 9 nm in all cases. The emission was recorded from the top (Optics: top) with a gain of 100 for GFP and 80 for NPN. OD was measured using 600 nm wavelength. The experiments were performed at 37˚C with fast continuous shaking and NPN signal measurements were collected with 1-minute intervals for 1 hour. GFP and OD measurements were taken at the beginning and at the end of the run. We considered the fluorescence values after stabilization of the signal. Thus, one time point was selected per experiment, approximately 45 minutes after the start of the measurements (ranging from 35 to 50 minutes). Wells containing bacteria in DMSO were used to measure the auto-fluorescence of the bacteria. These values were corrected for the OD and subtracted from NPN/OD fluorescence values.

## *In vivo* competitions

Nine- to 12-week-old specified opportunistic pathogen free (SOPF) C57BL/6 mice were pretreated with 25 mg of streptomycin by oral gavage 24 hours prior to infection with *S*.Tm strains to allow robust colonization [59]. Competitors and helper strains were grown separately overnight in LB containing the appropriate antibiotics. Prior to infection, the bacteria were grown to late exponential phase in LB without antibiotics. Cells were washed in PBS, diluted, and mixed to obtain a final inoculum provided to mice by oral gavage. Each 50 μl inoculum contained a total of c.a. $5 \times 10^7$ CFU consisting of the helper strain and 2 competitors at a 100:1:1 ratio. Fecal samples were collected daily, homogenized in 1-ml PBS by bead beating, and bacterial populations were enumerated by selective plating on MacConkey agar containing the appropriate antibiotics. In addition, samples were frozen for determination of the LCN2 concentration. Competitions were allowed to proceed for 3 days p.i. and then the mice were euthanized. To calculate the competitive indexes, population sizes of the 2 competing strains were enumerated by selective plating. The ratio of these 2 values was normalized to the ratio of the 2 competing strains in the corresponding inoculum. To determine the

inflammatory state of the gut, serial dilutions of fecal samples were analyzed using the Mouse LCN2-2/NGAL DuoSet ELISA kit (R&D Systems Minneapolis, MN) according to the manufacturer's instructions.

### Ethics statement

All animal experiments were approved by the legal authorities (Basel-Stadt Kantonales Veterinäramt, license #30480) and follow the 3R guidelines to reduce animal use and suffering to its minimum.

### Statistical analysis

Statistical analysis was conducted using GraphPad Prism 8.0.2.

**For Figs 1–3 and S2, S6, S9, and S12 Figs**, an important source of noise between repetitions was day-to-day variations in the induction level of the HilD regulon. Such variation is clearly visible in Fig 2D. Small changes in media composition may explain this phenomenon. This generates substantial variability in NPN uptake and stress sensitivity measurements between independent experiments. Therefore, to be able to compare mutants not always studied in parallel, we normalized the datasets using values from WT internal controls always performed in parallel with a given mutant or condition. A value of 1 means value for a given mutant equals value for the WT. After normalization to the WT, outliers were identified using the ROUT method [60] with Q = 1%. Statistical significance of differences between mutants and WT strains in parallel experiments was assessed using the nonnormalized dataset in Wilcoxon matched pairs signed rank tests. The normalized dataset was used in Mann–Whitney tests when comparing mutants or conditions. In S2 **and** S5 **Tables**, we followed the **Benjamini–Hochberg procedure** to control the FDR ($\alpha = 0.05$) [61], meaning that only comparisons yielding *p*-values equal or below the largest *p*-value below its corresponding Benjamini–Hochberg critical value were considered statistically significant.

Violin plots show the empirical distribution of the data, the median, and quartiles. The number of independent biological replicates per condition is indicated below the figures or in figure legends ($n = x$). In experiments where $n <= 9$, single data points are shown on top of the violin plot.

## Supporting information

**S1 Fig. Comparative proteome of S.Tm cells sorted according to the expression of P*prgH*::*gfp*. (A)** Histogram showing the P*prgH*::*gfp* reporter expression pattern in WT, Δ*hilD*, and Δ*hilE* strains. **(B)** Volcano plot showing differential abundance of proteins in sorted GFP+ (ON) and GFP− (OFF) cells of S.Tm WT reporter strain. S1 Table lists all these proteins and their functions and provides details about fold change, statistics, and loci. Source data are provided as a source data file (S1 Data). S.Tm, *Salmonella* Typhimurium; WT, wild-type.
(EPS)

**S2 Fig. Control of GFP expression from pM965 and NPN uptake in the absence of arabinose (corresponding to Fig 1). (A)** Histogram showing the *gfp* expression pattern in strains WT P*prgH*::*gfp* (red) and WT carrying the plasmid pM965 P*rpsM*::*gfp* constitutively expressing *gfp* (blue). **(B, C)** NPN fluorescence corrected by the optical density at 600 nm and normalized to WT pBAD24. (B) Strains harboring pBAD24. (C) Strains harboring p*hilA*. **S2 Table** lists *p*-values calculated for relevant comparisons. For comparisons against the WT, *p*-values were calculated using the raw data in paired Wilcoxon tests. For comparisons between mutants, *p*-values were calculated using the normalized data in unpaired Mann–Whitney tests. Source

data are provided as a source data file (S1 Data). NPN, N-phenyl-1-naphthylamine; WT, wild-type.
(EPS)

**S3 Fig. Growth rate and HS resistance in different genetic backgrounds.** Time-lapse microscopy analysis of WT P*prgH*::*gfp* after HS (50.5˚C, 15 minutes) and untreated control. The cells were observed for 12 hours posttreatment. **(A)** Proportion of cells expressing the HilD regulon (GFP+) from overnight cultures in LB. Triangles: cells used from control experiments, inverted triangles: cells after HS (t = 0). **(B)** Division time of cells expressing or not the HilD regulon from control experiments. **(C, D)** Proportion of cells able to grow in control conditions (C) or after HS (D). **(E)** Superimposed phase contrast and epifluorescence images of representative fields of view in control (upper panels) and HS (lower panels) experiments at t = 0, 3 hours or 7 hours posttreatment. Scale bar corresponds to 5 μm. *p*-Values were calculated using ANOVA and corrected by a Tukey HSD post hoc test. Three independent replicates. Source data are provided as a source data file (S1 Data). HS, heat shock; LB, Lysogeny broth; WT, wild-type.
(PDF)

**S4 Fig. Discrimination of cells according to membrane potential revealed by the $DiOC_2(3)$ staining.** **(A)** Flow cytometry plots showing events positioned according to intensities of their red (y-axis) and green fluorescence (x-axis) signals. Unstained WT control allowed delimiting the gate for stained cells. WT cells treated with 100 μm CCCP and 30 μm $DiOC_2(3)$ were used to delimit cells with low membrane potential (red) among the stained cells (green). This gating strategy has been used in all independent repetitions to calculate the fraction of cells with low membrane potential in different *S*.Tm populations (Fig 2). **(B)** Fluorescence microscopy imaging of cells treated the same as for flow cytometry shows the $DiOC_2(3)$ staining of individual cells (all cells green, high membrane potential cells green and red) and the loss of membrane potential after CCCP treatment (no red cells). Source data are provided as a source data file (S1 Data). CCCP, carbonyl cyanide 3-chlorophenylhydrazone; DiOC2(3), 3,3′-diethyloxa-carbocyanine iodide; *S*.Tm, *Salmonella* Typhimurium; WT, wild-type.
(EPS)

**S5 Fig. Membrane potential in the absence of stress revealed with $DiSC_3(5)$.** **(A)** Red fluorescence signal from all cells (Total) or cells expressing (GFP+) or not (GFP−) the HilD regulon from WT (left panel) or *ΔhilE* (right panel) strains carrying the reporter P*prgH*::*gfp* (representative experiment). As negative control, 100 μm CCCP was as used to reduce the membrane potential in all cells. **(B)** Mean (left) and median (right) of the red fluorescent signal due to accumulation of $DiSC_3(5)$ in WT or *ΔhilE* GFP+ or GFP− populations. *p*-Values were calculated using the paired Wilcoxon test. **(C)** Imaging of $DiSC_3(5)$-stained cells carrying the P*prgH*::*gfp* reporter by fluorescence microscopy. The loss of red fluorescence signal revealed the reduction in membrane potential upon CCCP treatment (lower panels). Source data are provided as a source data file (S1 Data). CCCP, carbonyl cyanide 3-chlorophenylhydrazone; DiSC3(5), 3,3′-dipropylthiadicarbocyanine iodide; WT, wild-type.
(EPS)

**S6 Fig. Validation of the cytometry analysis of cells exposed to TE.** **(A)** Normalized frequency of dead *ΔhilE* reporter cells stained with either Sytox blue or PI after treatment with 100 mM Tris-10 mM EDTA. *n* = x indicates the number of repetitions. **(B)** Normalized frequency of cells stained with either Sytox blue (blue dots) or PI (red dots) after treatment with 100 mM Tris-10 mM EDTA measured by flow cytometry. The graph shows the results for WT, *ΔhilD*, and *ΔhilE* carrying the chromosomal reporter P*prgH*::*gfp* or the plasmidic P*rpsM*::

*gfp* (constitutive GFP expression from pM965). The dataset is normalized by values obtained with the WT reporter strain. For comparisons against the WT, *p*-values were calculated using raw data in paired Wilcoxon tests. For comparisons between mutants or conditions, *p*-values were calculated using normalized data in unpaired Mann–Whitney tests. $n = 10$ repetitions. **(C)** Reduction of the GFP positive fraction (ΔGFP+ in percentage) among WT, *ΔhilD*, or *ΔhilE* cells negative for Sytox blue (blue dots) or PI (red dots)) treated with 100 mM Tris–10 mM EDTA compared to distilled water control. In these control experiments, the GFP was constitutively expressed (P*rpsM*::*gfp*). Significance of the deviation of the median from 0 estimated by Wilcoxon signed rank test. There was no significant loss of GFP + cells after treatment compared to control. $n = x$ indicate the number of repetitions. In all panels, *p*-values are indicated within the graph below a bar marking the 2 compared conditions. **(D)** Imaging of WT cells by fluorescence microscopy during exposure to 100 mM Tris-10 mM EDTA (time points 0, 30, and 60 minutes) in the presence of PI or Sytox blue in a CellAsic microfluidic device. Both dyes accumulate within cells exposed to lethal stress, while almost no cells were stained at the beginning of the treatment. Source data are provided as a source data file (S1 Data). PI, propidium iodide; TE, Tris-EDTA; WT, wild-type.
(PDF)

**S7 Fig. Single cell level analysis of lethal TE exposure in a microfluidic device (quantitative analysis of S1 Movie). (A)** Total amount of WT P*prgH*::*gfp* cells expressing GFP (ON; green bar) or not (OFF; gray bar), or stained by PI (PI+; red bar) at t0, 30, and 60 minutes of exposure to 100 mM Tris-10 mM EDTA and 30 μg/ml PI in distilled water. **(B)** Proportion of ON, OFF, or PI stained cells among cells that were OFF or ON at t0. **(C, D)** Distribution of fluorescence intensity in cells after 0, 30, and 60 minutes of TE exposure. The dashed lines indicate the cutoff intensities used to bin GFP+ (ON) and GFP− (OFF) cells (C), and PI+ and PI− cells (D). Cutoff intensities were determined according to the distribution at t0 for the GFP signal and at 30 minutes for the PI signal when positive and negative populations were clearly differentiated. Source data are provided as a source data file (S1 Data). TE, Tris-EDTA; WT, wild-type.
(EPS)

**S8 Fig. Time-lapse microscopy analysis of cells exposed to lethal concentration of TE and control.** Time-lapse microscopy analysis of WT P*prgH*::*gfp* treated with 100 mM Tris 10 mM EDTA and untreated control. **(A)** Violin plots showing the fraction of cells able to form microcolonies. **(B)** Fraction of GFP+ cells in regrowing and total population after treatment with 100 mM Tris-10 mM EDTA or untreated control. The *p*-values were calculated using the unpaired Mann–Whitney test, $n = 6$ repetitions. Source data are provided as a source data file (S1 Data). TE, Tris-EDTA; WT, wild-type.
(EPS)

**S9 Fig. Death after exposure to lethal concentration of TE in the absence of arabinose (corresponding to Fig 2).** Cytometry analysis of cells stained by Sytox blue or PI after exposure to 100 mM Tris–10 mM EDTA. Data normalized by WT pBAD24. **(A)** Strains harboring pBAD24. **(B)** Strains harboring p*hilA*. S5 Table lists *p*-values calculated for relevant comparisons. For comparisons against the WT, *p*-values were calculated on the raw data using paired Wilcoxon tests. For comparisons between mutants, *p*-values were calculated using the normalized data and the unpaired Mann–Whitney test. The FDR ($\alpha = 0.05$) was controlled by the **Benjamini–Hochberg procedure**. Source data are provided as a source data file (S1 Data). TE, Tris-EDTA; WT, wild-type.
(EPS)

**S10 Fig. Overexpression of *hilA* decouples the expression of virulence from sensing environmental stress (corresponding to Fig 3). (A)** Proportion of cells expressing GFP from P*prgH*::*gfp* in WT cells harboring pBAD24 or p*hilA*. Growth in presence of 1 mM Arabinose (Ara) induced expression of *hilA* from p*hilA* and subsequent expression of GFP from P*prgH*::*gfp* in c.a. 90% of the cells independently from pretreatment with sublethal TE concentration (0.04X TE). **(B)** Representative histograms from flow cytometry analysis. Source data are provided as a source data file (S1 Data). TE, Tris-EDTA; WT, wild-type.
(EPS)

**S11 Fig. Competitions 1 and 2, fecal loads during virulent infections (corresponding to Fig 4). (A, B)** CFUs in feces obtained by selective plating for competition 1 (**A**) and 2 (**B**). Dashed lines represent the detection limits, and bars indicate the medians. Source data are provided as a source data file (S1 Data). CFU, colony-forming unit.
(EPS)

**S12 Fig. Competition 2 and 3 in the absence of inflammation. A.** Deleting *invG* does not increase stress resistance. Normalized proportion of cells stained by Sytox or PI after exposure to 100 mM Tris-10 mM EDTA (TE). Mutants are compared to WT controls performed in parallel. For comparisons against the WT, *p*-values were calculated using paired Wilcoxon tests. For comparisons between mutants, *p*-values were calculated using the unpaired Mann–Whitney test on normalized data. **B to C.** Competitions 3 and 4 using avirulent (Avir) Δ*invG* Δ*ssaV* mutants. **C.** Experimental setup. **D.** Competitive indexes calculated from the relative proportions of each competitor in fecal samples. The dashed line represents a 1:1 ratio between HilD+ and HilD− competitors. Values below this line indicate that the HilD− strain outgrew the HilD+ strain. **E.** Intestinal inflammation estimated by measuring LCN2 concentration in the feces. Concentrations above $10^2$ ng/g are usually detected in feces from inflamed gut. Dots correspond to individual mice. Filled dots (black): competition 3 (Avir (HilD+) versus Avir Δ*hilD* (HilD−)); empty dots: competition 4 (Avir Triple Mutant (HilD+) versus Avir Δ*hilD* Triple Mutant (HilD−)). Two independent inocula per condition. **F and G.** CFUs in feces obtained by selective plating for competition 3 (**F**) and 4 (**G**).The *p*-values were generated with 1-way ANOVA and corrected by a Sidak multiple comparisons test. Bars represent the median. Source data are provided as a source data file (S1 Data). CFU, colony-forming unit; LCN2, Lipocalin 2; WT, wild-type.
(EPS)

**S1 Movie. Killing of S.Tm by TE analyzed at the single-cell level in a microfluidic device.** WT P*prgH*::*gfp* cells at late exponential phase in LB were loaded into a CellASIC ONIX microfluidic chip and exposed to exhausted LB 60 minutes before starting treatment with 100 mM Tris-10 mM EDTA and 30 μg/ml PI in distilled water. HilD ON cells (green) were prone to die, therefore losing GFP and turning red due to membrane disruption and staining of their DNA by PI. LB, Lysogeny broth; PI, propidium iodide; S.Tm, *Salmonella* Typhimurium; TE, Tris-EDTA; WT, wild-type.
(AVI)

**S1 Table. Proteomic analysis of the HilD regulon on sorted S.Tm cells.** Cells from WT P*prgH*::*gfp* in late exponential phase were sorted according to GFP fluorescent signal and their respective proteome were analyzed by mass spectrometry and compared. The table lists genes differentially expressed in GFP+ cells compared to GFP− cells (q value <0.05; −0.5>Log2 ratio>0.5). The corresponding volcano plot is presented in S1 Fig. S.Tm, *Salmonella* Typhimurium; WT, wild-type.
(XLSX)

**S2 Table. Statistical analysis Fig 1D and 1E and S2 Fig.**
(XLSX)

**S3 Table. Parameters for estimations of death rates.** This table lists the parameters used to calculate the death rates of WT, *hilE*, and *hilD* strains harboring P*prgH*::*gfp* exposed to lethal TE treatment. TE, Tris-EDTA; WT, wild-type.
(XLSX)

**S4 Table. CFU counts after lethal TE treatment.** Control experiments showing that PI or Sytox blue staining of dead cells upon lethal TE exposure correlates to the amount of dead cells deduced from the amount of colonies after plating on LB agar medium. CFU, colony-forming unit; LB, Lysogeny broth; PI, propidium iodide; TE, Tris-EDTA.
(XLSX)

**S5 Table. Statistical analysis (Fig 2H and 2I and S9 Fig).**
(XLSX)

**S6 Table. Strains and plasmids used in this study.**
(XLSX)

**S7 Table. Primers used in this study.**
(XLSX)

**S1 Data. Numerical values underlying all the figures.**
(XLSX)

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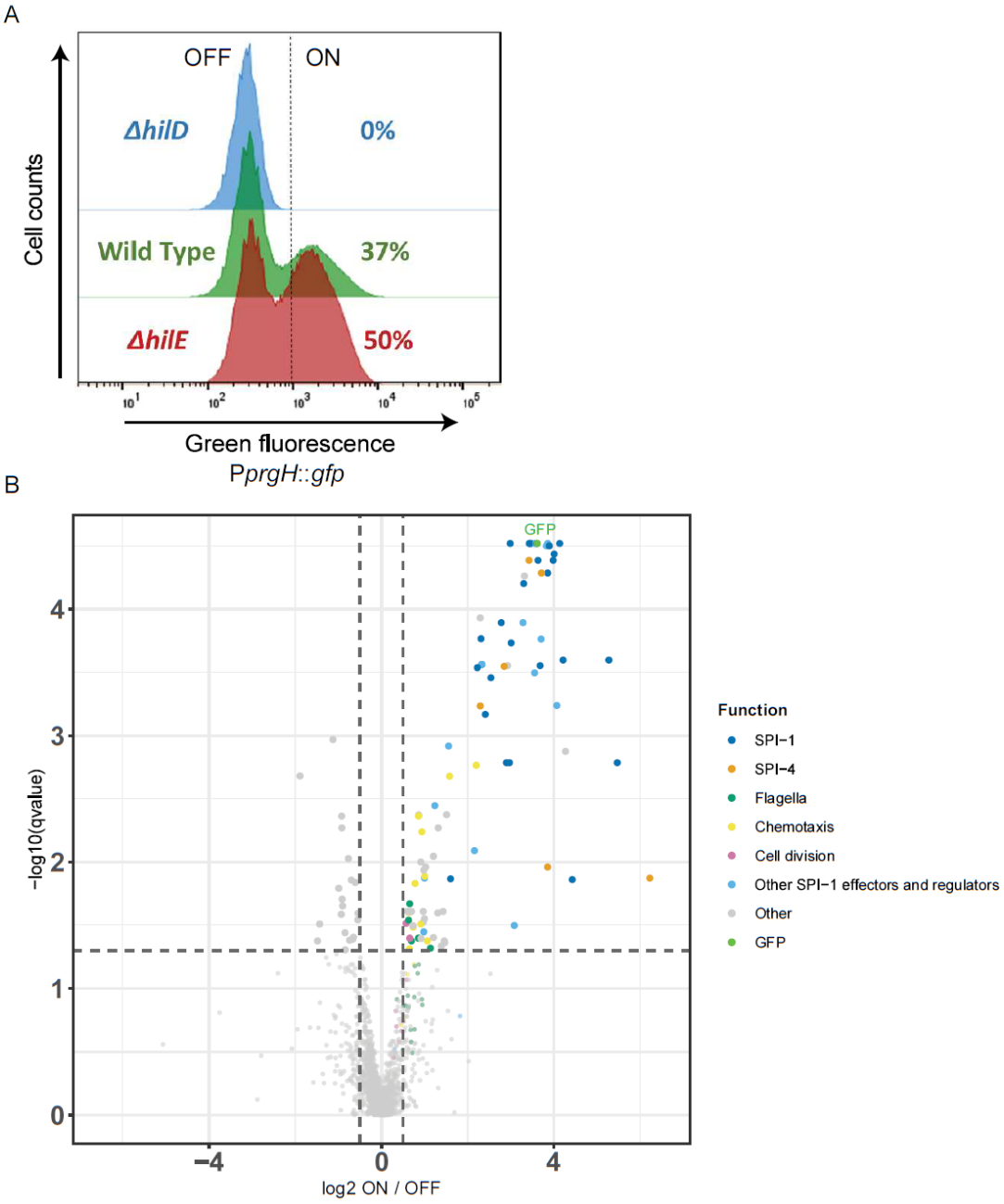
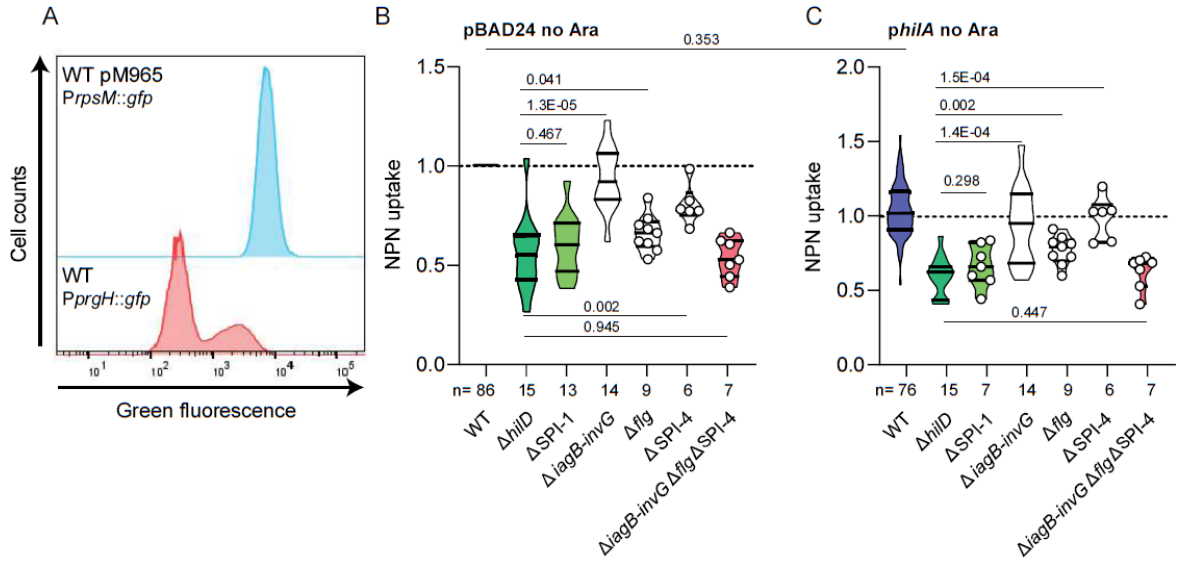
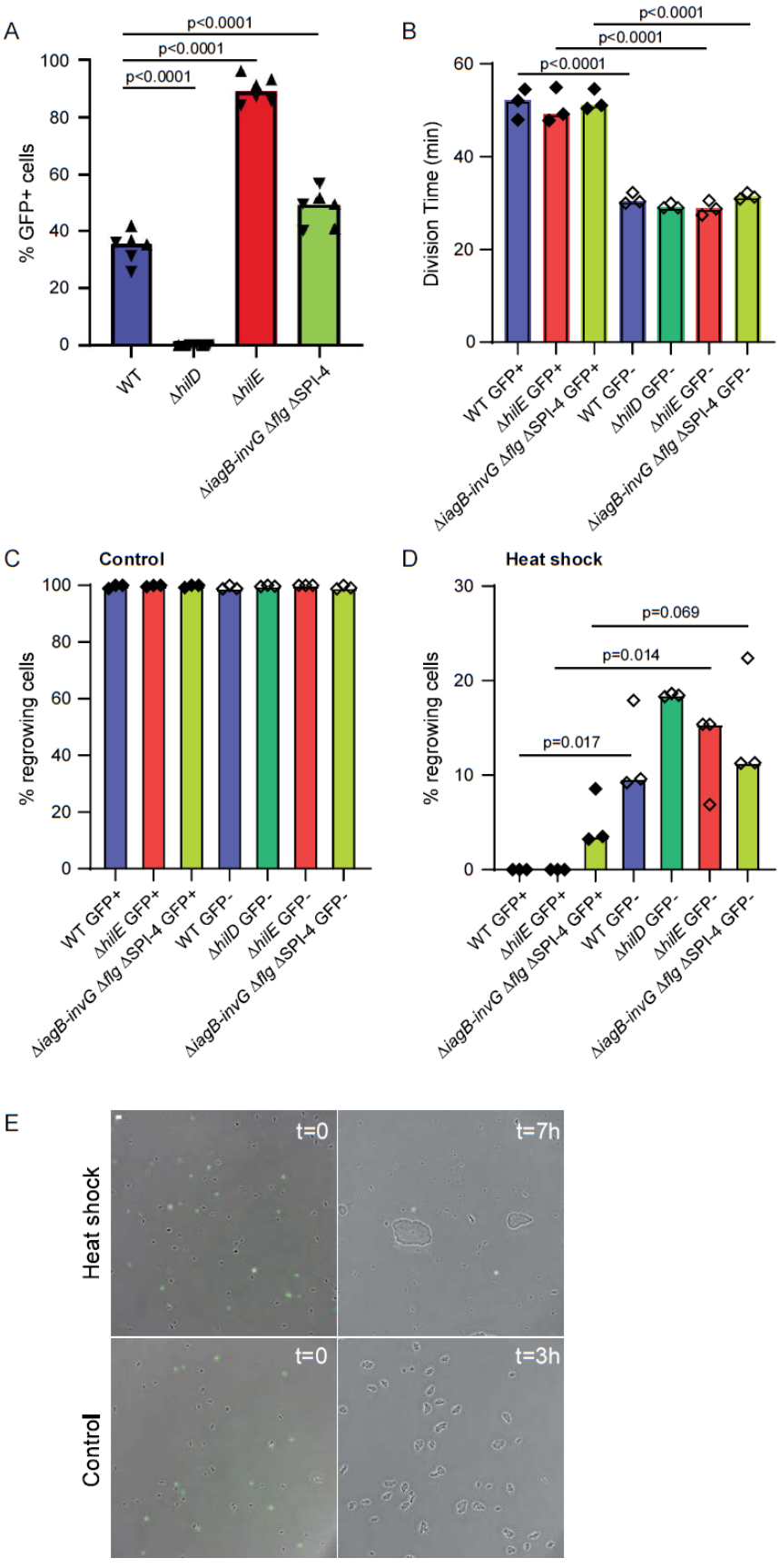
S1 Fig. Comparative proteome of *S.* Tm cells sorted according to the expression of P*prgH::gfp.*



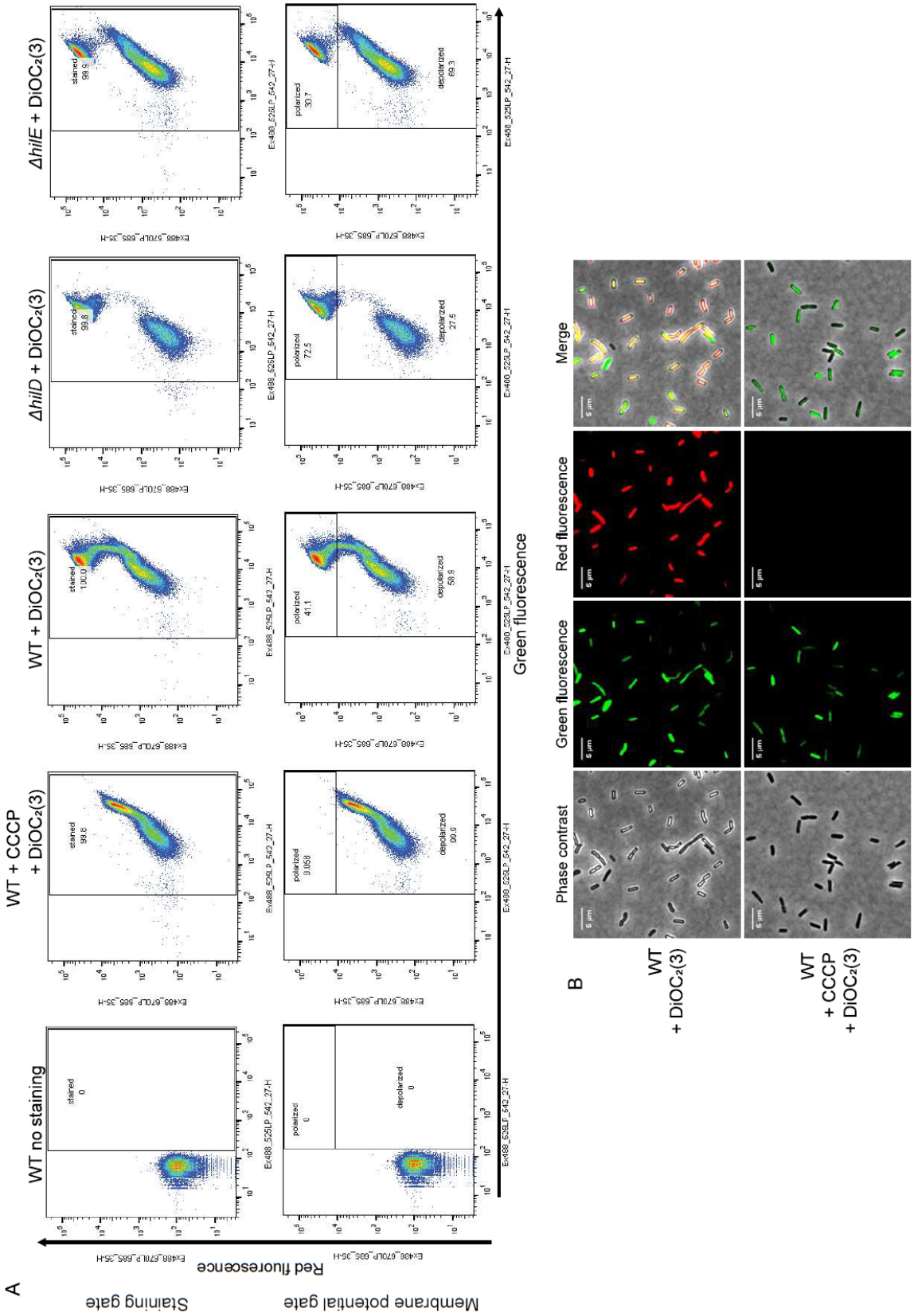
S2 Fig. Control of GFP expression from pM965 and NPN uptake in the absence of arabinose (corresponding to Fig 1).



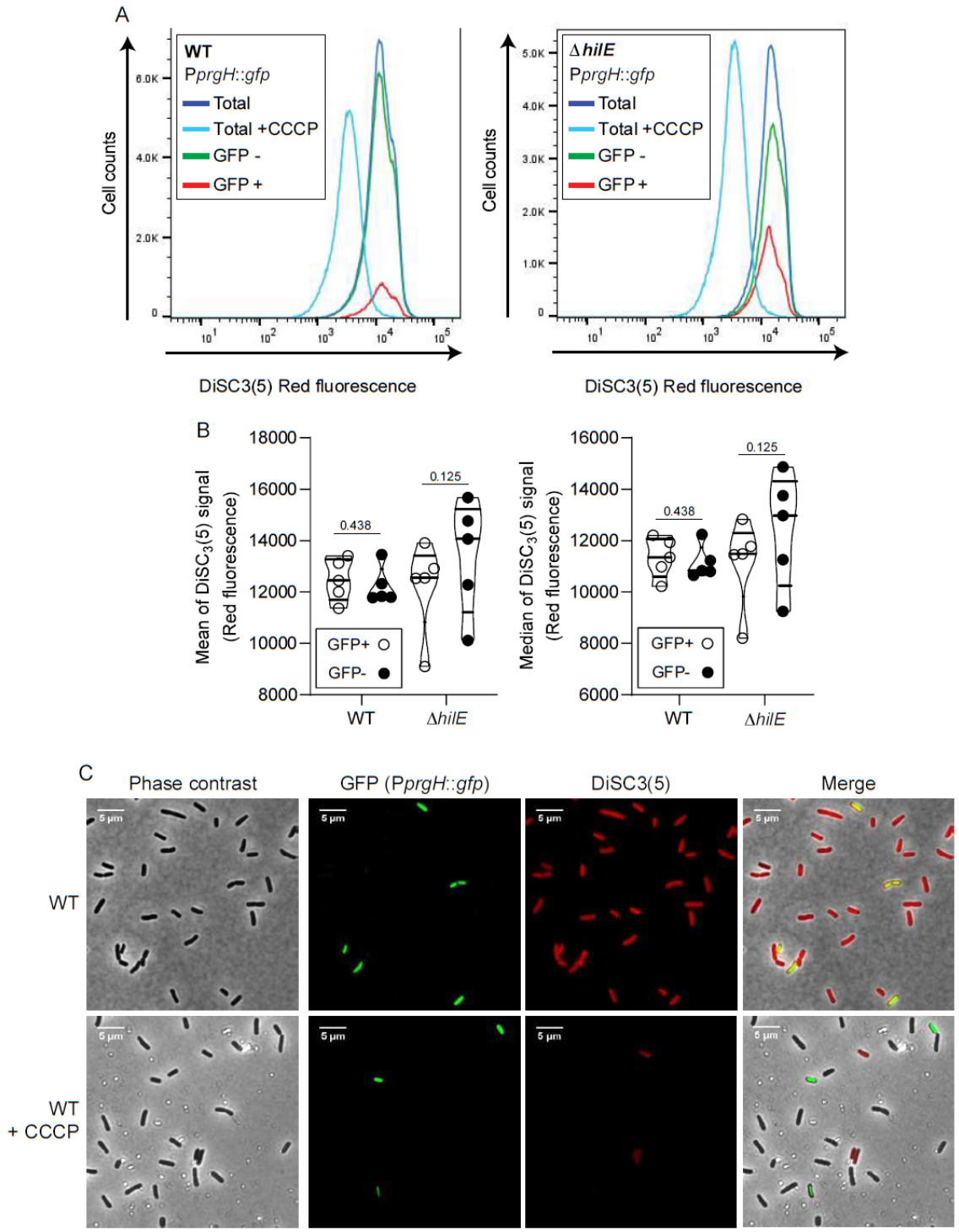
S3 Fig. Growth rate and HS resistance in different genetic backgrounds.



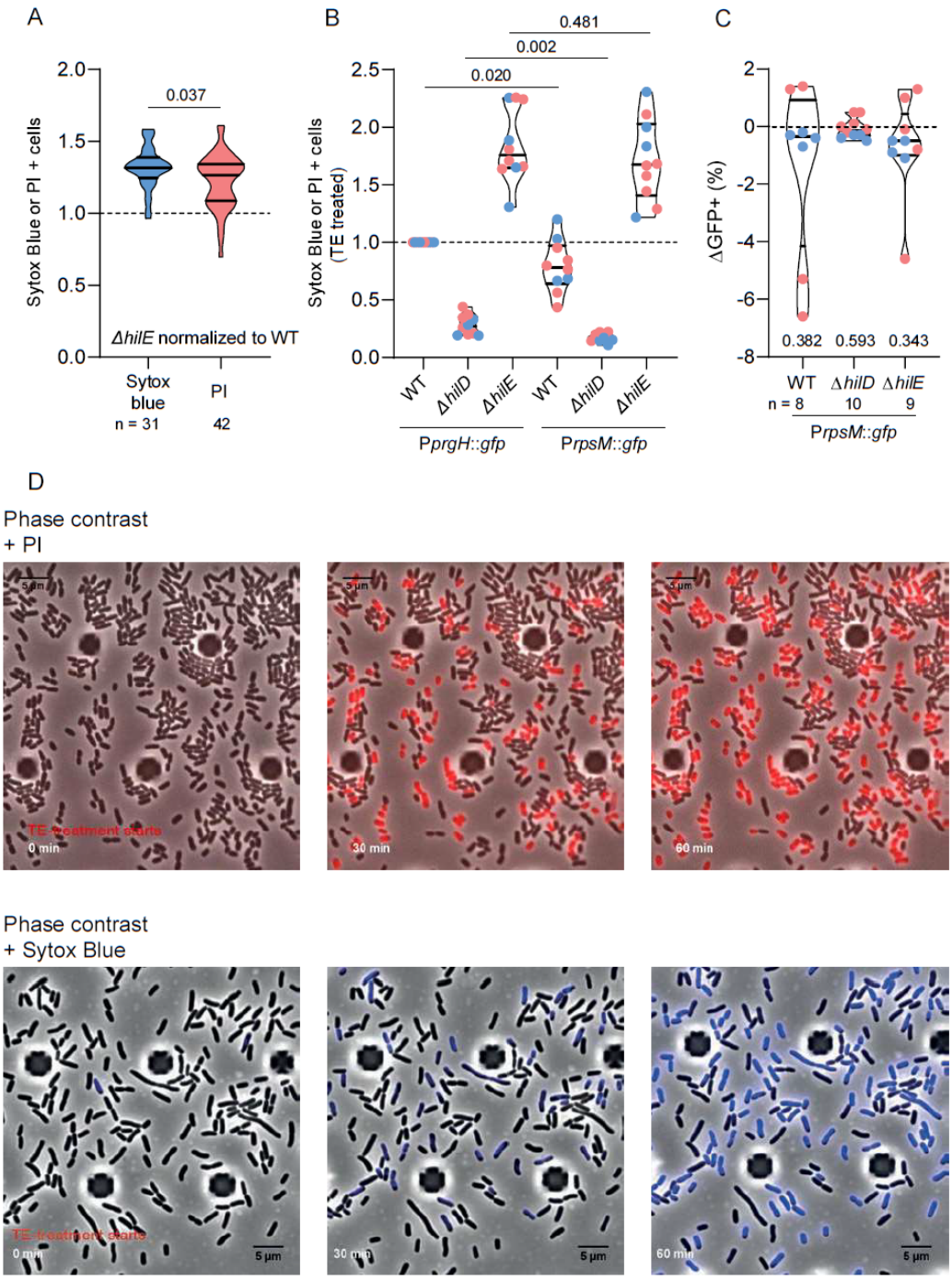
S4 Fig. Discrimination of cells according to membrane potential revealed by the $DiOC_2(3)$ staining.



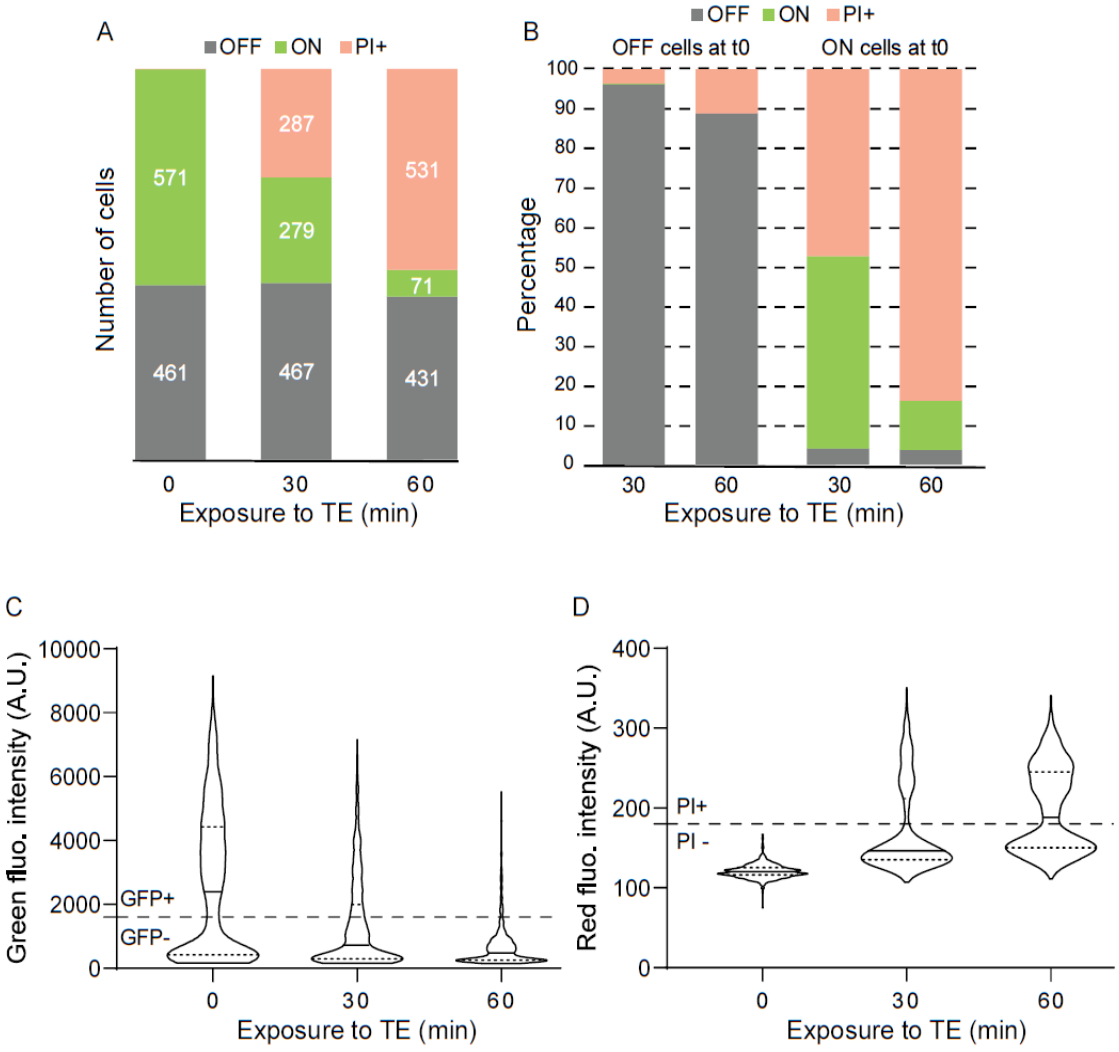
S5 Fig. Membrane potential in the absence of stress revealed with $DiSC_3(5)$.



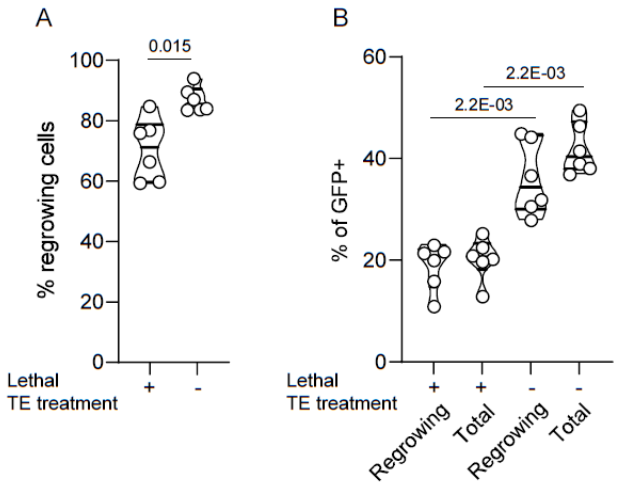
S6 Fig. Validation of the cytometry analysis of cells exposed to TE.



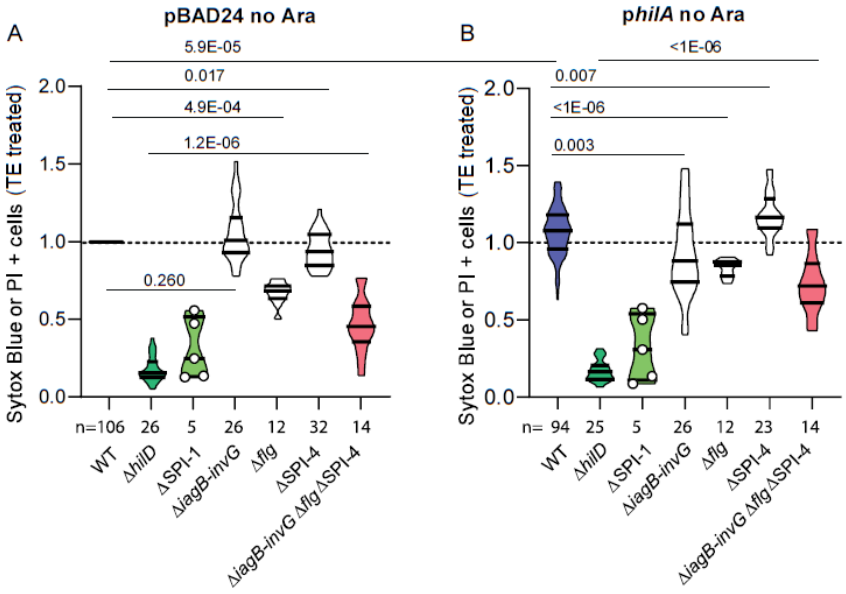
S7 Fig. Single cell level analysis of lethal TE exposure in a microfluidic device (quantitative analysis of S1 Movie).



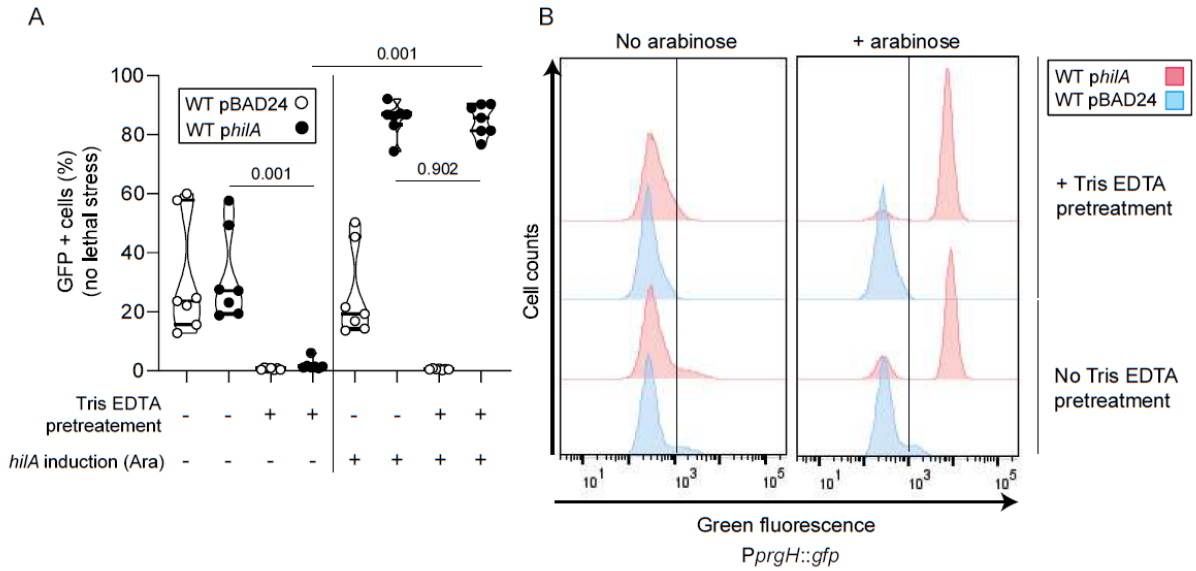
S8 Fig. Time-lapse microscopy analysis of cells exposed to lethal concentration of TE and control.



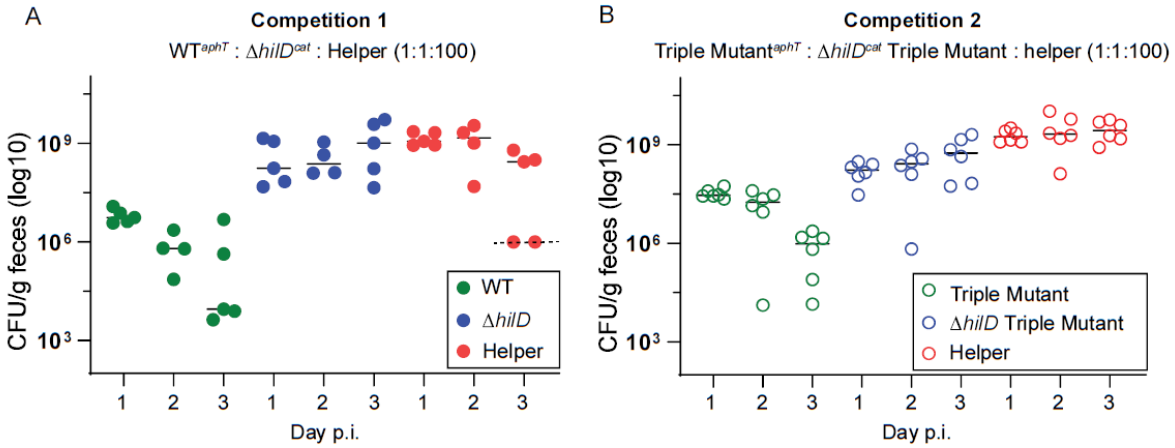

S9 Fig. Death after exposure to lethal concentration of TE in the absence of arabinose (corresponding to Fig 2).



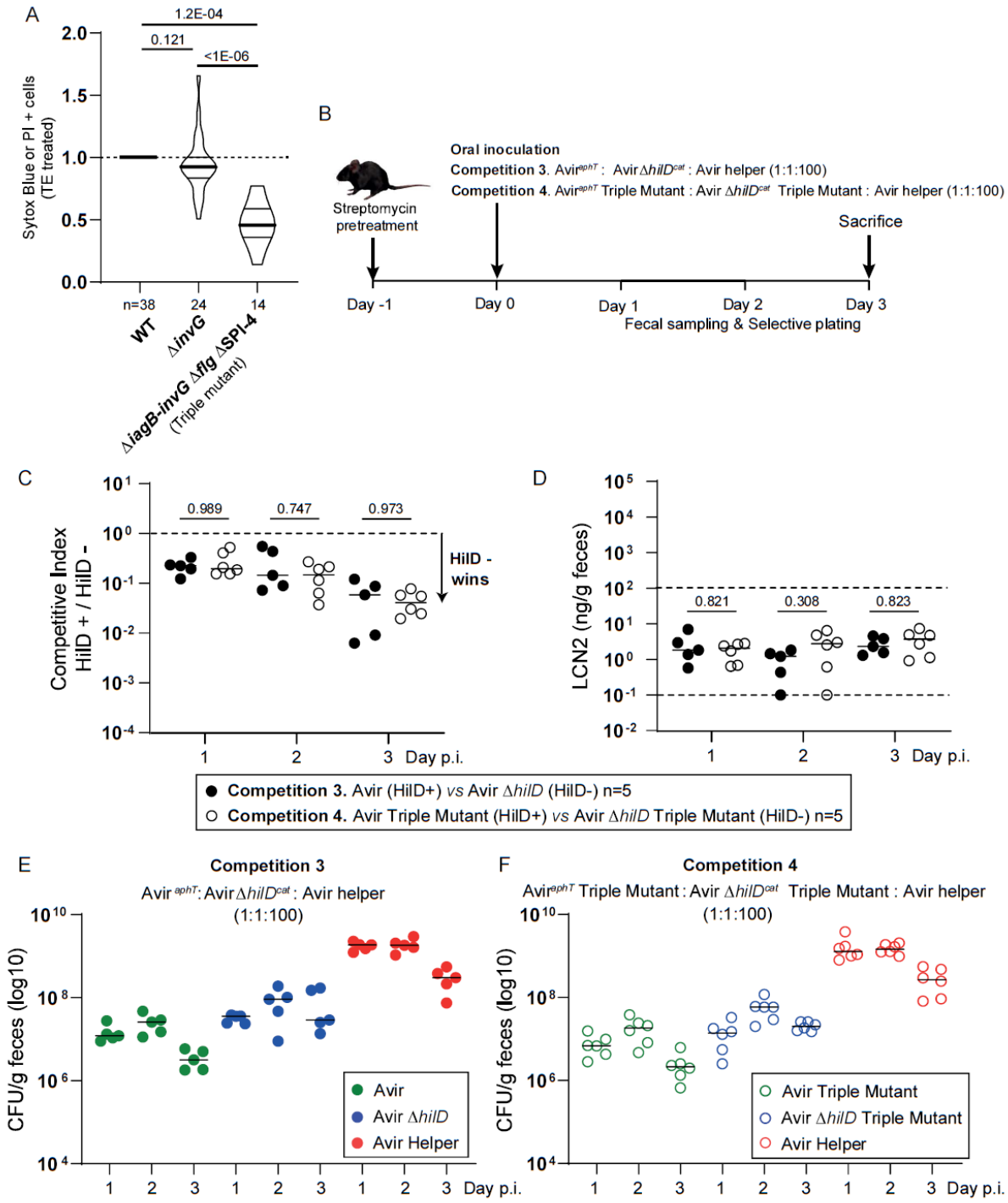
S10 Fig. Overexpression of *hilA* decouples the expression of virulence from sensing environmental stress (corresponding to Fig 3).




S11 Fig. Competitions 1 and 2, fecal loads during virulent infections (corresponding to Fig 4).



S12 Fig. Competition 2 and 3 in the absence of inflammation.



S1 Table. Proteomic analysis of the HilD regulon on sorted *S.* Tm cells.

| Higher production in GFP+ vs. GFP- cells | | | | | | |
|---|---|---|---|---|---|---|
| **Uniprot Accession #** | **Gene_Name** | **Protein_Description** | **log2ratio** | **q-value** | **Function** | **Locus** |
| A0A0H3NGY0 | *trg* | Methyl-accepting chemotaxis protein III | 0.94 | 5.8E-03 | **Flagelum and chemotaxis systems** | SL1344_1556 |
| A0A0H3NCE6 | *cheB* | Protein-glutamate methylesterase/protein-glutamine glutaminase | 0.78 | 1.5E-02 | | SL1344_1852 |
| A0A0H3NCQ6 | *cheM* | Methyl-accepting chemotaxis protein II | 0.63 | 2.5E-02 | | SL1344_1854 |
| A0A0H3NMF8 | *cheW* | Purine binding chemotaxis protein | 0.91 | 3.1E-02 | | SL1344_1855 |
| A0A0H3NDZ5 | *cheA* | Chemotaxis protein | 0.73 | 3.3E-02 | | SL1344_1856 |
| A0A0H3NCF1 | *motB* | Motility protein B | 0.65 | 4.9E-02 | | SL1344_1857 |
| A0A0H3NHX2 | *motA* | Motility protein A | 1.00 | 1.3E-02 | | SL1344_1858 |
| A0A0H3NCH8 | *fliZ* | Flagella biosynthesis regulatory protein | 0.70 | 4.2E-02 | | SL1344_1884 |
| A0A0H3NCT3 | *fliB* | Lysine-N-methylase (Ec 2.1.1.-) (Lysine N-methyltransferase) | 0.65 | 2.1E-02 | | SL1344_1887 |
| A0A0H3NMJ6 | *fliC* | Flagellin | 1.14 | 4.8E-02 | | SL1344_1888 |
| A0A0H3NCI3 | *fliS* | Flagellar secretion chaperone | 0.86 | 4.0E-02 | | SL1344_1890 |
| A0A0H3NE42 | *fliL* | Flagellar protein | 0.63 | 2.9E-02 | | SL1344_1904 |
| A0A0H3NEZ8 | *fljB* | Flagellin | 1.51 | 4.2E-03 | | SL1344_2756 |
| A0A0H3NLZ8 | SL1344_3112 | Methyl-accepting transducer domain-containing protein | 2.20 | 1.7E-03 | | SL1344_3112 |
| A0A0H3NG60 | SL1344_3189 | Methyl-accepting chemotaxis protein II | 1.58 | 2.1E-03 | | SL1344_3189 |
| A0A0H3NS38 | *aer* | Aerotaxis receptor protein | 1.07 | 4.2E-02 | | SL1344_3190 |
| A0A0H3NMQ9 | *tcp* | Methyl-accepting chemotaxis citrate transducer | 0.86 | 4.3E-03 | | SL1344_3542 |
| A0A0H3NF77 | *avrA* | Type III secretion system effector protein-regulator of *Salmonella*-induced inflammatory response | 2.41 | 6.8E-04 | **SPI-1** | SL1344_2845 |
| E1WAB4 | *orgB* | Oxygen-regulated invasion protein | 2.31 | 1.7E-04 | | SL1344_2849 |
| E1WAB5 | *orgA* | Oxygen-regulated invasion protein | 3.01 | 1.8E-04 | | SL1344_2850 |
| A0A0H3NQZ5 | *prgK* | Lipoprotein | 3.61 | 3.0E-05 | | SL1344_2851 |
| A0A0H3NGZ3 | *prgJ* | Type III secretion system apparatus | 5.27 | 2.5E-04 | | SL1344_2852 |
| A0A0H3NF82 | *prgI* | Type III secretion system apparatus | 3.86 | 5.2E-05 | | SL1344_2853 |
| A0A0H3NL53 | *prgH* | Type III secretion apparatus component | 3.43 | 3.0E-05 | | SL1344_2854 |
| E1WAC0 | *hilD* | Transcriptional regulator | 2.97 | 1.6E-03 | | SL1344_2855 |
| A0A0H3NF89 | *hilA* | Invasion protein regulator | 2.22 | 2.9E-04 | | SL1344_2856 |
| A0A0H3NR00 | *sptP* | Type III secretion system effector protein | 2.99 | 3.0E-05 | | SL1344_2858 |
| E1WAC5 | *iacP* | Probable acyl carrier protein | 5.47 | 1.6E-03 | | SL1344_2860 |
| E1WAC6 | *sipA* | Cell invasion protein | 3.98 | 4.1E-05 | | SL1344_2861 |
| A0A0H3NGZ6 | *sipD* | Pathogenicity island 1 Type III secretion system apparatus-part of the Translocon | 3.44 | 3.0E-05 | | SL1344_2862 |
| E1WAC8 | *sipC* | Cell invasion protein | 4.01 | 3.7E-05 | | SL1344_2863 |
| A0A0H3NF87 | *sipB* | Pathogenicity island 1 Type III secretion system effector protein | 4.13 | 3.0E-05 | | SL1344_2864 |
| A0A0H3NL58 | *sicA* | Type III secretion-associated chaperone | 3.89 | 3.2E-05 | | SL1344_2865 |

| | | | | | | |
|---|---|---|---|---|---|---|
| A0A0H3NF95 | *spaS* | Type III secretion system secretory apparatus | 1.60 | 1.4E-02 | | SL1344_2866 |
| A0A0H3NF93 | *spaP* | Type III secretion system secretory apparatus | 2.54 | 3.5E-04 | | SL1344_2869 |
| A0A0H3NL63 | *spaO* | Surface presentation of antigens protein (Associated with type III secretion and virulence) | 4.21 | 2.5E-04 | | SL1344_2870 |
| A0A0H3NF97 | *invJ* | Surface presentation of antigens protein (Associated with type III secretion and virulence) | 2.90 | 1.6E-03 | | SL1344_2871 |
| A0A0H3NGZ8 | *invC* | Secretory apparatus ATP synthase (Associated with virulence) | 3.63 | 4.1E-05 | | SL1344_2873 |
| A0A0H3NF99 | *invB* | Chaperone protein for type III secretion system effectors | 3.47 | 3.0E-05 | | SL1344_2874 |
| A0A0H3NL68 | *invA* | Secretory apparatus of type III secretion system | 2.78 | 1.3E-04 | | SL1344_2875 |
| A0A0H3NFA2 | *invE* | Cell invasion protein | 3.68 | 2.8E-04 | | SL1344_2876 |
| A0A0H3NR16 | *invG* | Type III secretion system secretory apparatus | 3.30 | 6.3E-05 | | SL1344_2877 |
| A0A0H3NH00 | *invF* | AraC-family regulatory protein | 4.42 | 1.4E-02 | | SL1344_2878 |
| E1WAE4 | *invH* | Invasion lipoprotein | 2.33 | 2.7E-04 | | SL1344_2879 |
| A0A0H3N9B0 | *slrP* | Type III secretion system effector protein | 2.32 | 2.7E-04 | **T3SS-1 effectors** | SL1344_0776 |
| A0A0H3NJU0 | *gtgA* | Hypothetical bacteriophage encoded virulence protein | 1.55 | 1.2E-03 | | SL1344_0965 |
| A0A0H3NBV1 | *pipC* | Chaperone protein | 3.70 | 1.7E-04 | | SL1344_1029 |
| A0A0H3NA16 | *sopB* | Type III secretion system effector protein | 3.85 | 3.0E-05 | | SL1344_1030 |
| A0A0H3NDT2 | *sopE2* | Type III secretion system effector protein | 3.55 | 3.2E-04 | | SL1344_1784 |
| A0A0H3NIK3 | *sopA* | Type III secretion system effector protein | 3.56 | 3.0E-05 | | SL1344_2043 |
| A0A0H3NGI8 | *sopE* | Type III secretion system effector protein | 3.28 | 1.3E-04 | | SL1344_2674 |
| A0A0H3NH64 | *sopD* | Type III secretion system effector protein | 3.83 | 3.2E-05 | | SL1344_2924 |
| A0A0H3NKG5 | *siiA* | Type I secretion-related protein | 2.29 | 5.8E-04 | **SPI-4** | SL1344_4193 |
| A0A0H3NIH1 | *siiB* | Hypothetical integral membrane protein | 3.71 | 5.2E-05 | | SL1344_4194 |
| A0A0H3NPA2 | *siiC* | Hypothetical type-I secretion protein | 3.42 | 4.1E-05 | | SL1344_4195 |
| A0A0H3NIT9 | *siiD* | Membrane fusion protein (MFP) family protein | 3.85 | 1.1E-02 | | SL1344_4196 |
| A0A0H3NVH0 | *siiE* | Large repetitive protein | 2.84 | 2.8E-04 | | SL1344_4197 |
| A0A0H3NKH1 | *siiF* | Hypothetical type-1 secretion protein | 6.22 | 1.3E-02 | | SL1344_4198 |
| A0A0H3NK15 | *putP* | Sodium/proline symporter | 1.20 | 9.0E-03 | **Other membrane preoteins** | SL1344_1063 |
| A0A0H3NB04 | SL1344_1263 | Hypothetical outer membrane protein | 3.32 | 5.5E-05 | | SL1344_1263 |
| A0A0H3NLA5 | *hyaB2* | Uptake hydrogenase-1 large subunit | 0.92 | 4.0E-02 | | SL1344_1467 |
| A0A0H3NIX7 | SL1344_4247 | Hypothetical membrane protein | 4.27 | 1.3E-03 | | SL1344_4247 |
| A0A0H3NMH1 | SL1344_1867 | Hypothetical lipoprotein | 0.69 | 2.5E-02 | | SL1344_1867 |
| A0A0H3NHJ8 | *minE* | Cell division topological specificity factor | 0.66 | 4.0E-02 | **Cell division** | SL1344_1744 |
| A0A0H3NDZ0 | *dedD* | Cell division protein | 0.57 | 3.0E-02 | | SL1344_2333 |
| A0A0H3NIM6 | *rtsB* | Regulator of *flhDC* | 4.07 | 5.8E-04 | **Regulators** | SL1344_4250 |
| A0A0H3NPF5 | *rtsA* | Regulator of T3SS-1 | 2.16 | 8.1E-03 | | SL1344_4251 |
| A0A0H3N7D0 | *nhaR* | Transcriptional activator protein | 1.24 | 3.6E-03 | | SL1344_0041 |

| | | | | | | |
|---|---|---|---|---|---|---|
| A0A0H3NHT8 | *sinR* | LysR-family transcriptional regulator (SPI-6 associated) | 3.08 | 3.2E-02 | | SL1344_0300 |
| A0A0H3NKV1 | *ssrB* | Two-component response regulator | 0.98 | 3.6E-02 | | SL1344_1325 |
| A0A0H3NCU0 | *slyA* | Transcriptional regulator | 1.00 | 1.3E-02 | | SL1344_1376 |
| A0A0H3NDU3 | SL1344_2283 | Hypothetical receptor/regulator protein | 1.32 | 5.4E-03 | | SL1344_2283 |
| A0A0H3NBY6 | *phoH* | Phosphate starvation-inducible protein | 1.39 | 4.6E-02 | **Phosphate regulon** | SL1344_1064 |
| A0A0H3NIH0 | *lsrF* | 3-hydroxy-5-phosphonooxypentane-2,4-dione thiolase | 0.60 | 2.5E-02 | **Regulation, transport and modification of AI-2** | SL1344_4027 |
| A0A0H3NV05 | *lsrG* | (4S)-4-hydroxy-5-phosphonooxypentane-2,3-dione isomerase | 1.00 | 2.8E-02 | | SL1344_4028 |
| A0A0H3N8Z2 | *asnB* | Asparagine synthetase B | 0.91 | 1.0E-02 | **Other** | SL1344_0662 |
| A0A0H3NJJ2 | *ybjD* | Toprim domain-containing protein | 0.98 | 1.2E-02 | | SL1344_0878 |
| A0A0H3NJR9 | SL1344_0945 | Predicted bacteriophage protein | 1.45 | 4.2E-02 | | SL1344_0945 |
| A0A0H3NAR7 | SL1344_1177 | Predicted bacteriophage protein | 2.92 | 2.8E-04 | | SL1344_1177 |
| A0A0H3NCI0 | SL1344_1265 | Putative DNA/RNA non-specific endonuclease (Fragment) | 2.29 | 1.2E-04 | | SL1344_1265 |
| A0A0H3NBN9 | *hdeB* | Acid stress chaperone | 1.31 | 2.5E-02 | | SL1344_1492 |
| A0A0H3NBG4 | *fdnG* | Formate dehydrogenase N (Nitrate-inducible, alpha subunit) | 0.97 | 3.0E-02 | | SL1344_1500 |
| A0A0H3NE12 | *fadI* | 3-ketoacyl-CoA thiolase | 1.47 | 4.3E-02 | | SL1344_2358 |
| A0A0H3NK61 | SL1344_2438 | Uncharacterized protein | 0.86 | 4.2E-03 | | SL1344_2438 |
| A0A0H3NG39 | *ribB* | 3,4-dihydroxy-2-butanone 4-phosphate synthase | 0.69 | 2.5E-02 | | SL1344_3168 |
| A0A0H3NT12 | *pckA* | Phosphoenolpyruvate carboxykinase (ATP) | 0.97 | 2.5E-02 | | SL1344_3467 |
| A0A0H3NH73 | *yhjH* | EAL domain-containing protein | 1.43 | 2.5E-02 | | SL1344_3576 |
| A0A0H3NPB1 | *yjcE* | Hypothetical sodium/hydrogen exchanger family protein | 0.73 | 3.2E-02 | | SL1344_4205 |
| A0A0H3NVK9 | *adi* | Arginine decarboxylase | 1.21 | 4.0E-02 | | SL1344_4233 |
| **Lower production in GFP+ vs. GFP- cells** | | | | | | |
| **Uniprot Accession #** | **Gene_Name** | **Protein_Description** | **log2ratio** | **q-value** | **Function** | **Locus** |
| A0A0H3NN23 | *mgtB* | Magnesium transport ATPase, P-type 2 | -0.70 | 1.4E-02 | **Magnesium regulon** | SL1344_3728 |
| A0A0H3NHI8 | *mgtC* | Virulence factor required for growth in low Mg2+ | -0.93 | 2.6E-02 | | SL1344_3729 |
| A0A0H3NJN5 | *corA* | Magnesium transport protein | -0.55 | 2.9E-02 | | SL1344_3906 |
| A0A0H3NL17 | *mgtA* | Magnesium transport ATPase, P-type | -0.61 | 1.5E-02 | | SL1344_4387 |
| A0A0H3N8R3 | *phoR* | Phosphate regulon sensor protein | -0.92 | 5.4E-03 | **Phosphate regulon** | SL1344_0393 |
| A0A0H3NHQ1 | *phoU* | Phosphate-specific transport system accessory protein | -0.92 | 4.3E-03 | | SL1344_3820 |
| A0A0H3NU57 | *pstB* | Phosphate import ATP-binding protein | -0.99 | 1.6E-02 | | SL1344_3821 |
| A0A0H3NN94 | *pstS* | Phosphate-binding protein | -1.13 | 1.1E-03 | | SL1344_3824 |
| A0A0H3NCQ5 | *ftsL* | Cell division protein | -0.66 | 4.1E-02 | **Cell division** | SL1344_0121 |
| A0A0H3NAQ3 | *ycfS* | Hypothetical exported protein | -0.84 | 4.9E-02 | **Other** | SL1344_1152 |
| A0A0H3NB36 | *sufB* | Uncharacterized protein | -1.89 | 2.1E-03 | | SL1344_1304 |
| A0A0H3NG75 | orf319 | Hypothetical pathogenicity island protein | -0.77 | 9.4E-03 | | SL1344_1323 |

| | | | | | | |
|---|---|---|---|---|---|---|
| A0A0H3NBY2 | *oppA* | Periplasmic oligopeptide-binding protein | -0.54 | 2.5E-02 | | SL1344_1677 |
| A0A0H3NF20 | *yejG* | Uncharacterized protein | -1.48 | 4.2E-02 | | SL1344_2197 |
| A0A0H3NS69 | *garL* | 5-keto-4-deoxy-D-glucarate aldolase | -0.74 | 4.2E-02 | | SL1344_3222 |
| A0A0H3NH07 | *glpG* | Rhomboid protease | -0.84 | 3.6E-02 | | SL1344_3491 |
| A0A0H3NH34 | *ugpB* | Glycerol-3-phosphate-binding periplasmic protein | -0.91 | 2.0E-02 | | SL1344_3523 |
| A0A0H3NHW7 | *recQ* | ATP-dependent DNA helicase | -1.43 | 3.1E-02 | | SL1344_3912 |
| A0A0H3NUZ6 | *sbp* | Periplasmic sulphate binding protein | -0.65 | 3.9E-02 | | SL1344_4012 |
| A0A0H3NIP3 | *yjbB* | Hypothetical membrane protein | -0.90 | 2.2E-02 | | SL1344_4124 |

S2 Table. Statistical analysis Fig 1D and 1E and S2.

| **Without arabinose** | | | | | |
|---|---|---|---|---|---|
| **Comparison** | **p value** | **test** | **Rank** | **BH critical value** | **Significant?** |
| *ΔiagB-invG Δflg* ΔSPI-4 philA Ara- vs *ΔiagB-invG* philA Ara- | 0.012 | Mann-Whitney | 13 | 0.030 | Yes |
| *ΔiagB-invG Δflg* ΔSPI-4 philA Ara- vs *Δflg* philA Ara- | 0.016 | Mann-Whitney | 14 | 0.032 | Yes |
| *ΔiagB-invG Δflg* ΔSPI-4 philA Ara- vs ΔSPI-4 philA Ara- | 0.001 | Mann-Whitney | 10 | 0.023 | Yes |
| WT p*hilA* Ara- vs *Δflg* p*hilA* Ara- | <1E-06 | Mann-Whitney | 4 | 0.009 | Yes |
| WT p*hilA* Ara- vs *ΔhilD* p*hilA* Ara- | <1E-06 | Mann-Whitney | 1 | 0.002 | Yes |
| WT p*hilA* Ara- vs *ΔiagB-invG* p*hilA* Ara- | 0.116 | Mann-Whitney | 17 | 0.039 | No |
| WT p*hilA* Ara- vs *ΔiagB-invG Δflg* ΔSPI-4 p*hilA* Ara- | <1E-06 | Mann-Whitney | 2 | 0.005 | Yes |
| WT p*hilA* Ara- vs ΔSPI-1 p*hilA* Ara- | <1E-06 | Mann-Whitney | 3 | 0.007 | Yes |
| WT p*hilA* Ara- vs ΔSPI-4 p*hilA* Ara- | 0.547 | Mann-Whitney | 21 | 0.048 | No |
| *ΔhilD* pBAD24 Ara- vs *Δflg* pBAD24 Ara- | 0.041 | Mann-Whitney | 16 | 0.036 | No |
| *ΔhilD* pBAD24 Ara- vs *ΔiagB-invG* pBAD24 Ara- | 1.3E-05 | Mann-Whitney | 5 | 0.011 | Yes |
| *ΔhilD* pBAD24 Ara- vs *ΔiagB-invG Δflg* ΔSPI-4 pBAD24 Ara- | 0.945 | Mann-Whitney | 22 | 0.050 | No |
| *ΔhilD* pBAD24 Ara- vs ΔSPI-1 pBAD24 Ara- | 0.467 | Mann-Whitney | 20 | 0.045 | No |
| *ΔhilD* pBAD24 Ara- vs ΔSPI-4 pBAD24 Ara- | 0.002 | Mann-Whitney | 11 | 0.025 | Yes |
| *ΔhilD* philA Ara- vs *Δflg* philA Ara- | 0.002 | Mann-Whitney | 12 | 0.027 | Yes |
| *ΔhilD* philA Ara- vs *ΔiagB-invG* philA Ara- | 1.4E-04 | Mann-Whitney | 7 | 0.016 | Yes |
| *ΔhilD* philA Ara- vs *ΔiagB-invG Δflg* ΔSPI-4 philA Ara- | 0.447 | Mann-Whitney | 19 | 0.043 | No |
| *ΔhilD* philA Ara- vs ΔSPI-1 philA Ara- | 0.298 | Mann-Whitney | 18 | 0.041 | No |
| *ΔhilD* philA Ara- vs ΔSPI-4 philA Ara- | 1.5E-04 | Mann-Whitney | 8 | 0.018 | Yes |

| | | | | | |
|---|---|---|---|---|---|
| ΔiagB-invG Δflg ΔSPI-4 pBAD24 Ara- vs *Δflg* pBAD24 Ara- | 0.031 | Mann-Whitney | 15 | 0.034 | Yes |
| ΔiagB-invG Δflg ΔSPI-4 pBAD24 Ara- vs *ΔiagB-invG* pBAD24 Ara- | 6.9E-05 | Mann-Whitney | 6 | 0.014 | Yes |
| ΔiagB-invG Δflg ΔSPI-4 pBAD24 Ara- vs ΔSPI-4 pBAD24 Ara- | 0.001 | Mann-Whitney | 9 | 0.020 | Yes |
| WT pBAD24 Ara- vs WT philA Ara- | 0.353 | Wilcoxon | 7 | 0.050 | No |
| WT pBAD24 Ara- vs *Δflg*pBAD24 Ara- | 0.004 | Wilcoxon | 3 | 0.021 | Yes |
| WT pBAD24 Ara- vs *ΔhilD* pBAD24 Ara- | 1.2E-04 | Wilcoxon | 1 | 0.007 | Yes |
| WT pBAD24 Ara- vs *ΔiagB-invG* pBAD24 Ara- | 0.173 | Wilcoxon | 6 | 0.043 | No |
| WT pBAD24 Ara- vs *ΔiagB-invG Δflg* ΔSPI-4 pBAD24 Ara- | 0.016 | Wilcoxon | 4 | 0.029 | Yes |
| WT pBAD24 Ara- vs ΔSPI-1 pBAD24 Ara- | 2.4E-04 | Wilcoxon | 2 | 0.014 | Yes |
| WT pBAD24 Ara- vs ΔSPI-4 pBAD24 Ara- | 0.031 | Wilcoxon | 5 | 0.036 | Yes |

| **With arabinose** | | | | | |
|---|---|---|---|---|---|
| **Comparison** | **p value** | **test** | **Rank** | **BH critical value** | **Significant?** |
| *ΔiagB-invG Δflg* ΔSPI-4 philA Ara+ vs *ΔiagB-invG* philA Ara+ | 1.0E-04 | Mann-Whitney | 9 | 0.020 | Yes |
| *ΔiagB-invG Δflg* ΔSPI-4 philA Ara+ vs *Δflg* philA Ara+ | 0.154 | Mann-Whitney | 19 | 0.043 | No |
| *ΔiagB-invG Δflg* ΔSPI-4 philA Ara-+ vs ΔSPI-4 philA Ara+ | 0.001 | Mann-Whitney | 12 | 0.027 | Yes |
| WT p*hilA* Ara+ vs *Δflg* p*hilA* Ara+ | 0.009 | Mann-Whitney | 14 | 0.032 | Yes |
| WT p*hilA* Ara+ vs *ΔhilD* p*hilA* Ara+ | 1.7E-06 | Mann-Whitney | 3 | 0.007 | Yes |
| WT p*hilA* Ara+ vs *ΔiagB-invG* p*hilA* Ara-+ | 2.1E-05 | Mann-Whitney | 6 | 0.014 | Yes |
| WT p*hilA* Ara+ vs *ΔiagB-invG Δflg* ΔSPI-4 p*hilA* Ara+ | 1.0E-05 | Mann-Whitney | 4 | 0.009 | Yes |
| WT p*hilA* Ara+ vs ΔSPI-1 p*hilA* Ara+ | <1E-06 | Mann-Whitney | 1 | 0.002 | Yes |
| WT p*hilA* Ara+ vs ΔSPI-4 p*hilA* Ara+ | 0.053 | Mann-Whitney | 17 | 0.039 | No |
| *ΔhilD* pBAD24 Ara+ vs *Δflg* pBAD24 Ara+ | 0.060 | Mann-Whitney | 18 | 0.041 | No |
| *ΔhilD* pBAD24 Ara+ vs *ΔiagB-invG* pBAD24 Ara+ | 1.1E-05 | Mann-Whitney | 5 | 0.011 | Yes |
| *ΔhilD* pBAD24 Ara+ vs *ΔiagB-invG Δflg* ΔSPI-4 pBAD24 Ara+ | 0.244 | Mann-Whitney | 20 | 0.045 | No |
| *ΔhilD* pBAD24 Ara+ vs ΔSPI-1 pBAD24 Ara+ | 0.880 | Mann-Whitney | 22 | 0.050 | No |
| *ΔhilD* pBAD24 Ara+ vs ΔSPI-4 pBAD24 Ara+ | 1.5E-04 | Mann-Whitney | 10 | 0.023 | Yes |
| *ΔhilD* philA Ara+ vs *Δflg* philA Ara+ | 1.0E-06 | Mann-Whitney | 2 | 0.005 | Yes |
| *ΔhilD* philA Ara+ vs *ΔiagB-invG* philA Ara+ | 0.728 | Mann-Whitney | 21 | 0.048 | No |
| *ΔhilD* philA Ara+ vs *ΔiagB-invG Δflg* ΔSPI-4 philA Ara+ | 0.031 | Mann-Whitney | 16 | 0.036 | Yes |

| | | | | | |
|---|---|---|---|---|---|
| *ΔhilD* philA Ara+ vs ΔSPI-1 philA Ara+ | 6.9E-05 | Mann-Whitney | 8 | 0.018 | Yes |
| *ΔhilD* philA Ara+ vs ΔSPI-4 philA Ara+ | 5.2E-05 | Mann-Whitney | 7 | 0.016 | Yes |
| ΔiagB-invG Δflg ΔSPI-4 pBAD24 Ara+ vs *Δflg* pBAD24 Ara+ | 0.026 | Mann-Whitney | 15 | 0.034 | Yes |
| ΔiagB-invG Δflg ΔSPI-4 pBAD24 Ara+ vs *ΔiagB-invG* pBAD24 Ara- | 1.9E-04 | Mann-Whitney | 11 | 0.025 | Yes |
| ΔiagB-invG Δflg ΔSPI-4 pBAD24 Ara- vs ΔSPI-4 pBAD24 Ara+ | 0.002 | Mann-Whitney | 13 | 0.030 | Yes |
| WT pBAD24 Ara+ vs WT philA Ara+ | <1E-06 | Wilcoxon | 1 | 0.007 | yes |
| WT pBAD24 Ara+ vs *Δflg*pBAD24 Ara+ | 0.004 | Wilcoxon | 4 | 0.029 | yes |
| WT pBAD24 Ara+ vs *ΔhilD* pBAD24 Ara+ | 2.4E-04 | Wilcoxon | 2 | 0.014 | yes |
| WT pBAD24 Ara+ vs *ΔiagB-invG* pBAD24 Ara+ | 0.375 | Wilcoxon | 7 | 0.050 | No |
| WT pBAD24 Ara+ vs *ΔiagB-invG Δflg* ΔSPI-4 pBAD24 Ara+ | 0.031 | Wilcoxon | 5 | 0.036 | Yes |
| WT pBAD24 Ara+ vs ΔSPI-1 pBAD24 Ara+ | 2.4E-04 | Wilcoxon | 3 | 0.021 | yes |
| WT pBAD24 Ara+ vs ΔSPI-4 pBAD24 Ara+ | 0.031 | Wilcoxon | 6 | 0.043 | yes |

S3 Table. Parameters for estimations of death rates.

| | **WT P*prgH*::*gfp*** | **Δ*hilE* P*prgH*::*gfp*** | **Δ*hilD* P*prgH*::*gfp*** |
|---|---|---|---|
| % ON initial (30' in distilled water)* | 27.5 | 46 | 0 |
| %dead cells after lethal TE treatment* | 26 | 33 | 6 |
| Difference % ON TE vs Water* | -6.9 | -5.2 | n.d. |
| % ON final (30' lethal TE treatment) | 20.6 | 40.8 | n.d. |
| **Death rate ON cells in TE (%)** | **44.6** | **40.6** | n.d. |
| **Death rate OFF cells in TE (%)** | **19.0** | **26.5** | **6** |
| *Median values from flow cytometry experiments | | | |

S4 Table. CFU counts after lethal TE treatment.

| | | Biological replicate #1 | | | Biological replicate #2 | | |
|---|---|---|---|---|---|---|---|
| | | Sytox Blue* | PI* | Plating** | Sytox Blue* | PI* | Plating** |
| 100 mM Tris- 10 mM EDTA (lethal TE treatment) | WT | 24 | 22 | 63 | 29 | 31 | 55 |
| | *ΔhilD* | 8 | 8 | 44 | 10 | 9 | 17 |
| | *ΔhilE* | 33 | 23 | 70 | 35 | 33 | 71 |
| * **Sytox Blue/PI** : % of dead cell = % of stained cells after TE treatment | | | | | | | |
| ** **Plating**: % of dead cell = ((CFU no stress - CFU TE) / CFU no stress)*100 | | | | | | | |

S5 Table. Statistical analysis (Fig 2H and 2I and S9 Fig).

| Without arabinose | | | | | |
|---|---|---|---|---|---|
| **Comparison** | **p value** | **test** | **Rank** | **BH critical value** | **Significant?** |
| *ΔiagB-invG Δflg* ΔSPI-4 philA Ara- vs *ΔiagB-invG* philA Ara- | 0.021 | Mann-Whitney | 17 | 0.0386 | Yes |
| *ΔiagB-invG Δflg* ΔSPI-4 philA Ara- vs *Δflg* philA Ara- | 0.041 | Mann-Whitney | 14 | 0.0318 | Yes |
| *ΔiagB-invG Δflg* ΔSPI-4 philA Ara- vs ΔSPI-4 philA Ara- | <1E-06 | Mann-Whitney | 11 | 0.0250 | Yes |
| WT p*hilA* Ara- vs *Δflg* p*hilA* Ara- | <1E-06 | Mann-Whitney | 18 | 0.0409 | Yes |
| WT p*hilA* Ara- vs *ΔhilD* p*hilA* Ara- | <1E-06 | Mann-Whitney | 2 | 0.0045 | Yes |
| WT p*hilA* Ara- vs *ΔiagB-invG* p*hilA* Ara- | 0.003 | Mann-Whitney | 3 | 0.0068 | Yes |
| WT p*hilA* Ara- vs *ΔiagB-invG Δflg* ΔSPI-4 p*hilA* Ara- | <1E-06 | Mann-Whitney | 15 | 0.0341 | Yes |
| WT p*hilA* Ara- vs ΔSPI-1 p*hilA* Ara- | <1E-06 | Mann-Whitney | 22 | 0.0500 | No |
| WT p*hilA* Ara- vs ΔSPI-4 p*hilA* Ara- | 0.007 | Mann-Whitney | 4 | 0.0091 | Yes |
| *ΔhilD* pBAD24 Ara- vs *Δflg* pBAD24 Ara- | <1E-06 | Mann-Whitney | 5 | 0.0114 | Yes |
| *ΔhilD* pBAD24 Ara- vs *ΔiagB-invG* pBAD24 Ara- | <1E-06 | Mann-Whitney | 6 | 0.0136 | Yes |
| *ΔhilD* pBAD24 Ara- vs *ΔiagB-invG Δflg* ΔSPI-4 pBAD24 Ara- | 1.2E-06 | Mann-Whitney | 7 | 0.0159 | Yes |
| *ΔhilD* pBAD24 Ara- vs ΔSPI-1 pBAD24 Ara- | 0.257 | Mann-Whitney | 21 | 0.0477 | No |
| *ΔhilD* pBAD24 Ara- vs ΔSPI-4 pBAD24 Ara- | <1E-06 | Mann-Whitney | 8 | 0.0182 | Yes |
| *ΔhilD* philA Ara- vs *Δflg* philA Ara- | <1E-06 | Mann-Whitney | 16 | 0.0364 | Yes |
| *ΔhilD* philA Ara- vs *ΔiagB-invG* philA Ara- | <1E-06 | Mann-Whitney | 9 | 0.0205 | Yes |
| *ΔhilD* philA Ara- vs *ΔiagB-invG Δflg* ΔSPI-4 philA Ara- | <1E-06 | Mann-Whitney | 10 | 0.0227 | Yes |
| *ΔhilD* philA Ara- vs ΔSPI-1 philA Ara- | 0.229 | Mann-Whitney | 17 | 0.0386 | Yes |
| *ΔhilD* philA Ara- vs ΔSPI-4 philA Ara- | <1E-06 | Mann-Whitney | 14 | 0.0318 | Yes |
| ΔiagB-invG Δflg ΔSPI-4 pBAD24 Ara- vs *Δflg* pBAD24 Ara- | 0.003 | Mann-Whitney | 11 | 0.0250 | Yes |
| ΔiagB-invG Δflg ΔSPI-4 pBAD24 Ara- vs *ΔiagB-invG* pBAD24 Ara- | <1E-06 | Mann-Whitney | 18 | 0.0409 | Yes |
| ΔiagB-invG Δflg ΔSPI-4 pBAD24 Ara- vs ΔSPI-4 pBAD24 Ara- | <1E-06 | Mann-Whitney | 2 | 0.0045 | Yes |
| WT pBAD24 Ara- vs WT philA Ara- | 5.9E-05 | Wilcoxon | 2 | 0.0143 | Yes |
| WT pBAD24 Ara- vs *Δflg*pBAD24 Ara- | 4.9E-04 | Wilcoxon | 4 | 0.0286 | Yes |
| WT pBAD24 Ara- vs *ΔhilD* pBAD24 Ara- | <1E-06 | Wilcoxon | 1 | 0.0071 | Yes |
| WT pBAD24 Ara- vs *ΔiagB-invG* pBAD24 Ara- | 0.260 | Wilcoxon | 7 | 0.0500 | No |

| | | | | | |
|---|---|---|---|---|---|
| WT pBAD24 Ara-<br>vs *ΔiagB-invG Δflg* ΔSPI-4 pBAD24 Ara- | 1.2E-04 | Wilcoxon | 3 | 0.0214 | Yes |
| WT pBAD24 Ara- vs ΔSPI-1 pBAD24 Ara- | 0.063 | Wilcoxon | 6 | 0.0429 | No |
| WT pBAD24 Ara- vs ΔSPI-4 pBAD24 Ara- | 0.017 | Wilcoxon | 5 | 0.0357 | Yes |

| **With arabinose** | | | | | |
|---|---|---|---|---|---|
| **Comparison** | **p value** | **test** | **Rank** | **BH critical value** | **Significant?** |
| *ΔiagB-invG Δflg* ΔSPI-4 philA Ara+<br>vs *ΔiagB-invG* philA Ara+ | <1E-06 | Mann-Whitney | 9 | 0.0205 | Yes |
| *ΔiagB-invG Δflg* ΔSPI-4 philA Ara+<br>vs *Δflg* philA Ara+ | 0.001 | Mann-Whitney | 17 | 0.0386 | Yes |
| *ΔiagB-invG Δflg* ΔSPI-4 philA Ara-+<br>vs ΔSPI-4 philA Ara+ | 4.2E-05 | Mann-Whitney | 15 | 0.0341 | Yes |
| WT p*hilA* Ara+ vs *Δflg* p*hilA* Ara+ | 0.194 | Mann-Whitney | 20 | 0.0455 | No |
| WT p*hilA* Ara+ vs *ΔhilD* p*hilA* Ara+ | <1E-06 | Mann-Whitney | 1 | 0.0023 | Yes |
| WT p*hilA* Ara+ vs *ΔiagB-invG* p*hilA* Ara-+ | 1.1E-04 | Mann-Whitney | 16 | 0.0364 | Yes |
| WT p*hilA* Ara+<br>vs *ΔiagB-invG Δflg* ΔSPI-4 p*hilA* Ara+ | <1E-06 | Mann-Whitney | 8 | 0.0182 | Yes |
| WT p*hilA* Ara+ vs ΔSPI-1 p*hilA* Ara+ | 2.9E-06 | Mann-Whitney | 12 | 0.0273 | Yes |
| WT p*hilA* Ara+ vs ΔSPI-4 p*hilA* Ara+ | 0.376 | Mann-Whitney | 21 | 0.0477 | No |
| *ΔhilD* pBAD24 Ara+ vs *Δflg* pBAD24 Ara+ | <1E-06 | Mann-Whitney | 2 | 0.0045 | Yes |
| *ΔhilD* pBAD24 Ara+ vs *ΔiagB-invG* pBAD24 Ara+ | <1E-06 | Mann-Whitney | 3 | 0.0068 | Yes |
| *ΔhilD* pBAD24 Ara+<br>vs *ΔiagB-invG Δflg* ΔSPI-4 pBAD24 Ara+ | <1E-06 | Mann-Whitney | 4 | 0.0091 | Yes |
| *ΔhilD* pBAD24 Ara+ vs ΔSPI-1 pBAD24 Ara+ | 0.009 | Mann-Whitney | 19 | 0.0432 | Yes |
| *ΔhilD* pBAD24 Ara+ vs ΔSPI-4 pBAD24 Ara+ | <1E-06 | Mann-Whitney | 5 | 0.0114 | Yes |
| *ΔhilD* philA Ara+ vs *Δflg* philA Ara+ | <1E-06 | Mann-Whitney | 11 | 0.0250 | Yes |
| *ΔhilD* philA Ara+ vs *ΔiagB-invG* philA Ara+ | 0.002 | Mann-Whitney | 18 | 0.0409 | Yes |
| *ΔhilD* philA Ara+<br>vs *ΔiagB-invG Δflg* ΔSPI-4 philA Ara+ | 0.585 | Mann-Whitney | 22 | 0.0500 | No |
| *ΔhilD* philA Ara+ vs ΔSPI-1 philA Ara+ | 1.2E-05 | Mann-Whitney | 13 | 0.0295 | Yes |
| *ΔhilD* philA Ara+ vs ΔSPI-4 philA Ara+ | 3.2E-05 | Mann-Whitney | 14 | 0.0318 | Yes |
| ΔiagB-invG Δflg ΔSPI-4 pBAD24 Ara+<br>vs *Δflg* pBAD24 Ara+ | <1E-06 | Mann-Whitney | 10 | 0.0227 | Yes |
| ΔiagB-invG Δflg ΔSPI-4 pBAD24 Ara+<br>vs *ΔiagB-invG* pBAD24 Ara- | <1E-06 | Mann-Whitney | 6 | 0.0136 | Yes |
| ΔiagB-invG Δflg ΔSPI-4 pBAD24 Ara-<br>vs ΔSPI-4 pBAD24 Ara+ | <1E-06 | Mann-Whitney | 7 | 0.0159 | Yes |
| WT pBAD24 Ara+ vs WT philA Ara+ | <1E-06 | Wilcoxon | 1 | 0.0071 | Yes |
| WT pBAD24 Ara+ vs *Δflg*pBAD24 Ara+ | 4.9E-04 | Wilcoxon | 4 | 0.0286 | Yes |

| | | | | | |
|---|---|---|---|---|---|
| WT pBAD24 Ara+ vs *ΔhilD* pBAD24 Ara+ | <1E-06 | Wilcoxon | 2 | 0.0143 | Yes |
| WT pBAD24 Ara+ vs *ΔiagB-invG* pBAD24 Ara+ | 1.3E-03 | Wilcoxon | 5 | 0.0357 | Yes |
| WT pBAD24 Ara+ vs *ΔiagB-invG Δflg* ΔSPI-4 pBAD24 Ara+ | 2.4E-04 | Wilcoxon | 3 | 0.0214 | Yes |
| WT pBAD24 Ara+ vs ΔSPI-1 pBAD24 Ara+ | 0.063 | Wilcoxon | 6 | 0.0429 | No |
| WT pBAD24 Ara+ vs ΔSPI-4 pBAD24 Ara+ | 0.587 | Wilcoxon | 7 | 0.0500 | No |

S6 Table. Strains and plasmids used in this study.

| Name | Relevant genotype | Background | Collection # | Reference |
|---|---|---|---|---|
| WT | SL1344 | SL1344 (SB300) | MDBZ0000 | (Hoiseth & Stocker, 1981) |
| WT$^{aphT}$ | *aphT* | | MDBZ0357 | (Grant et al., 2008) |
| *ΔhilD* | *ΔhilD* | | MDBZ0011 | (Sturm et al., 2011) |
| *ΔhilD$^{cat}$* | *hilD::cat* | | MDBZ0015 | (Sturm et al., 2011) |
| *ΔhilE* | *ΔhilE* | | MDBZ0012 | (Sturm et al., 2011) |
| WT P*prgH::gfp* | PprgH::*gfp cat* | | MSBZ0092 | (Sturm et al., 2011) |
| *ΔhilD* P*prgH::gfp* | *ΔhilD,* P*prgH::gfp cat* | | MSBZ0093 | (Sturm et al., 2011) |
| *ΔhilE* P*prgH::gfp* | *ΔhilE,* P*prgH::gfp cat* | | MSBZ0094 | (Sturm et al., 2011) |
| ΔSPI-1 | ΔSPI-1::*cat* | | MSBZ0098 | (Diard et al., 2013) |
| *ΔinvG* | *ΔinvG* | | MSBZ0252 | This study |
| Δ*iagB-invG* | Δ*iagB-invG*::*cat* | | MSBZ0234 | This study |
| Δ*flg* | Δ*flg*::*cat* | | MSBZ0242 | This study |
| ΔSPI-4 | ΔSPI-4::*cat* | | MSBZ0143 | This study |
| Δ*iagB-invG* Δ*flg* ΔSPI-4 | *ΔiagB-invG Δflg*::*aphT* ΔSPI-4::*cat* | | MSBZ0397 | This study |
| Triple Mutant $^{aphT}$ | *ΔiagB-invG Δflg* ΔSPI-4 *aphT* | | MDBZ0924 | This study |
| Triple Mutant *hilD$^{cat}$* | *ΔiagB-invG Δflg* ΔSPI-4 *hilD*::*cat* | | MDBZ0926 | This study |
| Avir helper | Δ*invG* Δ*ssaV* | | MDBZ0811 | This study |
| Avir$^{aphT}$ | Δ*invG* Δ*ssaV aphT* | | MSBZ0965 | This study |
| Avir Δ*hilD$^{cat}$* | Δ*invG* Δ*ssaV hilD::cat* | | MSBZ0967 | This study |
| Avir$^{aphT}$ Triple Mutant | Δ*invG* Δ*ssaV* Δ*iagB-invG* Δ*flg* ΔSPI-4 *aphT* | | MSBZ0974 | This study |
| Avir *hilD$^{cat}$* Triple Mutant | Δ*invG* Δ*ssaV* Δ*iagB-invG* Δ*flg* ΔSPI-4 *hilD::cat* | | MSBZ0975 | This study |

| Plasmid | Relevant genetic features | Resistance | Reference |
|---|---|---|---|
| pM965 | P*rpsM*::*gfp bla* | Ampicillin | (Stecher et al., 2004) |
| pBAD24 | *bla* | Ampicillin | (Guzman et al., 1995) |
| philA | P*bad*::*hilA bla* | Ampicillin | (Lostroh et al., 2000) |
| pKD46 | P*bad red, gam, exo bla* | Ampicillin | (Datsenko & Wanner, 2000) |
| pKD3 | *cat* | Chloramphenicol | (Datsenko & Wanner, 2000) |
| pKD4 | *aphT* | Kanamycin | (Datsenko & Wanner, 2000) |
| pCP20 | *flp, bla* | Ampicillin | (Datsenko & Wanner, 2000) |

S7 Table. Primers used in this study.

| Name | Sequence |
|---|---|
| Del_SPI4_up | ATATCAGGAGACAACATGGAAGACGAAAGTAATCCGTGGCTGTGTAGGCTGGAGCTGCTTC |
| Del_SPI4_dw | CAAGCGCTGCTTATTTTACATTAATAATTTATCCGGAGAACATATGAATATCCTCCTTAG |
| Ver_SPI4_up | GCGGTAGCGTTCACTTTC |
| Ver_SPI4_dw | AAGCAGTACCACCTGATAAC |
| Del_flg_up | CATTTGCGGAGGAGATATGCTCGACAGGCTCGATGCCGCCTTTGTGTAGGCTGGAGCTGCTTC |
| Del_flg_up | GGCAGCGACTACGTGGACTTGAGCAATTTAAAAGAGATTGTCCATATGAATATCCTCCTTAG |
| Ver_flg_for | TGAACGGAGCGTGACAAC |
| Ver_flg_rev | CGTAGCTGATTAGTGATAAAC |
| Del_iagB-invG_up | TCTGAGAGAGGAGATATGCATTATTTTTTTATCATCGTAATGTGTAGGCTGGAGCTGCTTC |
| Del_iagB-invG_dw | TCAATTGGCAGACAAATGAAGACACATATTCTTTTGGCCACATATGAATATCCTCCTTAG |
| Ver iagB up | ATTACCGTTAGTGCTGGTTG |
| Ver invG up | TTCGCCGCGGAAATTATCAA |
| Ver invG dw | ATGTCTGCCGGGACAATATT |
| Ver_SPI1_up | GTCTGTCAGTTATTACCGC |
| Ver_SPI1_dw | GGAATTGGTTCAGATCGGC |
| | |
| Ver_WITS13 | GCTAAAGACACCCCTCACTCA |
| Ver_ygdA | GGCTGTCCGCAATGGGTC |
| Ver_hilD_up | TCTCGATAGCAGCAGATTAC |
| Ver_hilD_dw | CAGTATAAGCTGTCTTCCG |
| Ver_hilE_up | GGTTGTTTTCGATATGACAAAAG |
| Ver_hilE_dw | CATGCTGTACGATCGGGAA |
| Ver_ssaV_up | GTCAGATACTCAAGCAAACCTTCTTAGACGAAG |
| Ver_ssaV_dw | GTAAAAGGAGATAGCAAAGCTTTGCTGCCATTAATC |

Instances of infectious diseases have been documented throughout history and are an integral part of the human existence (Nelson & Williams, 1928). For most of the time, the cause of emerging diseases was not understood and often attributed to unnatural causes. However, many centuries of scientific advancement and more careful patient observation brought understanding of the infection processes of many diseases. Moreover, a milestone discovery gave hope for a universal treatment of bacterial derived diseases. Series of experiments carried in the 1930s and 1940s by Alexander Fleming, Howard Florey and Ernst Chain at the Oxford University demonstrated that a mold produced penicillin can inhibit growth of many pathogenic bacteria (Fleming, 1929). The following decades can be described as the golden era of antibiotics discovery. Many people believed that total eradication of pathogenic bacteria is possible, and horrors of infectious diseases will become a vague memory known only from history textbooks. Despite warnings of Fleming, antibiotics became used on enormous scale not only in human health sector but also in agriculture, often in uncontrolled way. Such misuse led to a rapid spread of antibiotic resistance. Soon researchers realized that observing drug resistance is just a matter of time after a new substance was introduced. The quest for finding new antibiotics became a desperate race against rapidly evolving bacteria. Pathogens resistant to multiple drugs became a serious threat to public health as they are very challenging and extremely expensive to treat. Rising concern of emergence of pan-resistant bacterial strains (pathogens resistant to all available antibiotics) will turn many infections untreatable. Based on predictions of the World Health Organization (WHO) by the year 2050 the major cause of death will be due to untreatable infections (O'Neill report from 2016). Such dramatic turn of events in less than a century since discovery of penicillin, sets researchers on a challenging quest for alternative therapies, shifting away from antibiotic treatment. The driving force behind antibiotic resistance emergence is evolution. Every time a pathogen is exposed to a new drug, the selective pressure drives emergence of resistant mutants. Acquired mutations can render sensitive strain resistant in multiple ways (Blair et al., 2015). Instead of working against the evolutionary forces, perhaps it is time to better understand them and design novel strategies that favor desirable evolutionary outcomes. Knowledge gained by asking fundamental questions about what drives the emergence of virulence in the first place, can give rise to new ways of fighting resistant pathogens.

A novel direction in design of therapeutical treatment is brought by the so-called anti-virulence therapies. These approaches rely on the hypothesis that since anti-virulence drugs aim to

disarm pathogen rather than kill it, these drugs should generate much weaker selection for resistance than traditional antibiotics. However, the resistance to anti-virulence drugs has been already reported for some anti-virulence treatments (Maeda et al., 2012; Smith et al., 2012). Resistance to ant-virulence drugs can be defined as recovery of VF expression after treatment (Allen et al., 2014). Unlike in case of traditional antibiotics where acquired resistance is always beneficial for pathogen (prevents killing by antibiotic) the resistance against anti-virulence drugs is much more nuanced. If the resistance to anti-virulence treatment evolves, it is not always easy to predict if resistant mutants will be counter selected, or rather spread and become a clinical problem. The selection of which VF to target is of crucial importance for the effectiveness of the strategy. To address these problems, we must first understand the consequences of VF expression for the pathogen fitness. Lack of research focus on the cost and benefits of the VF expression in different conditions results in a deep knowledge gap which we would like to address in our research.

Studies of infection processes often focus on the interplay between pathogens virulence and host defense. However, how expression of broad repertoire of VFs by bacteria affects their own ability to withstand hostile environment conditions remains poorly understood. In *S*. Tm the cost of virulence expression drives the emergence of attenuated mutants harboring mutations in genes encoding positive regulators of virulence in the chronic mice infection (Diard et al., 2013). Moreover, virulence attenuated mutants have been identified in a *S.* Tm population genomics analysis of patient isolates (Marzel et al., 2016) and in farming animals (Tambassi et al., 2020). Until now, the only described cost of virulence in *S*. Tm was a two-fold reduction of the growth rate in cells expressing the HilD regulon (Sturm et al., 2011). Growing evidence suggests that likely several components are contributing to observed growth retardation. In their study Sturm et al., constructed a mutant lacking a T3SS-1 translocon and several effectors, which they found significantly increased growth rate of virulent *S*. Tm cells. It is estimated for a virulent *S.* Tm cell to express between 20-200 T3SS-1 apparatuses (Schlumberger et al., 2005) and about 3-10x $10^4$ effector proteins (Winnen et al., 2008) amounting to a large fraction of the overall *S*. Tm proteome. However, the observed growth rate of the mutant with deleted translocon and effectors remained lower than the growth rate of phenotypically avirulent cells (Sturm et al., 2011) suggesting contribution of other factors to observed growth retardation. Another single cell level study by Sanchez-Romero found flagella to be a contributing factor to growth rate defect in *S*. Tm cells which express it (Sánchez-Romero & Casadesús, 2021). These results suggest that the retarded growth of phenotypically virulent *S*. Tm cells is most likely multifactorial and possibly can be contributed

to several HilD-regulated functions. The *S.* Tm virulence expression being embedded inside the complex regulatory network, might be reminiscence of the *prf* virulence regulon of *Listeria monocytogenes* coordinating metabolism and virulence expression (Bruno & Freitag, 2010). Interestingly in *L. monocytogenes* the PrfA regulated virulence genes were found to significantly impair the growth rate of bacteria expressing them (Vasanthakrishnan et al., 2015). The comprehensive reporter study monitoring expression of individual VFs like T3SS-1, flagella, and SPI-4 encoded adhesin, on a single cell level would be of a great value to our understanding of contribution of individual VFs to *S.* Tm virulence. Results presented in this thesis can deepen the understanding of *S.* Tm virulence as a costly trait for the individual cells by investigating the stress resistance of virulent cells.

Virulence expression in *S.* Tm can be downregulated as a result of exposure to stress conditions destabilizing membrane homeostasis such as heat (Sirsat et al., 2011), CAMPs (Bader et al., 2003), bile (Hernández et al., 2012) or protein misfolding (Palmer & Slauch, 2020). We hypothesized that the membrane of VFs expressing cells is intrinsically unstable, thus rendering virulent cells more prone to the effect of membrane targeting stress conditions. In this study we used the chromosomal P*prgH:gfp* reporter inserted in the locus *putP* (Hautefort et al., 2003), to monitor virulence expression. To confirm that this reporter is a good proxy for expression of the entire HilD regulon we separated green fluorescent protein (GFP) expressing subpopulation from the non-expressing cells using fluorescence activated cell sorting and compared the proteome profile of these subpopulations. The results indicated the co-expression of the T3SS-1 together with flagella, chemotaxis and SPI-4 encoded adhesin, confirming previously published results (Petrone et al., 2014; Martínez-Flores et al., 2016). However, one needs to keep in mind this might not be true for all cells. The recent study by Sanchez-Romero employed a dual reporter system to simultaneously monitor the ON/OFF state of both flagella and SPI-1 using time lapse microscopy (Sánchez-Romero & Casadesús, 2021). Authors showed that in fact, SPI-1 and flagellar regulons display independent switching, forming four distinct subpopulations: SPI-1-ON and flagella ON; SPI-1 ON and flagella OFF; SPI-1 OFF and flagella ON; and finally, SPI-1 OFF and flagella OFF. Therefore, it is important to keep in mind that in our study we did not independently monitor other HilD-regulated functions using independent reporters, but rather used the P*prgH:gfp* reporter as a proxy for the entire HilD regulon. Notably, the mutants emerging in the *in vivo* experiments (Diard et al., 2013) and in patient isolates (Marzel et al., 2016) were found to be *hilD* or other virulence regulation mutants. If T3SS-1 gene expression was the main cost, the emergence of *hilA* mutants would be expected, similarly if flagella was the main contributor, for example

*flhDC* mutants would be expected. Therefore, it is likely that during infection, *S.* Tm expresses several components of the HilD regulon in the same cell. This suggests that the fitness cost of virulence should be contributed to the entire HilD regulon collectively, rather than individual HilD-regulated functions.

In our study we describe a novel factor contributing to the cost of virulence in *S.* Tm. We discovered that cells expressing HilD regulon show increased membrane permeability to the hydrophobic compound NPN. This compound shows weak fluorescence in the aqueous environments but gives a strong signal in hydrophobic environments such as the inner leaflet of the outer membrane and the inner membrane (Tsuchido et al., 1989). In growth conditions triggering HilD regulon expression (i.e., late exponential phase in lysogeny broth (LB)) we observed that WT and Δ*hilE* mutants had significantly higher NPN signal than Δ*hilD* strain, suggesting that membrane of cells expressing HilD regulon is more permeable. This observation could have interesting implications in therapy. Perhaps certain drug candidates could penetrate the membrane of these phenotypically virulent cells more easily than membranes of cells which are not expressing VFs. This would allow to target virulent subpopulation with higher efficiency in the potential treatment.

To evaluate the relative contribution of different HilD-regulated functions, we tested the deletion mutants of T3SS-1 components (*iagB-invG*), *flg*, and SPI-4. The first mutant is lacking majority of structural components of T3SS-1, however, the SPI-1 encoded regulators: *hilA, hilC* and *hilD* remain intact. Presence of these regulators ensures undisturbed functioning of other HilD/HilA co-regulated functions. The *flg* mutant is lacking the *flgBCDEFGHIJ* operon. These genes are under control of the class 2 promoter in the hierarchical regulatory cascade of flagellar gene expression (Chilcott & Hughes, 2000). The expression of flagellar master regulators FlhDC is affected by the binding of HilD to the P5 promoter site preceding *flhDC* (Singer et al., 2014). Additionally, the FliZ protein, expression of which is under control of class 2 promoter, is activating HilD on the post-transcriptional level. However, to the best of our knowledge, the *flgBCDEFGHIJ* genes, which we deleted, do not participate in the cross talk between SPI-1 and flagella. For this reason, we decided to delete these genes, as their deletion should not impact the expression of SPI-1 genes. The SPI-4 mutant lacks the giant adhesin important for *S.* Tm attachment to the epithelial cells (Barlag & Hensel, 2015). In the proteomic analysis comparing the P*prgH:gfp* expressing cells with their non-expressing counterparts in the WT *S.* Tm, we found that both SPI-1 and SPI-4 proteins were abundantly expressed in the GFP positive cells. This could suggest that these proteins constitute a large

fraction of the proteome of these cells. However, our results indicated that individual deletions of either *iagB-invG* or SPI-4, were not sufficient to rescue the membrane permeability to the level of Δ*hilD* mutant. It is possible that deletion of T3SS-1 translocated effectors in addition to *iagB-invG* would improve the membrane integrity. With the structural components of T3SS-1 deleted, the effector proteins can accumulate inside the cells potentially disturbing the membrane homeostasis. Interestingly, deletion of *flg* did reduce the membrane permeability to the level of Δ*hilD* strain. It has been described for *Pseudomonas putida* that deletion of flagella released a metabolic burden from the bacterium and improved its tolerance to certain stresses (Martínez-García et al., 2014). Moreover, in *E. coli* MG1655, a strain which does not possess a *bona fide* T3SS; expression of flagella was found to increase the intrinsic death rate (Fontaine et al., 2008). In their recent study Lyu et al. hypothesized that since flagellar rotation and the efflux pumps are the two major processes relying on the proton motive force (PMF) as a driving force, the production of flagella would deplete the PMF energizing the efflux (Lyu et al., 2021). Authors showed that the flagella expressing cells were more sensitive to the ciprofloxacin, and they proposed that due to the cost of the PMF to drive flagellar rotation, the motile cells were less capable of employing efflux to remove intracellular antibiotics. In this view, expression of flagella could render cells more sensitive to certain stress conditions such as presence of antibiotics. As flagella expression was shown to partake in the cost of growth retardation in cells which express it (Sánchez-Romero & Casadesús, 2021) it cannot be excluded that it also impacts the membrane integrity. In the conditions applied in our study, the cost of flagellar expression outweighed the cost of expression of either T3SS-1 or SPI-4 adhesin. The cumulative triple mutant *iagB-invG flg* SPI-4 also showed reduced membrane permeability on the level of avirulent *hilD* mutant. The triple mutant displayed even lower membrane permeability than the *flg* mutant suggesting the cumulative contribution of these HilD regulated functions to membrane integrity.

We further evaluated the constructed mutants using overexpression of HilA from the arabinose induced promoter on the multi copy plasmid. HilA is a positive regulator of virulence and impacts expression of T3SS-1 and SPI-4. In WT overexpressing HilA, we observed increased membrane permeability **(Fig. 8)**, which was rescued by deletion of *iagB-invG.* In this condition the overexpression of T3SS-1 could lead to accumulation of mislocalized secretin proteins. It was reported for *Yersinia enterocolitica* and *E. coli* mutants lacking functional phage-shock protein (Psp), that overexpression of secretin can lead to membrane disruption (Horstman & Darwin, 2012, Jovanovic et al., 2009). The Psp response is triggered upon aberrant localization of outer membrane secretin proteins or other conditions disrupting the inner

membrane like disruption of the PMF (Flores-Kim & Darwin, 2014; Darwin, 2005). Mislocalized secretins can insert into the inner membrane and form an aberrant pores which impact the PMF (Guilvout et al., 2006). Upon overexpression of HilA, the *flg* deletion did not lower the membrane permeability. The possible explanation is that in these conditions, HilA represses flagella expression by inhibition of *flhD* (Thijs et al., 2007). In other tested conditions (empty vector pBAD24 with addition of arabinose or uninduced p*hilA*) we observed a reproducible pattern of NPN signal between tested mutants, confirming flagella to be the most important contributor to membrane permeability. The increased NPN signal in HilD expressing cells suggested that the membrane of these cells can be disrupted, potentially rendering these cells more sensitive to the stress conditions targeting the membrane. Uncovering the mechanistic basis underlying membrane defects in phenotypically virulent subpopulation would be of a great interest to establish.

In their recent study Hockenberry et al., using the P*prgH:gfp* reporter *S.* Tm strain, investigated the membrane potential using fluorescent PMF indicator Mitoview. The authors observed that P*prgH:gfp* + cells maintain a higher membrane potential than the P*prgH:gfp* – counterparts in log phase growth (Hockenberry et al., 2021). However, we were not able to reproduce a similar result. We measured the membrane potential in the absence of stress condition using 3,3'-Dipropylthiadicarbocyanine Iodide ($DiSC_3(5)$), a red fluorescent hydrophobic probe accumulating in the polarized cell membranes. We observed no differences in $DiSC_3(5)$ accumulation between HilD expressing and non-expressing cells, suggesting that the membrane of phenotypically virulent cells was not inherently depolarized. This observation contrasting with results of Hockenberry et al. would suggest another mechanism underlying increased membrane permeability. It would be interesting to further evaluate this mechanism in future experiments for example by measuring the ATP levels in both sub-populations. In our study we decided to further investigate the consequences of increased membrane permeability by exposing *S.* Tm cells to stress conditions targeting membrane integrity.

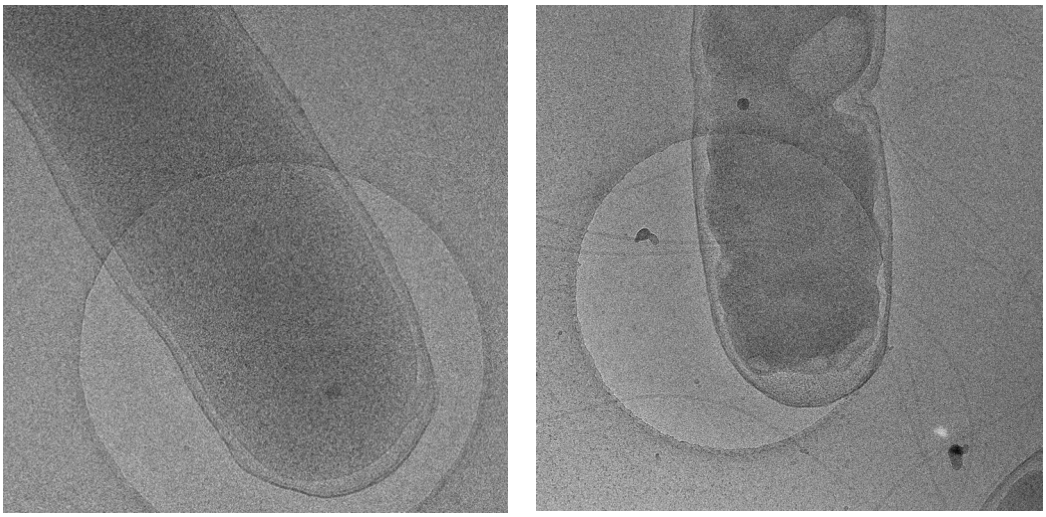


**Fig. 8. Cryogenic transmission electron microscopy (Cryo-TEM) of *S.* Tm.** The left side image is a *hilD* mutant cell, non-expressing virulence factors, where outer and inner membranes are mostly smooth. The right side image shows cell overexpressing HilA from plasmid (~90% P*prgH:gfp* ON cells in the population). Here the outer membrane is smooth but in many bacterial cells the inner membrane is disrupted with small as well as large intracellular vesicles. Cryo-TEM pictures were acquired by BioEM lab, Biozentrum, Basel.

One of the conditions we used in this study to investigate the impact of stress targeting the membrane on virulence expressing cells was 100mM Tris 10mM EDTA (TE). EDTA has a profound effect on the permeability barrier of Gram-negative enteric bacteria as it removes, by chelation, divalent cations stabilizing LPS. Tris and EDTA work synergistically in removing LPS from enteric bacteria (Vaara, 1992). However, Tris and EDTA are synthetic compounds which do not naturally occur in the host. As such the condition applied in the study is not physiological, however, certain molecules present *in vivo*, act via similar mechanism to destabilize the bacterial membrane. The effect on the outer membrane of EDTA is similar as to treatment with polymyxin or cationic agents (Vaara & Vaara, 1983). Therefore, the effect of stress condition we applied in this study is mimicking the effects of some naturally occurring antimicrobials. In our experiments we used two complementary approaches, flow cytometry and microscopy, to quantify the proportion of dead cells after TE treatment and the fraction of cells expressing HilD regulon (GFP+ cells) among the survivors. We found that TE exposure led to cell death, with HilD expressing cells showing a higher probability to be affected by the stress. In our proteomic analysis we did not find any membrane stress response components to be significantly expressed in the GFP+ subpopulation, suggesting that HilD expressing cells were not intrinsically more stressed than their non-expressing counterparts. We evaluated how different HilD regulated functions impact TE stress resistance by testing previously

created deletion mutants. Similar to the NPN results, deletion of *flg* operon and cumulative triple mutation *iagB-invG flg* and SPI-4 increased resistance to TE exposure. In our experimental conditions, expression of flagella was an important contributor impacting *S.* Tm stress resistance. The overexpression of HilA drastically increased sensitivity to TE. In this condition, individual deletion of *iagB-invG* and cumulative deletion *iagB-invG* flg SPI-4 restored some resistance when compared to WT, while individual deletions *flg* or SPI-4 had no effect. It is possible that depending on the type of inflicted stress, certain subpopulations of *S*. Tm could be affected differently. For example, exposure to antibiotics which need to be removed using energetically costly efflux pumps, would be more harmful to bacteria expressing functional flagella (Lyu et al., 2021). Assessing the relative contribution of individual HilD regulated VFs to the overall fitness cost for phenotypically virulent bacteria is very challenging as these traits interfere with each other. Moreover, it is possible that the relative amount of different membrane embedded VFs per cell also plays a role in membrane integrity maintenance in different conditions. However, that would be very difficult to determine on the single cell level. Taking all these technical challenges, considering entire HilD regulon, rather than individual functions, as a contributing factor to increased stress sensitivity could be a good strategy for the development of future treatments. Pre-treatment with sub-lethal TE led to reduced expression of HilD regulon and subsequently increased resistance to the lethal TE exposure. This result is in line with previously reported down-regulation of SPI-1 genes in response to stress targeting the outer membrane (Sirsat et al., 2011; Bader et al., 2003; Hernández et al., 2012). The regulatory link between membrane homeostasis and VFs expression indicates the importance of tight regulation of membrane embedded VFs for maintenance of membrane integrity. Our results demonstrated that the cost of virulence in *S.* Tm is strongly envelope related. We further evaluated importance of increased sensitivity of HilD expressing cells *in vivo*.

The triple mutant lacking majority of the structural components of T3SS-1 (*iagB-invG*), the *flg* and the SPI-4, showed increased resistance to TE treatment but did not prevent the growth defect associated with HilD expression in the ON cells. This result was very surprising since T3SS-1 and flagella are known to contribute to the reduced growth in phenotypically virulent cells (Sturm et al., 2011; Sánchez-Romero & Casadesús, 2021). Interestingly, results described by Sánchez-Romero show that while the SPI-1 ON state caused a growth retardation, combined expression of both flagella and SPI-1 led to growth arrest. Authors used the dual reporter system to monitor SPI-1 expression with GFP and flagella with mCherry (*sipB::gfp, fliC::mCherry*). Potentially, the co-expression of both fluorescent proteins is costly

for the cell impeding its growth. As a result of this strong growth retardation, population expressing both SPI-1 and flagella was relatively small (~1.3%). It is likely that the contribution of effectors expression (SopE, SopE2, SopB, SipA, SptP, SopA, SpvB, SpvC) also impacts the growth rate of HilD expressing cells in this case (Sturm et al., 2011). Presumably the quadruple mutant lacking *iagB-invG, flg,* SPI-4 and effectors would be both, fast growing and stress resistant. This observation lets us conclude that the stress sensitivity and growth impediment are two separate costs of virulence expression. It cannot be excluded that each of these costs contributes differently to the overall cost of *S.* Tm virulence under different condition. The fact that the cumulative triple mutant is stress resistant but slow growing at the same time, allowed us to de-couple these two effects and design the *in vivo* study to investigate the impact of stress sensitivity in the physiological conditions. The obtained results suggested that indeed, stress sensitivity represents a burden for *S*. Tm cells expressing HilD in the presence of inflammation. Gut inflammation is known to impose a thigh bottleneck on the *S*. Tm population in the lumen especially at a day 2 post infection, when it is estimated that only about 6000 *S.* Tm survive this population drop (Maier et al., 2014). Under such extreme conditions both, stress resistance and growth rate are likely to be of a great importance for pathogen's survival.

Understanding the cost of virulence can help us to design better anti-virulence strategies. These novel approaches remain controversial, as they do not eliminate the pathogens but rather disarm them. However, in the light of the ongoing global crisis related to antibiotic resistance, the anti-virulence approaches are gaining popularity (Martínez et al., 2019). Many bacterial virulence related traits are being investigated as potential targets for such therapies. As the production of VF is under control of regulatory mechanism, therefore, interference with regulatory mechanism could affect functioning of many VFs. The quorum sensing systems which are often involved in regulation of production of VF, constitute an important target for anti-virulence strategies (Defoirdt, 2018). The most concerning threat related to targeting of virulence regulation is the possibility of increasing virulence by selecting for constitutive expression of regulated VFs (Joelsson et al., 2006). Moreover, the assembly of VFs is essential to their proper functioning, making the bacterial machinery involved in VFs assembly another interesting target for anti-virulence strategies (Kahler et al., 2018). Other features playing an important role in the assembly of several VFs are bacterial functional membrane microdomains (FMMs) (García-Fernández et al., 2017). FMMs are the raft-like compartments of the prokaryotic cell membranes, similar to lipid rafts, which are important features of the membrane organization in eukaryotic cells. They are thought to function as oligomerization

platforms and facilitate protein interactions in bacterial membranes (Lopez & Koch, 2017). Next to interfering with production and assembly of VFs another attractive approach is based on targeting functioning of these VFs. Such strategies involve, for example neutralization of bacterial toxins (Kong et al., 2016). Careful selection of the VF to target is of crucial importance as it determines effectiveness of a given strategy in terms of its evolutionary robustness. A suitable target should be a VF whose disruption does not impair the fitness of the pathogen. In that case, the resistant mutants will recover fitness, leading to increased selection for resistance which should be avoided (Allen et al., 2014). Moreover, the detailed understanding of the dynamics of production and mechanism of action of considered VF is necessary. This knowledge would be fundamental to investigate the possible mechanisms of resistance. It is essential that the potential to develop resistant variants needs to be systematically addressed within an evolutionary ecology framework.

Similarly to the traditional antimicrobials, resistance to anti-virulence drugs seems to be inevitable and has been already reported (Maeda et al., 2012; Smith et al., 2012; García-Contreras et al., 2013; Zhu et al., 1998; Koch et al., 2005). However, the frequency at which these resistant mutants emerge may differ completely compared to antibiotic resistance. Especially targeting bacterial cooperative traits such as biofilm production seem to hold a lot of promise in this regard. In their study André and Godelle, computed the mathematical model to calculate the fixation probability of a mutant resistant to a drug targeting a cooperative trait and to predict the speed of resistance evolution. Authors showed that evolution of resistance in this case is several orders of magnitude slower than in case of antibiotic resistance (André & Godelle, 2005). The reason for that lies within the unit of organization generating resistance which is being targeted. Individual bacteria with a very rapid evolutionary rate have different adaptive properties than organized subpopulation fulfilling a certain cooperative function. As such, these treatment strategies target a smaller number of organization units with lower evolutionary potential, instead of billions of individual bacteria. Therefore, strategies aiming at disorganizing cooperative traits rather than killing every individual hold strong potential in terms of decreased probability of resistance emergence compared to traditional antibiotics (André & Godelle, 2005). Moreover, unlike resistance against traditional antibiotics which is always beneficial for the pathogen as it restores its survival, the resistance against anti-virulence strategies seems to be much more nuanced. The relative fitness of resistant mutant might be in fact decreased in comparison to a sensitive counterpart and thus be counter selected. Therefore, the relative fitness of possible resistant mutants should be also studied in detail.

A recent study by Dieltjens et al. illustrates an example of targeting a cooperative trait in *S.* Tm as a robust anti-virulence strategy. For *S.* Tm, biofilms play important role in the pathogen survival both, inside the host (e.g., colonization of gallstones (Marshall et al., 2014)), and outside the host (e.g., in food industry (Steenackers et al., 2012)). The biofilm formation relies on production of the EPS. The main components of EPS are cellulose (Zogaj et al., 2001) and curli fimbriae (Römling et al., 1998). Production of EPS facilitates attachment and protects against eradication by antibiotics, disinfectants, removal by mechanical cleaning or the host immune system (Steenackers et al., 2012). Dieltjens et al. showed that production of EPS is a cooperative trait, and that its inhibition may lead to counter-selection of resistance (Dieltjens et al., 2020; West et al., 2006). Authors developed 5-aryl-2-aminoimidazole-based inhibitors of EPS production of *S.* Tm biofilm. The previous reports showed that 2-aminoimidazoles can inhibit biofilm formation in a subcutaneous model in rats (Peeters et al., 2019) and have low cytotoxicity against mammalian cell lines (Steenackers et al., 2014). To test the evolution of resistance authors used WT *S.* Tm in a serial passage evolution experiment exposing cells to 50 µM of the EPS inhibitor. The biofilms in petri dishes were grown for 48 hours, scrapped off and re-inoculated. In over 20 passages (40 days) no change in EPS inhibition was observed. In a control setup using the conventional antimicrobials resistant clones emerged after few days. As authors did not find any resistant mutants in their evolutionary experiments, to investigate the fitness of potential resistant strain they screened the *Salmonella* reference (SAR) collection of natural isolates (Boyd et al., 1993). In this collection they identified two strains greatly differing in sensitivity to the EPS inhibitor. Competition experiments between the sensitive and resistance strain in either presence or absence of the EPS inhibitor showed that the resistant strain was counter selected. These findings are in line with mathematical predictions showing that anti-cooperative treatments should lead to slower emergence of resistance as the treatment is targeting groups of bacteria with lower evolutionary potential, rather than individuals (André & Godelle, 2005). This example showed that interference with the cooperative trait in *S.* Tm can be effective therapeutical strategy presenting additional advantage of resistant variants being counter selected during treatment (Dieltjens et al., 2020).

Similarly, virulence expression of *S.* Tm as a costly cooperative trait, is likely to be evolutionary unstable and therefore present an attractive target for the anti-virulence therapy. The cost of virulence expression is known to drive emergence of attenuated mutants during chronic infection in mice (Diard et al., 2013). Moreover, virulence attenuated mutants have been identified among the patient isolates (Marzel et al., 2016). However, virulence expression is

effectively stabilized by the tight regulatory network and bimodal expression of VFs, preventing fixation of attenuated mutants during infection (Fàbrega & Vila, 2013; Diard et al., 2013). In this case, the emergence of avirulent mutants is a relatively slow process and as such not likely to prevent infection progress. A detailed understanding of the cost of VF expression for the phenotypically virulent subpopulation, could allow to identify and modulate environmental factors to reinforce the evolution of *S*. Tm toward attenuation. It can be speculated that the virulence regulatory network of *S*. Tm is a reminiscent of the Prf regulon in *Listeria monocytogenes*. The Prf regulon controls virulence expression based on environmental inputs using the PrfA protein as a transcriptional activator of VFs expression. Vasanthakrishnan et al. showed that expression of virulence traits imposes a significant fitness cost on the pathogen impairing the *Listeria* growth rate (Vasanthakrishnan et al., 2015) which is a similar observation to what was found to be the case for *S*. Tm (Sturm et al., 2011). Taking these similarities, it would be interesting to survey the recent strategies proposed to fight these pathogens. In their study Tran et al. targeted a master regulator of *L. monocytogenes* virulence – PrfA thus interfering with the regulation of virulence (Tran et al., 2022). The use of small molecule inhibitor, preventing activation of PrfA led to clearance of the pathogen from replication vacuoles in infected macrophages. However, the impact of virulence inhibition on the growth rate of bacteria and emergence of resistance were not addressed. Another study by Marini et al. led to virulence attenuation in *L. monocytogenes* after treatment with sublethal concentration of Cannabis sativa L. essential oil (Marini et al., 2018). The essential oils represent a major group of plant antimicrobials and contain a mixture of secondary metabolites including alcohols, terpenes, ethers, aldehydes or phenols (Cannas et al., 2016). The essential oil components are explored as acting in synergy with antibiotics in treatment of antibiotic resistant pathogens (Langeveld et al., 2014). After exposure to the essential oil, listeriae were non motile and showed downregulation of both flagellar genes as well as *prfA* virulence activator. Moreover, bacteria showed significant reduction in the biofilm production and ability to invade the Caco-2 cells (Marini et al., 2018). This example shows a significant attenuation of virulence traits of *L. monocytogenes* after exposure to the mixture of compounds present in essential oil of Cannabis sativa L. In *S*. Tm inhibition of T3SS-1 has been explored over the years as a potential anti-virulence strategy. Timely and sequential delivery of effector proteins via T3SS-1 is crucial for successful host infection by *S.* Tm (Fàbrega & Vila, 2013). In line with that, T3SS-1 can be inhibited at different stages of infection thus blocking progression of disease. Inhibitors of T3SS-1 in *S*. Tm are summarized in **Table 1** (Hussain et al., 2021).

**Table 1. List of molecules tested for the inhibitory activity against T3SS-1 (Hussain et al., 2021)**

| Inhibitor(s) | Class | Target | Reference |
|---|---|---|---|
| INP0403 (ME0053) | Salicylidene acylhydrazide | Downregulated expression of SPI-1 | (Layton et al., 2010) |
| INP0400 | Salicylidene acylhydrazide | Targets T3SS-1 needle assembly | (Negrea et al., 2007) |
| INP0007 and derivatives | Salicylidene acylhydrazide | Inhibit T3SS-1 proteins | (Layton et al., 2010) |
| INP0010 (ME0052) and INP0031 (ME0055) | Salicylidene acylhydrazide | Interact with FolX, Tpx and WrbA | (Wang et al., 2011) |
| TTS29 and analogs | 2-imino-5-arylidene thiazolidinone | Target secretin components of T3SS-1 basal bocy | (Felise et al., 2008) |
| Cytosporone B | Polyketide | Indirectly decreases expression of global T3SS-1 gene regulator | (Li et al., 2013) |
| Licoflavonol | Prenylated flavonoids | Regulates the transcription of SicA/InvF genes | (Guo et al., 2016) |
| Paeono | A phenolicompound C | Blocks SipA translocation into the host cell. | (Lv et al., 2020) |
| Thymol | monoterpene phenol Fusaric | Inhibits the gene expression of effectors or translocation machinery or can directly inactivate machine components | (Zhang, Liu, Qiu, et al., 2018) |
| Fusaric acid | a toxin produced by fungi *Fusarium oxysporum* | Affects SicA/InvF and inhibits SPI-1 effectors. | (Crutcher et al., 2017) |
| Syringaldehyde | hydroxybenzaldehyde | SipA, SipB and SipC expression inhibition. | (Lv et al., 2019) |
| Cinnamaldehyde | aldehyde | Affects the regulatory genes of SPI-1 and reduces transcription of different SPI-1 genes. For | (Liu et al., 2019) |

| | | | |
|---|---|---|---|
| | | example, sipA and sipB | |
| Sanguinarine chloride | An extract from bloodroot plant *Sanguinaria canadensis* | Decreases the production of SipA and SipB | (Zhang, Liu, Wang, et al., 2018) |
| Myricetin | Natural plant extract | Decreases the transcription level of SPI-1 genes by hilD-hilC-rstA-hilA regulatory pathway | (Lv et al., 2021) |

Among the listed substances, several act by downregulating virulence genes. Paenol, which is an active ingredient from the dried root bark of the plant from peony family, acts through the *hilA* regulatory pathway inhibiting expression of SPI-1 related genes (Lv et al., 2020). Another compound decreasing expression of SPI-1 related genes is also of natural origin. Syringaldehyde, obtained from stems of plant *Hibiscus taiwanesis* causes decreased transcription of *hilD, hilC, rtsA, invF, hilA, sipA, sipB* and *sipC* genes (Lv et al., 2019). Myricetin is another compound affecting expression of regulatory genes *hilD, hilC, rtsA* and *hilA* and thus transcription of SPI-1 related genes (Lv et al., 2021). Importantly, all the listed compounds act by inhibiting T3SS-1 and are therefore targeting *S*. Tm virulence which is a cooperative trait. It would be of great value to further test these compounds within the evolutionary framework and investigate the emergence of resistant mutants.

Another approach proposed by Dhouib et al. is targeting not directly VFs, but rather machinery involved in proper protein folding which is crucial to VF assembly. The DsbA enzyme that can be found in several Gram-negative bacteria, including *S.* Tm, catalyzes disulfide bond formation and participates in structural assembly of multiple VFs. The importance of DsbA in the assembly of functional VFs makes it an attractive target for anti-virulence strategies. Both *S*. Tm and uropathogenic *E. coli*, encode not only the *dbsA* but also several accessory homologues of that protein. It was shown that two types of DbsA inhibitors - phenylthiophene and phenoxyphenyl derivatives, can inhibit not only DsbA but also its homologs in *S*. Tm (Totsika et al., 2018). In the follow up study authors also challenged evolutionary robustness of the DsbA inhibitors (Dhouib et al., 2021). The experimental evolution involving serial passaging of *S*. Tm in the presence of DsbA inhibitors or ciprofloxacin as a control for 10 days, showed that no resistance to DsbA inhibitors emerged while resistance to ciprofloxacin appeared rapidly. These findings suggest that strategy relying on inhibition of VF assembly are evolutionary robust in the in vitro setup, supporting their potential as evolutionary-proof

anti-virulence strategy. So far, studies which directly tested evolutionary robustness of anti-virulence drugs remain scarce, however, they should become a standard practice in the design of novel antimicrobial strategies.

Important aspect that should be considered when designing potential anti-virulence strategies should be the fitness cost of a given VF to the pathogen. Careful selection of the target VF would impact the evolutionary robustness of the strategy. So far, in *S*. Tm the only described cost of virulence expression was a two-fold growth reduction in comparison to phenotypically avirulent cells (Sturm et al., 2011). This observation can have important implications for the potential anti-virulence therapy. For instance, if a given therapy leads to downregulation of virulence genes expression, it might as well improve the growth rate of targeted cells and thus increase their fitness in this aspect. The improved growth rate of a sensitive subpopulation could enhance the counterselection against the potential resistant mutants. It will be of a crucial importance for the design of future therapies to study in detail the dynamic between sensitive and resistant cells. Our study shed the new light on understanding the cost of virulence expression in *S.* Tm. We found that phenotypically virulent and avirulent *S.* Tm subpopulations differ not only in their growth rate but also their membrane permeability and sensitivity to the outer membrane stress exposure. We showed that this newly found cost of virulence expression is independent of growth retardation and plays a role in *in vivo* infection model. These observations could have important consequences for potential therapeutical treatments. Since we observed two subpopulations reacting differently to the stress exposure it cannot be excluded that they can also react differently to the treatment. Most of the research studies have been performed on a bulk of cells thus averaging the outcome, however, recent years brought a new perspective on the phenotypic heterogeneity. Fact that genetically isogenic cells show phenotypic differences can have important consequences for therapy design. It appears that in case of *S*. Tm phenotypically virulent subpopulation shows increased membrane permeability which renders it more sensitive to stress. Increased membrane permeability can facilitate penetration of certain drugs or allow membrane targeting molecules to inflict higher damage specifically on cells expressing VFs. Targeting the membrane as a therapeutical strategy would be advantageous as it omits necessity of drug delivery inside of the cell. Understanding the mechanistic basis underlying increased membrane permeability of HilD expressing *S*. Tm cells would be of a great interest. As *S.* Tm encodes two VFs which are expressed in a bimodal way: T3SS-1 and flagella, it would be interesting to investigate in details membrane properties of subpopulations depending on which VF they express. A recent study showed that despite having an intertwined regulatory networks, T3SS-1 and flagella

show independent switching leading to presence of four distinct subpopulations: flagella ON and T3SS-1 ON, flagella ON and T3SS-1 OFF, flagella OFF and T3SS-1 ON, flagella OFF and T3SS-OFF (Sánchez-Romero & Casadesús, 2021). In their study, the authors sorted each of the four subpopulations and investigated their ability to infect HeLa cells. Interestingly it was observed that all four subpopulations individually showed reduced invasion than when combined. These findings suggest that all four subpopulations might be important for optimal invasion. It was previously described that T3SS-1 OFF cells play an important role in stabilization of cooperative virulence in *S.* Tm (Diard et al., 2013). It is possible that cells which do not express flagella might also play an important role for the invasion process which we are yet to understand. Our discovery, that *S.* Tm subpopulations differing in expression of HilD regulon, show different stress resistance can have important consequences for design of evolutionary-proof therapies. Mathematical framework describing emergence of resistance to anti-virulence therapies emphasizes that the targeted unit of organization is of crucial importance (André & Godelle, 2005). It is possible that four *S*. Tm subpopulations described by Sánchez-Romero and Casadesús represent larger organization units acting in cooperation during infection process. In this view each subpopulation differing in VF expression would represent such unit with lower evolutionary potential in comparison to billions of individual cells. Understanding principles governing cooperation between these organization units would be highly important and should be addressed in the future studies. In case of *S.* Tm, we do not yet fully understand if all four subpopulations show distinct features in terms of stress resistance. It would be very interesting to address this question in a double reporter study.

A high throughput screen of candidate compounds could be a good starting point to design a successful anti-virulence strategy against *S*. Tm. Importantly, monitoring the behavior of both, phenotypically avirulent and virulent subpopulations, in the presence of investigated compound would be of a great importance. It would be worthwhile to include drugs which failed previous trials aiming to clear the entire pathogen population. Anti-virulence strategies aim rather to disarm the pathogen rather than to eliminate it. Many previously studied compounds which failed to kill *S*. Tm, or have been designed for another purpose, can therefore be good candidates to reconsider as potential anti-virulence compounds. Such drug repurposing could save a large amount of time and money invested in developing already existing compounds. Certain drugs have been already repurposed to use as anti-virulence treatments in strategies targeting quorum sensing (Defoirdt, 2018). This includes the anti-cancer drug 5-fluorouracil (Ueda et al., 2009), the anthelmintic drug niclosamide (Imperi, Massai, Pillai, et al., 2013), and the antimycotic drug flucytosine (Imperi, Massai, Facchini, et al., 2013). These examples show

that it is possible to successfully reuse existing drugs for anti-virulence strategies without investing into novel drug development. Moreover, as virulence of *S*. Tm is regulated based on numerous environmental clues, it can be possible to design the environment components supporting the attenuation of virulence. It was shown that compounds secreted by *Bifidobacterium bifidum* interfere with attachment and invasion by *S.* Tm. leading to significant downregulation of important virulence regulators expression, including *hilA* and *hilD* (Bayoumi & Griffiths, 2012). These results suggest that probiotic *Bifidobacteria* strains can help to control *S*. Tm infection and potentially supplement the anti-virulence treatment. Other molecules present in the gut environment, like SCFA (Hockenberry et al., 2021) are also known to attenuate virulence of *S*. Tm. Modulation of ecological factors to create conditions supporting virulence attenuation could be a valuable addition to the anti-virulence strategy. Moreover, the residual commensal microbiota which are contributing to colonization resistance and preventing *S.* Tm cells from overtaking the intestine lumen, are also an important contributor limiting the *S.* Tm infection progress. In that regard the advantage of using the anti-virulence strategy specifically targeting the HilD expressing cells, should preserve the residual microbiota further boosting effectiveness of therapy. Many traditional antibiotics are not only killing the desired pathogen but also clearing beneficial microflora in the process. Our work demonstrated that certain types of conditions, like the stress targeting cell membrane, can selectively impact phenotypically virulent *S.* Tm cells. This observation can pave the way to effective strategies which can be applied on target bacteria present in mixed communities. We could show that impaired membrane integrity in itself is a significant selective force which plays a role during infection in the mouse model and can lead to emergence of virulence attenuated mutants with increased resistance to stress. Therefore, identification of compounds acting specifically on the HilD expressing cells could give rise to novel evolutionary robust anti-virulence therapies against *S*. Tm.

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# CHAPTER II

## Investigation of *Salmonella* Typhimurium adaptative fitness during early colonization of the gut ecosystem

*Role of a population bottleneck in transmission of cooperative virulence in S. Tm*

The ability of pathogen to colonize the susceptible host population ultimately depends on its ability to survive and replicate within the host, and to effectively spread between the hosts (Cressler et al., 2016). Inside the host, pathogens need to overcome various challenges including surviving the environmental factors, acquiring nutrient sources, competing with other members of microbial community, or escaping the host immune system. The ability to rapidly adapt to the hostile environment is of crucial importance for pathogens survival. Diversifying selection, favoring evolution of novel variants has been described as an important factor in several within host studies (Price et al., 2013; Marvig et al., 2015; Lieberman et al., 2011). Purifying selection leads to the loss of disadvantageous alleles, whereas mutations conferring fitness advantage can increase in frequency within a population (Didelot et al., 2016). Within-host evolution is a continuing process that occurs during each infection driving adaptation to a new host which represents a unique individual with a specific microflora and immune system. Adaptations to specific within-host environment can lead to successful infection at the scale of individual host. However, ultimately to ensure its spread among the host population, pathogen needs to infect the next host through the transmission process. During the process of transmission from a donor to recipient, the genetic diversity of pathogen can be lost due to the bottleneck, which randomly reduces the effective population size and can lead to genetic drift. Thus, only a limited number of individuals will be able to colonize a new host. Our understanding of the bottleneck effect on pathogen evolution remains limited. Both, survival within host and transmission between hosts, are the integral parts of a pathogen life cycle and as such, contribute to the emergence and spread of new pathovars.

Transmission is of crucial importance for the pathogen to spread among the population of susceptible host. However, this step can be also viewed as the population bottleneck for a pathogen in a case when only a few individual pathogens are successfully colonizing the secondary host. Population bottlenecks strongly reducing the size of pathogens population can occur at different stages of a pathogen lifecycle. The size of the transmission bottleneck will impact the diversity of pathogen strains transmitting from host to host. For *Staphylococcus aureus* several examples showed mixed colonization of the host by several bacterial lineages (Cespedes et al., 2005; Mongkolrattanothai et al., 2011; Votintseva et al., 2014). Such mixed colonization could be the effect of two or more different transmission events, but it is also possible that the different lineages were transmitted during a single colonization event. This

would indicate loose transmission bottleneck allowing diverse strains to be transmitted from one host to another (Didelot et al., 2016). In other cases, even despite tight transmission bottleneck, a certain level of pathogen diversity can be maintained. In the study investigating transmission of Influenza A virus, Sigal et al., found that during a single transmission event, several mutations which evolved *de novo* during within-host evolution are likely to be transmitted to recipient host (Sigal et al., 2018). Therefore, a certain level of genetic diversity of influenza A virus was maintained regardless of recurring transmission events. Our knowledge about the size of transmission bottlenecks for different pathogens, and their meaning for a pathogen diversity and evolution remains limited. Transmission bottlenecks in nature remain challenging to study as the key parameters, like the size of the pathogen inoculum initiating infection, are difficult to estimate. Despite being a key aspect of pathogen life cycle transmission is rarely investigated experimentally. Development of techniques such as genetic barcoding (Varble et al., 2014) is improving quality of the *in vivo* studies in the animal models (Abel et al., 2015) bringing new opportunities to systematically investigate pathogen transmission.

Transmission bottlenecks are especially important for pathogens expressing cooperative traits, to ensure transmission of cooperative genotype to the next host (Cremer et al., 2012). The cooperative traits are sensitive to the emergence of cheaters in the population. In extreme cases, if cheaters outcompete the cooperative genotype the total population can collapse due to loss of the public good production (West et al., 2006). An interesting example of the cooperative trait is the virulence of *S.* Tm. In this case, host gut inflammation is a common good that helps *S.* Tm to outcompete microbiota and increases the likelihood of pathogen transmission to another host (Stecher et al., 2007). Inflammation is triggered thanks to a subpopulation of *S.* Tm cells expressing T3SS-1 and its cognate effector proteins. The latter injected inside the host cytoplasm initiate the onset of gut inflammation. This beneficial for the pathogen outcome comes with a significant fitness cost for the cells expressing T3SS-1 (T3SS-1 ON). These cells have reduced growth rate in comparison to T3SS-1 OFF counterparts (Sturm et al., 2011), and can be killed by the host immune system upon host tissue invasion (Ackermann et al., 2008). Due to the high cost of virulence expression, this trait is prone to the emergence of the non-virulent mutants (cheaters), benefitting from the inflammation and not contributing to its onset (Diard & Hardt, 2017). If the frequency of cheaters is too high, inflammation will recede leading to regrowth of microbiota and decrease of *S.* tm population in the gut. However, virulence is stabilized by the presence of genetically virulent cells not expressing T3SS-1 which slows down the rise of defectors (Diard et al.,

2013). Mathematical modeling of *S.* tm population growth dynamics predicted that a decreased proportion of genetically virulent T3SS-1 OFF cells would accelerate the fixation of cheaters (genetically non-virulent cells). The model predicted proportion of ~35% T3SS-1 ON cells to be optimal for *S.* tm to trigger the inflammation and prevent the rise of cheaters. These modeling predictions show that the relative proportion of T3SS-1 ON and OFF cells is of high importance for *S.* tm infection as well as transmission (Diard et al., 2013). Since both T3SS-1 ON and T3SS-1 OFF cells are genetically virulent, the cooperative genotype should be transmitted to the secondary host via fecal shedding. However, if the frequency of cheaters in the gut lumen from where the pathogen is transmitted is high enough, it can impair the successful spreading of the virulent genotype to another host (Diard et al., 2014). In a recent study, Bakkeren et al. addressed the question of whether HGT could play a role in the maintenance of the virulent genotype in the host gut and favor its transmission to the next host (Bakkeren et al., 2022). In case of S. tm, cheater mutants carry the mutation in the virulence regulator *hilD.* Such mutants were isolated in patient samples (Marzel et al., 2016) and farm animals (Tambassi et al., 2020). Authors cloned the functional *hilD* allele on the conjugative plasmid highly transferrable in the host gut to see if it can restore virulence in the attenuated cheater mutants. Indeed, it was the case for a limited time, however, the mobile *hilD* allele was also susceptible to deactivating mutations, and virulence loss was observed on the level of the vector. Authors concluded that HGT was not able to restore the virulence of the attenuated population and other factors are likely to play a role in ensuring transmission of virulent genotype to the next host (Bakkeren et al., 2022). In their experiments, authors established that early transmission ensures the transfer of a sufficient number of cooperators to the next host to trigger the disease, whereas the long-term colonization was detrimental to cooperation due to the accumulation of cheaters. This suggests the important role of transmission timing for spread of cooperators. Moreover, the tight transmission associated with population bottlenecks favors the transmission of cooperative genotype. The previous study of the cooperative production of iron-scavenging siderophores in *P. aeruginosa* investigated the virulence of the pathogen depending on whether inoculum contained a single clone or a mix of clones. Authors found that in the mixed infections cheaters were more prevalent leading to a decreased level of virulence in an insect host. On the contrary, a single clone inoculum contained a higher fraction of cooperators, and such infection resulted in more rapid host death (Harrison et al., 2006). This example illustrates that transmission bottleneck limiting the number of clones transferred to the secondary host can stabilize cooperation.

*Role of microbiota in shaping S. Tm evolution during early gut colonization*

Pathogens are constantly undergoing adaptation to their environment. Whole genome sequencing of bacterial isolates allowed to investigate evolution of pathogens during relatively short timescales of within-host evolution (Didelot et al., 2016). The genetic diversity of pathogens evolving during host colonization can be attributed to different factors. Point mutations are one source of diversity. Studies comparing pairs of genomes of *Helicobacter pylori* sampled from the same host showed a high rate of within-host point mutations with ~30 mutations per year per genome (Kennemann et al., 2011). The point mutation rate differs between species due to differences in per site mutation rates, length of the genome, and efficacy of the DNA mismatch repair system. Another mechanism promoting genetic diversity is phase variation where in certain loci frequently occurring reversible mutations modulate gene expression (Moxon et al., 2006). In addition, acquisition of genetic sequences from other organisms via HGT can lead to faster diversification of the genome. Most of the mutations arising during within-host evolution are likely either neutral or detrimental. Such mutations can be fixed at a low frequency or rapidly lost (Tanner & Kingsley, 2018). However, the novel mutation can be selected for if it provides an advantage to pathogen survival within the host or improves transmission to another host. Adaptations to within-host lifestyle can relate to many aspects of pathogen life including the ability to process different nutrients, withstand the presence of antimicrobials or host immune system. This diversity generated within the host is often lost upon transmission to the next host, due to population bottleneck. Because of the random sampling occurring in this process, the most niche-adapted clones might not be passed to the next host. The effect of a population bottleneck depends on the initial genetic diversity of the population, and the size of the population bottleneck determining what fraction of the initial population will colonize the secondary host. Our understanding of the evolutionary dynamics of *S.* Tm population, as a consequence of consecutive cycles of within-host evolution and transmission, remains limited and requires further investigation.

The phase of early colonization of the host by *S.* Tm remains largely understudied. In order to trigger the disease symptoms, the pathogen first needs to reach a sufficiently high density in the gut lumen of the host (Barthel et al., 2003). However, upon *S.* Tm entry, this niche is preoccupied by a dense microbial community providing colonization resistance against incoming pathogens. The microbiota community composition is a critical factor determining host susceptibility to infection (Stecher et al., 2010; Ubeda et al., 2017). Presence of certain microbial species like *Bifidobacterium bifidum* in the host gut was associated with increased

colonization resistance against *S.* Tm (Bayoumi & Griffiths, 2012). Recent studies have started to shed light on the mechanism used by different commensal species to confer colonization resistance against *S*. Tm. The study by Hockenberry et al., showed that SCFA which are abundant microbial metabolites, decrease the growth rate of virulence expressing *S*. Tm cells on a single-cell level (Hockenberry et al., 2021). Bacteriocins produced by a specific bacterial species can restrict the colonization and replication of other species (Ducarmon et al., 2019). These short, toxic peptides can have multiple mechanism of action. Microcins produced by *E. coli* Nissle 1917 were able to restrict *S*. Tm growth both *in vitro* and *in vivo* (Sassone-Corsi et al., 2016). These microcins can bind siderophores and as microcin-siderophore complexes be taken up by *S*. Tm. However, the inhibitory effect on *S*. Tm was observed only during the iron depletion associated with intestinal inflammation. Competition for nutrients is another critical aspect restricting pathogen growth within the intestinal lumen. The more closely related are two species, the more likely it is that their nutrient preferences are overlapping. Commensal species of Enterobacteriaceae that are closely genetically related to *S*. Tm are common component of the intestinal microflora in humans and other mammals (Rogers et al., 2021). Collectively, these microbiota-derived factors contribute to the creation of a very challenging environment for *S*. Tm to concur. How pathogen can overcome these obstacles remains largely unknown. Most of the *in vivo* studies of *S.* Tm are carried in antibiotic-pretreated mice. This experimental practice helps to reach high densities of *S.* Tm in the gut lumen otherwise restricted due to colonization resistance. However, such an experimental setup does not allow the study of interactions between the pathogen and microbiota. For this reason, our understanding of what are the specific determinants improving *S.* Tm fitness during early colonization phase remains scarce. In our study, we would like to address this knowledge gap by studying the evolutionary dynamics of the *S*. tm population during early colonization.

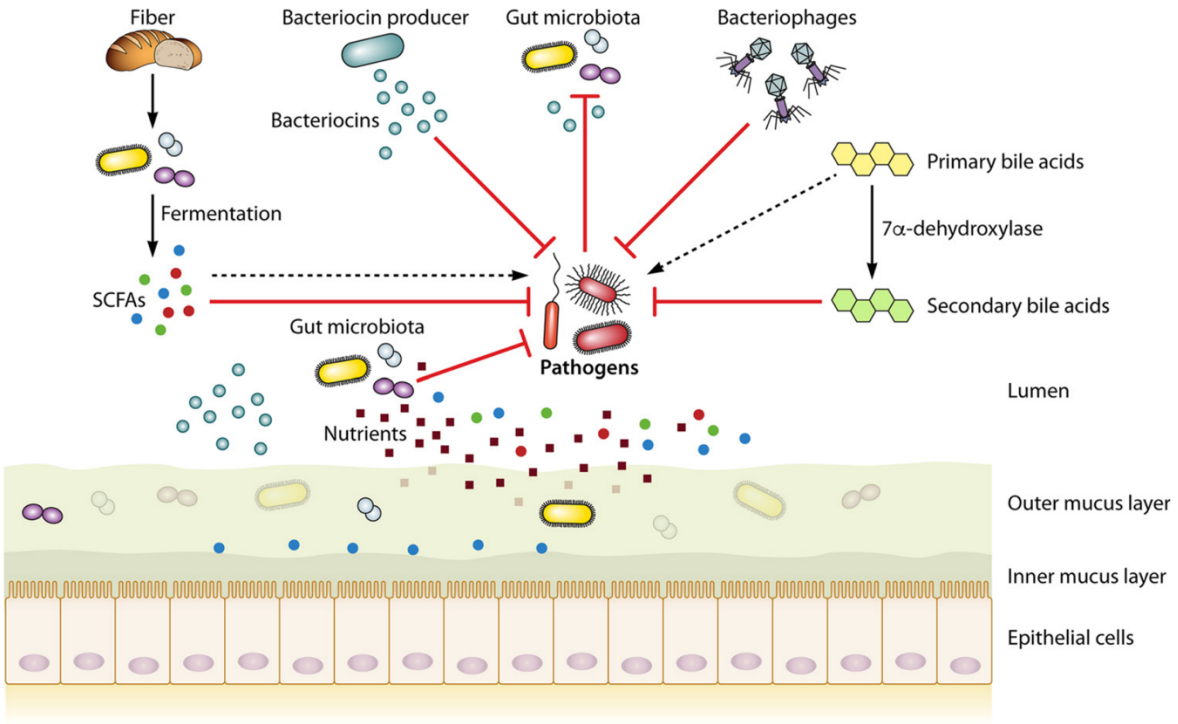


**Fig. 1. Factors contributing to microbiota-derived colonization resistance.** Fiber obtained from the host diet is digested by the gut commensals into SCFAs. Certain microbiota species can produce bacteriocins targeting the pathogen. Competition for nutrients can further impair growth of the pathogen. Moreover, host-derived factors like bile salts production also prevent pathogen growth in the gut. (Ducarmon et al., 2019)

## Aims of the project

The aim of this project was to identify traits improving the fitness of *S.* Tm during the colonization of mice with low complexity microbiota (LCM). Within-host evolution leads to the fixation of mutations providing *S.* Tm with an advantage for survival inside the host. These mutations can improve for example ability to digest nutrients present within the host gut, outcompete microbiota, or resist host immune barriers.

First step of the project is to design the appropriate *in vivo* experimental setup. Most of the studies of *S.* Tm are performed in antibiotic pre-treated mice. This experimental practice allows to reach high *S.* tm colonization in the animal gut, otherwise prevented due to colonization resistance provided by the microbiota (Van Der Waaij et al., 1971; Van Der Waaij & Berghuis, 1974; Stecher & Hardt, 2011). However, to study within-host evolution which is often driven by interspecies competition between *S.* Tm and microbiota for niche and resources, we need an experimental setup involving presence of microbiota in the mice gut. We decided to use the LCM mice which permit *S.* Tm colonization (Stecher et al., 2010) and would allow us to study the diversification of the pathogen as a result of competition with other species. Using these mice, we designed an experimental setup involving five consecutive cycles of within-host evolution and transmission. Such structure of experiment will allow us to investigate within-host evolution in the timescales relevant for colonization and determine fate of the evolved clones during transmission event to the next host.

Secondly, we want to study the dynamics of within-host evolution over the course of colonization. To investigate the structure of the population a technique of mixed inoculation employing strains comprising neutral genetic markers in the genome can be applied. Development of wild-type isogenic tagged strains (WITS) libraries which can be quantitatively tracked using qPCR technique brought new possibilities to study the population dynamics (Maier et al., 2014). We employ the mixture of 7 isogenic WITS tagged WT *S.* Tm strains. Representation of each WITS tagged strain will be monitored by quantification of the tagged strains composition present in the animal feces over time using qPCR. Diversity loss of barcodes over time will indicate the fixation of advantageous mutations in the population. Furthermore, we can monitor if these evolved clones can successfully colonize the secondary host. Systematic measurements of WITS composition over time would provide information on population dynamic of *S.* Tm during infection. Moreover, we want to investigate clones evolved during the experiment in more detail. The whole genome sequencing will allow us to determine

specific mutations which emerged in *S*. Tm clones. The aim is to understand what mutations increase fitness of *S.* Tm within-host and that can be passaged during transmission to another host. This new knowledge could provide new insights into mechanisms of pathogenesis and host adaptation of *S.* Tm.

*S. Tm can colonize the gut lumen of LCM mice*

We sought to investigate emergence and fixation of new mutations during the early colonization of the host gut. To achieve that goal, we needed the animal host which would fulfill the following criteria: be inhabited by a limited number of commensal bacteria in the gut and be susceptible to *S.* Tm colonization. These requirements were met by the LCM mice as their gut is inhabited by several microbial species which are permissive to *S.* Tm colonization (Stecher et al., 2010). Using the LCM mice, we designed an experimental setup including 5 consecutive cycles of within-host growth, separated by 4 transmission events. On the day 0, animals annotated E, F, G and H (n=4) hosted in two separate cages were inoculated via oral gavage with a mixture of equal amounts of 7 WITS tagged strains (Maier et al., 2014). The LCM mice were monitored for 3 days after infection, and sacrificed on the day 3 post infection (p.i.). The short colonization period is limiting time of days p.i. when the host gut is inflamed allowing for observation of pre-inflammatory adaptation of *S.* Tm to the new environment. Bacteria present in the feces of animals on day 3 p.i. were administered via oral gavage to naïve animals (n=4) initiating the second cycle of infection. The 5 cycles of within-host infection were included in the experiment resulting in the total length of 16 days. The schematic overview of the experiment is depicted in the **Figure 2 (Fig. 2).**

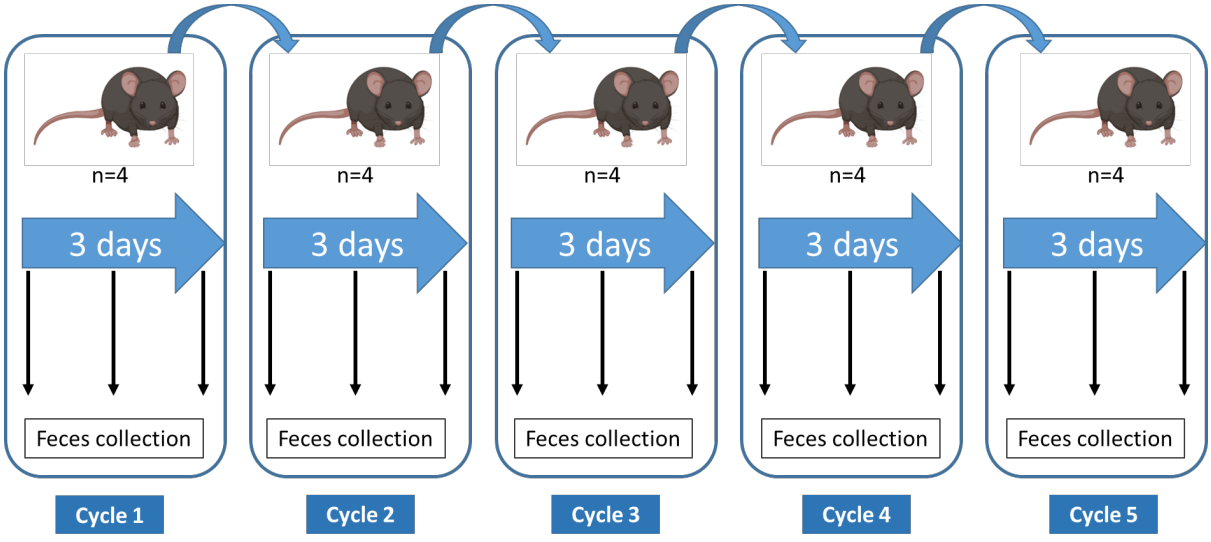


**Fig. 2. Schematic overview of the experimental setup.** Experiment consists of 5 consecutive cycles; each cycle involves n=4 LCM mice. Animals were annotated E, F, G, and H. In the beginning of the experiment each animal was administered via oral gavage a mixture of equal amounts of 7 WT isogenic tagged strains. After 3 days of within-host evolution animals were sacrificed. Bacteria present in the

feces of a given mouse on day 3 of cycle 1 were administered via oral gavage to the corresponding animal in the next cage initiating cycle 2. This protocol was repeated 4 times, resulting in 5 consecutive cycles of within-host colonization and 4 transmission events. Each individual cycle is represented by the blue frame, black vertical arrows represent sampling of animal feces on each day of experiment, blue horizontal arrows illustrate length of each cycle (3 days) and blue winged arrows connecting individual cycles depict transmission events.

To monitor the *S.* Tm colonization of the LCM mice throughout the experiment, animal feces were collected. The used *S*. Tm strain (SB300) is carrying a streptomycin resistance cassette on the plasmid pRSF1010. Therefore, *S.* Tm colonies could be selected on the MacConkey agar supplemented with streptomycin allowing to calculate the amount of *S*. Tm present in the gram of feces (CFU / g feces). **Figure 3 (Fig 3)** illustrates CFU / g feces collected on each day p.i. *S*. Tm was able to successfully colonize the gut of LCM mice. On the day 1 of cycle 1, and the day 1 of cycle 2, the CFU / g of feces reached value around $10^4$-$10^5$. On the day 2 of cycle 1, and day 2 of cycle 2 this value increased to ~$10^7$-$10^8$ CFU / g of feces, and on respective day 3 of these two cycles CFU / g of feces reached $10^9$. This suggest that in the cycles 1 and 2, *S.* Tm needed 3 days to establishing the niche for its replication. Interestingly, this effect was not observed in the cycles 3-5 where from the 1$^{st}$ day p.i. the CFU / g of feces reached high values of ~$10^7$. This observation could mean that *S.* Tm undergone adaptation within the cycles 1-2, which transmitted to subsequent cycles improved colonization of the host gut. These results confirm that LCM mice are a suitable model to monitor *S.* Tm colonization and to further investigate population dynamics and emergence of mutations.

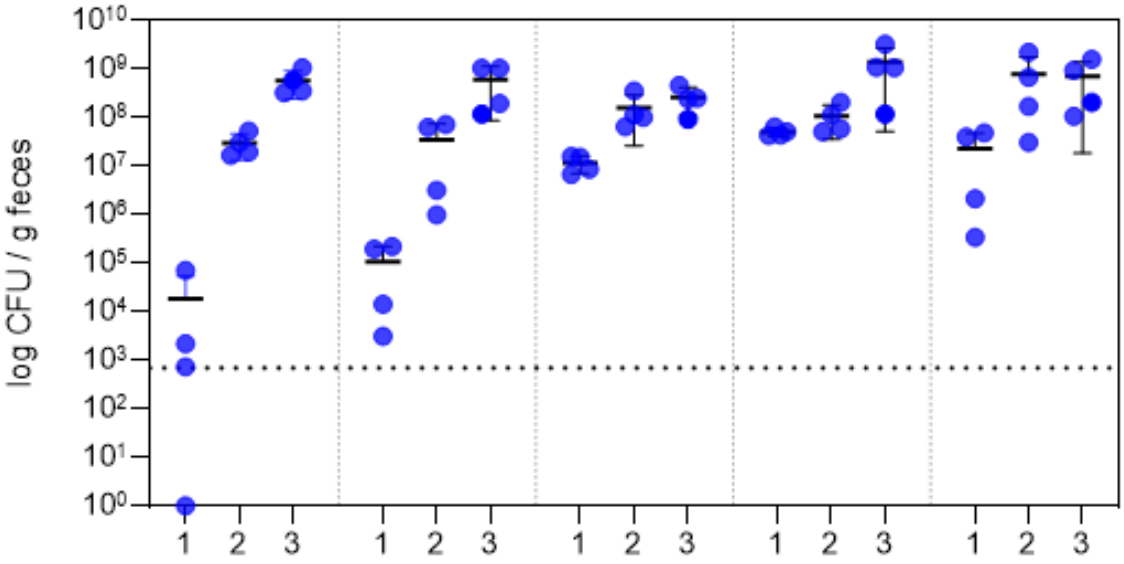


**Fig. 3. CFU / g of feces monitored throughout the experiment.** Dotted vertical lines represent transmission events separating consecutive cycles. Horizontal dotted line represents detection limit. The Y axis represents CFU / g of feces in log scale, on the X axis days on each cycle are depicted. Blue dots represent individual LCM mice (n=4 in each cycle).

*S. Tm triggers gut inflammation and onset of systemic disease in LCM mice*

Inflammation is of a crucial importance for *S.* Tm infection (Fàbrega & Vila, 2013). Thanks to the expression of VFs pathogen initiates onset of inflammation which drastically changes the environment within the gut towards conditions favorable to *S.* Tm and other Enterobacteriaceae (Stecher et al., 2007; Stecher et al., 2012). To evaluate if *S.* Tm colonizing LCM mice was able to trigger the onset of an inflammation, we measured amount of lipocalin-2 (LCN2) which is a marker of the gut inflammation (Raffatellu et al., 2009). **Figure 4 (Fig. 4)** illustrates the amount of LCN2 measured in ng / g of feces, on each day p.i. throughout the experiment. We could observe an increase of LCN2 indicating the onset of inflammation in each of 5 cycles of experiment. Interestingly, values of LCN2 seemed to increase in the cycles 3-5 in comparison to cycles 1-2. This is in line with faster gut colonization in the cycles 3-5 illustrated in **Figure 3 (Fig. 3).**

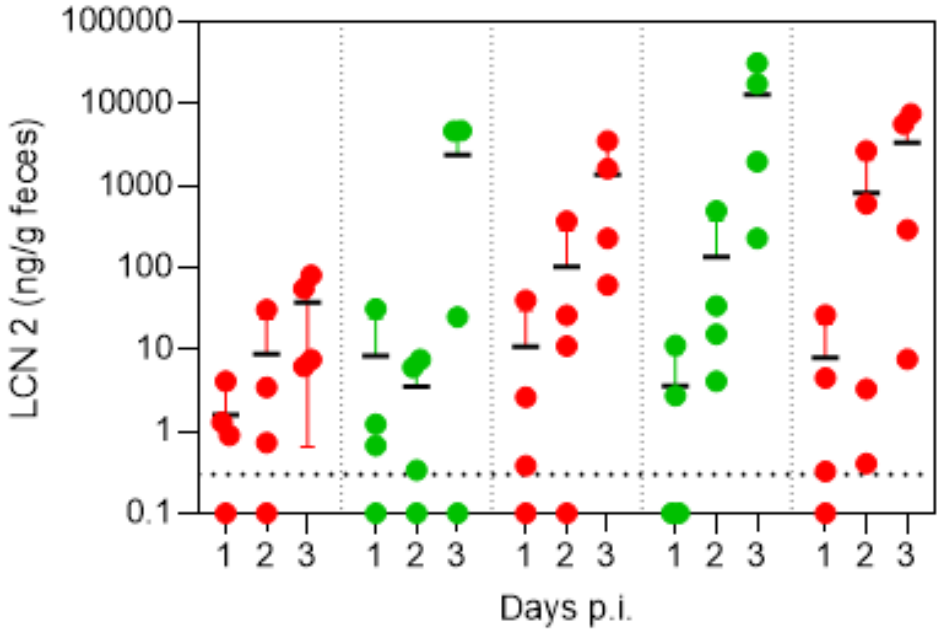


**Fig. 4. Lipocalin-2 (LCN2) ng / g of feces monitored throughout the experiment.** Dotted vertical lines represent transmission events separating consecutive cycles. The Y axis represents LCN2 in ng / g of feces in log scale, on the X axis days on each cycle are depicted. Dots represent individual LCM mice (n=4 in each cycle).

*S.* Tm can spread from the host gut to infect the systemic sites like lymph nodes, spleen or liver (Kaiser et al., 2013; Mastroeni & Grant, 2013). To determine if a systemic spread of *S.* Tm took place during the infection of LCM mice, the content of lymph nodes collected from each animal was plated on the MacConkey agar containing kanamycin. **Figure 5 (Fig. 5)** illustrates log CFU / lymph node monitored throughout the experiment. In all animals, except of one mouse from the cycle 2, *S.* Tm was present in the lymph nodes, indicating a successful systemic infection. Collectively, these results demonstrate that *S.* Tm strains present in each cycle are virulent and capable of triggering gut inflammation and spread to systemic sites within the host.

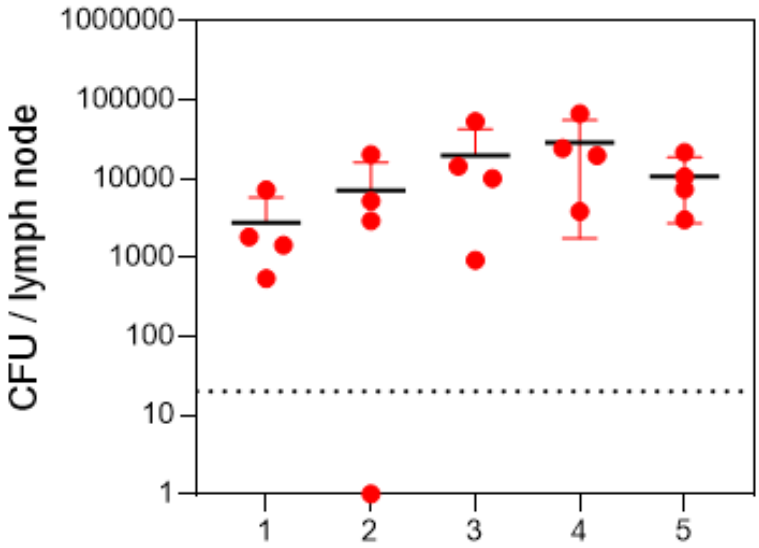


**Fig. 5. *S.* Tm isolated from the lymph nodes.** The Y axis represents CFU / lymph node depicted in log scale, numbers on the X axis correspond to number of the cycle. Each dot corresponds to single animal (n=4). Horizontal dotted line represents the detection threshold.

*Metabolism-related mutations benefit S. Tm within LCM mice*

To evaluate if the clones isolated from LCM mice on the day 3 p.i. of the cycle 5 carry any mutations, total of 12 clones were sequenced (3 per animal). The Illumina sequencing results identified 4 *de novo* mutations. Two of them were identified in a single animal E; the premature stop codon mutation in the *malE* gene was identified in strain with WITS13 tag, whereas the amino acid substitution in *malE* was identified in strain with WITS21. Isolate from the animal F was found to carry a premature stop codon in *malF* gene in strain with WITS2 tag. The strain isolated from animal G carried deletion within *gatR* gene of the strain with WITS1 tag. Remaining 8 of the sequenced clones did not carry *de novo* mutations. The isolated mutations are listed in the **Table 2**. Interestingly all identified mutations were found in genes related to the sugar metabolism. The *mal* operon expression enables *S.* Tm to uptake and digest maltose. The loss of function mutations in *malE* and *malF* genes suggest that *S.* Tm does not consume maltose in the LCM mice. This could be explained by either lack of maltose in the LCM gut, or this sugar is consumed by microbiota and therefore unavailable to *S.* Tm. The *gatR* gene is a repressor of galactitol utilization. Therefore, inactivation of this repressor would increase expression of the *gat* operon improving *S.* Tm ability to uptake and digest galactitol. This finding suggests that galactitol is an important carbon source for *S.* Tm in LCM mice.

**Table 2. List of identified metabolism-related mutations.** Clones isolated from LCM mice on day 3 p.i. of the cycle 5 were sent for Illumina sequencing. Results were annotated to the reference genome SL1344 NC_016810.1.

| Animal | WITS | Nucleotide of reference genome | Mutation |
|---|---|---|---|
| E | 13 | 4471260 | malE premature stop (W184) |
| E | 21 | 4471501 | malE E104K |
| F | 2 | 4469356 | malF premature stop (W378) |
| G | 1 | Deletion 3452453-3452785 | galactitol repressor (deletion aa 94-204) |

To evaluate if mutation in galactitol repressor would improve *S*. Tm ability to digest galactitol, we reconstructed *gatR* mutation in the genome using λ-Red gene replacement system (Datsenko & Wanner, 2000). Growth of the WT and Δ*gatR* strains in either M9 minimal medium or the M9 minimal medium supplemented with galactitol was measured in a plate reader. The OD600 measurements are illustrated in the **Figure 6 (Fig. 6).** While none of the strains was able to grow in M9 minimal medium, the Δ*gatR* strain was able to grow in a minimal medium supplemented with galactitol. WT strain in the minimal medium supplemented with galactitol was able to grow after 15 hrs, which could be due to the emergence of mutation facilitating digestion of galactitol. These data confirmed that a mutation within *gatR* gene improved the ability of *S*. Tm to use galactitol as a carbon source.

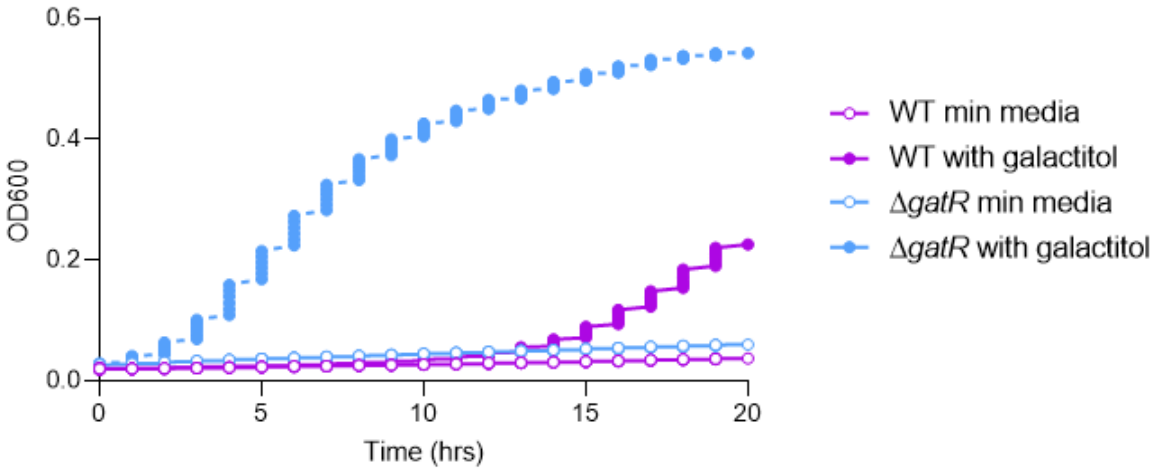


**Fig 6. Growth curves measured in M9 minimal medium with / without galactitol.** X axis represents time in hours (hrs), Y axis represents OD600. Plate reader measurement was carried for 20 hrs. Plotted growth curves contain data points from two independent experiments, each containing two technical replicates. Empty circles represent data points collected in M9 minimal medium, full circles represent

data points collected in M9 minimal medium supplemented with 1% w/v galactitol. WT strain is marked in blue, Δ*gatR* strain is marked in violet.

Thanks to the presence of WITS tags in the *S*. Tm genome we could quantify the relative proportion of individual labelled strains using quantitative PCR. The feces samples from animals E and F from day 2 of cycle 1 and from day 1 of cycle 5 were analyzed. The obtained results illustrated in **Figure 7 (Fig. 7)** show changes in relative proportion in the strains labelled with different WITS tags. Moreover, we analyzed the inoculum as it was administered to the mice at day 0 of cycle 1 (labelled Inoc), and enriched inoculum after an overnight cultivation in LB (labelled Mix). We found that in the animal E in the cycle 5, strains carrying WITS2, WITS13 and WITS21 showed an increased proportion in the overall population. In the animal F strains labelled with WITS2 and WITS21 showed an increased proportion in the population. The increased proportion of a given strain could be related to emergence and fixation of a beneficial mutation. The two different *malE* mutations identified in the animal E by Illumina sequencing, were found in strains carrying WITS13 and WITS21. Strains with these tags were found in an increased proportion in the population on day 1 of cycle 5. This could suggest that *de novo* mutations in *malE* were beneficial to *S*. Tm and thus strains carrying these mutations increased in frequency in the population. Similarly, in animal F the *malF* mutation has been identified in strain carrying WITS2. This strain was present at the high frequency in the population on day 1 of cycle 5. Collectively, the mutations we identified were found in strains present at high frequency in the population. This suggest that *de novo* mutations in the *mal* operon are beneficial to *S*. Tm in the LCM mice gut.

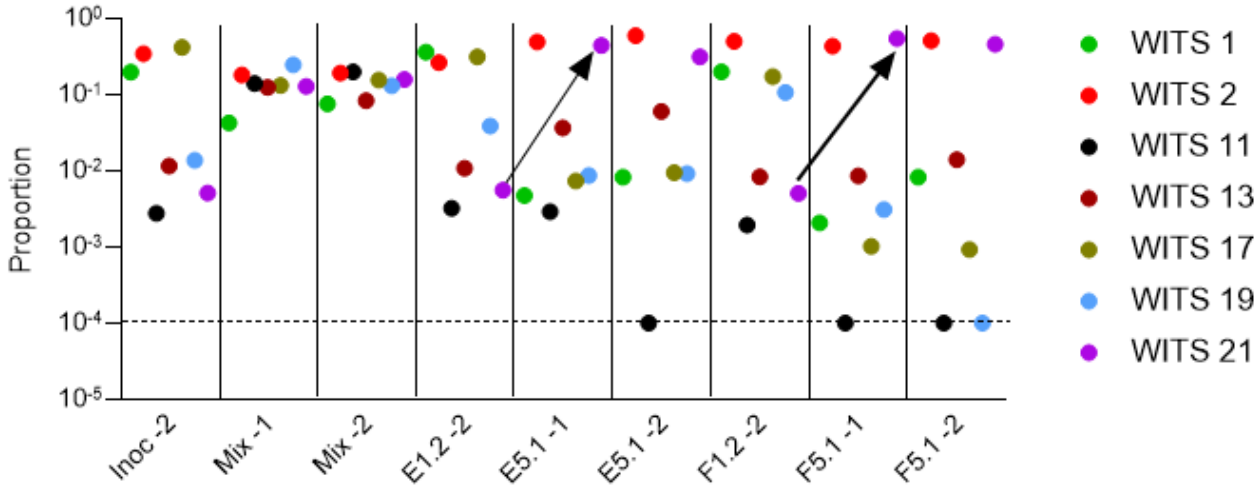


**Fig. 7. Relative proportion of WITS tagged strains.** Samples from animals E and F were analyzed by quantitative PCR (qPCR). X axis corresponds to different samples, Y axis represents relative

proportion of different WITS strains in log scale. Numbers associated with animal indicate in order: number cycle, day p.i. and dilution. Solid vertical lines separate datapoints from different samples. Dotted horizontal line indicates detection threshold. Solid circles represent different WITS chromosomal tags: green – WITS1, red – WITS2, black – WITS11, brown – WITS13, khaki – WITS17, blue – WITS19 and violet – WITS21.

## *Materials and Methods*

**Experiments presented in this chapter were performed by Dr. Andrea Rocker with exception of the mutant construction**

### Strains and media

All the strains and plasmids used in this study are listed in **Table 3**. *Salmonella enterica* sero-var Typhimurium SB300 (SL1344) (Hoiseth & Stocker, 1981) and derivatives were cultivated at 37˚C using LB liquid or solid media or M9 minimal medium. Strains isolated from mice were cultivated on MacConkey solid media. Antibiotic selection was performed with 100 µg/ml ampicillin 6 µg/ml chloramphenicol and 50 µg/ml kanamycin when needed. When stated, media was supplemented with 1% w/v galactitol. Used strains are listed in the **Table 3.**

**Table 3. List of strains used in the study**

| Strains | Genome | Reference |
|---|---|---|
| *S.* Tm SB300 | WITS 1 *aphT putAP::rpsM:gfp::cat* | (Grant et al., 2008) |
| *S.* Tm SB300 | WITS 2 *aphT* | (Grant et al., 2008) |
| *S.* Tm SB300 | WITS 11 *aphT* | (Grant et al., 2008) |
| *S.* Tm SB300 | WITS 13 *aphT* | (Grant et al., 2008) |
| *S.* Tm SB300 | WITS 17 *aphT* | (Grant et al., 2008) |
| *S.* Tm SB300 | WITS 19 *aphT* | (Grant et al., 2008) |
| *S.* Tm SB300 | WITS 21 *aphT* | (Grant et al., 2008) |
| *S.* Tm SB300 | WT SL1344 | (Hoiseth & Stocker, 1981) |
| *S.* m SB300 | Δ*gatR::aphT* | This study |

### Mutant constructions

*Salmonella* mutants used in this study were constructed by homologous recombination using the λ-Red gene replacement system as described in (Datsenko & Wanner, 2000). To select for recombinants, the chloramphenicol acetyltrasferase (cat) gene was amplified from the

pKD3 template plasmid using primers containing 40-bp region homologous to flanking regions of the target gene in the chromosome of *Salmonella*. The PCR product was transformed by electroporation into the recipient strain harboring pKD46 helper plasmid encoding λ phage *red, gam*, and *exo* genes controlled by an arabinose inducible promoter. Recombinant bacteria were selected on the LB plates containing chloramphenicol. Following recombination, the chloramphenicol resistance cassette was removed using the flippase encoded on the pCP20 helper plasmid. Correct gene replacement and resistance cassette deletions were confirmed by the PCR. Kanamycin resistance was obtained by integrating the *aphT* resistance cassette using Tn7-based tagging of *Salmonella* using pGRG36 Tn7 delivery plasmid as described in (McKenzie & Craig, 2006). Plasmids and primers used for the mutant construction are listed in the **Table 4**.

**Table 4. List of the plasmids and mutants used for the mutant construction**

| **Plasmids** | | |
|---|---|---|
| **Plasmid** | **Resistance** | **Reference** |
| pKD3 | Chloramphenicol | (Datsenko & Wanner, 2000) |
| pCP20 | Ampicillin | (Datsenko & Wanner, 2000) |
| pGRG36 | Ampicillin, Kanamycin | (McKenzie & Craig, 2006) |
| **Primers** | | |
| GalR_for | tatgaactcatttgagcgaagaaataaaattgtcgacctgatgggaattagccatggtcc | |
| GalR_rev | agacgatcattaattccacgcctgttttcgctaatgccgcgtaggctggagctgcttc | |
| GalR_up | ggaaagctttgcagaagcag | |
| GalR_down | gtataagtatgcgctggagc | |

Plate reader measurement of OD600

WT and Δ*gatR* strains were cultivated in 5 mL of LB supplemented with the appropriate antibiotics. The plate reader measurements were performed in a 96 well plate. 2µl of overnight culture were added to 200 µl of media (M9 minimal medium +/- 1% w/v galactitol). Optical density of strains was measured using the absorbance at 600 nm for duration of 20 hrs. Data points were collected in two independent experiments, each containing two technical replicates for each strain.

*In vivo* experiments

Experiment consisted of 5 consecutive cycles including 4 transmission steps. The 12-week-old low complexity microbiota (LCM) C57BL/6 mice (n=4). Individual WITS strains were grown

separately overnight in an LB containing the appropriate antibiotics. Prior to infection, the bacteria were grown to the late exponential phase in LB without antibiotics. Mixture of 100 µl of each of the WITS strains was prepared (total 700 µl), washed in PBS and diluted 1:100 in PBS. Each 50 µl of inoculum contained a total of c.a. $5 \times 10^7$ CFU an was provided to mice by an oral gavage. Fecal samples were collected daily, homogenized in 1-ml PBS by bead beating, and bacterial populations were enumerated by selective plating on MacConkey agar containing the appropriate antibiotics. In addition, samples were frozen for determination of the LCN2 concentration. To determine inflammatory state of the gut, serial dilutions of the fecal samples were analyzed using the Mouse LCN2-2/NGAL DuoSet ELISA kit according to the manufacturer's instructions. Infection was allowed to proceed for 3 days p.i. and then the mice were euthanized. On the day 3 p.i. 50 µl of the homogenized fecal pellet were administered to the next group of mice by an oral gavage.

Quantitative PCR analysis

All qPCRs were performed in 20 µl reaction volumes in 0.1-ml tubes in a QuantStudio3. Reactions contained 10 µl of SYBR Green PCR Kit reagent, 4 µl of RNase-free water, 2 µl of 1.5 mM forward and reverse primers, and 2 µl of template DNA (~100ng / µl; diluted from $10^0$ – $10^{-5}$). Reaction conditions were 95 °C for 10 min, 40 cycles of 95 °C for 15 sec, 60 °C for 30 sec, and 72 °C for 20 sec. It was not possible to perform a full standard curve for each primer pair on every rotor; however, the individual standards were included on each rotor run to ensure that the values obtained were in the range expected. (For detailed description of qPCR method, see (Grant et al., 2008). Primers used for the qPCR analysis are listed in the **Table 5.**

**Table 5. List of primers used for the qPCR analysis.**

| Primer | Sequence |
|---|---|
| WITS1 | acgacaccactccacaccta |
| WITS2 | acccgcaataccaacaactc |
| WITS11 | atcccacacactcgatctca |
| WITS13 | gctaaagacacccctcactca |
| WITS17 | tcaccagcccacccctca |
| WITS19 | gcactatccagccccataac |
| WITS21 | acaaccaccgatcactctcc |
| ydgA | ggctgtccgcaatgggtc |

Full Genome Sequencing

For the full genome sequencing, 3 colonies isolated from each animal included in the last experimental cycle were inoculated in 5 mL of liquid LB supplemented with kanamycin. DNA from 500uL of the ONC was purified using The DNeasy Blood&Tissue kit. Samples were sent for Illumina sequencing to MIGS using sequencing package 200 Mbp. Results were analyzed by software CLC Genomics Workbench v 20, *S.* Tm SL1344 reference genome NC_016810.1.

Bacteria are constantly adapting to their environment. For enteropathogens both stages: within-host growth, and transmission often associated with the outside host environments, are integral parts of the pathogen lifecycle. By changing their genomes bacteria can improve their fitness in a new environment. If the acquired mutation is beneficial for the pathogen in a given environment it should increase in frequency in the population, whereas if the new mutation is reducing pathogen fitness it should be counter selected (Didelot et al., 2016).

*S.* Tm lifestyle involves cycles of host colonization followed by passages through the environment outside the host. These two environments differ dramatically in terms of prevailing conditions. Within the animal host bacteria face relatively constant temperature conditions, the presence of microbiota, and host immune barriers. In this case, potential adaptations acquired within-host can enable the pathogen to digest available nutrients, compete with microbiota, or overcome host immune defenses (Didelot et al., 2016; Tanner & Kingsley, 2018). Previous studies identified several genes in *S.* Tm, which when inactivated, resulted in the increased growth rate, virulence, or both. Inactivation of *pcgL* (Mouslim et al., 2002), *phoN* (Miller et al., 1989), *grvA* (Ho & Slauch, 2001), *pnp* (Clements et al., 2002) genes increased the bacterial growth rate/ virulence in mice. However, it is not fully clear how these mutations confer their effect. In the case of the prolonged infection, a strain isolated after several weeks from a patient with persistent *Salmonella* infection carried mutations within *shdA* gene encoding a surface localized fibronectin-binding protein involved in the intestinal persistence (Kingsley et al., 2000; Kingsley et al., 2004). On the other hand, during transmission bacteria are exposed to very different environmental factors.

The majority of non-host environments are characterized by variable temperature and moisture, pH fluctuations, exposure to sunlight, low availability of nutrients, and presence of toxins. Interesting question regarding *S.* Tm environmental adaptation is how adaptations acquired in the within-host environment impact survival outside host environment and vice versa. The antagonistic pleiotropy hypothesis of niche specialization suggests that adaptive mutations that increase fitness in one niche will inevitably cause a loss of fitness in a dissimilar secondary environment (Lynch & Gabriel, 1987; Turner & Elena, 2000; Caley & Munday, 2003). Nilsson et al. in their study address this question in a laboratory setup. The authors used mice inoculated intraperitoneally with *S.* Tm strain LT2. After 3-4 days of infection cells harvested from the spleen were used to inoculate new mice. This cycling was repeated for 8-

10 cycles, corresponding to between 66 and 132 generations. To test if adaptations evolved in mice resulted in loss of fitness in the secondary environment, the competition experiments between evolved lineages and their ancestors were carried in LB at 30°C, 37C° and 42°C. Interestingly, the mouse evolved strains showed no general loss of fitness under laboratory conditions (Nilsson et al., 2004). However, as the competition experiments were carried out in a rich medium, perhaps it was similar to the within-host environment in terms of nutrient availability. An experimental setup with a less nutrient-rich medium would be interesting to test. Our understanding of how mutations evolved within the host impact *S*. Tm fitness outside the host remains limited.

Another related aspect concerns how the mutations evolved within one host can influence colonization of the secondary host. *S.* Tm can infect a wide range of animal species inhabiting various environments and consuming specialized diets. However, even individuals from the same species can consume very different food and have different microbiota species inhabiting the gut. Since *S*. Tm gets inside the host via digestion of contaminated food or water, the infection starts in the gastrointestinal tract of the host. In the early stages, the success of infection depends on *S.* Tm ability to overcome the colonization resistance of microbiota. Therefore, the inter-individual variation in the microbiota community composition is an important factor determining *S.* Tm colonization of the given host (Stecher et al., 2010; Thiemann et al., 2017). Whereas the adaptive potential of bacteria is remarkable, it remains unclear what are the specific determinants improving *S*. Tm survival withing the host gut in the early colonization stages. In our study we are address this knowledge gap in the mouse infection model.

The experimental setup consists of 5 consecutive cycles of within-host evolution divided by 4 events of transmission to the next host. Animals are infected via oral gavage, and with the same technique bacteria are transferred to the secondary host after 3 days of within host evolution. This experimental setup is not suitable for the study of how mutations that evolved within the host would impact *S*. Tm survival in the external environment. However, this experimental structure allows to sample impact of repeating colonization events, on the pathogen diversity. The LCM mice we use in the study share the same microbiota composition, therefore minimizing the individual-to-individual microbiota variability. Moreover, all animals are eating the same type of food thus eliminating dietary-related differences. In this experiment It is possible to track if *S.* Tm strain composition, evolved within first host, subsequently transferred to the secondary host would be maintained or lost due to the transmission

bottleneck. Thanks to monitoring *S*. Tm colonization in each host throughout the experiment, it can be determined, if the strains evolved with subsequent cycles would become better colonizers. To follow the population dynamics, 7 WITS tagged strains were employed (Maier et al., 2014). The relative abundance of differentially tagged strains will be monitored with qPCR. Since all the strains are barcoded, it would be possible to follow the emergence and fixation of mutations within the population reflected in a relative abundance of a given barcode. Resolution at which we can detect the fixation of the new mutants is limited. In a case when two cells with the same barcode, would develop two different beneficial mutations, the qPCR technique will not be able to differentiate between these clones. Moreover, the number of clones send for sequencing will further determine how many evolved mutations can be captured in the study. Since the microflora of the LCM mice is limited to several species, the colonization resistance in these animals is more infection-permitting compared to the conventional mice (Stecher et al., 2010). However, the colonizing *S.* Tm should compete with microbiota for the niche and nutrient resources. Therefore, the metabolism related mutations can improve the pathogen fitness in this setup. Moreover, since all animals share the same microflora, these metabolism related mutations in *S.* Tm should be beneficial for pathogen in all the hosts. The length of each experimental cycle permits the onset of inflammation, however, we tried to design the timeline where the time at which host gut is inflamed is not much longer than the time before onset of inflammation. With this we limit the possibility that clones evolved during inflammation would become dominant in the population preventing observation of mutations emerging in the earlier stage of gut colonization. The established experimental setup allows us to monitor emergence and fixation of mutants within the host, and their ability to colonize secondary host.

The use of LCM mice, colonized with a stable low-complexity gut microbiota represents an attractive experimental system. In these animals, the host gut microflora, which is absent from the antibiotic pretreated animals often used as experimental models, constitutes an important factor shaping *S.* Tm diversity within the host through competition with other bacteria (Rogers et al., 2021). At the same time, the present microbiota does not restrict *S.* Tm colonization and the onset of disease. Throughout the entire experiment *S.* Tm colonization in the host gut was monitored as the CFU / g of feces. Moreover, with exception of one animal in cycle 2, by the end of each cycle we detected the LCN-2 indicating an ongoing inflammation in the host gut. In addition, lymph nodes of all animals, except one animal in cycle 2, were colonized by *S*. Tm indicating the systemic spread of infection. Therefore, possible adaptations evolved during the host colonization contributed to successful *S*. Tm infection, as we detected symptoms of the

disease (gut inflammation). Interestingly, we observed a difference in how fast *S.* Tm can colonize the host gut between the experimental cycles. In the cycles 1-2 the pathogen was able to reach the high CFU / g of feces values more slowly than in the subsequent cycles 3-5. This suggest that potentially adaptations acquired during first 2 cycles rendered *S.* Tm a better colonizer. These results confirm that LCM mice are an infection-permissive host suitable to investigate *S.* Tm population dynamics throughout the experiment.

The nutrients available to *S.* Tm in the host gut are primarily shaped by the intrinsic microbiota communities which liberate simple sugars from complex diet and host derived polysaccharides (Ng et al., 2013). In this study, strains sequenced at the end of last cycle of the experiment have been found to carry mutations in the metabolic traits. The mutations we identified are within genes: *malE, malF* and *gatR.* By acquiring mutations within *malE* and *malF* genes, the maltose acquisition in *S.* Tm was impaired suggesting either lack of maltose within the host gut, or *S.* Tm preference for another carbon source. Interestingly, the *gatR* mutation is inactivating the galactitol utilization repressor, thus in fact increasing *S.* Tm ability to process galactitol. In their recent study, Eberl et al. showed that in $OMM^{12}$ mice, intrinsic *E. coli* by competing with *S.* Tm for galactitol utilization, was able to enhance the colonization resistance against the pathogen. This protective effect strongly dependent on the microbiota composition (Eberl et al., 2021). It is possible that in the case of LCM mice, competition for galactitol also plays an important role for the pathogen to establish the niche in the host gut. The *malE* mutation was found in two barcoded strains isolated from animal E (WITS 13 and WITS 21). The *malF* mutation isolated from the animal F was associated with strain tagged by WITS 2. Interestingly, the qPCR data showed that these three strains (WITS 13 and WITS 21 in animal E; and WITS 2 in animal F) were present at the high frequency in the population. This result suggests that mutations impairing maltose acquisition and processing are beneficial for *S.* Tm in LCM mice. The *gatR* mutation was isolated in the strain carrying WITS 1 barcode isolated from animal G, however, we do not have the qPCR data for this animal. Importantly, we sequenced only strains isolated on the last day of experiment, however, it would be very interesting to try to determine if the same mutations are present in the previous cycles. For example, if we could isolate the same type of *malE* mutation in a strain associated with the same WITS isolated from animal E in the earlier cycle, it would suggest that this strain evolved in the early cycle and got selected for and transmitted in the consecutive cycles. Another possibility would be *de novo* emergence of the same mutation separately in independent cycles. If the dominant strain emerged once and remained in a high proportion in the subsequent cycles, it would support the notion that mutation was selected early in the

experiment. Instead, if within each cycle different WITS tagged strains would dominate in the population, it would be possible that mutations emerged *de novo* in each cycle and did not impact the subsequent cycles.

The composition of microbiota in the host gut plays an important role in shaping the nutritional niche available to *S.* Tm. Identification of the microbiota present in the LCM mice gut using the sequencing of 16S rRNA genes, would further deepen our understanding of dynamics in this bacterial community. Another interesting question would be if a beneficial mutation emerged in the inflamed or non-inflamed host gut. VF-induced inflammation results in the formation of specific habitat within the host gut which supports growth of *S.* Tm and Enterobacteriaceae members. The increased luminal concentration of tetrathionate (Winter et al., 2010), nitrate (Lopez et al., 2015), oxygen (Rivera-Chávez et al., 2016), and lactate (Gillis et al., 2018) supports the growth of *S.* Tm, at the same time restricting the growth of other microbiota (Stecher et al., 2007). Due to the drastic changes in the gut milieu, the onset of inflammation can also correspond with changes in nutrient sources available to *S.* Tm. In their study Nuccio and Bäumler using the comparative genome analysis identified a metabolic network utilized by gastrointestinal pathogens. This *in silico* analysis providing a preview of the resources available to *S.* Tm (Nuccio & Bäumler, 2014). This metabolic network suggests that *S.* Tm has access to the numerous monosaccharides including glucose, gluconate, galactose, galactonate, trehalose, rhamnose, ribose, xylose, arabinose, idonate, 2,3-diketo-glutonate, hexunonate and galactitol. Interestingly, based on this *in silico* analysis it can be concluded that the predicted nutrient niche differs from the noninflamed gut where the microbiota depletes these resources (Caballero-Flores et al., 2020; Ng et al., 2013). Perhaps the mutations we identified at the end of cycle 5 are important for improving *S.* Tm survival during the gut inflammation. With our experimental design we aimed to restrict the time when the gut inflammation is ongoing, to limit fixation of mutants improving *S.* Tm fitness during inflammation. It would be interesting to reconstruct the identified mutations in the avirulent *S.* Tm which cannot trigger gut inflammation and compete such mutant against avirulent WT in the LCM mice. If a given mutation was beneficial specifically during conditions of inflamed gut it should not outcompete the avirulent WT without the inflammation in the same microbiota context. The stage of inflammation is in many ways beneficial to the pathogen for reasons mentioned earlier, however, it also drastically reduces the luminal population of *S.* Tm at the onset of the inflammation (Maier et al., 2014). Therefore, the pathogen could be under strong selection during the onset of inflammation leading to the emergence of beneficial mutations enhancing survival. It would be very interesting to investigate transcriptomic data from clones

isolated from the inflamed gut using RNAseq. Such an experiment could address not only what nutrient sources are consumed by *S.* Tm but also what stress resistance mechanism plays a role in the pathogen survival during early colonization. Additionally, performing the 16S rRNA sequencing to identify microbiota strains present in the LCM mice could help us predict what nutrients are available to *S.* Tm in the gut of these animals. This information would complement our understanding of identified mutations and how they benefit the pathogen growth.

Another important question relates to the fitness of newly evolved strains. To address this question, *de novo* mutations of *mal* operon and *gatR* repressor will be constructed and used in the competition experiments against WT in the naïve mice. It would be also interesting to use the evolved strains in competition against their ancestor WT strain, to determine their relative fitness. The advantage of metabolism-related mutations is likely to be strongly related to microbiota context of the host. Since the LCM mice have simple composition of microbiota species, it is possible that mutations beneficial in this specific context might not be advantageous in infection of host with the more complex microbiota. The experiment with mice carrying more complex microbiota infected with strains evolved in the LCM mice could address the question if the benefit of a given mutation is more universal or limited only to LCM host. Moreover, animal host is a complex model consisting of many niches with different environmental characteristics. Sequencing of strains isolated from the lymph nodes and comparing them with strains isolated from feces could bring new information on how *S.* Tm adapts to these distinct within-host niches. Another interesting aspect relates to the effect of transmission on the diversity of evolved strains. The mice we used share the same microbiome and diet, therefore should be a similar host for *S.* Tm and in such conditions, the strains evolved in one host should also confer advantage in another similar animal. However, the important aspect of our experimental design is the presence of a transmission bottleneck between the cycles of within-host growth. It cannot be excluded that transmission bottleneck plays a role as additional selection pressure and impacts which evolved mutants colonize the secondary host. Studies systematically addressing pathogen adaptation in experimental setup with recurring transmission bottleneck are lacking. Taken together our experimental setup allows the exploration of many interesting questions regarding how the parameters of the pathogen life cycle, like competition with microbiota, and recurring population bottlenecks, impact the evolution of the pathogen and its fitness.

The selective pressures (i.e., competition with microbiota, size of transmission bottlenecks) shape trajectories of pathogen adaptations during its life cycle. These modifications serve pathogen as a double-edged sword. On one hand, they provide genotypic and phenotypic diversity improving exploitation of the new niche or resistance to environmental insults. On the other hand, however, these changes can also lead to evolutionary dead end and the evolution of virulence attenuated clones in pursuit of short-term advantage. A better understanding of pressures shaping *S*. Tm evolution at the different stages of the infection cycle can help design novel therapies against this pathogen.

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