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Bistable regulation of *ttss-1* genes in *Salmonella* Typhimurium

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Es scheint, dass jede Wahrheit heute in zwei einander entgegengesetzte Unwahrheiten zerlegt auf die Welt kommt, und es scheint auch das eine Art sein, zu einem überpersönlichen Ergebnis zu gelangen. (*Robert Musil, Der Mann ohne Eigenschaften*)

Table of contents

Thesis summary

To date, *Salmonella* Typhimurium (*S.* Tm) represents one of the most prevalent human pathogens with implications in diarrheal diseases throughout the world. Consequences of *Salmonella* infections range from diarrhea to lethal systemic infections. Naturally, the human gut represents a reservoir for a number of commensal bacteria that contribute to the human diet and confer colonization resistance against invading bacteria. It is thought that *Salmonella* Tm can subvert the host's inflammatory response to circumvent colonization resistance and facilitate colonization of the gut.

Salmonella Tm virulence relies on a large set of different virulence factors. However, the gastrointestinal inflammation is primarily mediated by a Type Three Secretion System (TTSS-1) encoded on a 40 kb AT-rich region of the chromosome, termed as pathogenicity island 1(SPI-1). Remarkably, the virulence factors of SPI-1 are only expressed in a sub-fraction of the *S.* Tm populations. This phenomenon is observed in laboratory cultures as well as in the gut of the infected host. Bistable gene expression - the stable co-existence of two phenotypes of isogenic populations in a given environment - bases on noise in gene expression and the possibility of its amplification under certain prerequisites. Without doubt, stochastic gene expression plays a key role in the fate of individual cells and the phenotypic diversity in clonal populations will contribute to the overall fitness of the genotype. Besides virulence in *S.* Tm, bistability is observed in other cell differentiation processes, e.g. sporulation of *Bacillus subtilis* or persistence among several bacterial species. The latter differentiation processes usually represent a backup mechanism of the population to deal with unpredictable and dramatic changes in the environment.

So far it has remained unclear why *S.* Tm might benefit from bistable virulence factor gene expression. Normally, virulence factors are expected to enhance the fitness of a population in terms of an evolutionary advantageous strategy. Nevertheless, those considerations are made for the average of the population, but might disregard costs for the individual bacterium. Thus, in the first part of the presented thesis I focused on the question, whether virulence gene expression might entail fitness costs for the individual bacterium. We hypothesized that this could explain the necessity of bistable *ttss-1* expression. We used a number of single cell based fluorescence reporter strains in vitro experiments to monitor the bistable expression of *ttss-1* operons like e.g. *sicAsipBCDA* that encode for the translocon of the TTSS-1 and effector proteins. Our experiments revealed a significantly reduced growth rate accompanying *ttss-1* expression. Mutations in the regulatory cascade resulted either in *S.* Tm populations that were exclusively composed of individuals entirely lacking *ttss-1* expression or in those, which displayed virtually 100 % TTSS-1^+ individuals. Mutations leading to an increased fraction of the *ttss-1* expressing bacteria always correlated with compromised growth. Nevertheless, in LB batch cultures we could observe an increase of the TTSS-1^+ subpopulation entering the late logarithmic phase. We developed a mathematical model that explained the increasing

fraction of TTSS-1$^+$ bacteria in the late logarithmic growth phase of a given *S*. Tm culture by an increased rate of *ttss-1* induction and the concurrent decrease in growth rate of TTSS-1$^+$ bacteria.

The regulatory cascade that controls the expression of *ttss-1* genes is maintained by a set of hierarchically arranged SPI-1 encoded transcriptional activators, HilC, HilD and HilA. Besides this, the proper regulation of SPI-1 is maintained by a remarkable number of additional regulators affecting *ttss-1* expression in response to determinants ranging from metabolism and stress signals to motility. Thus, in a second approach we analyzed the *ttss-1* master regulator HilA. Despite the complexity of the regulatory network, all signals and information seemed to converge on HilA. In contrast to other SPI-1 specific transcription factors as HilD or HilC, HilA exhibits homology to response regulators of two component systems the major signal transduction pathways in bacterial cells. In a pull-down assay we could indentify the chemotaxis sensory kinase CheA as an interaction partner of HilA. As response regulators get usually phosphorylated by their cognate sensory kinase, we also analyzed phosphorylation of HilA, but could not find evidence for this kind of posttranslational modification. Nevertheless, Δ*cheA* mutants displayed enhanced levels of the HilA target operon *sicAsipBCDA*. Yet, HilA levels themselves remained unaffected by CheA. This established a novel level of co-regulating flagellar function and *ttss-1* expression.

Furthermore, we found evidence for posttranslational modifications of HilA. Besides phosphorylation, acetylation of proteins plays a major role in the transmission of signals throughout the cell. Mass spectrometric analyses revealed that two lysine residues K_{231} and K_{324} of HilA are subject to N_ε-acetylation by the acetylCoA-synthetase Acs. It is mainly involved in the central metabolism making acetate accessible for the citric acid cycle and thus for energy release. The modification of HilA also changed its activity and led to decreased transcription of *sicAsipBCDA*. Again, HilA levels remained unaffected. Both, the interaction with CheA and the posttranslational N_ε-acetylation changed the ratio of TTSS-1$^+$ to TTSS-1$^-$ fractions within the population and hence represent mechanisms to fine-tune bistability.

Finally, we observed that *S*. Tm responds to temperature changes. We found that temperatures below 30 °C as well as above 40 °C remarkably reduced the expression of SPI-1 virulence genes. Again, we conducted a series of single cell reporter assays monitoring the expression of the *sicAsipBCDA* operon. We observed that the ratio of TTSS-1$^+$ to TTSS-1$^-$ bacteria varied in response to temperature. As 40 °C describes an environmental cue, which can also occur during infection (fever), we speculated that fever might represent a host response sensed by the pathogen to avoid excessive disease and compromised host survival, which could limit pathogen transmission.

In conclusion, this work provides new insights into the regulation of *ttss-1* genes of *S*. Tm. It elucidated that *ttss-1* expression has intriguing consequences for the individual bacterial cell and

considers the growth cost by calculations of *ttss-1* initiation rates by a new mathematical model. Furthermore, it manifests the central role of HilA in the regulatory cascade in respect to integration of chemotaxis signals (CheA) and to information about the metabolic state of the bacterium by acetylation (Acs) and how those mechanisms can affect the ratio between TTSS-1 subpopulations. This represents a key advance in our understanding of the infection process.

Zusammenfassung

Bis heute stellt *Salmonella* Typhimurium (*S.* Tm) eines der weltweit am häufigsten vorkommenden menschlichen Pathogene dar. Folgen einer Salmonelleninfektion reichen von Durchfall bis hin zum Teil tödlich verlaufender systemischer Infektionen. Der menschliche Darm wird naturgemäß von einer Reihe symbiotischer Bakterien besiedelt, die zum einen zur Verbesserung der Verdauung beitragen und zum anderen eine sogenannte Kolonisationsresistenz gegenüber normalerweise darmfremden, invasiven Bakterien vermitteln. Es wird vermutet, dass *S.* Tm die im Wirtsorganismus (z.B. Mensch) ausgelöste Entzündung benützt, um die von den kommensalen Bakterien vermittelte Kolonisationsresistenz zu umgehen.

Die Virulenz von *S.* Tm stützt sich auf eine große Anzahl verschiedener Faktoren. In erster Linie wird jedoch die Magen-Darm-Entzündung durch das Typ-III-Sekretionssystem (TTSS-1) hervorgerufen, das auf einer 40 kb AT-reichen Region des Chromosoms, *Salmonella* Pathogenitäts Insel 1 (SPI-1), kodiert wird. Bemerkenswerterweise sind die Virulenzfaktoren von SPI-1 nur in einem Teil des *S.* Tm Population exprimiert. Dieses Phänomen wird sowohl in Laborkulturen sowie im Darm des infizierten Wirts beobachtet. Bistabile Genexpression - die stabile Koexistenz zweier Phänotypen einer isogenen Population unter identischen Umweltbedingungen – basiert auf *noise* (stochastisch auftretende Unregelmäßigkeiten) in der Genexpression und der Möglichkeit diesen zu amplifizieren. Zweifelsohne spielt stochastische Genexpression eine wichtige Rolle im Schicksal einer einzelnen Zellen und der phänotypischen Vielfalt in einer klonalen Population, die wiederum zur Fitness dieses Genotyps beitragen. Neben Virulenz in *S.* Tm ist Bistabilität in anderen Zelldifferenzierungsprozessen zu beobachten, z.B. während der Sporulation von *Bacillus subtilis* oder der Koexistenz von persistenten und nicht persistenten Individuen innerhalb verschiedener Bakterienarten. Letztere Differenzierungsprozesse beschreiben Versicherungsmöglichkeiten mit unvorhersehbaren und dramatischen Veränderungen in der Umwelt umzugehen.

Bisher war es unklar, warum *S.* Tm von einer bistabilen Expression der Virulenzfaktoren profitieren könnte. Normalerweise sorgen Virulenzfaktoren dafür die Fitness einer Population im Sinne eines evolutionären Vorteils zu steigern. Diese Überlegungen treffen für den Durchschnitt der Population zu, ignorieren aber gegebenenfalls die Kosten für einzelne Bakterien. So konzentriere ich mich im ersten Teil der vorliegenden Arbeit auf die Frage, ob die Expression von Virulenzfaktoren Fitnesskosten für die einzelnen Bakterien zur Folge haben. Wir verfolgten die Hypothese, dass diese Kosten bistabile *ttss-1*-Expression notwendig macht. Hierzu haben wir eine Reihe auf Einzelzellebene basierender Fluoreszenzreporterstämme in vitro Experimenten konstruiert, um die bistabile Expression des *ttss-1*-Operons, wie z.B. *sicAsipBCDA*, das das TTSS-1-Translokon und Effektor-Proteine codiert, mikroskopisch zu untersuchen. Unsere Experimente zeigten eine deutlich verringerte

Wachstumsrate begleitend zur *ttss-1*-Expression. Mutationen in den regulatorischen Kaskade resultierten entweder in *S.* Tm Populationen mit fehlender *ttss-1*-Expression oder aber mit nahezu 100% TTSS-1$^+$ Individuen. Solche Mutationen, die zu einem erhöhten Anteil des *ttss-1* exprimierenden Bakterien führten, korrelierten immer mit eingeschränktem Wachstum. Daraus schlussfolgernd müssten TTSS-1$^+$ von TTSS-1$^-$ Individuen letztlich verdrängt werden. In LB Flüssigkulturen beobachteten wir jedoch eine Zunahme der TTSS-1$^+$ Subpopulation. Wir entwickelten ein mathematisches Modell, dass den zunehmenden Anteil von TTSS-1$^+$ Bakterien in der spät-logarithmischen Wachstumsphase einer *S.* Tm Kultur mithilfe einer erhöhten *ttss-1*-Induktionrate und dem gleichzeitigen Rückgang der Wachstumsrate der TTSS-1$^+$ Subpopulation erklärt.

Die regulatorischen Kaskade, die die Expression der *ttss-1* Gene steuert, wird durch eine Reihe von hierarchisch angeordneter SPI-1 kodierter Transkriptionsaktivatoren, HilC, HilD und HilA, bestimmt. Zusätzlich wird SPI-1 durch eine bemerkenswerte Anzahl von zusätzlichen Regulatoren beeinflusst. Diese reichen von Stoffwechsel und Stresssignalen bis hin zu Faktoren der Motilität. So analysierten wir in einem zweiten Ansatz den TTSS-1$^-$Master-Regulator HilA. Trotz der Komplexität des regulatorischen Netzwerkes, schienen alle Signale und Informationen bei HilA zu konvergieren. Im Gegensatz zu anderen SPI-1$^-$spezifischen Transkriptionsfaktoren wie HilD oder HilC weist HilA Homologie zu *response regultors* von Zwei-Komponenten-Systeme auf, den wichtigsten Signaltransduktionswegen in Bakterienzellen. In einem Pull-Down-Assay konnten wir die *chemotaxis sensory kinase* CheA als Interaktionspartner von HilA identifizieren. Da in der Regel *response regulators* durch ihre spezifische *sensory kinase* phosphoryliert werden, haben wir auch die Phosphorylierung von HilA untersucht, konnten jedoch keine Hinweise für diese Art der posttranslationalen Modifikation feststellen. Dennoch scheinen Δ*cheA* Mutanten die Expression des von HilA regulierten Operon *sicAsipBCDA* zu erhöhen. Dabei blieb die HilA Konzentration an sich von CheA unbeeinflusst. Mit diesen Daten konnten wir eine weitere Schnittstelle in der Co-regulation zwischen Flagellen und *ttss-1*-Expression aufdecken.

Darüber hinaus fanden wir Hinweise auf posttranslationale Modifikationen von HilA. Neben Phosphorylierung spielt Acetylierung von Proteinen eine wichtige Rolle bei der Übertragung von Signalen in der Zelle. Massenspektrometrische Analysen ergaben, dass zwei Lysinreste K_{231} und K_{324} von HilA N_ε-Acetylierung durch die AcetylCoA-Synthetase Acs unterliegen. Deren Funktion besteht vor allem im zentralen Stoffwechsel. Hier macht es Acetat für den Zitronensäure-Zyklus und damit für die Energiefreisetzung zugänglich. Die Modifizierung von HilA beeinflusste dessen Aktivität und führte zu einer verminderten Transkription des *sicAsipBCDA* Operons. Auch hier blieb die HilA Konzentration unberührt. In beiden Fällen, der Interaktion mit CheA und die posttranslationale N_ε-

Acetylierung, änderte sich das Verhältnis von TTSS-1^+ zu TTSS-1^- Fraktionen innerhalb der Population. Beide beschreiben somit Mechanismen, die Bistabilität beeinflussen können.

Schließlich beobachteten wir, dass *S.* Tm auf Temperaturänderungen reagiert. Wir fanden, dass bei Temperaturen unter 30 °C sowie über 40 °C die Expression von SPI-1 Virulenzgenen merklich reduziert war. Wieder haben wir eine Reihe von Einzelzellreporterassays benützt, um die *sicAsipBCDA* Expression zu dokumentieren. Wir konnten beobachten, dass das Verhältnis von TTSS-1^+ an TTSS-1^- Bakterien in Abhängigkeit von der Temperatur variiert. Bei Temperaturen um 40 °C handelt es sich um Bedingungen, die auch während der Infektion (Fieber) auftreten. So vermuteten wir, dass Fieber eine Wirtsreaktion darstellt, die vom Erreger bemerkt und eine übertriebene, außer Kontrolle geratene Infektion, eine erhöhte Wirtssterblichkeit zu vermeiden, die die Übertragungsrate des Erregers verringern würde.

Zusammenfassend beschreibt diese Arbeit neue Erkenntnisse über die Regulation der *ttss-1* Gene von *S.* Tm. Sie beschreibt Kosten für das einzelne Bakterium, die mit der Expression der Virulenzgene einhergehen. Sie berücksichtigt die Wachstumskosten für die Population mit Hilfe eines neuen mathematischen Modells zur Berechnung der *ttss-1* Initiationsrate. Darüber hebt sie die zentrale Rolle von HilA innerhalb der regulatorischen Kaskade hervor, indem es Chemotaxis-Signale (CheA) sowie Informationen über den metabolischen Zustand der Zelle durch Acetylierung (ACS) inkorporiert und damit das Verhältnis zwischen TTSS-1 Subpopulationen beeinflusst. Dies stellt einen wichtigen Fortschritt in unserem Verständnis des Infektionsprozesses dar.

Chapter I

General Introduction

Introduction

Salmonella spp. in the World and in Science

For the majority of people, *Salmonella* represents an unwelcome companion of poultry and eggs in the western food industries. Sources of *S.* spp. infections are improperly treated meat and drinking water. After oral ingestion of food contaminated with *S.* spp., people usually develop disease symptoms ranging from gastroenteritis to typhoid fever due to infection. Therefore, *S.* spp. are referred to as pathogens. Nowadays, even in industrialized countries there are still high numbers of incidences per year and over one billion estimated cases worldwide (108). Especially people at developing countries suffer from Salmonellosis with three million deaths per year; mainly due to inadequate hygienic standards of food treatment and insufficient medical infra-structure (120, 121).

For this reason, *S.* spp. have been a subject of research for the last century since isolates have been first described by T. Smith and D. E. Salmon in 1885. During the first period of research, the attempt was to counteract its life threatening character. After the discovery of penicillin by A. Fleming (51) and the improving development of antibiotics, *S.* spp. lost the reputation as life threatening bacteria in the industrialized world. Though, they remain a major reason for massive use of antibiotics in high scale farming.

Today, *S.* spp. are mainly used as model organisms to investigate general principles and processes of bacterial infection. Thus, most studies of *S.* spp. take place at the host pathogen interface and ask how they affect each other. For this purpose, a number of model systems have been established in order to address these questions, ranging from cell culture to invertebrates, e.g. flies (*Drosophila melanogaster*), to more complex systems as mice (*Mus musculus*) or cattle (*Bovis tauris*). *Salmonella* emerged also as a useful tool to study functions of the native immune system within the gut (170). Besides this, a major topic deals with the problem of virulence gene expression within the infected host. In 2003, the lab of J. Hinton could demonstrate in vitro via usage of fluorescent reporter proteins, that only a fraction of a *S.* Typhimurium (*S.* Tm) population expresses the virulence genes of its pathogenicity island 1 (68). This phenomenon is also observed during the infection of mice (3).

Since these questions cannot be addressed and solved by deterministic gene regulation models, stochastics in gene regulation were introduced to explain the occurrence of phenotypic noise and the emergence of subpopulations under identical environmental cues (3, 11, 136, 161). Certain evidences show that the impact of bistability (the occurrence of two subpopulations simultaneously) in terms of differentiation and evolution is broader than originally anticipated. This reinforces the role of *S.* spp. as model organisms in biological science in order to achieve a more comprehensive understanding of how bacterial individuals emerge.

Salmonella **spp. - Classification**

Salmonella spp. are rod-shaped gram-negative, motile and facultative anaerobic bacteria that belong to the family of *Enterobacteriacea* a subdivision of γ-Proteobacteria that have the ability to perform an intracellular lifestyle. Besides infecting humans, *S.* spp. exhibit a rather broad spectrum of hosts, ranging from mammals and birds to reptiles. The usage of different vehicles and animal transport facilitated worldwide transmission of *S.* spp.

The genus *Salmonella* is divided in three different species *S. bongori, S. subterranean and S. enterica*, which is further subdivided in six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) (74). To assign the variances within subspecies, *S.* subspecies are classified in different serotypes (serovars), which refer to specific characteristics within the lipopolysaccharide (LPS, O-Antigen) and the flagellar (H-Antigen) antigens according to the Kauffman-White-scheme (86, 93). Prominent serovars are Typhimurium, Enteritidis, Typhi and Paratyphi, since they cause the highest number of reported Salmonellosis cases. Conventionally, *Salmonella* species are referred to genus and serotype so that *Salmonella enterica* subspecies *enterica* serovar Typhimurium is often used in the abbreviated form *Salmonella* Typhimurium (*S.* Tm). *S.* Tm has a broad host range, but serovars as *S.* Typhi (primates) or *S.* Gallinarum (poultry) are restricted to specific hosts.

The infection by ***Salmonella*** **Typhimurium**

Contaminated food and drinking water represent the primary source of an infection with *S.* Tm . Thus, *S.* Tm uses the gut as the port of entrance to the human body to cause Enteritis. Bacteria, which survive the acidic milieu of the stomach (10), invade non-phagocytic enterocytes of the intestine and M-cells within defined regions, the Peyer's patches (133). Besides the bacteria-induced endocytosis, immune cells like dendritic cells (DCs) sample the intestinal gut flora and facilitate the active uptake of *S.* Tm (104, 114). This second entrance pathway allows even non-invasive *S.* Tm strains still to infect systemically (65). Both ways are accompanied by immune responses. These are triggered by the pathogens virulence factors or via pathogen recognition by pathogen-associated molecular pattern receptors (TLRs) releasing chemokines and cytokines in order to provoke inflammatory responses (138, 160). Simultaneously, attraction and activation of leukocytes and injuries of the tissue architecture as e.g. edema are observed as well as supporting defense mechanisms as antimicrobial peptides and reactive oxygen species release (155, 170).

Clearly, the onset of an infection also represents substantial challenges for *S.* Tm. First of all, the human gut is colonized by a remarkable number of commensal bacteria (*Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Fusobacterium*, *Lactobacillus*, *Peptococcus*, *Peptostreptococcus* are predominant (106, 140)). They outnumber the cells of the human body by hundred fold. The majority of these commensals does not provoke harm to the host, but in contrast, supports digestion and thus the uptake of nutrients by human gut cells. Besides this, they are involved in immune responses and maintenance of a very *xenophobic* society that complicates infections by incoming pathogens. This makes commensals to contributors of health (156). The inflammation induced by *S.* Tm is a strategy to suppress commensal growth, while *S.* Tm can use these conditions for enhanced growth and transmission. The musosa sheds glycosylated proteins that can be utilized by *S.* Tm. In contrast Lipocalin secretion of epithelial cells impairs iron uptake of commensals but not of *S.* Tm (127). Recent studies give also rise to a specialized anaerobic respiration of which *S.* Tm takes advantage of, whereas the majority of commensals fail to accomplish this (171). Additionaly, *S.* Tm displays high resistance against antimicrobial peptides that are secreted by the mucosa (103, 153, 155, 170, 171).

What major attributes are required in order to invade host cells and to compete with commensals, so that a bacterium like *S*. Tm becomes a pathogen?

Virulence factors of *S*. Tm

S. Tm is closely related to the nonpathogenic isolates of *Escherichia coli* (e.g. *E. coli* K12). Just a number of additional genes can turn an inoffensive bacterium into a pathogen that gained the capability to invade eukaryotic cells and survive intracellular. Those regions, termed pathogenicity islands, have been acquired by horizontal gene transfer and differ from the remaining genome by an elevated AT ratio (61, 62, 113). Besides Pathogenicity islands (PAIs), pathogens feature a set of mobile genetic elements (e.g. plasmids, transposons) that ensure a fexible gene pool with regard to the host adaptation (64). Flexibility also includes mutations and downsizing the genome (64). It paves the way to an obligate intracellular lifestyle like *Mycoplasma* spp. perform (150).

Two prominent islands are crucial for the successful infection of *S*. Tm: *Salmonella* Pathogenicity Island 1 and 2 (SPI-1 in Fig. 1 and SPI-2,). Both islands encode proteins that assemble to a needle like complex spanning both bacterial membranes and eventually the cytoplasmic membrane of the host cell; Type Three secretion System 1 (TTSS-1 on SPI-1) and TTSS-2 (in the case of SPI-2). The architecture of such an apparatus is designated to transport proteins from the bacterial cytosol to the cytoplasm of a host cell. In addition, the pathogenicity islands carry information for transcription factors that mediate the appropriate expression of SPI-1 (resp. SPI-2) internal genes, effector proteins and their chaperones. Thereby pathogenicity islands determine the expression, the structure and functionality of these potent virulence systems.

Since the docking, invasion and intracellular survival of *S*. Tm are complex processes that cannot be guaranteed exclusively by TTSS-1 and TTSS-2, *S*. Tm harbors additional factors determining the success of infection. The chemotaxis/flagellar system contributes to it (146, 153, 154) as well as

further pathogenicity islands encoding for SPI-1 co-regulated adhesins (SPI-4) (57, 58) or effector proteins as e.g. SopB (SPI-5) (134, 174).

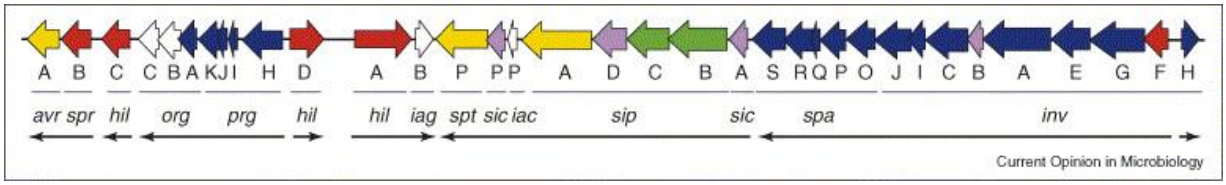


Figure 1, *Salmonella* Pathogenicity Island 1 (SPI-1). SPI-1 is a 40 kb island within the chromosome of *S.* Tm. Three big operons *spa-inv*, *prg-org* as well as *sic-sip* encode for effector proteins (yellow), needle complex structural genes (blue), translocon components (green and *sipD*), genes of unknown function (white) and the regulator *invF* (red), which assembles together with *sicA* (violet). In addition, the regulators *hilA*, *hilC* and *hilD* constitute their own operons (red); adapted from (44).

TTSS-1 and effector proteins

Life, of course, requires membranes, which separate the cytoplasm from the environment. In the case of gram-negative bacteria there are two membranes (inner and outer membrane) that have to be crossed in order to get from cytoplasm to the exterior or vice versa. Membranes consist mainly of fatty acids and other lipophilic components. On the one hand they define a barrier and thereby enable life; on the other hand proteins of the cytoplasm cannot easily diffuse to the exterior, which in terms of degradation, motility, communication or invasion and for many other processes represents an essential prerequisite. For these purposes, microorganisms employ a number of different more or less complex protein secretion systems. They range from simple systems spanning one membrane and require the Sec- or Tat pathways (reviewed in (50)) to huge machineries that are integrated in both membranes and bridge thereby the periplasm (130). They are classified according to their structure and functionality from Type I to Type VI secretion system plus the chaperon usher system (130), which is involved in pilus synthesis (164). Type Three and Four (TTSS and TFSS) are the most complex protein secretion systems and mediate the contact between bacteria and the host cell

cytoplasm. The TTSS is evolutionary and structurally highly conserved and related to flagella (29, 105). Besides *S*. Tm, it occurs in *Shigella flexneri* (122), *Yersinia* spp. (28, 29, 35, 147) and many other gram-negative pathogenic bacteria. TFSS mediates virulence e.g. in *Legionella* spp. (148). Although translocation of protein substrates is Sec independent, the assembly of the TTSS into membranes is not (Fig. 2). The insertion of the basal body spans inner and outer membrane and is characterized by a radial symmetry that constitutes a supramolecular structure with a tunnel, through which later on substrates are supposed to be secreted (105). The ring within the inner membrane is built up by PrgH and PrgK. InvG inserts into the outer membrane (55, 90, 105). The Type Three Secretion dependent phase begins with the secretion and assembly of needle components as PrgI. On the tip of it, the translocon (SipB, SipC, SipD) assembles (Fig. 2). Mutations of one of those translocon components results in bacteria being only able to secrete effector proteins into the surrounding media but fail to translocate them into the host cell cytoplasm. Although there are more than 10 different effector proteins that are translocated via the TTSS-1, the substrate pool is rather small compared to the overall protein pool within the cell. To limit substrate compatibility, effector proteins carry secretion signals located within the first 30 amino acids. However, the information encoded in the signal peptide is insufficient to guarantee specificity. Unlike sec signal peptides, they share no obvious conserved sequence patterns, but in 2009, R. Arnold and co-workers developed a sequence-based algorithm that is able to predict Type Three Secretion signals with a specificity of 71 % and 85 % selectivity (9). Additionally to that, accessory proteins (chaperones) bind effectors and guide them to the apparatus (39, 169). TTSS specific chaperons, e.g. InvB and SicP, are small, highly related, dimeric proteins that lack ATP binding and hydrolysis domains in contrast to other chaperons (90). The channel constituted by the TTSS is only capable to translocate small cargo proteins or lager ones, which are unfolded into smaller subdomains. Therefore, the chaperon effector complex is dissociated by the ATPase InvC, an accessory protein of the basal body (5, 6). Through the PrgI channel and the translocon within the host membrane, effector proteins can reach the target cell cytoplasm. Prior to the contact with cells, TTSS are already preformed in the bacterial envelope in ten to hundred copy numbers (91), but translocation happens only after contact with the eukaryotic cell

membrane (145, 169). Next to the SPI-1 encoded TTSS-1, *S*. Tm harbors a second TTSS encoded on SPI-2, which is similarly structured, but consists of its own specific subunits. It is involved in intracellular survival of *S*. Tm (reviewed in (1, 166)).

The specific activities of some key TTSS-1 effector proteins within the host cell cytoplasm are depicted in Table 1. Briefly, many of them affect polymerization of the actin cytoskeleton on the site of bacterial entrance to trigger the induction of massive membrane protrusions (ruffles). This is accomplished by mimicking host cell proteins or by host cell protein homology. SopE for example has Guanine exchange factor activity (GEF) and thus activates Rho GTPases as Cdc42 and Rac1 that initiate actin polymerization. Alternatively, Actin polymerization is attained by direct interaction with effector proteins. They act by decreasing the critical concentration for actin polymerization (SipA (144) and SipC (69)). There have been interactions described between SPI- effectors and effectors of SPI-2; e.g. SipA cooperates with SifA for correct positioning of the SCV (24). Other effectors like SptP can neutralize the action of other effectors (in triggering the depolymerization of actin). For this reason, effectors are hierarchical translocated according to their action during the invasion process (Table 1 (169)).

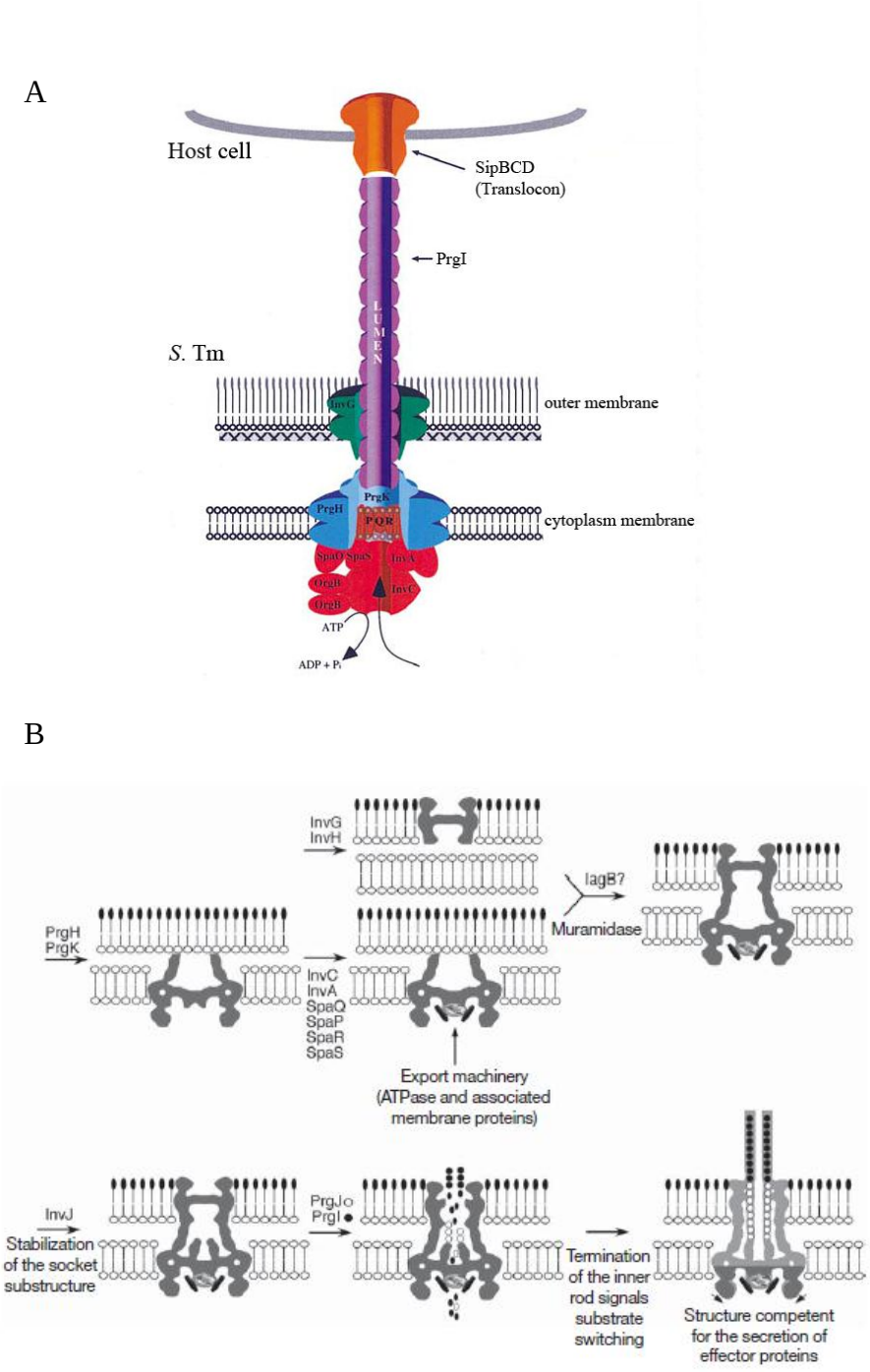


Figure 2, The TTSS-1 needle complex and its assembly (adapted from (90) and (55)). A) The basal body of TTSS-1 is composed of PrgH, PrgK and InvG (blue and green), Effector proteins are secreted through a channel (depicted in purple) constituted of several copies of PrgI. The translocon (SipB, SipC, SipD) inserts in the host membrane and facilitates translocation into the host cell cytoplasm. A number of accessory proteins assemble together to energize transport and avouch for substrate specificity. B) The assembly of the TTSS-1 into the membranes. The basal body (105) resp. socket (55) of the TTSS is a self-assembling machinery that is halfway sec-transport dependent. After

stabilization by InvJ and termination of the inner rod, the TTSS switches its signal specificity from apparatus proteins to effector proteins as e.g. SopE or SipA.

Table 1. Selected SPI-1 and co-regulated effector proteins that are translocated by TTSS-1

Effector	localization in genome	Main function within the host cell	references
AvrA	SPI-1	Inhibition of NFκB signaling pathway, anti-inflammatory	(36, 81)
SipA	SPI-1	Binds actin and increases actin polymerization according to lower the critical concentration for polymerization of actin filaments	(75, 128, 144, 145, 169, 180)
SipB	SPI-1	Involved in translocase activity of the TTSS-1 needle; induces autophagy in macrophages	(70, 72, 73)
SipC	SPI-1	Involved in translocase activity of the TTSS-1 needle, actin nucleation	(69, 112, 179)
SipD	SPI-1	Involved in translocase activity of the TTSS-1 needle, regulator of effector translocation	(27, 84, 129, 165)
SptP	SPI-1	GAP (GTPase activating Protein) for the RhoGTPases CDC42 and Rac1 (71), Tyrosine phosphatase activity	(54, 85, 169)
SopA	outside SPI-1	HECT-like E3 ubiquitin ligase	(128, 175, 177)
SopB	SPI-5	Inositol phosphatase, activates the RhoGTPase Cdc42	(14, 174, 178)
SopE	SopE Phage	GEF (Guanine exchange factor) for the RhoGTPases CDC42 and Rac1(149), initiates Actin filament formation	(53, 66)
SopE2	outside SPI-1	Similar to SopE	(157)

Chemotaxis and Flagella

Even simple organisms like *S.* Tm display the intriguing proficiency to move along concentration gradients. Two subsystems are required for this: (i) a chemotaxis system, which senses concentrations of attractants or repellents, (ii) flagella; basically a TTSS, which instead of translocating proteins assembles a hollow corkscrew-like appendage that rotates counter- or clockwise to achieve motility. It shares with TTSSs a basal body embedded in both membranes. This structure drives the rotation of the pilus due to energy yielded by proton motive force. *S.* Tm possesses several flagella distributed over the entire cell surface (peritrichous), which in terms of coordinated swimming bundle together to a single one. The motor is composed of three proteins FliM, FliN and FliG that also determines whether the flagellum rotates clockwise or counterclockwise. In *S.* Tm two different types of flagelin can be expressed, *fliC* and *fljB*. A regulatory process called phase variation decides, which type of flagellin is inserted into into the flagellum, since their transcription depends on the activity of a recombinase (Hin) that either turns promoter regions on or off. According to this, either *fliC* or *fljB* transcription is enabled in a mutually exclusive fashion.

Changes of e.g. nutrient concentrations sensed by the chemotaxis system are communicated by a phosphorelay system constituted of the sensory kinase CheA and the response regulator CheY. CheY displays conformational changes due to phosphorylation by CheA and increases its ability to bind to FliM of the motor complex (168). The counterclockwise rotation (straight swimming, "run") is thereby interrupted by a short clockwise rotation – tumbling -, where the bundle of flagella fall apart and the bacterium changes the direction by chance once the reset "run" is initiated. Several accessory proteins modulate the perception of stimuli by processes involving methylation of methyl accepting proteins (adaptation) and phosphatases of CheY that offset the effect of CheA. Furthermore, a second posttranslational modification of CheY affects motility. Barak et al. demonstrated that acetylation of lysine residues within the protein sequence of CheY is carried out by the acetyl-coA synthetase (Acs) (16, 98). Acs is required for the conversion of acetate to acetyl-coA, which is channeled into the tricarboxylic acid cycle. The acetylation hampers the interaction between CheY and CheA as well as

the interaction between CheY and FliM. According to this, the central metabolism also plays a key role in chemotaxis and motility (Fig. 4).

In conclusion, the importance of flagella driven chemotaxis is well established. It enables *S*. Tm and other motile bacteria to conquer easily new environments as the gut. It is not directly involved in the invasion of cells but a number of studies could show that the infection in mice is delayed (153, 154) and thus contributes to the infection and the inflammatory response.

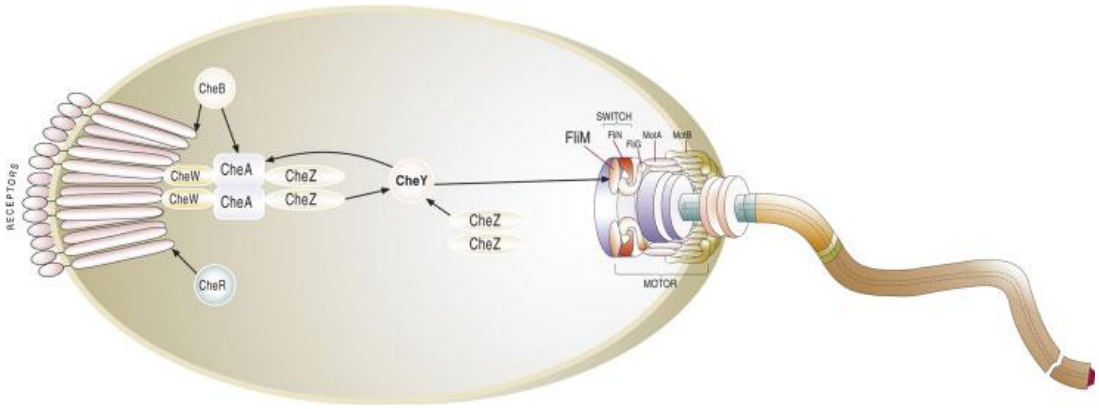


Figure 3, The Chemotaxis system and Flagella (40). Sketch of *S*. Tm chemotaxis system at the pole. Inversely flagella are distributed over the whole surface; here depicted and simplified as one flagellum. According to the distinct position of the chemotaxis system at the pole, *S*. Tm is able to sense concentration gradients over time. Basically, it consists of sensing proteins, adapting proteins and a phosphorelay system (CheA and CheY) that transfer the information to the flagellum consisting of motor (FliM, FliN, FliG) and a FliC or FljB polymer. Both systems are coupled by binding of phosphorylated CheY to FliM triggering tumbling of the flagellum to alter the direction of motility.

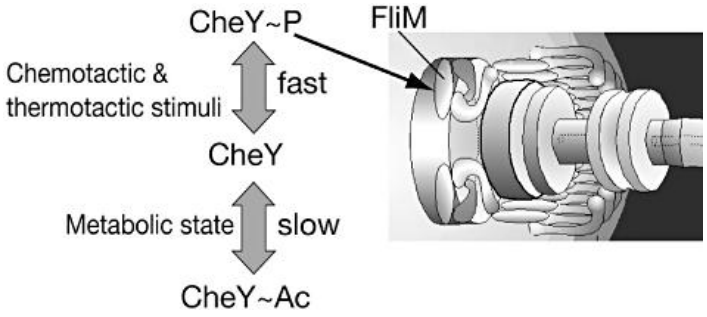


Figure 4, The chemotaxis response regulator CheY gets phosphorylated and acetylated (98). CheA frequently phosphorylates CheY in response to environmental changes. Additionally, CheY is acetylated by Acs, which mirrors the metabolic state of the cell. Phosphorylation normally fosters interaction with the flagella, whereas acetylation prevents it.

Regulation of Salmonella virulence

Basic principles of SPI-1 regulation

The expression of *S.* Tm virulence factors is a highly controlled process with more than a dozen regulators that work in concert to affect transcriptional levels of SPI-1 and SPI-1 associated genes (Fig. 5). Even within the pathogenicity island itself, the four regulators HilA, HilC, HilD and InvF ensure expression of the TTSS-1 and effector proteins. Furthermore, these regulators are subjected to other key regulators connected to maintenance and modulation of the current state of the bacterial cell. Thus the regulation of SPI-1 depends on a variety of processes ranging from metabolism to motility and many others that determine the precision of virulence gene expression. As SPI-1 or the phage remnants encoding for SopE (38) were originally acquired by horizontal gene transfer (88, 89), it reflects a higher AT ratio compared to the surrounding chromosome (113). Those regions derive mostly from phages, other mobile DNA elements or have been acquired by conjugation with second bacteria (124). With regard of this, it is not always actively incorporated into the genome by the bacterium. That makes insertion of foreign DNA to a risk for the organism. To prevent the transcription of possible threats, regions like SPI-1 are predominantly silenced by histon-like proteins as H-NS, Fis or Hha according to their low GC content (76, 113, 117, 142). An H-NS mutant showed

therefore an enhanced expression of SPI-1 genes (117). A similar function is described for the nucleoid-associated protein Fis that is time dependently expressed. *S.* Tm in fresh LB cultures show high levels of Fis that drop during growth. The expression profile thus correlates inversely with expression of SPI-1 genes (87). In addition to that, a number of environmental signaling pathways have impact on SPI-1 expression (44). The majority of them belong to two component systems (21, 26) and are common to several other Proteobacteria, e.g. EnvZ/OmpR that regulates the porin distribution within the outer membrane and thereby determines the diffusion rate of osmotically active substances (31, 80, 125). Furthermore it has been shown that Mg^{2+}, antimicrobial peptides as well as phosphate through PhoPQ respectively PhoBR bias SPI-1 expression negatively (56, 60, 118). PhoPQ is also involved in the expression of SPI-2 genes and in phagosome sensing. Therefore, it plays a key role in conferring resistance against the innate immune system of the host (25, 118, 126). This reflects its involvement in all stages of the *S.* Tm infection process. A further stress-associated two component system CpxAR contributes to antimicrobial stress resistance, invasion efficiency and to the survival within macrophages (78, 167).

As SPI-1 is silenced by H-NS, there need to be SPI-1 activators facilitating SPI-1 expression. Fur is involved in many global regulons in response to iron and has been shown to activate SPI-1 by repression of *hns* (45).

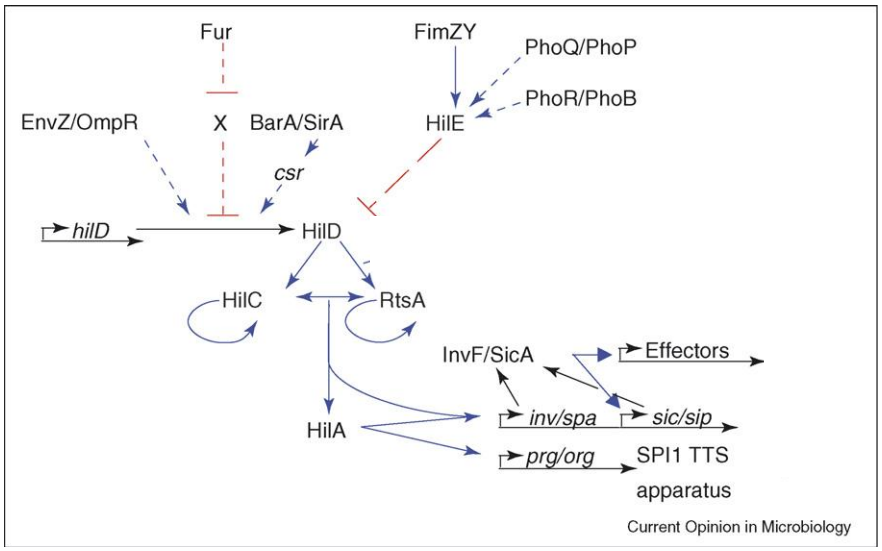


Figure 5, The regulatory cascade leading to the precise expression of SPI-1 genes (42). A complex cross-regulation upstream of the key regulator HilA includes a positive feedback loop (HilC, HilD, RtsA) and several members of global regulons. SPI-1 encoded regulators lead to the concerted expression of effector and TTSS structural proteins.

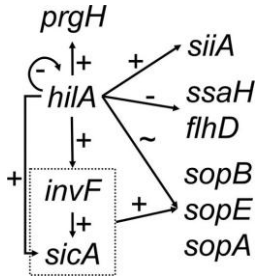
The central SPI-1 regulators

The regulators described above affect the expression of genes coding either for the TTS apparatus itself or for effector proteins that are designated to get translocated into the host cell cytoplasm. However, all of them control either directly or indirectly the promoters of *hilC*, *hilD*, *hilE*, *hilA* or *rtsA* (Fig. 5 (42)). Each of these regulators, with the exception of RtsA and HilE are found on SPI-1 itself and accomplish the auto-regulation of SPI-1. However, regulatory information is bundled to trigger the expression of the main positive regulator HilA (13, 42, 163), which was first identified in a mutant that showed a tenfold increase in ability to invade HEp-2 cells in 1995 (96). A point mutation in the promoter region at nt-378 of the *hilA* open reading frame led to a threefold higher expression of virulence genes (12). Because of the enhanced invasiveness it was called hyper invasive locus (*hil*). Since *hilC* and *hilD* have also substantial effects on virulence, they were termed accordingly. However, HilA drives the expression of all SPI-1 regulons except from *hilC* and *hilD* itself. Besides SPI-1 encoded genes, HilA regulates SPI-2 (*ssaH*) and SPI-4 (*siiA*) associated operons, flagellar

regulators (*flhD*), *sopE*, *sopA* and *sopB* (SPI-5, Fig. 5A and B; (163)). Focusing on the *sicAsipBCDA* and *inv-spa* operon within SPI-1, HilA activity is enhanced by the chaperon SicA and the transcription factor InvF, which assemble to act in concert and amplify transcription of *inv-spa*, *sopE* and *sicAsipBCDA* (102). Within the regulatory cascade a positive feedback loop involving HilD, HilC and RtsA is acting upstream of HilA (42). Even though the promoter region of *hilA* is a major target for silencing proteins as H-NS to prevent transcription (116), binding boxes for HilC and HilD were found that can derepress the key regulator *hilA* (Fig. 5B (4, 101, 102, 141, 143)). The action of HilC and RtsA is mainly restricted to increase the number of HilD molecules that finally contribute to the activation of *hilA*. This is supported by the observation that *hilC* and *rtsA* mutants are still able to express SPI-1 genes (to a lower extent), while the deletion of *hilD* leads to the entire loss of SPI-1 transcription (42, 136). Due to this hierarchical cascade-like structure with several internal positive loops that constitute superordinate positive feedback amplification, original signals from the environment are multiplied. Based on the interaction between activators, SPI-1 expression is highly balanced and concise.

In addition of SPI-1 silencing by Hns, *S.* Tm features further mechanisms to suppress SPI-1 expression. In contrast to the other core regulators, HilE is not a transcription factor. It basically binds to HilD in order to inhibit its transcriptional activity and thus prevents the transcription of *hilA* (18, 47). Recent studies revealed that besides transcriptional control, *hilD* expression is also controlled by mRNA stability, which differs according to Dam methylation (100).

A



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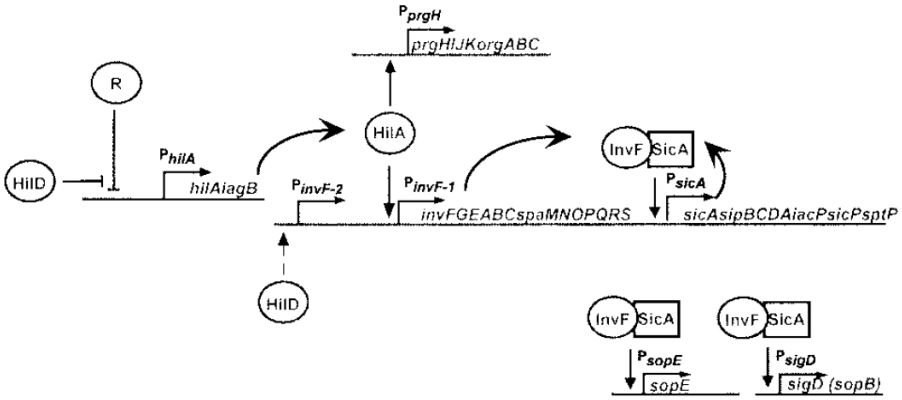

Figure 6, Regulation and the impact of *hilA* expression. A) HilA affects several operons outside SPI-1 and hence ensures co-expression of relevant genes e.g. on SPI-4 (*siiA*). It triggers also the expression of a potent transcription factor InvF that can assemble together with SicA to activate expression of further genes as *sopA, sopB* and *sopE* that are distributed throughout the genome (see also B). Flagella (*flhD*), SPI-2 (*ssaH*) and *hilA* itself are negatively affected by *hilA*. B) HilD acts as an activator for *hilA* that is normally suppressed by Hns (113, 117). This leads to a cascade consisting of HilA and InvF/SicA that trigger the expression of SPI-1 (associated) genes.

Cross-regulation between Flagella, Fimbriae and SPI-1

With regard to the importance of motility for *S.* Tm infection of mice (146), the bacterium harbors several mechanisms of cross regulation between SPI-1, the flagellar and fimbrial system (137). Functionally, they are all involved in host cell binding and invasion. Briefly, flagella feature several characteristics that are in common with the TTSS of SPI-1. They consist of a long helical filament and a rotary motor embedded within the membrane. Thus, they facilitate swimming in liquids, swarming

on surfaces, determine the pace of *S*. Tm reaching the site of invasion within the gut and have even been involved in host cellbinding (146, 154). Implications in the activation of inflammation according to cytokine release have also been reported (109). *S*. Tm possesses hair-like appendices, fimbriae, which facilitate attachment to surfaces as mannosylated glycoproteins (77). They are thought to contribute to colonization and persistence during intestinal infections (79). Recent studies now revealed a hierarchy between these systems, ranging from the expression of flagella that contribute to virulence expression and ending up in transcription of fimbriae (137). A key element seems to be the flagella master regulator FlhDC that activates *fliZ* and thus flagella biosynthesis. Beyond that, FliZ also activates *hilD* and hence SPI-1. Taking this together SPI-1 and flagellar expression are coordinately regulated by originally flagellar specific transcription factors. FlhDC itself is a target of controlled proteolytic degradation by the protease ClpXP (83). In addition, the operon *rtsAB* has substantial impact on the expression of both systems. In the previous chapter, the role of RtsA in SPI-1 expression was commented. *rtsB* as a part of the same operon share largely equal expression levels with *rtsA*. But in contrast to the connection between FlhDC and *hilD*, RtsA and RtsB have inverse effects on the expression of SPI-1 and flagella (43). Additionally to that, *rtsAB* and *flhDC* are concertedly regulated by Fis (87). Also the fimbrial system interferes with SPI-1 and flagella regulatory systems. Negative feedback loops derive from FimZ (19), which represses SPI-1 by activating the SPI-1 repressor *hilE* as well as *flhDC* and thus reduces flagella synthesis. This complex and nested way of cross-regulation with several negative and positive feedback loops is thought to manage a cascade-like expression pattern that is initiated during the infection (Fig. 7).

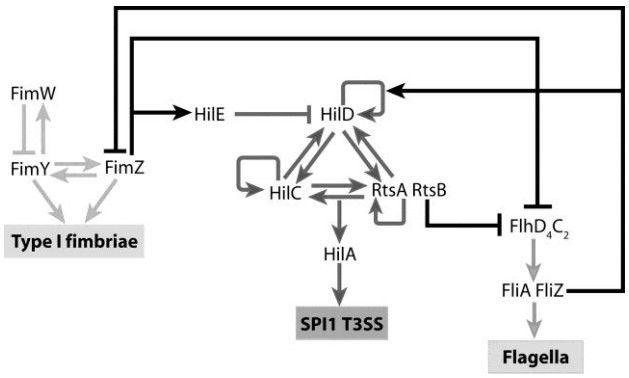

Figure 7, Crosstalk between SPI-1, Fimbriae and Flagella regulation (137). Central regulators in either specific regulatory cascade affect the expression of adjacent regulons. See text for details.

The impact of metabolism on the regulation of SPI-1 expression

S. Tm is a highly versatile bacterium that is able to grow on different substrates under aerobic as well as under anaerobic conditions, where it either applies respiration or fermentation to gain energy for anabolic processes as growth and virulence. Certainly, metabolic capacities are required during infection. Thus, metabolism can be considered as virulence factor, e.g. by defining the window, where expression of SPI-1 genes is possible. Glucose is one of the preferred substrates utilized by *S.* Tm. If the surrounding media is plentiful of glucose, systems that code for alternative degradation pathways are repressed by Crp (135). The cAMP levels within the cell depend on glucose. A decline of glucose implies increasing concentrations of cAMP, a cis-activator of Crp, so that decreased glucose levels result in the transcription of genes controlled by Crp (48). Interestingly, mutations of Crp or of the adenylate cyclase Cya, which generates cAMP, led to strongly reduced expression levels of SPI-1. The same effect can be observed in glucose complemented media. It has been shown that this is attributable to the expression of *sirA* (162). The *hilA* promoter is mostly affected by HilD but can also be bound and activated by the response regulator SirA, which gets phosphorylated by the sensor kinase BarA via unknown signals (123). Thereby, changes in sugar concentrations can be transmitted to SPI-1. Another system, CsrA (7, 94), affects motility, glycogen synthesis and gluconeogenesis (132) as well as SPI-1 (162). CsrA is a 61-amino-acid protein that binds the mRNA of target genes

and either promotes or inhibits translation of various genes. It can be sequestered by small regulatory RNAs as *csrB* and *csrC*, which antagonize CsrA action. Although the direct mechanism is unknown, CsrA affects SPI-1 gene expression (7). SirA (and indirectly Crp/Cya) down-regulates *csrC* and thus contributes to the action of CsrA.

In 2002, it has been shown that acetate in the medium induces expression of SPI-1 virulence genes. Acetate as a carbon source enters central metabolic pathways as activated derivatives, either acetyl-phosphate or acetyl-coA. The phosphorylated form of acetate could be shown to phosphorylate BarA, thus activating SirA and consequently *hilA* (Fig. 8 (95)). Finally in 2007, the global sugar repressor, Mlc, which also interacts with the sugar phosphotransferase system (PTS), was identified to increase SPI-1 expression by the repression of the SPI-1 major negative regulator HilE (99).

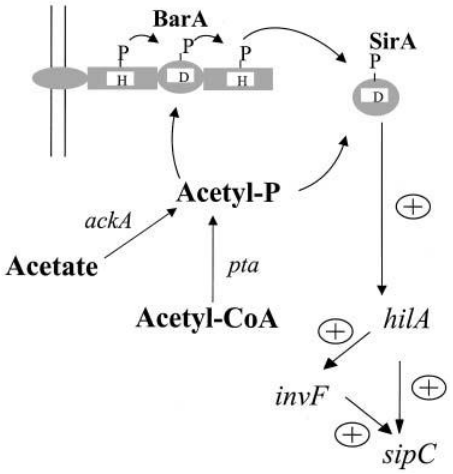

Figure 8, Effects of acetate and its derivatives on SirA and *hilA*. Acetate gets phosphorylated by the phosphotransferase AckA or alternatively acetyl-coA is converted to acetylphosphate by the acetyl-transacetylase Pta. In both cases, *hilA* is induced by the acetylphosphate dependent phosphorylation of the BarA/SirA two component system (95).

Overall, SPI-1 expression is affected by a large number of regulators ranging from SPI-1 encoded, specific regulators over *housekeeping* regulators (e.g. SirA, Crp, Fis) to a remarkable cross-regulation

with other complex systems like flagella or fimbriae. Concerted expression seems to be essential for SPI-1 virulence.

Additional mechanisms regulating SPI-1 expression

The environment underlies dramatic changes over and over again. For the residing bacteria this often represents life determining characteristics. Therefore, bacteria are equipped with complex sensory and regulatory systems that adapt gene expression accordingly. However, sometimes changes are not predictable or occur to fast to guarantee successful adaptation. This explains the value of other means of control like stochastic gene expression, which allows isogenic individuals to differently react on environmental challenges.

Phenotypic variation - the *lac* promoter in bacteria

In some cases, genetically identical individuals among a population exposed to one environment evolve different phenotypes yet. One of the first descriptions of variances within isogenic populations arose from the investigation of *E. coli* phage alpha susceptibility by M. Delbrück in 1945 (32, 34). Different burst sizes (number of phage particles within a burst) originating from individual *E. coli* cells were observed. But the variances between burst sizes could not be described by simple standard deviation. In contrast, they differed largely, ranging from 0 to over 1000 phage particles, whereas the vast majority was found at a size of 100 to 200. In conclusion, burst sizes were represented by a heavy-tailed distribution (Fig. 9). This strongly argued for variances in biological systems beyond simple standard deviations. Further phage based approaches by S. Benzer revealed unequal distribution of β-galactosidase among isogenic *E. coli* individuals (22). Also Novick and Weiner described this phenomenon of heterogeneity in β-galactosidase induction with the help of single cell dilutions and phage experiments already in 1957 (115). They found that bacteria are either “on” (expressing β-galactosidase) or “off” (no expression) termed All-or-none-phenomenon (115). Despite

the simple design of those experiments, they unambiguously demonstrated that isogenic populations consist of distinct individuals. With the advent of advanced fluorescence microscopy and fluorescence activated cell sorting (FACS), transcriptional studies at the single cell level established numerous additional examples of heterogeneous gene expression. Nevertheless, the *lac* operon is still a major subject of researchers to investigate phenotypic variations within bacteria (Fig. 10 (176)).

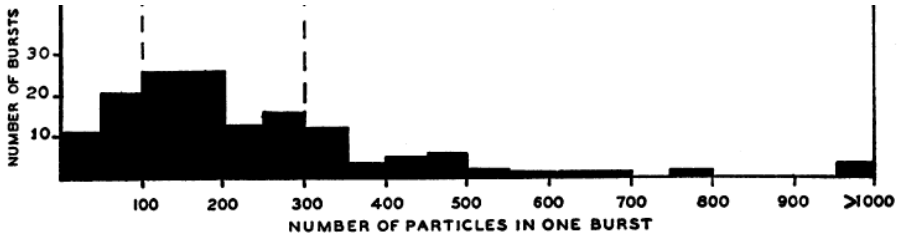

Figure 9, Phage experiment by M. Delbrück in1945. Different burst sizes (number of phages) of *E. coli* deriving from individual, isogenic *E. coli* (33).

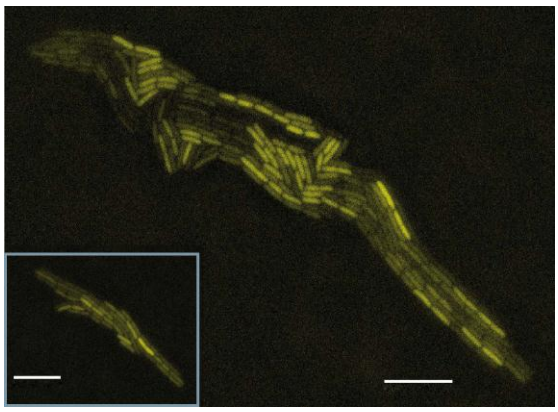

Figure 10, Bistability of the lac operon. A micro-colony of *E.coli* is constituted with a plasmid carrying *yfp* to display expression of the *lac* operon. The inset shows the expression before treatment with the non-metabolizable analog thiomethyl-β-galactoside (TMG), the big 2 hours after treatment. TMG is meant to induce the lac operon. Obviously, YFP levels are subject to fluctuations representing heterogeneity in the expression of the lac operon within individuals of the micro-colony, even though they are exposed to identical environmental cues. For details see (131).

Noise

Noise is a term, which describes stochastic fluctuations of gene expression within a cell or among individuals. During the last decade, noise has been proven to represent an important factor in gene regulation, which affects key cellular functions as metabolism or growth. Furthermore, it is associated with development and might even play a role in evolution (20, 41). As a measure the dimensionless coefficient of variation (standard deviation over mean) is often used. Promoters underlie (i) extrinsic and (ii) intrinsic noise (159). While (i) mainly describes fluctuations throughout the cell due to changes in the amount of e.g. polymerases, (ii) refers to fluctuations that contribute to the expression of a single promoter. In 2002 by Elowitz et al. published a landmark study using fluorescent CFP and YFP reporters to display stochastic gene expression of the *lac* promoter (46). *E. coli* cells harboring two plasmids with the *lac* promoter region driving either *cfp* or *yfp* expression were exposed to fully or subsaturated inducer concentrations. This means that either all or only some of the promoters are liberated from the *lac* repressor. Extrinsic noise is predominant, when all promoters are free. In this case *yfp* and *cfp* are expressed at the same level among all individuals leading to an "uni-colored" population; i.e. the transcription in both constructs is correlated. If the inducer concentration is not sufficient to release all promoters, intrinsic noise becomes predominant; either *cfp* or *yfp* is expressed leading to a population where bacteria phenotypically differ. They are either green, red or show intermediate pheotypes. The noise among both is no longer correlated. This simple but important experiment showed evidence for fluctuations directly within the expression of the *lac* operon and thus for the impact of noise. Both sources of noise are thought to contribute to affect the individual phenotype of a given bacterium. Nevertheless, which source of noise has a dominant impact may vary between regulatory systems (Fig. 11 (37)). Stochastic fluctuations can be observed in all cellular regulatory entities, gene expression (complying transcription and translation) as well as protein degradation. Several approaches were made to elucidate the impact of each on phenotypic variation (82). In an experiment using *gfp* reporter, transcription, initiation and translational rates were forced to vary. A gene with low transcription but high translation rates elicited large, variable and infrequent bursts leading to strong fluctuations within a cell. On the other hand, a gene with high transcription and low translation of a transcript rates produces small and frequent bursts. Merely weak fluctuations

in protein concentration have been observed, which lead to a smaller phenotypic variation in the population. In conclusion, a poorly transcribed and highly translated gene exhibits more noise than a highly transcribed and poorly translated one (82, 119).

A B

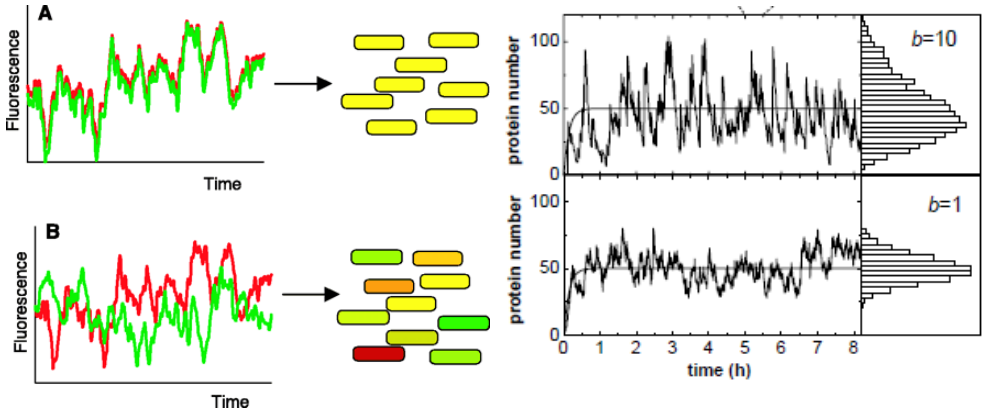

Figure 11, noise affecting the expression of the *lac* operon and the contribution to noise by transcription and translation. A) Schematic view of extrinsic (upper panel) and intrinsic noise (lower panel). *lacZ* was transcriptionally fused to *yfp* (green) and a copy to *cfp* (red). Afterwards they were inserted into the chromosome of *E. coli*. Under inducing conditions (IPTG) noise and thus expression of both gene duplicates is correlated (extrinsic noise is biggest). Below this, noise is not correlated between both operons and specific to each one (intrinsic noise is predominant) (46). In the latter case mixed populations can be observed. B) Illustration of the impact of transcription and translation on noise. A low transcription rate going along with high translation of the transcripts favors noise and therefore the phenotypic distribution (increased deviation). High transcription and low translation is less suitable to generate variances. For details see (119).

From noise to bistability

Stochastic gene expression converts isogenic bacteria to individuals that can be discrimnated. It is required to explain why subpopulations emerge but it is not sufficient. To evolve different phenotypes in an all-or-non fashion, the amplification of stochastic differences becomes necessary. Feedback loops are designated to accumulate components that trigger expression of either activators or repressors and are therefore termed positive or negative feedback loops. Positive feedbacks amplify

deviations in transcription rates and foster segregation into subpopulations, while the system is stabilized (the deviation is weakened) by negative feedbacks. Regulatory systems often include several cross-linked and auto-regulatory loops to respond appropriately to environmental changes (Fig. 12 (8, 52, 110)).

A positive feedback is a necessary condition to generate subpopulations. In addition, response elements (regulators) are supposed to react on stimuli with a sigmoidal-like response curve (Fig. 13 (151)). Sigmoidal response curves are often mirrored in cooperative binding of regulators as well as multimer formation. This creates a threshold that decide whether or not a phenotype is expressed (8). The ultrasensitive area of response (the steep slope in Fig. 13C) functions as the switch, i.e. effective concentrations below this threshold favor one state, while those above the threshold favor the other. Here it is important to introduce the term hysteresis (Fig. 13 (110)). It basically describes the dependency of a steady state response curve on the direction of parameter change (decrease or increase). It explains why two steady states can exist within a certain parameter interval (110). Regulatory systems like that are called bistable and determine the existence of stable subpopulations.

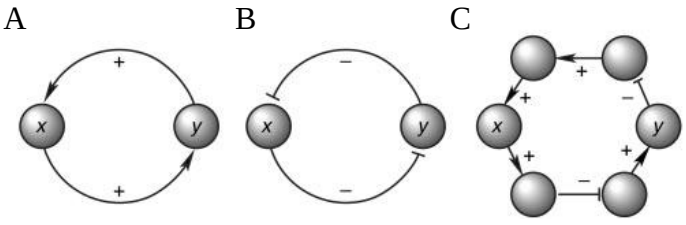

Figure 12, Examples of feedback loops that may trigger bistable expression. A) positive B) inhibitory (negative) and C) complex mutually inhibitory feedback loop. Adapted from (8), see text and (8) for details.

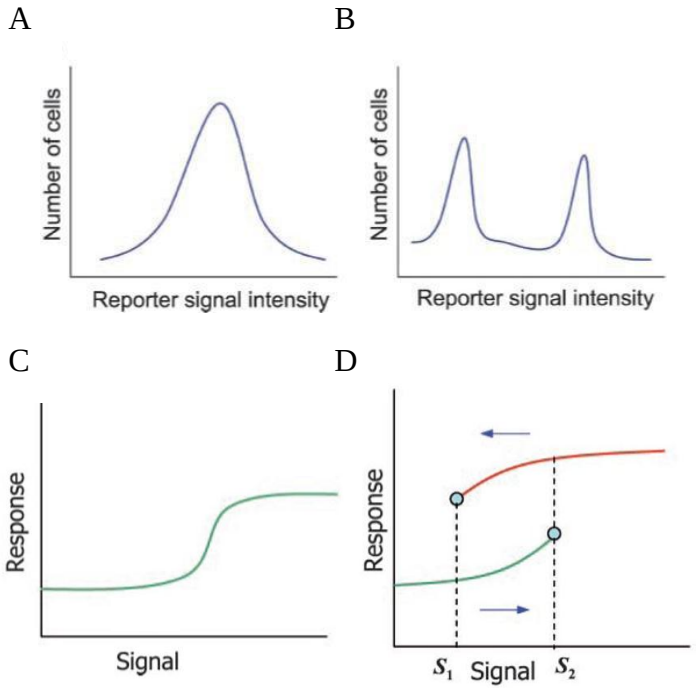

Figure 13, Occurrence of Bistability. A) Theoretical curve of a monostable and B) of a bistable population; C) sigmoidal like response curve of a hypothetical regulator. The steep part of the curve represents the ultrasensitive region, which acts as a threshold to destine the response of an individual cell. D) Hysteresis in a bistable system. In between the parameters S1 and S2 there are two co-existing stable steady states. It depends on the direction of change of signal intensity, whether the green or red response curve is applicable (adapted from (110)).

Further examples of phenotypic variability

Additional examples of individualism that do not base on gene polymorphisms are found among different areas of life in bacteria where regulation plays a key function. An observation that has not been linked to bacteria for a long period of time is aging. On a first glance *E. coli* cells divide into two identical daughter cells. But in fact they proliferate in a bacterium exhibiting old poles and in a bacterium that inherited the newer poles. It could be observed that the older pole possessing bacterium shows a decreased growth rate and even an enhanced mortality (30, 158). So far, the mechanism, which explains the weaknesses of an older bacterial cell is not understood (172). Obvious by contrast is the unequal division of *Caulobacter crescentus* that features swarming and stalked cells with

differential accumulation of age (2). It might be that the messenger molecule cyclic di-GMP plays a role.

Broader attention was spent to bacterial populations that displayed persistent subpopulations of *Staphylococcus aureus,* already described in 1944 (23). In this case, fractions within a population can survive in the presence of antibiotics, while the majority deceases. Interestingly, these so called persisters are dormant prior to stress and do not grow until antibiotics drop again below critical concentrations. That persisters evolve in the absence of antibiotics and are therefore determined by stochastic gene expression, could be shown for *E. coli* by Balaban et al. in 2004 (15). By this strategy, *E. coli* guarantee the survival of the population and transmission of genes to further generations similar to endospore forming bacteria like *Bacillus subtilis* (107).

Another interesting observation was also made for *E. coli*. Different strains often compete for resources. Some individuals of them develop a bomber phenotype (97) that imply the induction of the toxin colicin and is meant to extinguish competing strains, which are not resistant to colicin. The production of this toxin underlies stochastics (111) and displays an altruistic trait, since it inevitably leads to the death of the producer (bomber). A deterministic expression of colicin of all individuals of one strain would lead to the extinction of the genotype. A small fraction of suicidal *E. coli* makes competition more successful, because the resistant strain will continue to grow (97).

The chemotaxis system of bacteria can also be interpreted as a sort of individual behavior. Bacteria swim with a certain frequency of tumbling, which leads to random change of swimming direction. When bacteria are exposed to attractants tumbling is suppressed till they adapt to the environment (16, 40). However, adaptation times vary between different individuals of isogenic populations. It turned out, that each bacterium had a distinct adaptation time throughout the cell cycle (152). In conclusion, all of these examples demonstrate the phenomenon of individualism, which exclusively relies on phenotypic noise and feedback signaling in isogenic populations.

Arguments for phenotypic variation – bet hedging

Changes within an environment can be predicted to some extent, sensed and processed by the organisms, so that reaction time becomes feasible. But what is about the changes that cannot be foretold and are infrequent or contrary to growth and proliferation under present cues? Under those circumstances bacteria cannot respond properly on behalf of *considerations* and require alternative ways to prevent extinction in fluctuating environments. The incorporation of stochastics is termed bet-hedging alluding to gambling strategies. Briefly, bacteria do not know what the appropriate answer to upcoming environmental changes is and *guess* with different strategies. As a consequence of stochastic gene expression, subpopulations often emerge on the cost of compromised growth (e.g. persistence) or even suicidal extinction (e.g. colicin synthesis). Stochastic gene expression is therefore addressing *what would it be like if….* Or to quote a publication that console individuals compromised by phenotypic variation: "Thus, an offspring produced for a rare and little-populated eventuality may regret that their day of glory is unlikely to arise, but will be exactly compensated by the extent of their relative triumph should it do so." (30, 59). Bacteria often use a mixed strategy. If stress is sensed, but the optimal response is not clear, bacterial populations are likely to become heterogeneous (92, 173). The concept of mixed strategies depends mainly of the frequency and seriousness of environmental changes (30). As stated before, subpopulation formation in preparation for rare events is a costly issue and raises the question whether it is reasonable in terms of evolution. Multiple attempts have been undertaken to explain the advantages of this widespread phenomenon with the help of game theory and cost-benefit functions (30, 59, 92, 139, 173). The degree of success is given by the term evolutionary stable strategy (ESS) that is explained by a population that cannot be invaded by a new strategy (30). Indeed, those approaches showed that altruistic behavior can display an ESS. Sasaki and coworkers demonstrated that isogenic phenotypically diverse populations always scoop genetically polymorph populations displaying the same variations in phenotype. They reasoned that the cost of generating differences within the genotype is higher (139).

Beyond this, it is to question if an isogenic population that consists of bacteria differing in their phenotype is constituted of *individuals*. Classical definitions persist of genetic variances between individuals. Based on those ambiguities in terminology, scientists came up with different ways of looking at terminology and changed perspectives. In terms of evolution each individual is supposed to maximize its own fitness. Alternative strategies anticipate that isogenic populations follow a multicellular-like strategy (30, 63). This implies, selection happens on bacterial populations rather than on individual cells that merely show a fraction of the phenotype of the population. The big advantage following such a strategy is that a population (a multicellular-like society) is prepared for several events even though they have to bear costs. A pure population, which is not affected by the costs of stochastic gene expression, may be wiped out after dramatic environmental changes, while a mixed population exhibits survivors (30).

The bistable expression of SPI-1

Although, expression of SPI-1virulence genes is a controlled process being predictable under certain environmental cues as described in the first sections of regulation, it contains accidental factors, too. Using GFP reporter assays, J. Hinton and co-workers reported in 2003 that *prgI* (the gene encoding the needle of the TTSS) is expressed in approximately 50 % of the cells in vitro experiments (LB and cell culture assays (68)). Together with the help of fluorescent reporters, it is thereby possible to monitor expression of virulence genes in different environments. Moreover, mouse experiments could show that virulence genes are subject to stochastic gene expression and hence display phenotypic variations and bistability in vivo (3). Where bistability is caused, remains a miracle until recent days, even though there are several attempts to solve it. Yet, two publications dealing with the generation of bistability of virulence gene expression in *S*. Tm attracted interest. In 2008 C. A. Voigt and co-workers reported that the upstream regulators HilD and HilC do not display bistability (161). In fact, a split feedback loop regulated by the assembly and disassembly of the downstream regulator InvF/SicA is proposed to be responsible for the emergence of bistability (Fig. 14). However, the role of the key

regulator HilA (see also Fig. 5) was not elaborated, despite its central function in SPI-1 regulation (for further details (161)). In 2010 this observation and reasoning was partially disputed by the group of C. V. Rao. They clearly showed the bistable expression of *hilD* as well as of *hilA* and demonstrated for HilC and RtsA a rather negligible role with regard to the generation of bistability (Fig. 14, further details (136)). Both studies used GFP-based reporter systems, but the genetic constructs based on different lengths of *hilD* promoter regions. This might explain differences within their observations. Beyond that, neither publication could pinpoint the exact origin of SPI-1 bistability. Additionally, upstream regulators of *hilD* have not been brought into account so far. Furthermore, in both approaches plasmids instead of chromosomal reporters have been utilized. Considering, the importance of the ratio between promoter and regulator, plasmids represent a rather unfortunate choice in terms of monitoring bistable expression, since they interfere with the regulatory system by changing the ratio of regulator and promoter.

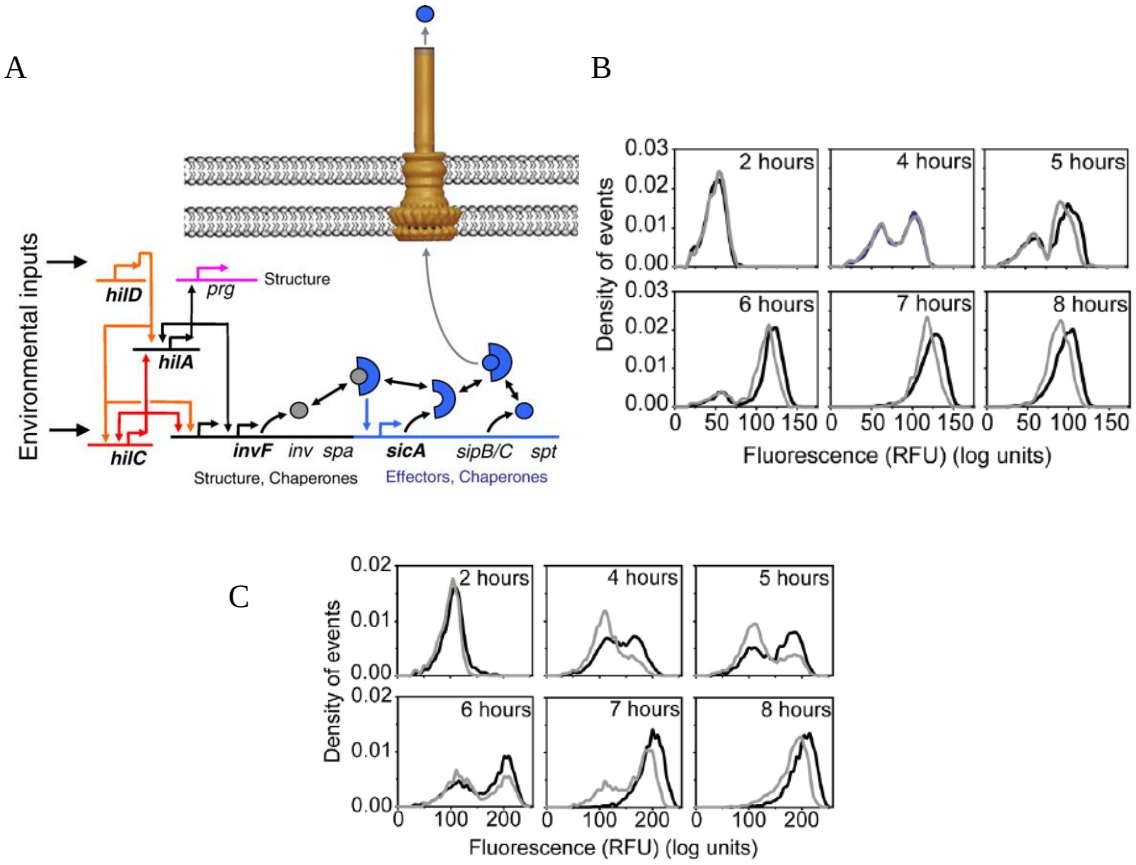

Figure 14, Bistability in *S*. Tm virulence. A) *hilD* and *hilC* drive the expression of *hilA* that triggers the expression of *invF* and *sicA*. Both these work together as activators of SPI-1 operons. Bistability is thought to be caused due to the peculiar assembly of InvF and SicA, (adapted from (161), see also for further details). B) FACS analysis of a culture expressing *gfp* under the *hilD* promoter after cultivation at indicated times. Two distinct subpopulations are observed (black curve). The deletion of *rtsA* and *hilC* did not alter the expression of *hilD* (grey curve). C) Same experiment as in B) studying the promoter of *hilA*. Bistability is not affected in a *ΔrtsA ΔhilC* strain (grey curve). Only a slight delay in *hilA* expression can be observed (adapted from (136)).

From the advantages of being different – beyond bet hedging: Division of labor

The world is not an exclusively cozy and friendly p*lac*e. Probably no one would claim that the world is free of menaces and challenges, no matter whether it is the perspective of an individual or the whole society or civilization. It has been an issue from the first steps in human history, when people competed with natural enemies for mainly food or shelter, to recent days, where our conflicts take place between different human societies and still deal about the same concerns, shelter and food. A closer look reveals that solving those problems or taking those challenges is seldom achieved by a single man; often it is a cooperation that favors solution and profit. A successful result attained by a group of people can have tremendous effects, since it can be able to shape identity or even be the fundament of a nation (e.g. the Swiss Confederation, which renounced from the Habsburg power). Often it determines life and for example in cases of environmental catastrophes, like floods and earthquakes, cooperation makes survival more likely.

But is this a human invention - to cooperate? For sure it is not. Cooperation between different species is common and well described within the literature. Even collaboration between various phyla of life is observed and wide spread (rhizobia (49), mycorrhiza (67), lichen (17), commensals within the human gut (156)).

A special case of collaboration could now be identified for *S*. Tm. The theoretical framework was already published in 2008 and describes a phenomenon called *self-destructive cooperation* (3). Reconsidering, that the gut flora is a serious barrier for invading pathogens, causing inflammation represents a necessary strategy to outnumber commensals. Based on the observation that *S*. Tm expresses virulence genes bistably, only the SPI-1+ subpopulation is capable to invade and cause inflammation. The majority of the invaders get thereby eliminated by the immune system. They sacrifice themselves for the SPI-1- fraction remaining in the gut lumen in order to colonize it during the inflammatory response. This behavior can easily be conceived as a sort of cooperation among individuals based on phenotypic variances.

Aims of the presented thesis

At the beginning of the thesis it had been clear that *S*. Tm expresses SPI-1 virulence genes in a bistable manner (68). However, consequences of the bistable expression of SPI-1 genes had not been unraveled yet. To analyze this we wanted to use ample cell reporter strategies to investigate the TTSS-1+ phenotype. Additionally, we constructed several mutants that are involved in the regulatory cascade of *ttss-1* genes and looked for their effects on bistability.We considered accompanying impacts that might give insights into the reason for bistability of *ttss-1* genes and affect the induction of *ttss-1* genes in vitro. Based on these results, it should be determined, if the self-sacrificing model that was proposed by M. Ackermann and W.D. Hardt can be supported by our findings (Chapter 2).

In a second approach, we wanted to analyze how HilA, the SPI-1 master regulator, is implementing different signals from global regulators of the *S*. Tm cell. Several publications describe the high level of cross-regulation of *ttss-1* genes with flagella and metabolic regulators. Most of these cross-talks happen on the level of mRNA, either transcription (e.g. Crp, FlhDC) or mRNA stability (e.g. CsrA). This is fairly investigated. However, the *ttss-1* key regulator HilA exhibits several features that are uncommon to simple transcription factors. The N-terminal domain displays characteristics of response regulators (OmpR/ToxR family) with an aspartate residue that is shared by homolog proteins. Normally this aspartate residue can be phosphorylated via a sensory kinase by environmental triggers in e.g. OmpR or PhoP. We questioned, if in the case of HilA sensory kinases exist and if, which effect it would have. Finally, mass spectrometric analysis of HilA should elucidate, if posttranslational modifications occur, how they are regulated and are there phenotypic characteristics according to modifications of HilA (Chapter 3 and 4).

Beyond these questions, we wondered about the adaptive interaction of *S*. Tm and its host mainly with regard on the temperature, which represents a substantial environmental cue. According to the inflammatory response that inevitably follows a *S*. Tm infection, *S*. Tm is exposed to increased temperatures up to over forty degrees. We asked how temperature can affect the expression of *ttss-1* genes as well as flagella and thereby help to clarify what happens to the virulence of *S*. Tm during

fever (Chapter 5). These questions were of importance for modeling bistability of SPI-1 expression, its regulators and its consequences for *S*. Tm infection biology.

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Chapter II

The cost of virulence: Retarded growth of *Salmonella* Typhimurium cells expressing type III secretion system 1.

The cost of virulence: Retarded growth of *Salmonella* Typhimurium cells expressing type III secretion system 1.

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Abbreviations: wt, wild type; *ttss-1*, SPI-1 encoded type III secretion system (genotype; >20 genes) ; TTSS-1[+], phenotype expressing *ttss-1*; MOI, multiplicity of infection. *S.* Tm, *Salmonella enterica* subspecies 1 serovar Typhimurium; Gfp, green fluorescent protein; μ, growth rate;

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Abstract

Virulence factors generally enhance a pathogen's fitness and thereby foster transmission. However, most studies of pathogen fitness have been performed by averaging the phenotypes over large populations. Here, we have analyzed the fitness costs of virulence factor expression by *Salmonella enterica* subspecies I serovar Typhimurium in simple culture experiments. The type III secretion system *ttss-1*, a cardinal virulence factor for eliciting *Salmonella* diarrhea, is expressed by just a fraction of the *S.* Typhimurium population, yielding a mixture of cells that either express *ttss-1* (TTSS-1^+ phenotype) or not (TTSS-1^- phenotype). Here, we studied in vitro the TTSS-1^+ phenotype at the single cell level using fluorescent protein reporters. The regulator *hilA* controlled the fraction of TTSS-1^+ individuals and their *ttss-1* expression level. Strikingly, cells of the TTSS-1^+ phenotype grew slower than cells of the TTSS-1^- phenotype. In spite of this growth penalty, the TTSS-1^+ subpopulation increased from < 10 % to approx. 60 % during the late logarithmic growth phase of an LB batch culture. This was attributable to an increasing initiation rate of *ttss-1* expression, in response to environmental cues (e.g. low oxygen pressure) accumulating during this growth phase, as shown by experimental data and mathematical modeling. Finally, *hilA* and *hilD* mutants, which form only fast-growing TTSS-1^- cells, outcompeted wild type *S.* Typhimurium in mixed cultures. These data demonstrated that virulence factor expression imposes a growth penalty in a non-host environment. This raises important questions about compensating mechanisms during host infection which ensure successful propagation of the genotype.

Author summary

Pathogenic bacteria require virulence factors to foster growth and survival of the pathogen within the host. Therefore, virulence factor expression is generally assumed to enhance the pathogen's fitness. However, most studies of pathogen fitness have been performed by averaging the phenotypes over large pathogen populations. Here, we have analyzed for the first time the fitness costs of virulence factor expression in a simple in vitro culture experiment using the diarrheal pathogen *Salmonella enterica* subspecies I serovar Typhimurium (*S.* Typhimurium). TTSS-1, the cardinal virulence factor for eliciting *Salmonella* diarrhea, is expressed by just a fraction of the clonal *S.* Typhimurium population. Surprisingly, time lapse fluorescence microscopy revealed that *ttss-1*-expressing *S.* Typhimurium cells grew at a reduced rate. Thus, the pathogen has to "pay" a significant "price" for expressing this virulence factor. This raises important questions about compensating mechanisms (e.g. benefits reaped through TTSS-1 driven host-interactions) ensuring successful propagation of the genotype.

Introduction

The ability to infect a host and elicit disease is dictated by the virulence factors expressed by a given pathogen. This may include, but is not limited to, protective factors neutralizing antibacterial defenses, enzymes involved in nutrient acquisition within the host, regulators of virulence factor expression and toxins or secretion systems for subverting host cell signal transduction. The coordinated expression of such virulence factors enhances colonization, growth/survival within the host and transmission. However, most studies of virulence factor function and pathogen fitness have been performed in bulk assays, averaging the phenotypes over large pathogen populations of genetically identical cells. In contrast, little is known about the potential advantages, costs or burdens arising from virulence factor expression by an individual cell of the pathogen population. Therefore, single cell analyses might be of significant interest, in particular if virulence factors which are expressed in a bistable fashion by some but not all members of a pathogen population, e.g. the *ttss-1* system of S. Typhimurium (1, 18, 41, 48, 62), as described in this paper.

Bistable gene expression is genetically encoded. In most cases, one particular genotype expresses one predictable phenotype in a given environment. However, in some cases, two different phenotypes are expressed by isogenic organisms living in the same environment. This is termed phenotypic variation, bimodal gene expression or bistability and represents a special case of gene expression (49). The importance of bistability for pathogenic bacterial fitness and evolution is just beginning to be understood.

Like other cases of gene expression, bistability is generally observed in response to particular environmental cues. The response is driven by a dedicated (set of) regulator(s), which responds to environmental signals (operon model of Jacob (29)). This response is subject to stochastic fluctuations. In particular in the case of regulators expressed in a few copies per cell, this can significantly affect the active regulator concentration thus randomizing the corresponding phenotype in a population (12, 40). In combination with non-linear responses (e.g. regulator multimerization, feedback loops), this can lead to formation of phenotypically distinct and stable subpopulations of

isogenic bacteria (12, 37, 38, 40, 49). In terms of evolution, two models may explain the advantage of bistability: i. in "bet hedging", the optimally adapted phenotype will prevail and ensure the survival of the shared genotype in a changing environment (24). ii. in "division of labor", both phenotypes cooperate to ensure survival of the shared genotype (1). In either way, the bistable expression of certain genes is thought to promote the survival of the genotype. However, it has remained poorly understood whether/how bistability may affect the lifestyle of pathogenic bacteria.

Salmonella enterica subspecies 1 serovar Typhimurium (*S*. Tm) is a pathogenic Gram-negative bacterium causing numerous cases of diarrhea, worldwide. Its' type III secretion system 1 (TTSS-1) was recently identified as an example for bistable gene expression (18, 41, 47, 62). TTSS-1 is a well-known virulence determinant of *S*. Tm required for eliciting diarrheal disease (4, 57, 59). The needle like TTSS-1 apparatus injects effector proteins into host epithelial cells, thus triggering host cell invasion and pro-inflammatory responses (16, 42, 58). TTSS-1 is encoded on a genomic island (*Salmonella* pathogenicity island 1 (SPI-1)), which also harbors genes for effector proteins and for several regulators of *ttss-1* expression, e.g. *hilA*, *hilC* and *hilD* (3, 43).

The bistable *ttss-1* expression is controlled by a complex regulatory network, which includes coupled positive feedback loops, controls the threshold for *ttss-1* induction and amplifies *ttss-1* expression (10, 41). Bistable *ttss-1* expression is observed in "*ttss-1* inducing" environments, i.e. the gut lumen of infected mice or in non-host environments, e.g. when *S*. Tm is grown to late logarithmic phase in LB (1, 18, 41, 48). This yields mixed populations of isogenic *S*. Tm cells that express *ttss-1* (TTSS-1^+ phenotype), or do not (TTSS-1^- phenotype), in a bimodal fashion. In the mouse gut, only the TTSS-1^+ cells can actively invade the mucosal tissue and efficiently trigger inflammation (1, 16). This inflammatory response may help to overcome the commensal microflora, thus enhancing *Salmonella* growth and transmission (26, 32, 36, 50, 52, 53, 63). Experimental data indicate that bistable *ttss-1* expression might represent an example of "division of labor" (1), but further data is required to settle this point. At any rate, *ttss-1* expression seems to be instrumental for eliciting diarrheal disease and

enhancing pathogen transmission. But the functional properties of the TTSS-1$^+$ phenotype are not well understood.

The complex setting of the infected animal gut has hampered the analysis of the TTSS-1$^+$ phenotype. In vitro experiments are essential for gaining detailed mechanistic insights. Here, we have analyzed the induction of *ttss-1* expression and its effects on the growth rate of the TTSS-1$^+$ phenotype by single cell reporter assays, competitive growth experiments and mathematical modeling. In such non-host environments, expression of the *ttss-1* virulence system expression imposed a growth penalty on the TTSS-1$^+$ cells. This may have important implications with respect to compensatory mechanisms during the infection of animal hosts.

Results

Single cell reporters for studying the TTSS-1$^+$ phenotype

We started our analysis of the TTSS-1$^+$ phenotype by probing *ttss-1* expression at the single cell level. For this purpose, we chose the *sicA* promoter (P_{sicA}), which controls expression of the chromosomal *sicAsipBCDA* operon (Suppl. Fig. S1C). This operon encodes key parts of the TTSS-1 virulence system. On the one hand, we employed a transcriptional *sipA-tsr*$_{venus}$ reporter gene cassette placing the reporter downstream of the *sicAsipBCDA* operon (Suppl. Fig. S1; (48, 62)). Due to its localization at the bacterial poles, the *tsr*$_{venus}$ reporter allows detecting < 10 proteins per cell (64). Thus, *sipA-tsr*$_{venus}$ provides a highly sensitive reporter for the TTSS-1$^+$ phenotype.

Next, we verified the performance of the *sipA-tsr*$_{venus}$ reporter. *sipA-tsr*$_{venus}$ expression was bistable and TTSS-1$^-$ and TTSS-1$^+$ individuals were distinguishable by the presence/absence of Tsr_{venus} spots at the bacterial poles ((64); Fig. 1A; Suppl. Fig. S1D). TTSS-1 expression and virulence were not compromised (Fig. 1B). The accurate response of *sipA-tsr*$_{venus}$ to *Salmonella* signaling cascades was established by disturbing known elements of the TTSS-1 gene regulation network and FACS analysis of *sipA-tsr*$_{venus}$ expression (Fig. 1C, D). In line with the published work on *ttss-1* regulation (Fig. 1D): i. Over-expression of positive TTSS-1 regulators increased the abundance of *tsr*$_{venus}$-expressing individuals (Fig. 1C; Suppl. Fig. S1D). In particular, *hilA*, *hilC* and *hilD* over-expression increased the fraction of *sipA-tsr*$_{venus}$ expressing individuals from ~20 % to 80-100 %. ii. The median signal intensity per *sipA-tsr*$_{venus}$ expressing cell increased when positive regulators were over-expressed (p*hilA*: 3.8±0.3-fold; p*hilC*: 4.0±0.1-fold; p*hilD*: 4±0.1-fold; median ± s.d.). iii. Control experiments in a *ΔhilA* mutant verified that expression of the TTSS-1$^+$ phenotype depended on the *ttss-1* master-regulator, HilA (Fig. 1C; open bars) and iv. The average HilA protein levels of the analyzed strains correlated positively with the fraction of *tsr*$_{venus}$-expressing individuals ($r^2 = 0.78$; quantitative Western blot; Fig. 1E). These data verified the accurate performance of the *sipA-tsr*$_{venus}$ reporter and demonstrated that *hilA*-dependent regulation affects both, the fraction of TTSS-1$^+$ individuals and the level of *ttss-1* expression per cell.

In addition, we employed p*sicA-gfp*, a reporter plasmid expressing *gfp* under control of the *sicA* promoter. This construct yielded brighter fluorescence than the chromosomal *sipA-tsr*$_{venus}$ and was better suited for FACS analysis. Again, this reporter yielded a bistable expression pattern (Fig. 1F). Using wt *S.* Tm p*sicA-gfp* we separated TTSS-1^{+} and TTSS-1^{-} subpopulations by FACS. Western blot analysis of the FACS-sorted subpopulations verified coincident expression of p*sicA-gfp* and the TTSS-1 protein SipC (Fig. 1F, G). This indicated that our fluorescent reporter constructs are faithful reporters of the bistable expression of the TTSS-1^{+} phenotype.

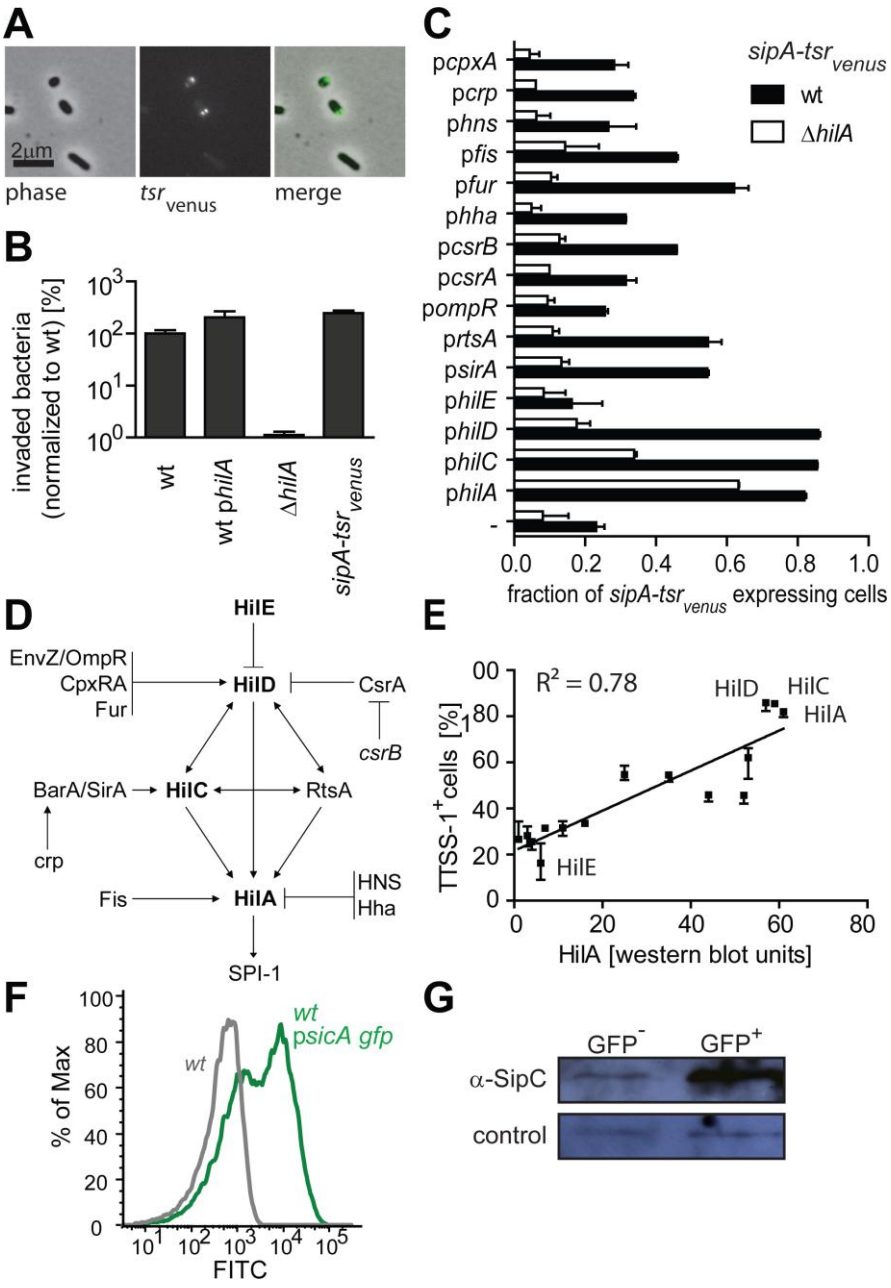

Figure 1. *sipA-tsr*$_{venus}$ as a single cell reporter for *ttss-1* expression. A) Bistable expression of *sipA-tsr*$_{venus}$ in wt *S.* Tm (M2001). Living bacteria (4 h in LB) were imaged by fluorescence- and phase contrast microscopy. Bar, 2 µm; B) Invasion into MDCK cells (3 indep. experiments; ±s.d.; Materials and Methods). C) Response of the *sipA-tsr*$_{venus}$ reporter to over-expression of known *ttss-1* regulators. Wt *S.* Tm (*sipA-tsr*$_{venus}$; M2001; black bars) or *ΔhilA* (*sipA-tsr*$_{venus}$; M2018; open bars) harboring the indicated regulator-expression plasmids (suppl. Table S2) were cultured for 4 h in LB and FACS-analyzed (triplicates ±s.d.). D) *ttss-1* regulation cascade depicting the regulators analyzed in C) and E; adapted from (2, 9, 11, 22, 44, 56)). E) Correlation between HilA protein levels and the fraction of

ttss-1 expressing individuals. The fraction of cells with the TTSS-1$^+$ phenotype (from C) was plotted against the average HilA expression (average of ≥ 3 independent quantitative Western blots per regulator and strain). F) Bistable expression of *psicA-gfp* in *S.* Tm SL1344 determined and separated by FACS; G) Western blot analysis of TTSS-1$^-$ and TTSS-1$^+$ subpopulations from F) using a polyclonal rabbit α-SipC antibody.

Time-lapse microscopy reveals retarded growth of TTSS-1$^+$ individuals

During our experiments, we observed that *hilA, hilC* and *hilD* over-expression led to reduced culture densities (e.g. OD_{600} for wt *sipA-tsr*$_{venus}$: 3.4 ±0.3 vs. wt *sipA-tsr*$_{venus}$ p*hilA*: 2.0 ±0.3; mean ± s.d.). This was a first hint suggesting that retarded growth might be a general feature of the TTSS-1$^+$ phenotype. However, it remained to be shown whether growth retardation occurs in wild type cells expressing normal levels of *hilA*, *hilC* and *hilD*.

The growth rate of the TTSS-1$^+$ individuals was analyzed by time-lapse microscopy. Wild type *S.* Tm harboring *gfp*- or *tsr*$_{venus}$-reporters for *ttss-1* expression were placed on an agar pad (LB, 1.5 % agarose), the TTSS-1$^+$ individuals were identified by fluorescence microscopy and growth was analyzed by time-lapse phase contrast microscopy (1 frame/30 min; Fig. 2A). Imaging did not impose detectable photo damage to the bacteria, as indicated by the unaltered growth rate. Strikingly, TTSS-1$^+$ individuals grew slower than TTSS-1$^-$ individuals (wt *S.* Tm *sipA-tsr*$_{venus}$ (M2001); μ_{T1+} = 0.77 h^{-1} vs. μ_{T1-} = 1.02 h^{-1}; p = 0.038 for the factor 'phenotype' in a two-way ANOVA; Fig. 2B). The negative control strain *ΔhilA sipA-tsr*$_{venus}$ yielded only TTSS-1$^-$ individuals which grew at the "fast" rate (μ_{T1-} = 1.01 h^{-1}; Fig. 2B). Thus, TTSS-1$^+$ individuals seemed to grow at a reduced rate.

To exclude potential artifacts attributable to the *sipA-tsr*$_{venus}$ reporter, we analyzed unmodified wild type *S.* Tm not harboring any reporter. Using a maximum likelihood approach, we identified two populations with distinct growth rates (likelihood ratio test for two populations versus one population, p < 0.001, μ_{slow} = 0.64 h^{-1} vs. μ_{fast} = 1.1 h^{-1}; Fig. 2C), very similar to the ones described above (Fig. 2B). Furthermore, unmarked mutants lacking the entire SPI-1 region (Δ*spi-1*) or the positive *ttss-1*

regulator *hilD* yielded exclusively fast growing cells, while deletion of the negative *ttss-1* regulator *hilE* yielded only slow growing cells (Fig. 2C). Finally, wild type *S*. Tm harboring p*sicA-gfp* or a chromosomal *gfp*-reporter for the TTSS-1 gene *prgH* (18) yielded slow growing TTSS-1$^+$ and fast growing TTSS-1$^-$ cells (μ_{T1+} = 0.44 h^{-1} vs. μ_{T1-} = 1.01 h^{-1}; p = 0.015 for the factor 'phenotype' in a two-way ANOVA; Fig. 2D). Bacteria expressing the p*sicA-gfp* or *prgH-gfp* reporters grew even slower than the TTSS-1$^+$ *sipA-tsr*$_{venus}$ bacteria or the slow-growing wt *S*. Tm subpopulation (Fig. 2BC). Presumably, this was attributable to the additional "burden" conferred by the GFP expression, as described, before (60).

Thus, the time-lapse microscopy experiments verified bistable *ttss-1* expression and revealed that the TTSS-1$^+$ phenotype has a reduced growth rate, even at wild type HilA and TTSS-1 levels (μ_{T1+} in the range of 0.7 h^{-1} vs. μ_{T1-} in the range of 1.0 h^{-1}). This was confirmed in a dye dilution assay (supplementary Fig. S2).

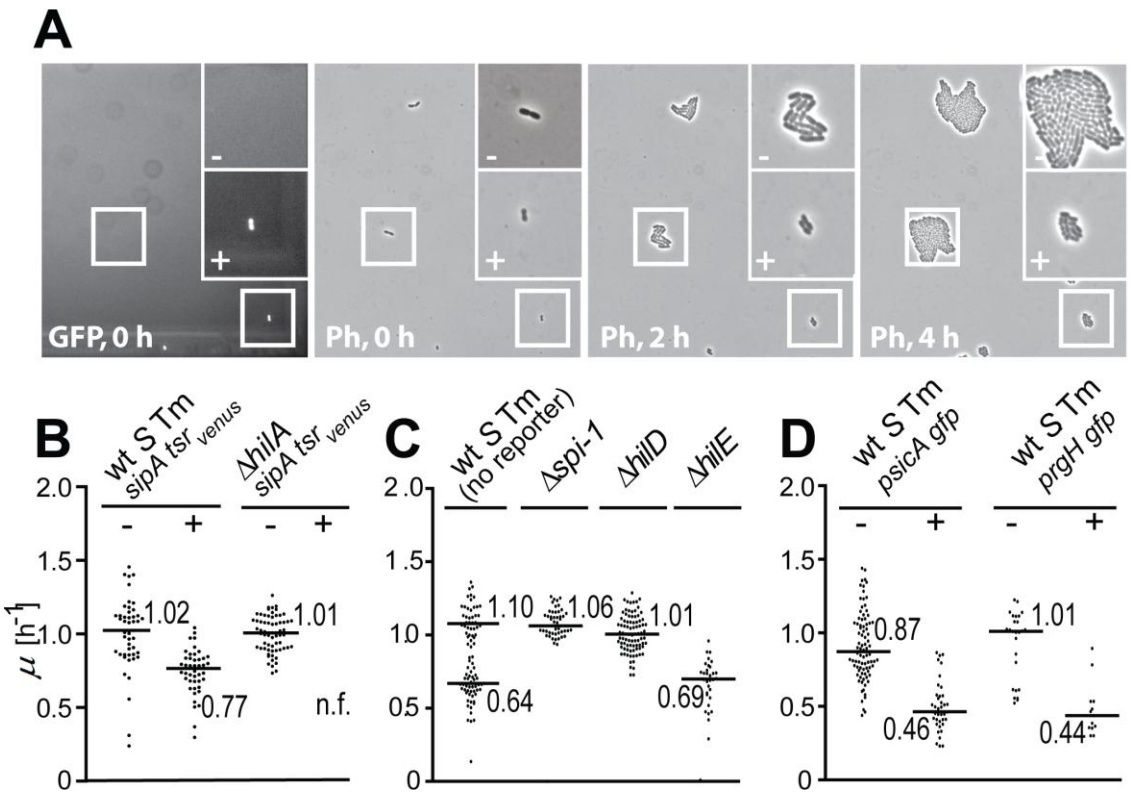

Figure 2. Time-lapse microscopy reveals retarded growth of TTSS-1$^+$ individuals. Bacteria (4h LB subculture, OD_{600} = 1), were placed on an agar pad (37°C) and imaged to detect *ttss-1* expression (fluorescence) and growth (phase contrast; 1 frame/30 min). A) Sample images from a typical time-lapse microscopy experiment with wt *S.* Tm (SL1344, p*sicA-gfp*). B)-D): Time-lapse microscopy experiments with wt *S.* Tm (M2001; *sipA-tsr*$_{venus}$) and an isogenic *hilA* mutant (M2018; *sipA-tsr*$_{venus}$; B); wt *S.* Tm (SL1344; no reporter) and mutants lacking *ttss-1*, *hilD* or *hilE* (no reporter); C); wt *S.* Tm (SL1344; p*sicA-gfp* and an isogenic wt reporter strain (SL1344 *prgH-gfp*; D). Each data point represents the growth rate of an individual micro colony. Data were from ≥3 independent experiments. Black line, median; Numbers, median growth rates.

Retarded growth and *ttss-1* induction determine the fraction of TTSS-1$^+$ individuals: a mathematical analysis

When monitoring growth and bistable *ttss-1* expression in a wt *S.* Tm (p*sicA-gfp*) culture, the fraction of TTSS-1$^+$ individuals began to rise after 2.5 h as soon as the culture entered the late logarithmic phase, increased in a linear fashion, and reached approx. 60 % after 7 h once the culture entered the stationary phase (Fig. 3A).

Our results implied that two different parameters affect the fraction of TTSS-1$^+$ individuals and the overall growth progression in the late logarithmic phase: i. Competitive growth. TTSS-1$^+$ individuals are constantly outgrown by the fast-growing TTSS-1$^-$ individuals ($\mu_{T1+} < \mu_{T1-}$; Fig. 2); this constantly reduces the size of the TTSS-1$^+$ subpopulation. ii. *ttss-1* induction. Presumably, initiation of *ttss-1* expression in TTSS-1$^-$ individuals compensates the "TTSS-1$^+$ losses" attributable to competitive growth and explains the increasing fractions of TTSS-1$^+$ individuals during the late logarithmic phase.

To infer the dynamic initiation rate r_i of *ttss-1* expression in the late logarithmic phase from our experimental data, we devised a mathematical model describing the growth of the TTSS-1$^+$ (N_{T1+}; growth rate μ_{T1+}) and the TTSS-1$^-$ population (N_{T1-}; growth rate μ_{T1-}) as a function of time (t):

$$dN_{T1+}/dt = \mu_{T1+} N_{T1+}(t) + r_i(t) N_{T1-}(t) \qquad (1)$$

$$dN_{T1-}/dt = \mu_{T1-} N_{T1-}(t) - r_i(t) N_{T1-}(t) \qquad (2)$$

It should be noted that the model does not include a term for “switching off” *ttss-1* expression. This was justified by our failure to observe “off switching” in the experiments shown in Fig. 2 and further supported by other data (see, below). During the late logarithmic phase, the relative abundance of the TTSS-1$^+$ individuals increased, and the fraction α of TTSS-1$^-$ individuals (N_{T1-}) decreased in a linear fashion (Fig. 3A):

$$\alpha(t) = N_{T1-}(t)/(N_{T1-}(t) + N_{T1+}(t)) \qquad (3)$$

Equation (2) can be rearranged to calculate $r_i(t)$ (see supplementary information for details):

$$r_i(t) = (\mu_{T1-} N_{T1-}(t) - (dN_{T1-} / dt)) / N_{T1-}(t) \quad (4)$$

With the data from Fig. 3A and by using equation (3) we could determine $N_{T1\text{-}}(t)$ and, after fitting an empirical function to $N_{T1\text{-}}(t)$, also $dN_{T1\text{-}}/dt$. Using equation (4), this allowed calculating $r_i(t)$ during the late logarithmic phase (see supplementary information for details). We found that the initiation rate (r_i) of *ttss-1* expression increased continuously during the late logarithmic phase, e.g. from 0.15 h^{-1} at 3.5 h to 0.48 h^{-1} at 6.5 h (Fig. 3B).

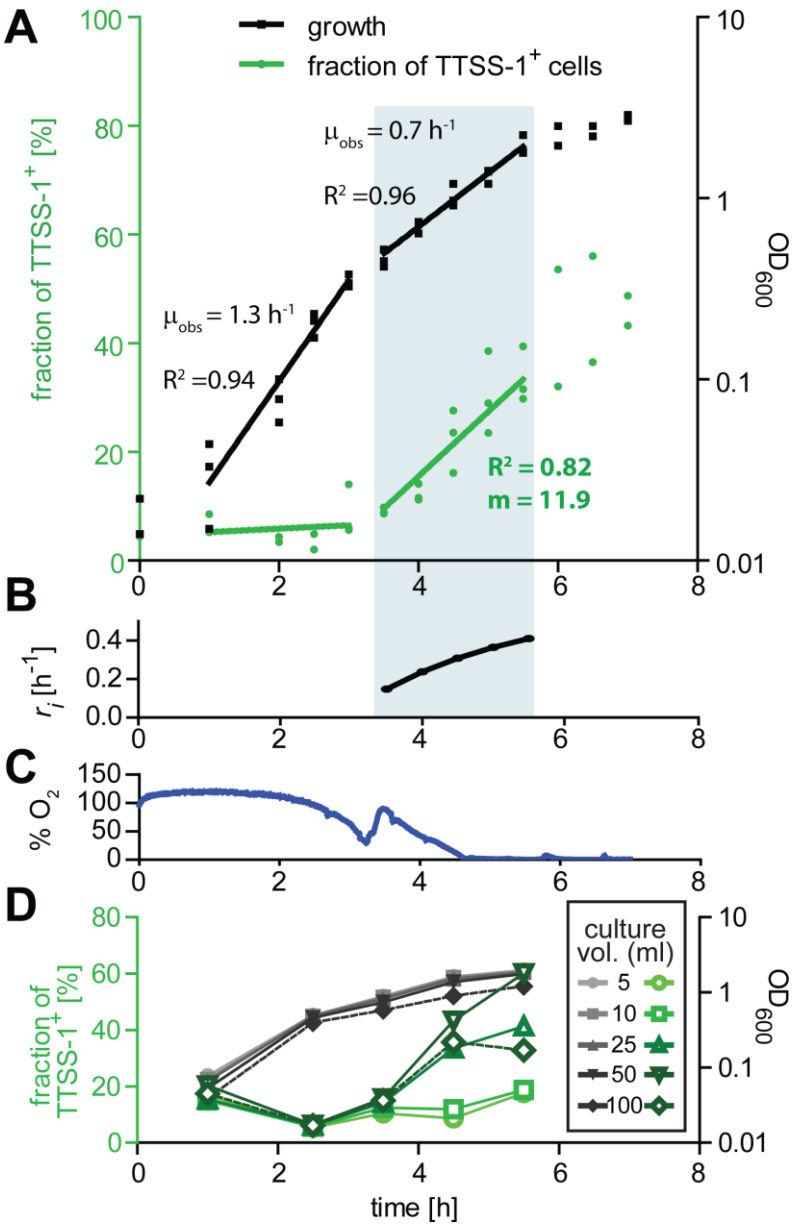

Figure 3. Time course experiment analyzing the initiation of *ttss-1* expression. A) Wt *S.* Tm (SL1344, p*sicA-gfp*) was sub-cultured under mild aeration in LB. Growth (OD_{600}, black) and *ttss-1* expression (FACS, green) was analyzed and fitted separately for early and late log phase. Gray: late logarithmic phase. B) Calculation of $r_i(t)$ during the late log phase using eq. 4 and data from A). C) pO2 during the experiment. D) Growth (OD_{600}, black) and *ttss-1* expression (FACS, green) in 250ml flasks (shaken 160 rpm, 37°C) harboring the indicated volume of LB (inoculation: 1/100 from a 12 h *S.* Tm p*sicA-gfp* culture).

Environmental signals affecting *ttss-1* expression in the late logarithmic phase

The initiation rate of *ttss-1* expression seemed to increase upon entry into the late logarithmic growth phase (Fig. 3A). Therefore, it might be induced by growth-related environmental signals (e.g. oxygen depletion, quorum signals, nutrient depletion, metabolite accumulation). To address this, we analyzed the partial oxygen pressure (pO_2) during growth. As expected, pO_2 declined to < 30 % relative aeration during the first three hours (Fig. 3C). After approximately 3.5 h, we detected a transient rebound of the oxygen pressure followed by a steady decline to < 3 % relative aeration during the next hour. This undulation of oxygen pressure is indicative of a change in the growth physiology at 3.5 h and was in line with the reduced growth rate (Fig. 3A, shaded area).

The data suggested that altered metabolism, nutrient availability, waste product accumulation, the reduced growth rate or the low oxygen pressure might represent cues inducing *ttss-1* expression. To test the role of pO_2, we performed batch culture growth experiments in identical 250 ml culture flasks filled with the indicated volumes of media (wt *S.* Tm p*sicA gfp* grown in 5, 10, 25, 50 or 100 ml LB; Fig. 3D). This setup allowed analyzing the effect of reduced pO_2 (i.e. in larger, poorly aerated culture volumes) at equivalent growth rates. We observed that the fraction of *ttss-1* expressing cells increased in larger culture volumes. Therefore, low oxygen tension might represent one environmental cue directly or indirectly inducing bistable *ttss-1* expression.

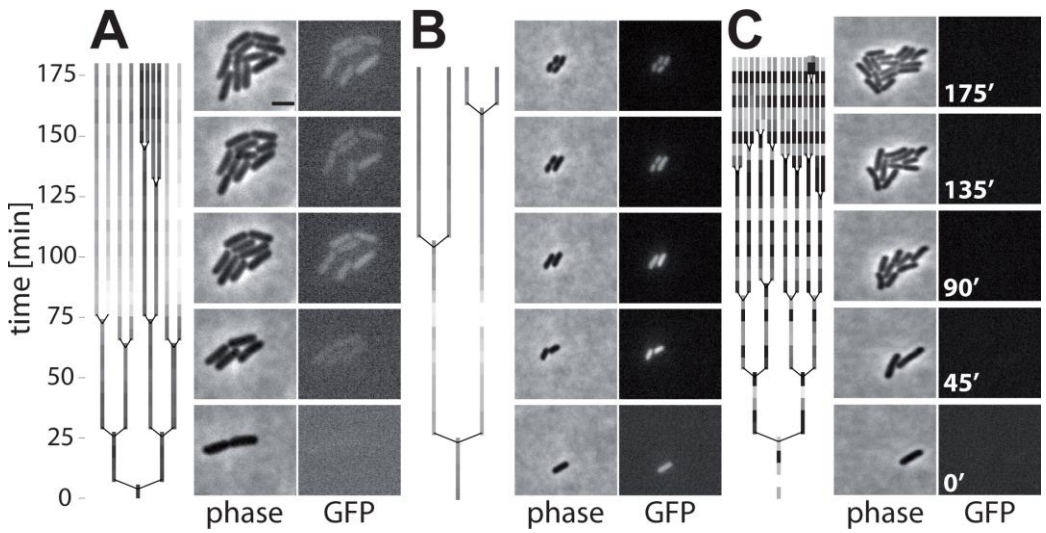

Figure 4. Time-lapse microscopy shows onset of *ttss-1* expression and concomitant growth retardation. Lineage trees with corresponding phase contrast and GFP images of *S.* Tm (M556; p*sicA-gfp*) grown on agar pads with spent LB. Coloring of the lineage trees reflects the relative mean GFP intensity of individual cells (dark= low; light= high; scaled to the highest fluorescence in tree). A) On-switching of *ttss-1* expression in a fraction of the micro colony. B) Micro colony uniformly expressing *ttss-1* throughout the assay. C) Micro colony not expressing *ttss*-1 throughout the assay. Scale bar, 2 µm; see also suppl. Fig. S3.

Time lapse microscopy detects the emergence and the reduced growth rate of TTSS-1$^+$ cells

In liquid culture, the initiation of *ttss-1* expression occurred in the late logarithmic phase. However, our initial time lapse microscopy data for bacteria sampled from this growth phase did not show initiation of *ttss-1* expression (Fig. 2). We reasoned that this might be attributable to the lack of inducing environmental signals, as these experiments had been performed on agar pads soaked with fresh LB medium. To test this hypothesis, we modified the time lapse microscopy experiment and imaged bacteria (*S.* Tm p*sicA-gfp*) placed on agar pads soaked with filter-sterilized spent medium taken from a culture at the same growth phase (OD_{600} = 0.9, see Materials and Methods). We analyzed growth of 191 micro colonies. At the beginning, 135 did not express *ttss-1*. But remarkably, we

observed 15 of 135 initially TTSS-1$^-$ micro colonies, in which individual bacteria induced *ttss-1* expression during the course of our imaging experiment (e.g. Fig. 4A, suppl. Fig. S3; suppl. movie S1). After induction, the TTSS-1$^+$ cells grew at a slower rate than their TTSS-1$^-$ siblings. In addition, we observed numerous TTSS-1$^+$ bacteria (56 micro colonies) and TTSS-1$^-$ bacteria (120 micro colonies) which did not "switch" their *ttss-1* expression status. In line with the results above, *ttss-1* expression and the interval between two cell divisions was negatively correlated (Fig. 4A,B,C, Spearman's rho = -0.747, $p < 0.0001$, N = 29).

These experiments support the stochastic initiation of *ttss-1* expression. But the initiation rate of *ttss-1* expression (< 0.04 h^{-1}) was lower than that predicted from the batch culture experiment shown in Fig. 3 (r_i = 0.15-0.48 h^{-1}). This might be attributable to the lack of some environmental cue, e.g. low oxygen pressure, as time lapse microscopy was performed at ambient atmosphere. Only two micro colonies showed a decrease in fluorescence as expected for "off-switching". Hence, the rate of off-switching is not substantial. This indicated that our mathematical model, which assumed that "switching off" the *ttss-1* expression would be negligible, was justified (equation (1) did not include $r_i(t)N_{T1\text{-}}(t)$). These experiments verified that *ttss-1* expression is initiated in a stochastic fashion under "inducing" environmental conditions and that the TTSS-1$^+$ phenotype exhibits a growth defect.

Handicap of wt *S*. Tm in a competitive growth experiment

Finally, we confirmed the growth penalty attributable to *ttss-1* expression in the late logarithmic phase in competition experiments. Wt *S*. Tm expresses *ttss*-1 in a bistable fashion and forms a significant fraction of slow-growing TTSS-1$^+$ cells during the late logarithmic phase (Fig. 3). This slows down the apparent growth of the total wild type population (see above). In contrast, *hilA* or *hilD* mutants, which do not express *ttss-1*, yield a pure population of fast-growing TTSS-1$^-$ cells (Figs. 1 and 2). Thus, in a mixed culture, *hilA* or *hilD* mutants should outgrow wt *S*. Tm. Indeed, both mutants out-competed the wt strain during the late logarithmic phase of the mixed culture (Δ*hilA*, Δ*hilD*; Fig. 5A,B). In contrast, a *hilE* mutant, which forms a larger fraction of TTSS-1$^+$ cells than wt *S*. Tm (Fig.

2), was outcompeted by wt *S.* Tm in this type of assay (Δ*hilE*, Fig. 5C). This verified the growth penalty of TTSS-1^{+} cells in LB batch cultures.

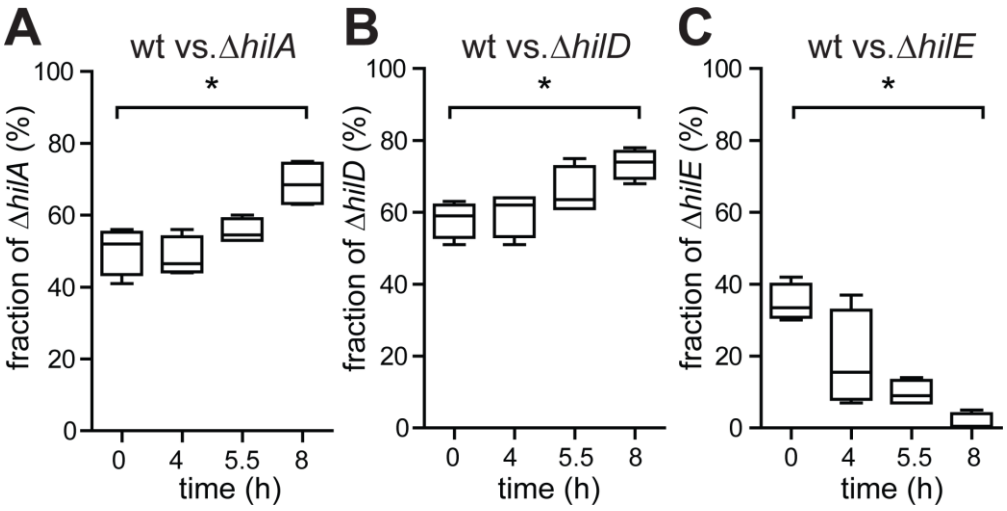

Figure 5. Competitive growth experiment confirming that *ttss-1* expression retards growth. A) Wt *S.* Tm (ATCC14028, km^{S}) and an isogenic *hilA* mutant (M2005, km^{R}), were used to inoculate a sub-culture at a ratio of approx. 1:1. Growth of the mixed culture was monitored via OD_{600}. B) Competitive growth between wt *S.* Tm and an isogenic *hilD* mutant (M2007, km^{R}), resp. an isogenic *hilE* mutant (M2008, cm^{R}), C). The fraction of wt *S.* Tm was determined by differential plating on LB agar (50 µg/ml kanamycin, resp. 30 µg/ml chloramphenicol) at the indicated time points. Data were derived from four experiments (± s.d., p = 0.014).

Discussion

The effect of virulence factor expression on the fitness of an individual pathogen cell has remained unclear. We have analyzed the fitness costs associated with the expression of *ttss-1*, which encodes a key virulence function of *S.* Tm. An in vitro system was chosen for a detailed analysis of the growth phenotype of TTSS-1$^+$ cells. We found that these cells have a reduced growth rate. This established that *ttss-1* expression represents a burden (and not an advantage) at the level of the individual cell, at least in the non-host environment of our assay system. The growth penalty affects the fraction of TTSS-1$^+$ individuals and the overall growth progression in a *S.* Tm culture. Mathematical modeling and experimental data demonstrated that this growth penalty and an increasing initiation rate of *ttss-1* expression during the late logarithmic growth phase were sufficient to explain the dynamic abundance of TTSS-1$^+$ and TTSS-1$^-$ individuals in a clonal *S.* Tm batch culture.

Evidence for bistability of *ttss-1* expression has only recently been accumulated. Under inducing conditions, single cell reporters for expression of *ttss-1* or effector proteins yielded cells in the "on" and cells in the "off" state (18, 41, 48, 55, 62). The regulatory network controlling *ttss-1* expression includes at least three positive feedback loops and this architecture is thought to set the threshold for initiating *ttss-1* expression and to amplify the level of expression (41, 55). The TTSS-1$^+$ phenotype can persist for several hours, even if the bacteria are shifted into environments normally not inducing *ttss-1* expression (histeresis; shift to fresh LB, Fig. 2). However, it should also be noted that it has not been possible to define unequivocally where stochasticity is introduced. In fact, stochastic initiation of *ttss-1* expression might hinge on different regulators in different environments.

TTSS-1$^+$ cells have at least two important characteristics. First, they express the virulence factors enabling host manipulation and elicitation of disease (16, 47, 58). Second, as we have found here, they grow at a reduced rate. *ttss-1* expression may represent a "burden" in itself, i.e. through partial disruption of the proton gradient by "leaky" TTSS assembly-intermediates and/or the metabolic energy required for biosynthesis of the TTSS. A typical TTSS-1$^+$ cell is estimated to express 20-200 TTS

apparatuses and approx. 3-10 x 10^4 effector proteins, amounting to a significant fraction of the total cellular protein (48, 62).

An alternative explanation for the reduced growth rate of TTSS-1^+ cells might reside in coordinated expression of a complex regulon. Several global regulators (e.g. crp, mlc, fur; (11, 29, 56)) and silencing proteins (hns, hha; (34, 35)) can control *ttss-1* expression. Moreover, HilA may control multiple loci apart from *ttss-1* (25). And we have observed co-expression of *ttss-1* and of *fliC*, which encodes a key structural component of the flagella, in the late logarythmic phase (suppl. Fig. S4). Accordingly, *ttss-1* expression might be one feature of a "differentiated" state which also includes adaptations reducing the growth rate. It is tempting to speculate that this state might be particularly adapted for mucosal tissue invasion. This would be an important topic for future research.

Interestingly, similar phenomena have been observed in other *ttss*-expressing pathogens. In *Pseudomonas aeruginosa*, growth in suboptimal media was shown to result in bistable *ttss* expression (39). But it remained unclear whether growth might be affected. In contrast, the plasmid-encoded TTSS of *Yersinia* spp. is well known to cause growth retardation in response to host cell contact or low calcium environments (6, 19). However, in this case, *ttss* induction seems to be uniform even in suboptimal media (61). Thus, bistability and growth retardation do occur in other *ttss* expressing bacteria, but specific adaptations may exist for each pathogen.

Which environmental cues induce *ttss-1* expression in *S*. Tm? *ttss-1* is expressed in the lumen of the host's intestine and in the late logarythmic phase in LB-batch culture. Low oxygen pressure is common to both environments and may represent an inducing signal (see Fig. 3C). In line with this hypothesis, *Shigella flexneri*, a closely related gut pathogen, can modulate the activity of its TTSS in response to low oxygen pressures typically observed at the gut wall (33). Similarly, HilA-mediated *ttss-1* expression is known to respond to oxygen pressure (3, 46). In addition, numerous other internal and external cues are known to affect *ttss-1* expression, including osmolarity, pH, growth rate, or the presence of short chain fatty acids like acetate (13, 15, 21, 25, 27, 28, 31). The sum of these environmental cues seems to determine the level of *ttss-1* induction. This might explain our

observation of a low, but detectable initiation rate of *ttss-1* expression on agar pads soaked with spent medium (Fig. 4). This environment should harbor most cues present in the late log culture medium, but lacks low oxygen pressure, which could not be established in the real time microscopy setup.

In summary, our findings indicate that the TTSS-1$^+$ phenotype is more complex than previously anticipated. Currently, we can only speculate how this affects the real infection and transmission in vivo. Our results suggest that the TTSS-1$^+$ subpopulation is constantly drained by the burdens inflicted by immune defenses within the infected gut mucosa (1) and by the reduced growth rate (this work). The latter should represent a competitive disadvantage against all other bacteria (commensals and TTSS-1$^-$ *S.* Tm cells) present in the gut lumen. Moreover, this burden should materialize even before invading the gut tissue. In order to explain the evolution of bistable *ttss-1* expression and the successful propagation of the *ttss-1* genotype, one has to predict that the TTSS-1$^+$ phenotype must confer some type of advantage. According to the "division of labor" model, the advantage might emanate from a "public good", i.e. the TTSS-1 induced gut inflammation fostering *Salmonella* growth in the gut lumen and enhancing transmission. Alternatively, the TTSS-1$^+$ phenotype might include (unidentified) features enhancing the survival and growth of the *ttss-1* expressing bacteria themselves, e.g. in permissive niches of the host's intestine or by enhancing the chances of chronic infection and long-term shedding. Identifying these mechanisms will represent an important step for understanding the evolution of bistable *ttss-1* expression.

Materials and Methods

Bacteria

All strains were derivatives of *Salmonella* Typhimurium SL1344 or ATCC14028 (see suppl. Tab. S1). All plasmids and primers are shown in suppl. Tab. S2 and S3. Bacteria were inoculated (1:100 in LB) from 12 h overnight cultures (LB, supplemented with the appropriate antibiotics) and grown under mild aeration for 4 h at 37 °C, if not stated otherwise. In Fig. 1C,E, the medium included 0.01% arabinose.

HilA was analyzed by quantitative Western blot using an affinity-purified rabbit α-HilA antiserum (Fig. 1E). Recombinant HilA was used for normalization. SipC was detected using an α-SipC serum (Fig. 1G).

For invasion, MDCK cells were grown in MEM (Invitrogen), infected for 30 min (MOI = 5; (8), washed and incubated in MEM (400 µg/ml gentamicin; 1 h). Intracellular bacteria were enumerated by plating.

FACS

Prior to analysis, fluorophore formation was ensured (2h, RT, 30 µg/ml chloramphenicol). Tsr_{venus} and Gfp emission was analyzed at 530 nm (supplement; FACSCalibur 4-color, Becton Dickinson). Bacteria were identified by side scatter (SSC). Data were analyzed with FlowJo software (Tree Star, Inc.). For Tsr_{venus} (Fig. 1), ln-transformed fluorescence values for 40000 events were median-normalized (subtraction) and compared to the similarly normalized data from the reporterless control strain, thus yielding the fraction of TTSS-1^+ individuals. For sorting bacterial cells, *S.* Tm (p*sicA-gfp*) cells were sorted by FACS (Aria Becton Dickinson, FACSDiva Software).

Time-lapse microscopy

Bacteria were placed on a 1.5% agarose pad equilibrated with LB, sealed under a glass coverslip and mounted (37°C temp. control; Axioplan2; Plan-APOCHROMAT 63x/1.4 oil; Zeiss or IX81, UPlanFLN 100x/1.3 Oil, Olympus). Reporter fluorescence (Exc. 470/20 nm; BP 495 nm; Em. 505-530

nm) and micro colony growth (phase contrast) were monitored and evaluated using Axiovision software (Zeiss). The slope of the ln-tranformed bacterial numbers (*t*) yielded the growth rate μ. For *sipA-tsr*$_{venus}$ and *prgH-gfp*, the micro colonies were scored visually as TTSS-1$^+$ or TTSS-1$^-$. To analyze differences in growth rates between TTSS-1$^+$ and TTSS-1$^-$ micro colonies, we performed a full-factorial analysis of variance with the two factors phenotype (fixed) and experiment (random). Variance was analyzed in SPSS 17.0 (SPSS Inc. - Chicago, IL).

Growth rates w/o reporter were analyzed via a maximum likelihood approach to test for two subpopulations with different growth rates. The growth rate measurements from five independent experiments (87 micro colonies) were combined. Using maximum likelihood, we fitted a bi-modal distribution (the sum of two normal probability density functions) and a unimodal (normal) distribution, and compared the two fits with a likelihood ratio test using R software (54).

In Fig. 4, cell growth and *ttss-1* expression were analyzed using a modified version of the cell tracking software described in (40). The first cell in each micro colony that could be observed over a whole division was used to analyze the statistical association between *ttss-1* expression and the interval between two divisions (by non-parametric correlation analysis using PASW Statistics 18.0.0). 157 micro colonies were analyzed to estimate the fraction of micro colonies in which all cells, none of the cells, and a fraction of the cells expressed *ttss-1*. These groupings were based on visual inspection of each micro colony.

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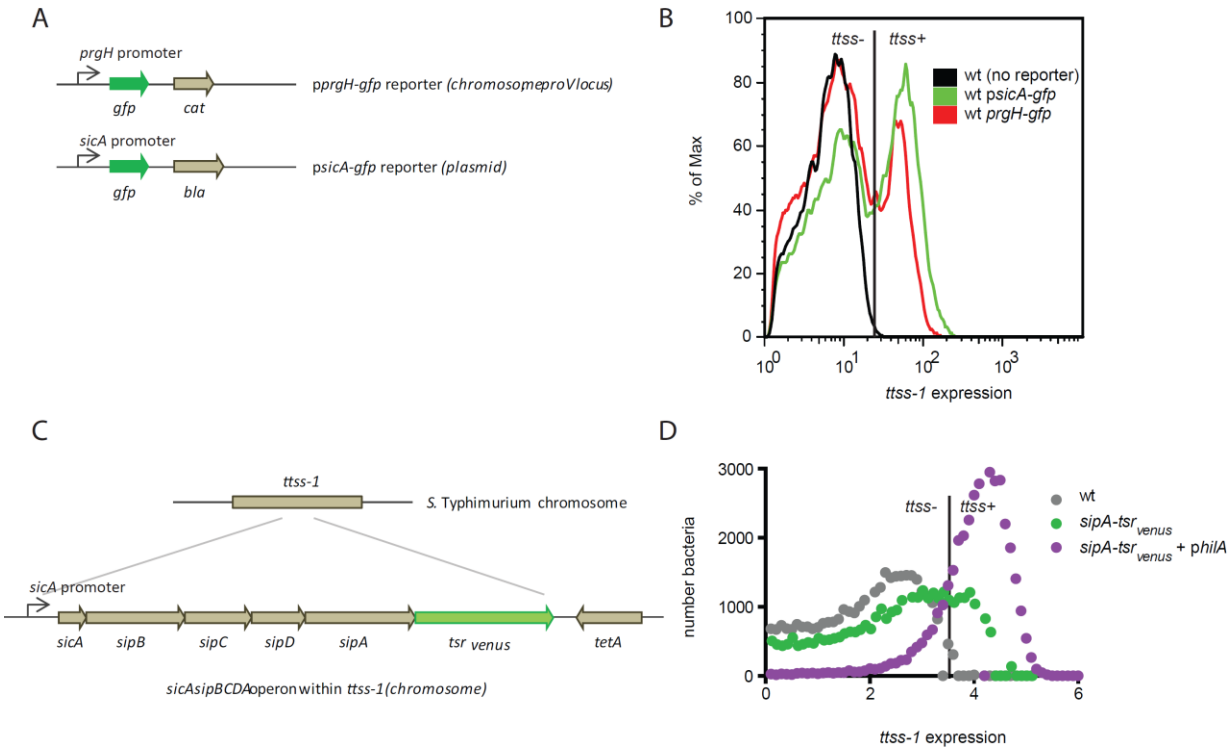
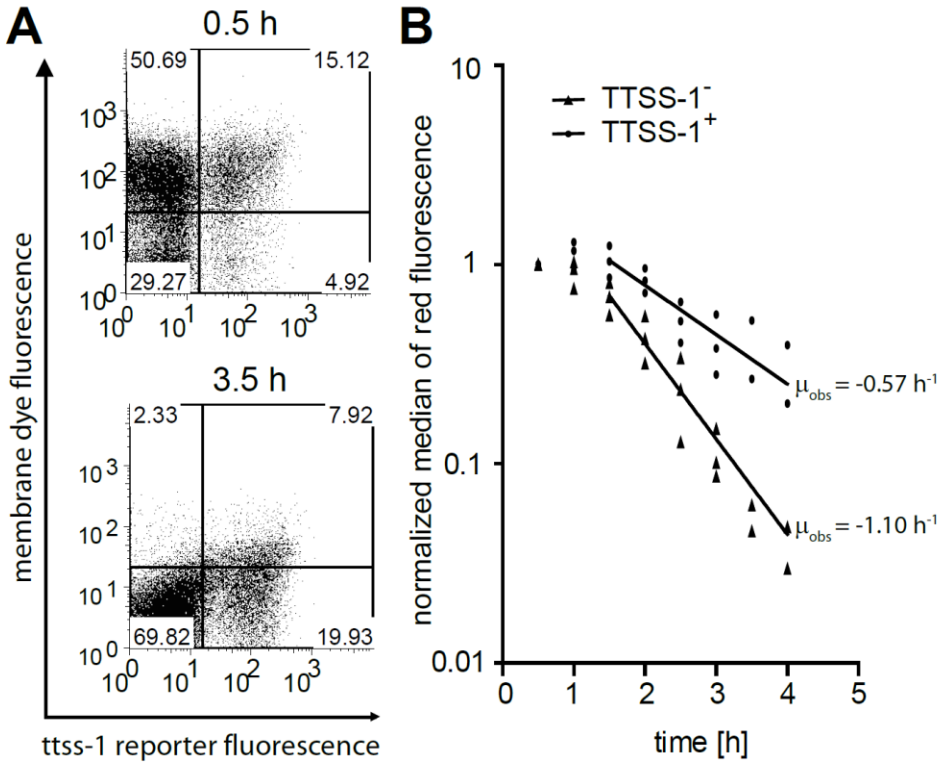
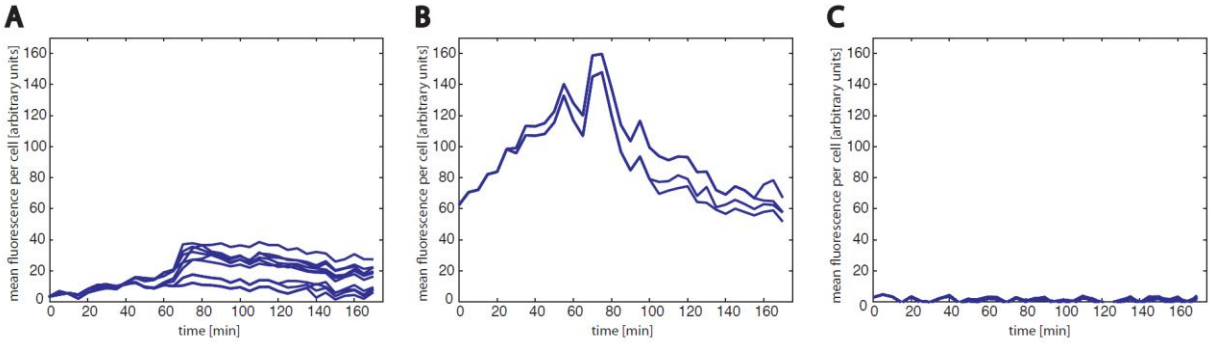
Supplementary Figures



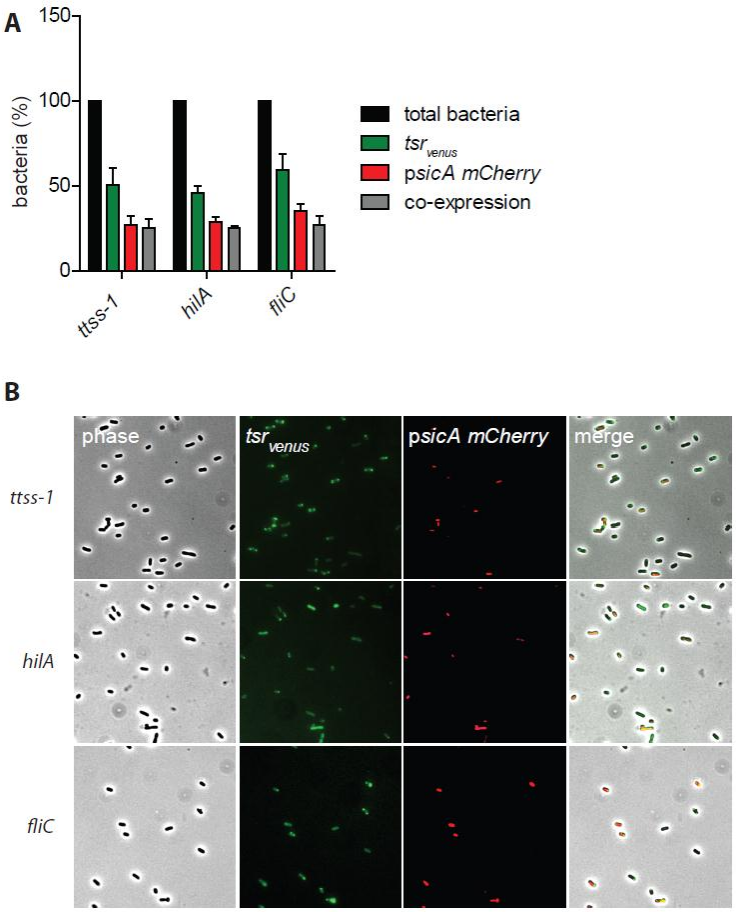
Suppl. Fig. S1. Graphical maps and bistable gene expression by the *gfp* and *venus* constructs. A) Transcriptional reporters for *prgH* and *sicA* expression. The *prgH* and *sicA* promoters are driving *gfp* expression. The *prgH-gfp* reporter is integrated into the chromosomal *proV* locus (18). The *sicA-gfp* reporter is plasmid-encoded (pM972; p*sicA-gfp*). B) Bistable *ttss-1* expression as detected using the *prgH-gfp* and *sicA-gfp* reporters. Wild type *S.* Tm SL1344 w/o reporter (black), harboring p*sicA-gfp* (green), or harboring *prgH-gfp* (red) were cultured for 4h in LB, *gfp* expression was analyzed by FACS and the results were plotted using FlowJo7.5 software (Materials and Methods). C) Transcriptional reporter for *sipA* expression. The *sipA-tsr*$_{venus}$ reporter was constructed by integrating pM2002 into the *S.* Tm chromosome at the 3'-end of the *sicAsipBCDA* operon. D) Bistable *ttss-1* expression profile of wild type *S.* Tm ATCC14028 w/o any reporter (gray), with the *sipA-tsr*$_{venus}$ reporter (green) or with the *sipA-tsr*$_{venus}$ reporter and p*hilA* (purple); data were analyzed by using MSExcel2007 and Prism5 software.



Suppl. Fig. S2. Dye dilution assay confirmed retarded growth of TTSS-1$^+$ individuals. A dye dilution assay served as a second, independent method for measuring growth of TTSS-1$^+$ individuals. In this type of assay, bacteria are labeled with a stable dye which is diluted by 2-fold during each cell division. Here, we used the membrane dye PKH26 and a *S.* Tm *wbaP* strain harboring a *ttss-1* reporter plasmid (SKI12, p*sicA-gfp*). This strain lacks the LPS O-side chain and allowed efficient membrane labeling of living cells with PKH26. It should be noted that the *wbaP* strain grew normally in LB-broth and efficiently invaded host cells, a hallmark of TTSS-1 function (23). A) SKI12 pM972 was sub-cultured (LB, 4h, OD_{600} = 1), washed three times with 4°C PBS, and incubated for 2 min at room temperature with 5 µM PKH26 (50 mM acetate buffer pH 5; Sigma-Aldrich). Excess dye was removed by washing three times with LB. Then, the bacteria were grown in LB, aliquots were removed at the indicated times and GFP- and PKH26 fluorescence were analyzed by FACS (PKH26 = red fluorescence). B) Dye-dilution rates of the TTSS-1$^+$ and TTSS-1$^-$ sub-populations. The median fluorescence intensity of the left (TTSS-1$^-$) and the right (TTSS-1$^+$) quadrants were plotted at each time point, analyzed. Line: exponential fit to the experimental data. The TTSS-1$^-$ individuals displayed an apparent PKH26 dilution rate of $t_{1/2}$ = 36 min (i.e. μ = 1.1 h^{-1}; Fig. 3B). The PKH26 dilution rate of the TTSS-1$^+$ individuals amounted to $t_{1/2}$ = 86 min (i.e. μ = 0.57 h^{-1}; Fig. 3B). This was in line with our results from time-lapse microscopy and confirmed that the TTSS-1$^+$ phenotype has a reduced growth rate.



Suppl. Fig. S3. Quantification of fluorescence intensity in time-lapse microscopy. Fluorescence was quantified over time for the growing micro colonies analyzed in Fig. 4. Each line shows fluorescence of a single cell, branching of lines indicates division events. A, B, and C correspond to A, B, and C in Fig. 4.



Suppl. Fig. S4. *fliC* is co-regulated with *ttss-1*. *S.* Tm possessing a transcriptional reporter for *ttss-1* (p*sicA mCherry*, plasmid) and either *sipA-tsr*$_{venus}$ or *hilA-tsr*$_{venus}$ or *fliC-tsr*$_{venus}$ (each on chromosome) were grown in LB to an OD_{600} of 1 and examined for co-expression by microscopy. The co-expression of p*sicA gfp* and *sipA-tsr*$_{venus}$ served as a direct positive control. We could observe a less efficient expression of *mCherry*, even though *gfp* and *mCherry* are driven by literally the same promoter (p*sicA*, see also Supplementary Figure S1). Most probably this is caused by the stability of the different fluorophores and the higher sensitivity of the *tsr*$_{venus}$ reporter. In the case of *hilA* and *fliC* we could determine a co-expression of *ttss-1* genes. At least all TTSS-1+ (p*sicA mCherry*) featured *hilA* and *fliC* expression. It was recently shown that FliC, which assembles to the flagella, underlies noisy gene expression (besides phase variation (5)) and emerges FliC+ and FliC- subpopulations (14). A) Quantification of four independent experiments; shown is the median ± s.d.; B) Representative microscopy pictures of the three strains.

Supplementary Tables

Supplementary Table S1. Bacterial strains

Strain	relevant genotype	reference
ATCC14028	wt *S.* Typhimurium (*S.* Tm)	American Type Culture Collection
JH3010	SL1344, *prgH-gfp* reporter; wt	(18)
SKI12	SL1344, *wbaP::cat*	(23)
M2000	SL1344, *sipA-tsr*$_{venus}$ reporter; wt	This study
M2001	ATCC 14028, *sipA-tsr*$_{venus}$ reporter; wt	(62)
M2005	ATCC 14028, *hilA::aphT*	This study
M2007	ATCC 14028, *hilD::aphT*	This study
M2008	ATCC 14028, *hilE::cat*	This study
M2018	ATCC 14028, *sipA-tsr*$_{venus}$, *hilA::aphT*	This study
M2076	ATCC 14028, *hilA-tsr*$_{venus}$	This study
M2080	SL1344, *hilA::aphT*	This study
M2821	SL1344, *fliC-tsr*$_{venus}$	This study
M556	SL1344, *sseD::aphT*	(17)
SL1344	wt *S.* Typhimurium (*S.* Tm)	(20)

Deletion of *hilA* was performed by insertion of a kanamycin resistance cassette as previously described (7). M2001, a wt ATCC14028 derivative carrying the *sipA-tsr*$_{venus}$ reporter (64) in the chromosome downstream of the *sipA* stop codon has been described, recently (62). M2018 was constructed by P22 transduction of the *hilA::aphT* allele into M2001. *ttss-1* regulators were amplified by PCR from SL1344 genomic DNA, cloned into pBAD24 and expressed under control of the p_{BAD} promoter.

Supplementary Table S2. Plasmids

Plasmid	feature of interest	reference
p*hilA*	Amp^r, pBAD-Myc/HisC with *hilA-myc/his* ORF under *ara* control; pCH112	(30)
p*hilC*	Amp^r, pBAD-Myc/HisC with *hilC-myc/his* ORF under *ara* control; pLS119	(45)
p*hilD*	Amp^r, pBAD-Myc/HisC with *hilD-myc/his* ORF under *ara* control; pLsS118	(45)
pM2010	Amp^r, pBAD24 with *hilE* ORF under *ara* control	this study
pM2011	Amp^r, pBAD24 with *sirA* ORF under *ara* control	this study
pM2015	Amp^r, pBAD24 with *rtsA* ORF under *ara* control	this study
pM2017	Amp^r, pBAD24 with *ompR* ORF under *ara* control	this study
pM2018	Amp^r, pBAD24 with *csrA* ORF under *ara* control	this study
pM2021	Amp^r, pBAD24 with *csrB* ORF under *ara* control	this study
pM2024	Amp^r, pBAD24 with *hha* ORF under *ara* control	this study
pM2025	Amp^r, pBAD24 with *fur* ORF under *ara* control	this study
pM2026	Amp^r, pBAD24 with *fis* ORF under *ara* control	this study
pM2028	Amp^r, pBAD24 with *hns* ORF under *ara* control	this study
pM2039	Amp^r, pBAD24 with *crp* ORF under *ara* control	this study
pM2042	Amp^r, pBAD24 with *cpxA* ORF under *ara* control	this study
p*sicA gfp*	Amp^r, pBR322ori with *promoter of sicA* was inserted into pM968, thus driving gfp expression from the *sicA* promoter	This study, (51)
p*sicA mCherry*	Amp^r, Cm^r, *gfp* of p*sicA gfp* has been replaced by *mCherry* and Cm^r was introduced	This study
pM2002	Tet^r, pSB377 with tsr_{venus} downstream of *sipA* (nt 1156-2058 of the orf) for homologous recombination into *S.* Tm chromosome	This study
pM2080	Tet^r, pSB377 with tsr_{venus} downstream of *hilA* (nt 114 to 1661 of the orf) for homologous recombination into *S.* Tm	This study

pM2819	Amp^r, pGP704 with tsr_{venus} downstream of *fliC* (nt 25-1485 of the orf) for homologous recombination into *S.* Tm	This study

Supplementary Table S3. Primer sequences

Primer	Sequence 5'-3'
SirA-EcoRI-fw	GTGAATTCTTGATCAACGTTCTTCTTG
SirA-XbaI-rev	GTTCTAGATCACTGGCTTGTTAACGTCTC
RtsA-EcoRI-fw	TCGAATTCATGCTAAAAGTATTTAATCCCTC
RtsA-XbaI-rev	GATCTAGATCAATTAACATATTGATGACGAG
OmpR-EcoRI-fw	GCGAATTCATGCAAGAGAATTATAAGATTCTGGTGG
OmpR-XbaI-rev	GCTCTAGATCATGCTTTAGAACCGTCCG
CsrA-EcoRI-fw	GCGAATTCATGCTGATTCTGACTCGTCGAG
CsrA-XbaI-rev	GCTCTAGATTAGTAACTGGACTGCTGGG
Fur-EcoRI-fw	GTGAATTCATGACTGACAACAATACCGC
Fur-XbaI-rev	GTTCTAGATTATTTAGTCGCGTCATCGTG
Fis-EcoRI-fw	GAGAATTCATGAAAGCGGAATGTGAACC
Fis-XbaI-rev	GATCTAGATTACTTCTCTGAAGATAGCGCG
Hha-EcoRI-fw	GCGAATTCATGTCTGATAAACCATTAACTAAAAC
Hha-XbaI-rev	GATCTAGATTAACGAATGAATTTCCATACTGAAG
Hns-EcoRI-fw	GCGAATTCATGAGCGAAGCACTTAAAATTCTG
Hns-XbaI-rev	GATCTAGATTATTCCTTGATCAGGAAATCTTCCAG
CsrB-NheI-fw	GAGCTAGCGAGTCGTACAACGAAGCGAACGTC
CsrB-HindIII-rev	GGAAGCTTCAAAAAAAAGGGAGCACTGTATAAACAG
Crp-EcoRI-fw	GCGAATTCATGGTGCTTGGCAAACCGCAAACAG
Crp-XbaI-rev	GCTCTAGAGTGATAAATCAGTCTTCGCCACATCG
Mlc-EcoRI-fw	GAGAATTCGTGGTTGCTGATAGTCAGCCTGGG
Mlc-XbaI-rev	GATCTAGAGGCAAGGGCAATCAGCTTGAGTTAGC
CpxA-EcoRI-fw	GCGAATTCATGATAGGAAGTTTAACCGCGCGCATC
CpxA-XbaI-rev	GATCTAGATTAGGTTCGCTTGTACAGCGGTAGCCACAGC
ΔhilA::kan-fw	TATTATAACTTTTCACCCTGTAAGAGAATACACTATTATCGTGTAGGCTGGAGCTGCTTC
ΔhilA::kan-rev	ACGATGATAAAAAAATAATGCATATCTCCTCTCTCAGATTATGGGAATTAGCCATGGTCC
ΔhilD::kan-fw	CCAGTAAGGAACATTAAAATAACATCAACAAAGGGATAATGTGTAGGCTGGAGCTGCTTC
ΔhilD::kan-rev	TTAATAAAAATCTTTACTTAAGTGACAGATACAAAAAATGATGGGAATTAGCCATGGTCC
ΔhilE::cat-fw	ATTGTCGGTATTTAATCTGGTATACAGAGACACCAACGAAGTGTAGGCTGGAGCTGCTTC
ΔhilE::cat-rev	ACAGCATCGCCCACTGCGAGTCCGCAAGCTTGTTTTGTCCATGGGAATTAGCCATGGTCC

Supplemtary Information

Supplementary Information 1. Mathematical model for calculating the rate of initiation r_i of *ttss-1* gene expression during the late log phase.

To infer the initiation rate (r_i) of *ttss-1* expression in the late logarithmic phase from our experimental data, we developed a mathematical model describing the growth of the TTSS-1$^+$ (N_{T1+}: number of TTSS-1$^+$ cells; μ_{T1+}: growth rate of these cells) and the TTSS-1$^-$ population (N_{T1-}: number of TTSS-1$^-$ cells; μ_{T1-}: growth rate of these cells) as a function of time (t). *ttss-1* expression is initiated in the TTSS-1$^-$ subpopulation at the rate r_i:

$$\frac{dN_{T1+}}{dt} = \mu_{T1+} N_{T1+}(t) + r_i(t) N_{T1-}(t) \quad (1)$$

$$\frac{dN_{T1-}}{dt} = \mu_{T1-} N_{T1-}(t) - r_i(t) N_{T1-}(t) \quad (2)$$

During the late logarithmic phase, the relative abundance of the TTSS-1$^+$ individuals increased, and the fraction α of TTSS-1$^-$ individuals (N_{T1+}) decreased dynamically (Fig. 3A):

$$\alpha(t) = \frac{N_{T1-}(t)}{N_{T1-}(t) + N_{T1+}(t)} \quad (3a)$$

At any time, the total number of individuals $N_{total}(t)$ consists of the two subpopulations $N_{T1-}(t)$ and $N_{T1+}(t)$:

$$N_{total}(t) = N_{T1-}(t) + N_{T1+}(t) \quad (3b)$$

From our experiments, we know the parameters μ_{T1-} and μ_{T1+} (Fig. 2), $\alpha(t)$ and $N_{total}(t) = OD_{600}(t)$ (Fig. 3A). Now, we can use these data to estimate $r_i(t)$.

Combining Eq. (3a) and Eq. (3b) yields an expression, with which we can calculate the dynamic progression of the TTSS-1$^-$ subpopulation:

$$N_{T1-}(t) = \alpha(t) \cdot N_{total}(t) \quad (3c)$$

As $\alpha(t)$ and $N_{total}(t)$ were measured in the experiment shown in Fig. 3A, we can take Eq. (3c) to calculate $N_{T1-}(t)$. To calculate $r_i(t)$ we have rearranged Eq. (2):

$$r_i(t) = \frac{\mu_{T1-} N_{T1-}(t) - \frac{dN_{T1-}}{dt}}{N_{T1-}(t)} \quad (4)$$

To obtain $r_i(t)$, we fitted an empirical function to the data for $N_{T1-}(t)$ yielding $N_{T1-}(t)$ = 1.76E-02 t(h)$^{2.53}$ and differentiated this function vs (t) to obtain dN_{T1-}/dt:

$$\frac{dN_{T1-}}{dt} = 2.53 \cdot 1.76 \cdot 10^{-2} \cdot t(h)^{(2.53-1)}$$

Using these values and Eq. (4) allowed us to calculate r_i (t) during the late logarithmic phase (Fig. 3B).

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Chapter III

The *Salmonella* Typhimurium Type Three Secretion System 1 regulator HilA is bond and inactivated by the chemotaxis sensory kinase CheA

The *Salmonella* Typhimurium Type Three Secretion System 1 regulator HilA is bond and inactivated by the chemotaxis sensory kinase CheA

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Abbreviations: *S*. Tm, *Salmonella enterica* subspecies 1 serovar Typhimurium; wt, wild type; SPI-1. *Salmonella* pathogenicity island 1; SPI-1+, SPI-1-, phenotype of *S*. Tm expressing SPI-1 genes or not, respectively; *ttss-1*, SPI-1 encoded type III secretion system (genotype; >20 genes); *gfp*, green fluorescent protein; DEAE, Diethylaminoethylcellulose; nt, nuctleotide; orf, open reading frame;

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Abstract

Salmonella enterica subspecies 1 serovar Typhimurium (*S.* Tm) is a well-known diarrheal pathogen. Gut infection and enteropathogenesis rely on the coordinated expression of numerous virulence factors, including flagella and the SPI-1 encoded type III secretion system (TTSS-1). However, the mechanisms coordinating virulence factor expression are still not entirely clear. Here, we have focused on HilA, a key regulator of *ttss-1* expression. HilA is a member of the PhoP/OmpR response regulator family. Typically, the members of this protein family act as transcriptional activators in response to specific stimuli and become activated by cognate sensory-kinases. However, in the case of HilA, no cognate sensory kinase had been identified and it had remained unclear, whether HilA is regulated at the post-translational level. Here, we have performed HilA-affinity purification experiments and identified CheA as a HilA-binding protein. CheA is well established as the sensory kinase of CheY, a response regulator controlling flagella-mediated chemotaxis. Disruption of *cheA* enhanced *ttss-1* transcription transcription, as demonstrated by GFP- and β-Galactosidase reporter assays, as well as Western blotting. In contrast, *hilA* expression was not affected in the *cheA* mutant. Based on these data, we propose that CheA dampens HilA activity at the post-translational level. This represents an additional mechanism coordinating *ttss-1* expression and flagellar function. To the best of our knowledge, this is the first mechanism coordinating both systems at the post-translational level.

Introduction

Salmonella enterica subspecies 1 serovar Typhimurium (*S.* Tm) represents an important pathogen causing diarrhea. Virulence thereby relies on coordinated expression of numerous virulence factors. For eliciting host cell invasion and enteric disease, the TTSS-1 and flagella are of key importance (43, 46, 47).

Virulence factor expression by *S.* Tm is controlled by a complex network of different regulators (11, 13, 14, 40). Some regulators integrate virulence factor expression with global regulatory processes of the bacterium (e.g. Crp, Mlc, Fur, Hns, CsrA (2, 14, 33, 37, 38, 49)), while others seem to fine-tune the expression of complex virulence factors themselves (e.g. HilA, HilD, InvF (11, 50)). HilA has emerged as a master regulator of *ttss-1* expression by *S.* Tm and the transcription of the *hilA* gene is known to be regulated by a complex set of regulators.

Many target genes regulated by HilA are located on an island on the chromosome of *S.* Tm, termed *Salmonella* pathogenicity island 1 (SPI-1, (50)). It encodes mainly for a Type Three Secretion System (TTSS-1), which triggers the invasion into the intestinal epithelium. SPI-1 harbors the operon *sicAsipBCDA*, which is essential for TTSS-1 dependent infection, transcription of the needle complex genes (SicA/InvF), translocon assembly at the tip of the needle (SipB,C,D (21, 39)) as well as inducing actin rearrangements (SipA and SipC (20, 36, 42)). In the absence of HilA, *S.* Tm does not express a functional TTSS-1 and is thus not able to infect host cells (3, 4).

HilA is composed of a C-terminal domain of unknown function and an N-terminal domain with homology to response regulators of the OmpR/ToxR response regulator family. In most cases, response regulators are activated by a cognate sensory kinase, which modulates the activity of the response regulator (29, 45). OmpR for example is specifically regulated by EnvZ (16). The flagellar system provides another prominent example. Sensory kinases act in response to environmental or intracellular signals by auto- phosphorylation of an intrinsic histidine. This phosphate group is then transmitted to an aspartate of the appropriate response regulator. This posttranslational regulation of the transcription factor activity of the response regulator enables bacteria to react within seconds to

environmental challenges like changes in nutrient concentrations or osmolarity by switching between straight swimming and tumbling (10, 29). In this way, most well established response regulators are controlled by sensory kinases. However in the case of HilA, no cognate sensory kinase had been identified. This raised the question whether or how the activity of HilA might be regulated at the post-translational level.

The coordinated transcription of *ttss-1* and of the flagellar system has been well established (43). Former studies revealed links between flagellar and virulence gene regulation by the operon *rtsAB* and many more (12). Briefly, RtsA contributes to the transcription of *hilA*, while RtsB represses transcription of *flhDC*, which encodes one of the key regulators in flagella biosynthesis. Via transcriptional activation of *fliZ* and *fliA*, this represses *ttss-1* expression at the transcriptional level by down regulation of *hilD* (12, 41). Beyond this, further synchronization is facilitated by Fis (28) and by mRNA stability control via CsrA (25). Our study now elicits an additional interaction. Here we have analyzed HilA interaction partners. We found that the chemotaxis sensory kinase CheA interacts with HilA and that this attenuates HilA-mediated *ttss-1* expression at the post-translationald level. This establishes a novel mechanism coordinating virulence factor expression by *S*. Tm.

Materials and Methods

Strains and plasmids. All strains used in this study are isogenic derivatives of *Salmonella enterica* serovar Typhimurium SL1344 (22) or ATCC14028 (Table 1). The *cheA* mutant was constructed using the lambda red recombination system (7). The kanamycin resistance cassette of pKD4 was amplified by PCR using the primers 5'CAAGCGTACCCACATCGCCAAAAGCGGAACCGAGGTGATAGCTGTAGGCTGGAGCTGCTTCGA3' and 5'CTTACATTACTCATACCGGTCATATTATTCCCTTCTCACTCAACATATGAATATCCTCCTTA3'. The 1.7 kb PCR product was electroporated into SL1344 harboring pKD46 and Δ*cheA::aphT* mutants were selected by plating on LB-Agar (50 μg / ml Kanamycin).

The suicide plasmid pM1303 carried *lacZ* downstream of a truncated *sipA* (nt 1156-2058 of the orf) and was transferred by conjugation into SL1344 to generate the reporter strain M1309. To create the suicide plasmid pM2002, pVS152Tsr (54) was cleaved by the restriction endonucleases *Eco*47III and *Xma*I. The tsr_{venus} carrying fragment was introduced into pM1300 (cleaved by *Msl*I and *Xma*I) downstream of a truncated *sipA* fragment (nt 1156-2058 of the orf), to finally create pM2002. By homologous recombination pM2002 was introduced into the genome of ATCC14028 to generate the reporter strain M2001. The *cheA* mutation was introduced in both strains by phage transduction P22 to obtain M2084 and M2529. All mutations were verified by PCR or DNA sequencing.

Culturing of bacterial strains. SL1344 derivatives were always cultured under limited aeration on a steady wheel in LB broth at 37 °C. 12 h overnight cultures (stationary phase) were inoculated in fresh LB with an OD_{600} of 0.02 and grown again to late logarithmic phase (OD_{600} of 1.0 – 1.2). All assays, unless specified otherwise, were performed with bacteria originating from this growth phase.

Pulldown assay for identifying HilA interaction partners. $HilA_{his6}$ was overexpressed in *Escherichia coli* BL21 harboring plasmid pCH112 (35) cultured aerobically in LB broth at 37 °C and induced with 0.1 % arabinose for several hours. Purification was done by using the C-terminal His_6-tag. The wash buffer was composed of 50 mM Hepes pH 8.0, 500 mL NaCl, 5 % glycerol and 20 mM imidazole. The elution was performed with the same buffer harboring 200 mM imidazole. To further

purify HilA to 95 %, the elution fraction was applied to a DEAE sepharose column (50 mM Hepes pH8.0, 5 % glycerol) and eluted with the same buffer harboring increasing concentrations of NaCl. Purity was confirmed by SDS PAGE. The purified HilA was covalently coupled to Aminolink beads (Thermo Scientific) by following the supplier's protocol. A *S*. Tm wt lysate was prepared by bacterial lysis (French Press) and centrifuged at 17 000 g (Sorval SS34) and the supernatant was added to HilA bound beads and incubated for 1 hour at 4 °C slightly shaking at 14 rpm to maintain floating of beads. After several wash steps with PBS, proteins have been eluted with 8 M urea. As a negative control all steps were performed in parallel with Tris base coupled beads. Afterwards a Tricin gel was used to analyze the eluted proteins. HilA specific bands were isolated and the corresponding proteins were identified by mass spectrometry (MALDI TOF) after trypsin digestion.

FACS. Prior to analysis, GFP maturation was ensured (2h, 37 °C, 30 µg/ml chloramphenicol). This allowed fluorophore formation but inhibited any protein biosynthesis. GFP emission was analyzed at 530 nm (LSRII, Becton Dickinson). Bacteria were identified by side scatter (SSC). Data were analyzed with FlowJo software (Tree Star, Inc.). With help of a reporterless control strain (SL1344) the specific GFP signal from the reporter plasmid pM972 (p*sicA gfp*) carrying strains has been identified and allowed assignement of TTSS-1^{+} and TTSS-1^{-} individuals.

Microscopy. Bacteria with the reporter tsr_{venus} were placed on a 1.5% agarose pad to guarantee a plain focus level and sealed under a cover slip (Axioplan2; Plan-APOCHROMAT 63x/1.4 oil; Zeiss or IX81, UPlanFLN 100x/1.3 Oil, Olympus). Reporter fluorescence (Exc. 470/20 nm; BP 495 nm; Em. 505-530 nm) and bacteria (phase contrast) were monitored and evaluated using Axiovision software (Zeiss).

Statistical analysis. Calculation of p-value was performed using the exact Man-Whitney U test (software: GraphPad Prism) determining significant differences between groups with at least 4 replicates. Values of $p < 0.05$ were considered as significantly different between two groups.

Table 1 Strains and plasmids

Strains and plasmids	*genotype / characteristics*	*resistance*	*reference*
strains			
M1309	SL1344, *sipA::pM1303*	Sm, Tet	This study
M2001	ATCC14028, *sipA* tsr_{venus}	Tet	This study
M2084	ATCC14028, Δ*cheA::aphT*, *sipA* tsr_{venus}	Sm, Kan, Tet	This study
M2087	SL1344, Δ*cheA::aphT*	Sm, Kan	This study
M2080	SL1344, Δ*hilA::aphT*	Sm, Kan	This study
M2529	M1309, Δ*cheA::aphT*	Sm, Kan, Tet	This study
SL1344	*S.* Tm *wt*	Sm	(22)
ATCC14028	*S.* Tm *wt*		American Type culture collection
BL21(DE3)	*E. coli*, F^- *ompT gal dcm lon hsdS*$_B$(r_B^- m_B^-) *λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*		(48)
plasmids			
pKD4	pANTSγ, contains an FRT-flanked *aphT* cassette		(7)
pKD46	pBAD18, λ red system		(7)
pCH112	p*hilA*; pBAD-Myc/His (Invitrogen) with $hilA_{myc/his6}$ ORF under ParaBAD control	Amp	(35)
pM972	p*sicA gfp*; pBR322ori with promoter of *sicA* driving transcription of *gfp*	Amp	This study
pM2002	pSB377 with tsr_{venus} downstream of *sipA* (nt 1156-2058 of the orf) for homologous recombination into *S*. Tm chromosome	Tet	This study
pM1303	**sipA::M45::LacZ*	Tet	This study
pM2868	p*cheA*, pBAD24 derivative	Amp	This study
pVS152Tsr	p*lac* tsr_{venus}	Amp	(54)

Results

HilA interacts with CheA

HilA is a member of the OmpR/ToxR protein family. Its N-terminal domain shows homology to OmpR and PhoP, which both get phosphorylated at a conserved aspartate residue by their particular sensory kinases (EnvZ and PhoQ) in response to environmental cues (osmolarity, Mg^{2+}/antimicrobial peptides). This aspartate residue is also conserved in HilA (D67, Fig 1A). However, so far no cognate sensory kinase has been reported for HilA. To identify possible HilA binding partners, we performed a pull-down assay. $HilA_{His6}$ (p*hilA*) was covalently coupled to beads and incubated with lysate of wild type *S.* Tm SL1344. As a negative control we incubated an equivalent amount of *S.* Tm lysate with TrisCl buffer-coupled beads (Materials and Methods). This analysis revealed CheA as a prominent binding partner of HilA (determined by SDS PAGE and MALDI TOF, Fig 1B). Mass spectrometric analysis of a band at 83 kDa revealed the ATP binding subunit of the Clp protease, ClpA. We did not further follow ClpA as an interaction partner, because of its broad spectrum of targets within bacterial cells and its involvement in general protein turnover regulation (30). Besides this, the Clp Protease has already been described to suppress SPI-1 expression indirectly by degrading the SPI-1 and flagellar activator FlhDC (24, 27). Thus, we focused on CheA as a potential regulator of virulence gene expression by *S.* Tm.

As a part of the chemotaxis complex CheA conveys external signals, e.g. sugar concentrations, in phosphorylating itself and transfers this phosphate to its cognate response regulator CheY. This affects interaction of CheY with FliM (5) and allows *S.* Tm to move along chemical gradients. The HilA interaction with CheA suggested that CheA regulates the activity of HilA. Cross regulation between flagellar and SPI-1 system, has already been described at the transcriptional level, e.g. via FlhDC/FliA (9, 25, 32).

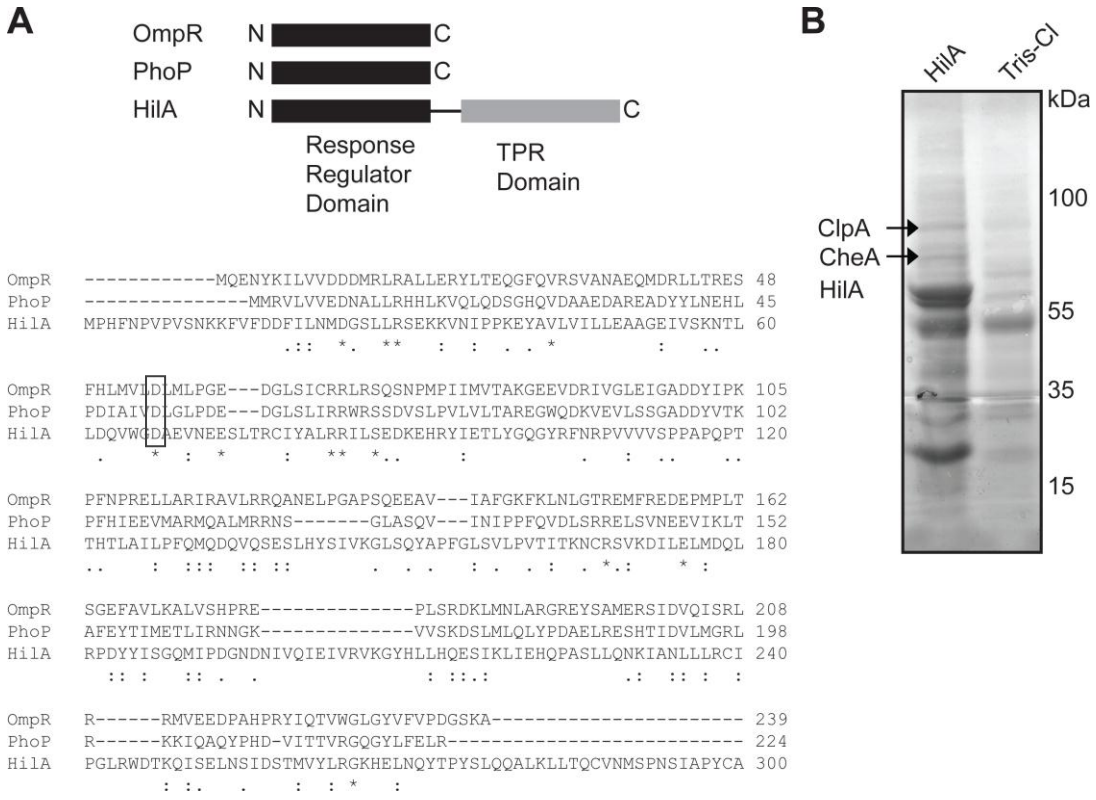

Figure 1, HilA shares sequence similarities with PhoP and OmpR and interacts with CheA. A) Alignment of the protein sequences of OmpR, PhoP and HilA. The N-terminal domain of HilA is similar to the transcriptional response regulators OmpR (belonging to the two-component system EnvZ/OmpR) and PhoP (PhoQ/PhoP). The C-terminal part of HilA is not present in OmpR or PhoP and its function is unknown. In all three sequences an aspartate residue is conserved (box), which has been shown to be phosphorylated in the case of OmpR and PhoP. **B)** Coomassie stained tricin gel after pull-down assay. $HilA_{His6}$ over-expressed from plasmid p*hilA* was purified by affinity and anion exchange chromatography and covalently coupled to Aminolink beads (Thermo Scientific). As a control, beads were coupled to Tris base. After that, both columns were loaded with supernatant of wt *S.* Tm lysate of a late logarithmic phase LB culture (OD_{600} = 1.2), washed several times with 25 mM PBS. The interacting proteins were then eluted by 8 M urea. Bands were analyzed by mass spectrometry (MALDI TOF). CheA appeared at 70 kDa.

Construction of a *cheA* mutant

To analyze a possible role of CheA in HilA mediated *ttss-1* expression, we constructed a mutant by replacing the coding sequence for CheA by a kanamycin resistance cassette (Materials and Methods). As expected, the *cheA* mutant showed impaired swarming motility on 0.3 % LB agar plates. Motility was recovered by complementation with Δ*cheA* p*cheA* (Fig. 2).

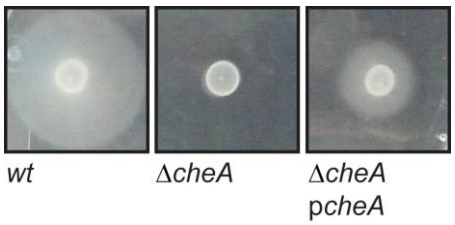

Figure 2, CheA mutant loses its swarming motility. The *cheA* mutant we applied in our assays forfeits its motility (swarming LB agar, 0.3 % agar agar). By p*cheA* (pM2860) we could reconstitute the wt phenotype. The motility agar was supplemented with 0.01 % arabinose. The latter induces *cheA* expression by p*cheA*.

A Δ*cheA* mutant expresses elevated levels of SipC

To analyze the role of CheA in *ttss-1* expression, we used three different assays monitoring the expression of the HilA-induced operon *sicAsipBCDA*. This operon features a SPI-1 specific chaperon (SicA), effector proteins (SipA, SipC) as well as subunits of the TTSS-1 translocon (SipB, SipC, SipD). First, we performed Western Blot and β-Galactosidase assays for monitoring global changes in *sicAsipBCDA* expression. Western blot analysis revealed increased levels of SipC expression by Δ*cheA*, while HilA levels remained unaffected (Fig. 3A,B). Similar results we observed using a transcriptional *sicAsipBCDA-lacZ* fusion in the chromosome of wt or a Δ*cheA* mutant. Again we observed an enhanced activity of the target operon in the absence of *cheA* (Fig. 1C). The data show that CheA has no detectable effect on the expression of HilA itself but rather reduces the expression of the HilA-controlled operon *sicAsipBCDA*.

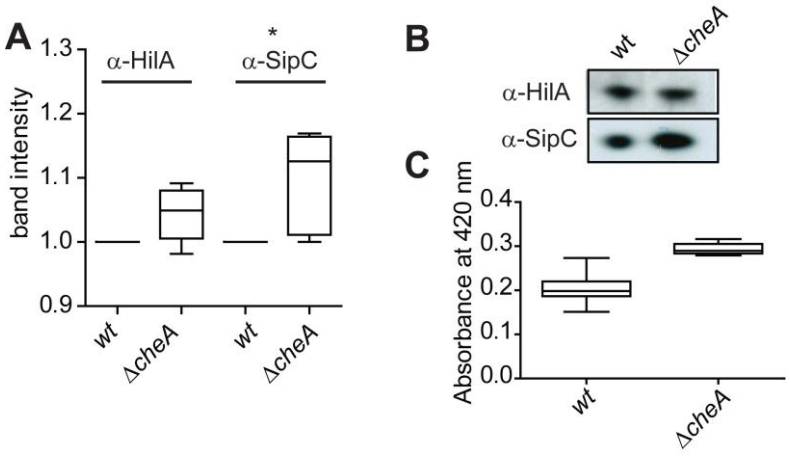

Figure 3, Phenotypic characteristics of a Δ*cheA* mutant. A) Quantitative analysis of the HilA and SipC concentration in a late logarithmic phase LB culture of *S*. Tm wt or Δ*cheA*. Cultures of an OD_{600} equal to 1.2 were harvested and bacterial lysates were analyzed by Tricin gel electrophoresis and Western blot. The graph represents four independent experiments. **B)** Representative Western Blot of the data shown in A). **C)** β-Galactosidase assay (Materials and Methods). M1309 (*sicAsipBCDA-lacZ*) and M2529 (*sicAsipBCDA-lacZ ΔcheA*) were cultured similar to the description in A). The data shown represent 10 independent data points.

Single cell reporter assays corroborate increased expression of the *sicAsipBCDA* operon in a Δ*cheA* mutant

The expression of *ttss-1* is known to occur in a bistable fashion (1, 19, 42, 53). Thus, under inducing conditions the *S*. Tm population in a typical culture is composed of cells expressing *ttss-1* and cells, which do not. Western blot and *lacZ* fusions are naturally restricted to average expression levels of the entire culture. For this reason, we have verified our data using fluorescence based reporters. This allowed us to follow expression on a single cell level. Therefore, we created a strain that harbored tsr_{venus}, a highly sensitive fluorescence reporter (54) transcriptionally fused to the *sicAsipBCDA* operon. Tsr is an integral membrane protein located at the poles of *S*. Tm. This enabled highly sensitive detection (even of weak signals) by microscopy (Fig 4A). We have grown *S*. Tm (*sicAsipBCDA* tsr_{venus}) and Δ*cheA* (*sicAsipBCDA* tsr_{venus}) under inducing conditions and evaluated

reporter expression by fluorescence microscopy. This revealed a slightly but significantly increased number of *ttss-1* expressing bacteria (TTSS-1+ phenotype) in the absence of *cheA* (Fig 4B). Using an alternative reporter, i.e. a plasmid expressing *gfp* from the *sicA* promoter, led to equivalent results (Fig. 4CD).

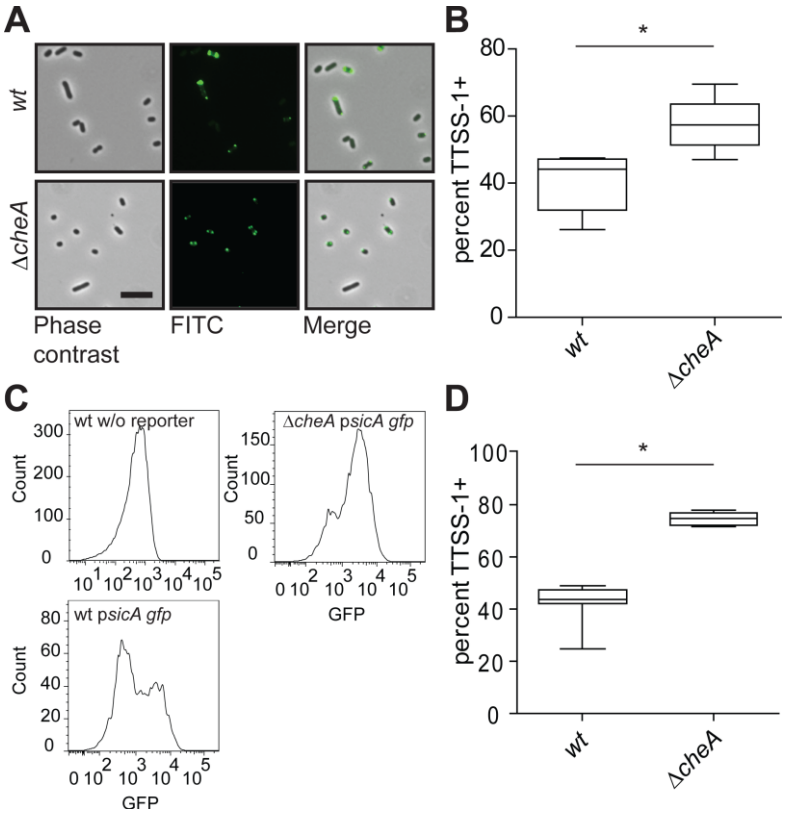

Figure 4, A Δ*cheA* mutant yields an increased fraction of *ttss-1* expressing cells. A) Microscopic picture of *S.* Tm harboring a transcriptional fusion of tsr_{venus} to *sicAsipBCDA* (wt, M2001) and an isogenic derivative lacking *cheA* (M2084). Bacteria were cultured in LB broth and investigated in the late logarithmic phase (OD_{600}= 1.2). Images were taken by phase contrast and fluorescence microscopy (Venus; Exc. 470/20 nm; BP 495 nm; Em. 505-530 nm). Not all bacteria display expression of the *sicAsipBCDA* operon. **B)** Quantification of the data shown in panel A). The median of four independent experiments including at least 100 bacteria per strain is indicated by the horizontal line. TTSS-1+ refers to cells expressing tsr_{venus} **C,D)** FACS analysis of wt *S.* Tm (SL1344) and *S.* Tm Δ*cheA* (M2087) harboring p*sicA gfp* (pM972). p*sicA gfp* expression occurs in a bistable fashion. The wt *S.* Tm w/o reporter identifies the background signal. Δ*cheA* (p*sicA gfp*) yields a higher proportion of TTSS-1+ cells than the isogenic wild type (p*sicA gfp*) strain. The median of 4 independent experiments is shown in D). TTSS-1^+ refers to GFP-positive bacteria, p = 0.02.

Discussion

In *S.* Tm, virulence factor expression is regulated by an intricate network of regulators. The transcriptional regulators involved are already quite well understood. Much less is known about the contributions of post-transcriptional control. We found that HilA, the master regulator of *ttss-1* expression, and CheA, a key regulator of bacterial chemotaxis can bind to each other and that *cheA* attenuates HilA-mediated *ttss-1* expression. This represents a novel, post-translational mechanism coordinating both systems.

It is interesting to speculate about the role of the CheA-HilA-interaction during an animal infection. Work in bovine and murine animal models has established that both, flagella-mediated chemotaxis and the expression of *ttss-1* contribute to host colonization and disease (43, 46, 47). The available data suggests that flagella contribute to homing and attachment to host cells and that they might trigger pro-inflammatory TLR5-mediated tissue responses (8, 9, 17, 26, 34, 43, 44, 55), while the *ttss-1* drives the subsequent invasion into epithelial cells of the host's intestine and triggers massive enteropathy during the first phase of the infection (6, 15, 42, 52). This indicates that both systems must be functionally coordinated during host infection. CheA-mediated control of HilA activity might contribute to this, e.g. by coordinating *ttss-1* expression with the detection of chemical gradients within the host's intestine.

HilA belongs to the PhoP/OmpR family of response regulators. However, it appears to be an unusual family member. First, it harbors an additional C-terminal domain of unknown function which is lacking from the homologs. This domain displays a tetratricopeptide repeat (TPR) and might therefore engage in protein-protein interactions (23). Future work will have to address the functional role of this domain.

Second, we could not observe phosphorylation of HilA (data not shown), even though it displays the conserved residue D67. This residue of PhoP/OmpR-family response regulators is typically phosphorylated by the cognate sensory kinase. It is of future interest whether the CheA-HilA interaction is accompanied by phosphorylation in vivo or whether the interaction itself leads to

changes in the activity of HilA. Third, the chromosomal region encoding *hilA* does not harbor the gene for a cognate sensory kinase. This is somewhat unusual, as many members of two-component systems are encoded together within one operon to warrant transcriptional co-regulation of sensory kinase and response regulator. Nevertheless, there are a number of examples of "orphan" two-component systems, where both partners are encoded in different chromosomal loci. In *Caulobacter crescentus* 57 % of the two-component system genes are orphan (45). The fact that partners are not always encoded next to each other has impeded the assignment of the cognate binding partners and the functional understanding of the respective two-component systems. In fact, it is not always clear, if there is a cognate partner.

It is interesting to note that CheA interacts with two different response regulators, CheY and HilA, and therefore represents a "branching point" (Fig. 5). This is reminiscent to ArcA, an oxygen-response regulator interacting with two different sensory kinases ArcB and the stress sensor CpxA. Branched two-component systems in either direction are known and apparently necessary (31). From an evolutionary perspective, it seems likely that CheY was the "original" target of CheA as the flagellar system was already present in the last common ancestor of *E. coli* and *Salmonella* spp., some 100 Mio years ago (51). SPI-1, encoding *hilA* and the entire *ttss-1* were acquired later when the *E. coli* and the *Salmonella* lineages diverged (18). The subsequent integration of *ttss-1* expression with central regulatory mechanisms has probably helped to limit *ttss-1* expression at "unfavorable" sites, i.e. in the absence of host cells suitable for infection. This pertains to the established network of transcriptional regulators (13), but also to the CheY-mediated modulation of HilA activity described, here and may explain how CheA has emerged as a "branching point" in *S*. Tm two component system signaling (Fig. 5). It is plausible that such branching points represent a general mechanism for integrating horizontally acquired genes (or other genes) into existing regulatory circuits.

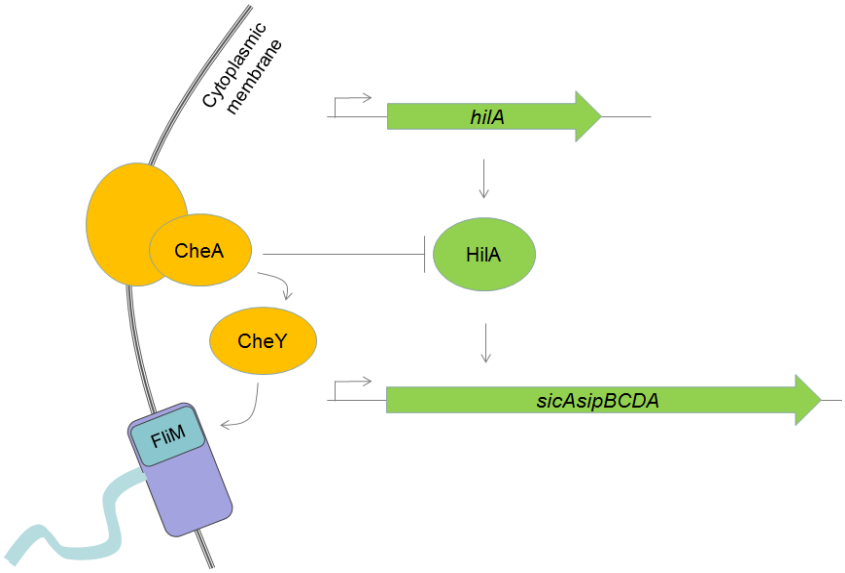

Figure 5, Schematic overview of CheA - regulator of flagellar rotation and of HilA activity. CheA reduces the activity of HilA and hence decreases the efficiency of *sicAsipBCDA* transcription. HilA acts positively on *sicAsipBCDA* but is negatively affected by CheA.

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Chapter IV

The *Salmonella* Typhimurium virulence regulator HilA is acetylated by the AcetylCoA-Synthetase Acs

The *Salmonella* Typhimurium virulence regulator HilA is acetylated by the AcetylCoA-Synthetase Acs

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Author's contributions: Design of the project, performing experiments in Fig. 2, 3, 4, 5, evaluation of the data, writing the manuscript, A. Sturm; Mass spectrometry and evaluation, A. Schmidt; performing experiment in Fig. 2, A. Rommel; Evaluation of Fig. 3E, S. Kreibich; evaluation of data in Fig. 4 and 5, M. Zimmermann; writing the manuscript and discussion, W.D. Hardt.

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Abbreviations: *S.* Tm, *Salmonella enterica* subspecies 1 serovar Typhimurium; wt, wild type; SPI-1. *Salmonella* pathogenicity island 1; SPI-1+, SPI-1-, phenotype of *S.* Tm expressing SPI-1 genes or not, respectively; *ttss-1*, SPI-1 encoded type III secretion system (genotype; >20 genes); *gfp*, green fluorescent protein; nt, nuctleotide; orf, open reading frame; MOI, multiplicity of infection

Manuscript in Preparation

Abstract

Posttranslational protein modification by acetylation has only been recognized, recently. N_{ε}-acetylation of lysine residues represents a fast and effective way to respond to changes of the metabolic state of the cell. We found evidence that the expression of *Salmonella* Typhimurium Pathogenicity Island I (SPI-1) is controlled by this mechanism. SPI-1 encodes the Type Three Secretion System 1 (TTSS-1) that mediates the translocation of effector proteins and thereby triggers invasion into host cells and enteric disease. TTSS-1 expression is induced in response to several environmental cues and tightly controlled by an elaborate network of regulators which converges on the central *ttss-1* transcription factor HilA. However, *ttss-1* regulation by HilA is still not completely understood. By mass spectrometry, we have found that HilA is N_{ε}-acetylated at several lysine residues in vivo. Acetylation at the HilA residues K_{231} and K_{324} was diminished in the absence of the acetyl-coA synthetase Acs, an enzyme channeling acetate into the citric acid cycle. *Acs* mutants displayed enhanced expression of the *ttss-1* operon *sicAsipBCDA*, as indicated by transcriptional *gfp*-reporters and by Western Blot data. However, HilA protein levels remained at wild type levels. This suggested that HilA-activity is negatively regulated by lysine acetylation. In conclusion, *acs*-dependent HilA acetylation was found to couple *ttss-1* expression to the central carbon metabolism and represents the first example of *ttss-1* control by posttranslational protein modifications.

Introduction

The human pathogen *Salmonella* Typhimurium (*S*. Tm) is known for its ability to cause gastrointestinal disease. Mucosal infection requires the Type Three Secretion System 1 (TTSS-1) that mediates bacterial uptake by translocation of effector protein into host cell cytoplasm. The components of the TTSS-1 are encoded on a 40 kb region of the *S*. Tm chromosome, *Salmonella* Pathogenicity Island I (SPI-1). SPI-1 also encodes transcription factors regulating *ttss-1* expression in response to diverse environmental signals (e.g. antimicrobial peptides, Fe or osmolarity (12, 14, 19, 24, 25)) This regulatory system hinges on HilA, the master transcription factor for *ttss-1* expression. Besides TTSS-1, successful infection of the host's intestine requires a number of additional virulence factors. The expression of these virulence factors correlated with that of *ttss-1*. This seems to be achieved at the level of global regulatory pathways that facilitate the expression of the TTSS-1, flagella and fimbria (FlhDC, FimYZ (8, 11, 13)), stress sensors (e.g. CpxA (23)), iron associated genes (Fur (14)) and biofilm determinants (SirA (2, 36, 37)). Several transcriptional regulators are known to connect virulence factor expression to carbon metabolism (e.g. CsrA, Cya, Crp, Mlc (2, 15, 28, 36)) thus linking virulence to environmental and metabolic cues.

The vast majority of signaling inputs for *ttss-1* expression converge on HilA. In contrast to other SPI-1 transcription factors, HilA exhibits two different domains, an N-terminal response regulator domain of the OmpR/ToxR familiy of two component regulatory systems and a C-terminal TPR domain of unknown function. In contrast to various other two component systems, no cognate sensory kinase has been identified for HilA. This has caused the question whether low HilA activity might be regulated. Truncation studies could already show that transcription activity is exclusively driven by the response regulator domain (4). Thus, it had remained unclear whether HilA activity is regulated by post-translational mofications. However, phosphorylation of the conserved aspartate residues, which are typically phosphorylated in response regulators, has not yet been identified. Besides Phosphorylation, Acetylation has emerged as a common way to modulate charge and conformation of proteins. By these changes stability, binding capacity, protein-protein interaction or DNA affinity can be regulated (22). In the most cases acetylation is regulated by the Acetyltransferase Pat and the Deacetylase CobB (32, 34, 38). Mass spectrometric analysis of the *S*. Tm proteome identified 191 proteins with lysine N_{ε}-acetylation modification (38). Alterations in carbon

utilization dramatically affected acetylation of metabolic enzymes, i.e. populations cultured by glucose-based glycolysis exhibits higher fractions of acetylated lysines in the overall protein pool than by citrate-based gluconeogenesis (38). By acetylation activity of metabolic enzymes are related to substrate variances (35). Besides enzymes of the central carbon metabolism, the SPI-1 proteins SipC, SpaS and HilD were also shown to carry N_{ε}-acetyl-residues. However, the role of these modifications had remained unclear. In contrast, N_{ε}-acetylation has been shown to regulate chemotaxis.

The AcetylCoA synthetase Acs was shown to acetylate lysines of the response regulator CheY of the chemotaxis system. This modification decreases the interaction with its sensory kinase CheA thus rendering CheY less susceptible to phosphorylation (5, 27). Acs is an enzyme of the central carbon metabolism, which transforms acetate into acetyl-CoA and makes thus acetate accessible during aerobic growth (33). CheY has been so far the only known target of protein acetylation by Acs in bacteria. Therefore, CheY allows integration of environmental signals (from chemotaxis response) and the state of central carbon metabolism. The former depends on CheY phosphorylation and the latter on N_{ε}-acetylation. This raised the question whether other virulence determinants might also be regulated by N_{ε}-acetylation. Here we have analyzed the N_{ε}-acetylation of HilA and its consequences for *ttss-1* expression.

One role of acetate in SPI-1 regulation has already been demonstrated. The phosphorylated form of acetate was shown to induce virulence genes as *hilA* and the downstream regulator *invF* by phosphorylating the two component system BarA/SirA ((26). *BarA* and *sirA* mutants displayed inverse defects (1). Here we show that lysine N_{ε}-acetylation of HilA represents mechanism, by which acetate metabolism is coupled to *ttss-1* virulence gene expression. Furthermore, these findings establish that *ttss-1* genes are not exclusively regulated by transcription, but also by posttranslational modifications.

Materials and Methods

Strains and plasmids. All strains used in this study are isogenic derivatives of *Salmonella enterica* serovar Typhimurium SL1344 (21) (Table 1). The *acs* mutation was generated by lambda red recombination (10). The chloramphenicol carrying region of pKD3 was amplified by PCR with the Primers 5' ATGCGACATATTATTAACATCCTACAAGGAGAACAACAGCGTGTAGGCTGGAGCTGCTTC3' and 5' CCGGATAAAACGCGTTCGCGTTGCCATCCGGCATTTATGGATGGGAATTAGCCATGGTCC3'. Subsequently the PCR fragment was electroporated into *S.* Tm SL1344 harboring pKD46 and recombinant colonies were selected by plating on LB agar (30 µg Chloamphenicol). Afterwards, the Δ*acs::ca*t allele was transferred into *S.* Tm SL1344 by P22 transduction. The mutation was verified by PCR.

Table 1 Strains and plasmids

Strains and plasmids	*genotype / characteristics*	*resistance*	*reference*
strains			
M2527	SL1344, Δ*acs::cat*	Sm, Cm	This study
SL1344	*S.* Tm *wt*	Sm	(21)
plasmids			
p*sicA gfp*	pBR322ori with promoter of *sicA* controlling *gfp*	Amp	This study
p*ssaG gfp*	pBR322ori with promoter *of ssaG* controlling *gfp* expression	Amp	(20)
p*hilA*	pCH112, arabinose inducible *hilA-*$_{his6}$		(29)

Culturing of bacterial strains. SL1344 derivatives were cultured aerobically in LB broth at 37 °C. 12 h overnight cultures (stationary phase) were inoculated in fresh LB with an OD_{600} of 0.02 and grown under mild aeration to late logarithmic phase (OD_{600} of 1.0 – 1.2). All assays were performed with bacteria originating from this growth phase, if not stated otherwise. For acetate induction studies, bacteria were cultured in M9 minimal media with 4 g/L glucose or with 4 g/L glucose and 1 g/L acetate.

FACS. To analyze *sicAsipBCDA* expression, bacteria harboring the reporter plasmid pM972 (expresses *gfp* controlled by the *sicA* promoter) were cultured in LB to an OD of 1-1.2. In FACS, single bacterial cells were identified by side scatter (SSC) and GFP emission was analyzed at 530 nm (LSRII, Becton Dickinson). Data were analyzed with FlowJo software (Tree Star, Inc.). A reporterless control strain (SL1344) was used to define the background fluorescence signals.

Size Exclusion Chromatography (SEC). The multimerization state of HilA was elucidated by size exclusion chromatography (ÄktaFPLC). For this purpose, C-terminal his_6-tagged HilA (pCH112) was over-expressed in SL1344 and M2527 (induced with 0.01 % arabinose). Purification was performed at RT in 50 mM Hepes pH 7.5 further containing 500 mM NaCl, 5 %Glycerol and 10 mM Imidazol, respectively 200 mM Imidazol for elution from a Ni-NTA column. To finally determine the molecular weight of HilA or multimers thereof, samples were separated in 50 mM TrisCl pH 7.5 on a Superdex 200 10/300 GI column with a flow rate of 0.5 mL / min at 4 °C.

HPLC analysis of M9 media. To determine changes in acetate concentration 20 µl samples of M9 media with either glucose only or glucose and acetate were taken after indicated time periods and analyzed by HPLC (Agilent1100 Series). Separation was performed in 5 mM H_2SO_4 on an Aminex HPX-87-H column (300 mm x 7.8 mm, Biorad) with isocratic flow (0.6 mL /min) at 60 °C during 30 min. Peaks were detected by RID (Refractive Index Detector).

Modified Gentamicin Infection assay. For quantitative analysis of invasiveness of *S.* Tm strains, bacteria were transformed with pM975, carrying the promoter of the SPI-2 gene *ssaG* and a *gfp*, which is expressed within the host cell. First, HeLa cells were cultured in DMEM medium (37 °C, 5 % CO_2) and infected with bacteria grown to the late logarithmis phase (OD 1-1.2; MOI of 39) for 20 minutes at 37 °C. Afterwards cells were washed 3 times with HBSS and incubated at 37 °C in DMEM (400 mg / ml gentamicin) for another 4 hours to allow proper intracellular *gfp* expression (37 °C, 5 % CO_2). Afterwards cells were fixed with PFA and incubated in 20 % (w/v) sucrose in PBS over night at 4 °C. Nuclei were stained with DAPI and the fraction of infected cells was analyzed by automated fluorescence microscopy and automated image analysis using the cell classifier software as described recently (30).

Quantitative Western Blot analysis. Bacteria were grown under TTSS-1 inducing conditions in LB to an OD_{600} of 1 to 1.2. Bacterial cells were harvested by centrifugation. Pellets were disrupted with French Press analyzed by Tricin Gel Electrophoresis and blottingd on Nitrocellulose membranes. Detection of HilA and SipC bands was performed using polyclonal rabbit α-HilA (Neosystems), a polyclonal rabbit α-SipC and a secondary goat α-rabbit HRP conjugate (Biorad). Signal intensities of HilA and SipC bands were evaluated by AlphaImager Software.

Results

Mass spectrometric analysis of lysine N_ε acetylation of HilA

Acetate can represent a carbon source for *S*. Tm and regulate SPI-1 expression. We hypothesized that lysine N_ε-acetylation might be involved. For this reason, we analyzed HilA by mass spectrometry. First, we overexpressed *hilA* (p*hilA*) in *S*. Tm SL1344 and purified the recombinant protein via its C-terminal His_6-tag by Ni-NTA chromatography. To exclude further contaminations, the elution fraction was run on an SDS PAGE. After tryptic digestion of the protein bands, the sample was analyzed by mass spectrometry. We identified five acetylated lysine residues (K_{90}, K_{231}, K_{324}, K_{456} and K_{533}, representative mass spectrum of AK_{324}EHAIK in Fig 1A).

Acs facilitates lysine N_ε-acetylation at K_{231}

Earlier study had shown that the chemotaxis response regulator CheY is also N_ε-acetylated and that this modification was introduced by the acetyltransferase AcetylCoA-Synthetase (Acs). To evaluate if Acs plays a similar role in HilA modification, we overexpressed HilA (p*hilA*) in *S*. Tm Δ*acs*, and analyzed N_ε -acetylation levels of the five modified lysine residues of the recombinant regulator. The mass spectrometric analysis of purified HilA revealed a reduced acetylation, i.e. a fivefold reduced acetylation of K_{231}^{Ac}. K_{324}^{Ac} were also slightly reduced in a Δ*acs* mutant (Tab. 2). Even though HilA and CheY belong to the same family of response regulators, the Acs modified residue K_{231} of HilA display no equivalent site in the protein sequence of CheY, while K_{324} of HilA shares an equivalent residue in the sequence of CheY (K_{92}, Fig. 1B).

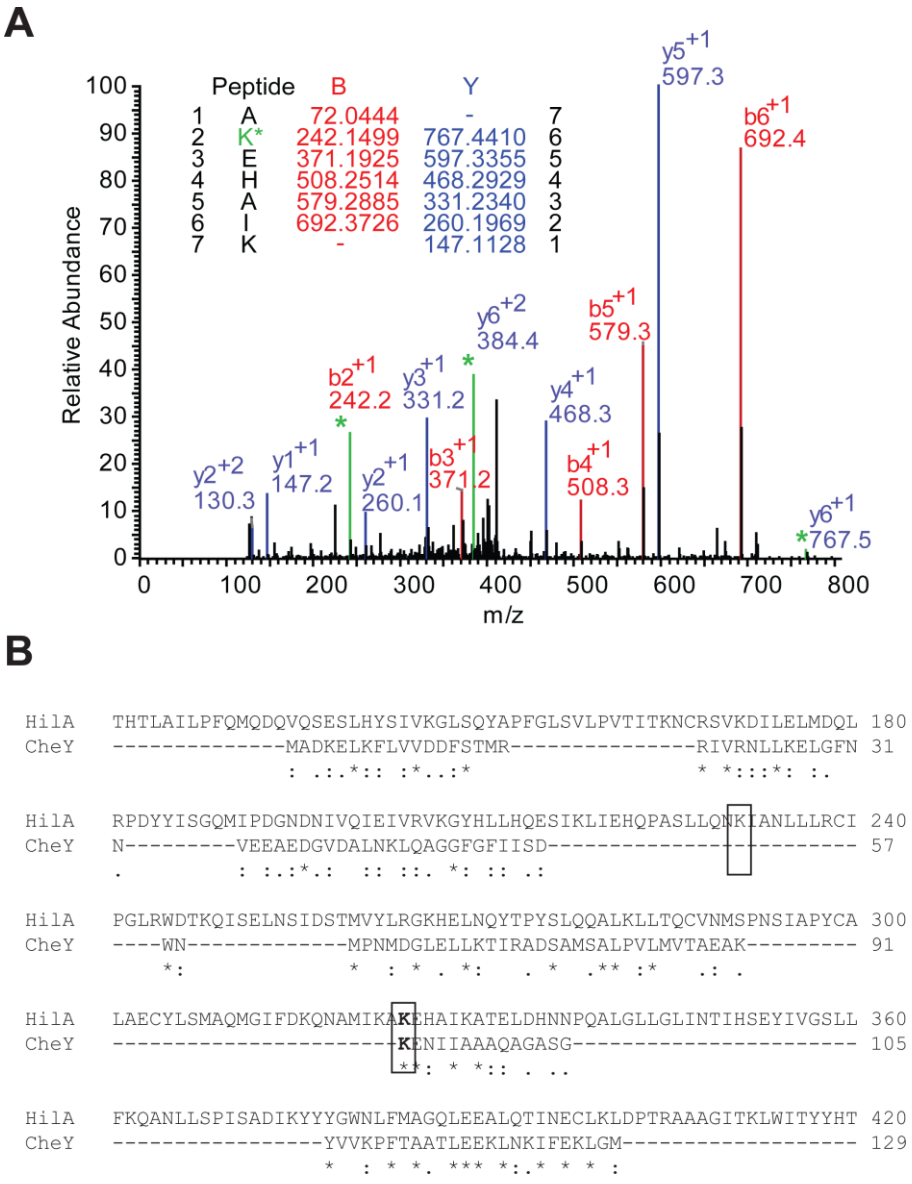

Figure 1, N_{ε}-acetylation of HilA. A) A his_6-tagged HilA was overexpressed in *S.* Tm wt harboring p*hilA-his*$_6$ and purified by affinity chromatography (Ni-NTA). SDS PAGE purified protein was digested by trypsin to yield maximum purity for mass spectrometry (sequence coverage > 80 %, Tab. 2). The mass spectrum used for the analysis of the peptide $AK_{324}EHAIK$ is shown. This peptide shows acetylation of residue K_{324}. **B)** CheY displays a lysine residue (K_{92}) at an equivalent position as lysine K_{324} of HilA. Both residues seems to be acetylated (27). K_{231} of HilA exhibits no equivalent site in the protein sequence of CheY.

Table 2 MS analysis of tryptic HilA identifies five acetylated lysines.

Peptide	Degree of acetylation in Δ*acs*
ILSEDK$_{90}^{Ac}$EHR	0.954993
LIEHQPASLLQNK$_{231}^{Ac}$IANLLLR	0.20893
AK$_{324}^{Ac}$EHAIK	0.416869
GK$_{456}^{Ac}$HELAR	2.137962
FK$_{533}^{Ac}$NEDNIWFK	1.819701

Acetylation does not affect HilA homodimer formation

HilA has a molecular weight of 63 kDa. Based on its sequence homology to the OmpR/ToxR family, its function as a transcription factor was supposed to depend on Dimer formation. This raised the question whether the acetylation might regulate HilA activity by disrupting HilA homodimer assembly. To address this question, *hilA* (p*hilA*) harboring a C-terminal his$_6$-tag was expressed in wt SL1344 and Δ*acs*, purified by Ni-NTA affinity chromatography and afterwards analyzed by Size Exclusion Chromatography (SEC). Using the molecular weight standards like Ferritin (440 kDa, 10 ml), Aldolase (158kDa, 13.1 ml), Conalbumin (75 kDa, 14.78 ml) and Ovalbumin (44 kDa, 14.56 ml), we determined for HilA a prominent peak at 13.9 ml, which is equivalent to a molecular weight of approximately 115 kDa. Similar results were obtained for HilA from wt SL1344 and Δ*acs*. In both cases the peak roughly matched the weight of two HilA molecules. Thus, acetylation did not affect the assembly to a HilA dimer, at least not under the conditions of our assay (Fig. 2).

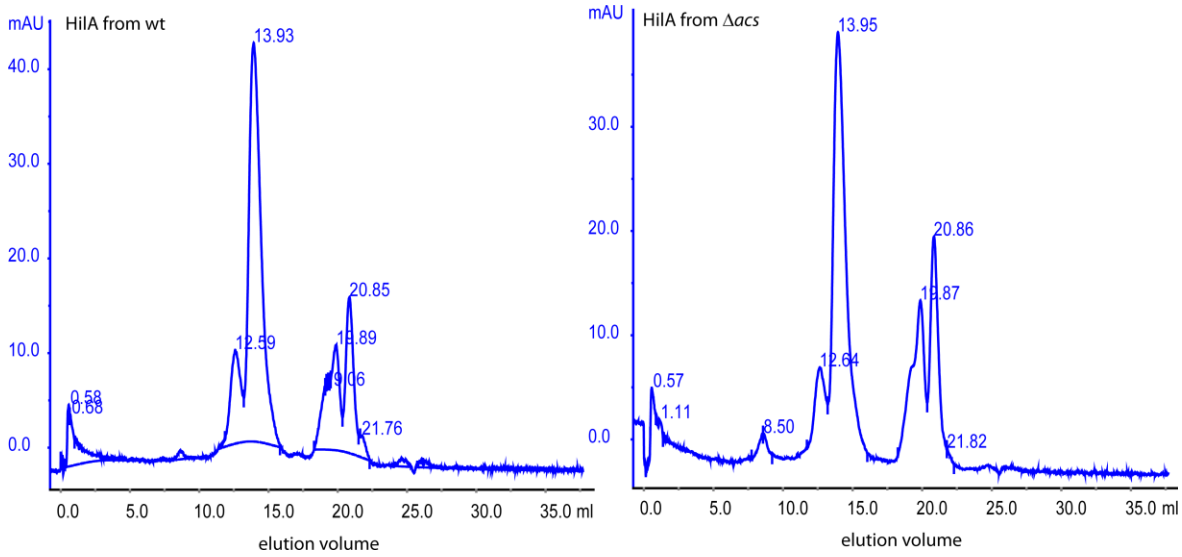

Figure 2, HilA assembles to homodimer in wt and Δ*acs*. Chromatograms are depicted of Size exclusion chromatography of $HilA_{his6}$ after over-expression of p*hilA* and purification by Ni-NTA. Samples either derived from wt or were rised in the Δ*acs* mutant. The prominent peak at an elution volume of 13.9 ml reflects in both samples the dimeric form of HilA. Additional peaks (19.9 ml, 20.9 ml) display probably co-eluted proteins from the Ni-NTA chromatography. The peak at 12.6 ml represents a molecular weight of approximately 220 kDa and matches roughly a potential tetrameric HilA. The molecular weight of HilA was calculated by the equation $K= (V_e-V_0)/(V_c-V_0)$, where V_0 represents the column void volume (8.52 ml), V_e the elution volume of marker proteins or HilA and V_c depicts the column volume (Superdex 200 10/300 GI, 25 mL). A calibration curve was generated using this equation inserting the molecular weights of the four marker proteins and their elution volume (see text).

Acs lowers *sicAsipBCDA* expression via HilA

The occurrence of Acs-dependent modifications of HilA, raised the question whether Acs would affect HilA dependent *ttss-1* expression. To address this question, we cultured wt SL1344 and an isogenic Δ*acs* mutant (M2527), and analyzed HilA and SipC levels by Western blot. A quantitative analysis revealed that HilA levels remained unchanged while SipC levels were elevated in the mutant (p_{SipC} = 0.03, Fig. 3AB). Equivalent results were obtained by FACS analysis using the transcriptional *ttss-1* reporter plasmid p*sicA* gfp (p = 0.008, Fig. 3CD). Finally, we have analyzed whether elevated *sipC* expression translates into increased invasiveness, a hallmark of *ttss-1* expression. HeLa Kyoto cells were infected with *S.* Tm wt (p*ssaG gfp*) and Δ*acs* (p*ssaG gfp*) and the invasion efficiency was analyzed in a modified gentamicin protection assay (Materials and

Methods (30)). In fact, Δ*acs* was slightly more invasive than the isogenic wt strain (p = 0.03, Fig. 3E). In conclusion, these data were in line with the hypothesis that Acs-mediated HilA modifications might control *ttss-1* mediated invasion.

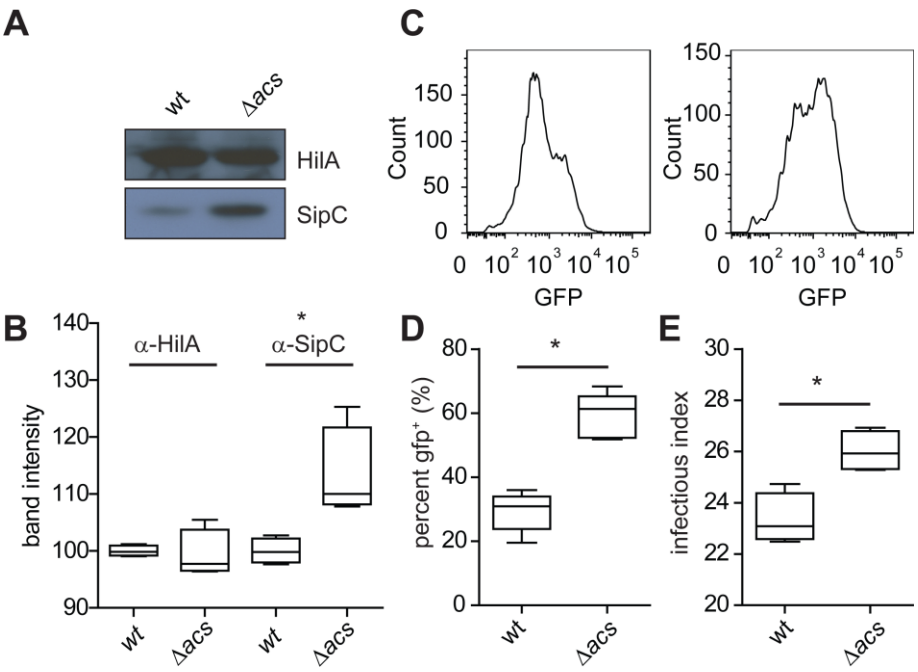

Figure 3, Elevated *sicAsipBCDA* expression in an *acs* mutant A) Western blot analysis of wt and Δ*acs*. Bacteria were grown in LB broth till late logarithmic phase (OD_{600} = 1.2) and afterwards analyzed by Tricin gel electrophoresis and Western blot. Rabbit α-HilA and rabbit α-SipC were used to determine intracellular protein concentrations. **B)** Densitometric analysis of Western blots of four independent experiments. **C)** and **D)** Gfp reporter assay to quantify p*sicA* expression at the single cell level. SL1344 and M2527 harboring both p*sicA gfp* (pM972) to visualize expression of the *sicAsipBCDA* operon, were grown to an OD_{600} of 1.2 as described before and GFP was analyzed by FACS. **E)** Invasion assay; HeLa Kyoto cells were infected with wt and Δ*acs*, both harboring p*ssaG gfp* (pM975). HeLa cell invasion efficiency was analyzed in a modified gentamicin protection assay (Materials and Methods (30)).

Acetate affects HilA activity

In former studies it had been shown that acetate was able to induce *ttss-1* virulence genes (26) by activating the BarA/SirA two component system. Because Acs is involved in utilizing acetate to generate acetyl-CoA, we wondered if acetate induction of *ttss-1* expression might involve Acs. Therefore we analyzed *ttss-1* expression of a Δ*acs* mutant in the presence or absence of acetate. We cultured *S.* Tm the Δ*acs* mutant harboring p*sicA gfp* in minimal media supplemented with either only glucose or glucose and acetate. In this experiment, we also had to consider the activity of SirA and BarA, which is known to activate *ttss-1* in response to acetate. This activity can be suppressed by glucose, because SirA/BarA is subject to catabolite repression (Crp,(36)). We did not detect any differences between the growth rates in either medium, but both, wt and Δ*acs*, showed increased levels of p*sicA gfp* expression in the presence of acetate. As expected in the absence of acetate, wt SL1344 and Δ*acs* expressed only very low levels of the p*sicA gfp* reporter. If acetate was added, reporter expression was increased. In this media, reporter expression by Δ*acs* was more pronounced than in wt SL1344. To exclude artifacts attributable to metabolite accumulation or depletion during the growth of the culture, we analyzed the media by HPLC. These analyses verified that neither wt nor Δ*acs* depleted acetate in either culture (Fig. 5). In conlclusion these data demonstrated that Acs represses *ttss-1* expression in the presence of acetate.

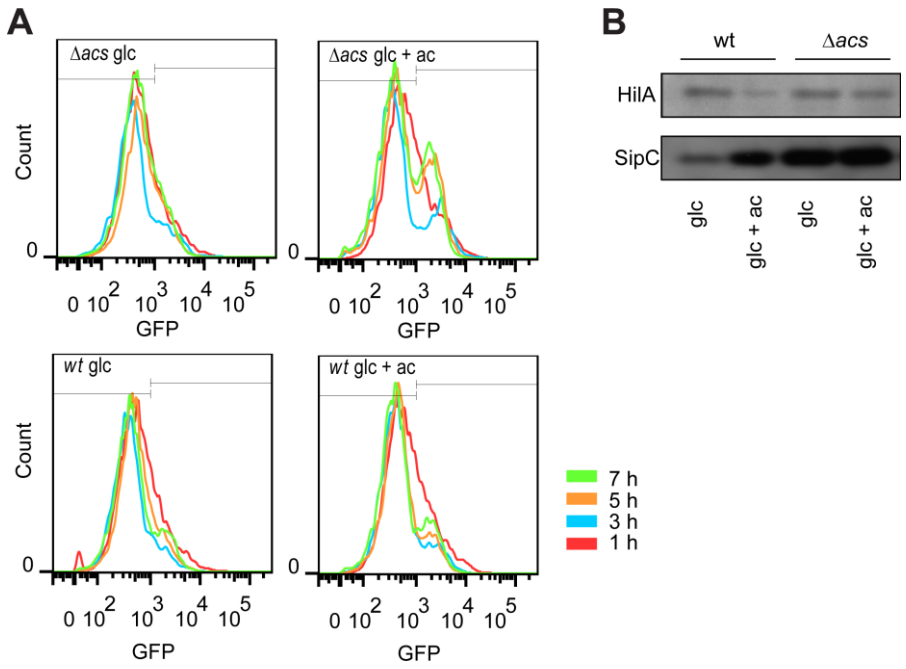

Figure 4, Δ*acs* responds stronger to acetate. *S.* Tm wt and Δ*acs* harboring p*sicA gfp* were cultured in M9 minimal medium supplemented with glucose (4 g/L) or additionally to glucose (4 g/L) with acetate (1g/L) and afterwards analyzed by FACS to determine the fraction of GFP+ cells (= *sicA* expression). **B)** Western blot analysis of end point (t = 7h) concentrations of HilA and SipC of cultures shown in A).

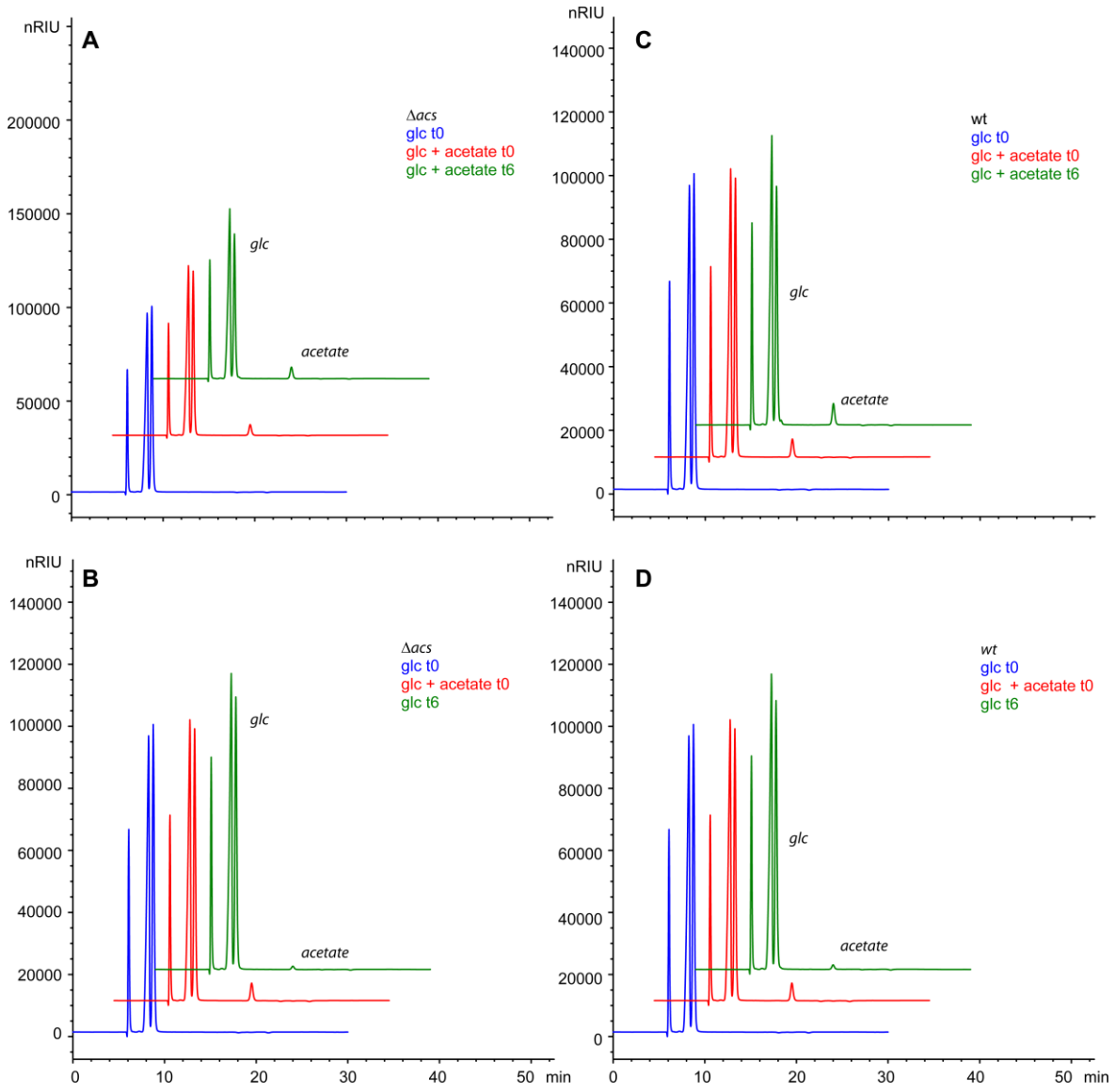

Figure 5, Acetate levels remain unaltered when wt SL1344 or Δ*acs* are grown in M9 supplemented with glucose and acetate. HPLC chromatograms of **A)** Δ*acs* in M9 glucose + acetate and **B)** M9 glucose only, **C)** wt in M9 glucose + acetate and **D)** in M9 glucose only. In blue and red the initial media concentrations of glucose and acetate are depicted as reference. In M9 glucose medium no acetate peak could be detected. Acetate concentrations did not change when it was added to the medium at the beginning (A and C, compare red and green). Neither wt nor Δ*acs* did consume acetate, so acetate concentration levels remained unaffected.

Discussion

The mechanisms controlling *ttss-1* expression are still not completely understood. So far, the community has focused on regulation at the transcriptional level. Here, we describe a first example of *ttss-1* regulation by post-translational lysine N_{ε}-acetylation. We found five N_{ε}-acetylation sites in the central *ttss-1* regulator HilA and demonstrated that N_{ε}-acetylation of K_{231} was dependent on Acs, an enzyme required for channeling acetate into the central carbon metabolism under aerobic growth conditions. In an *acs* mutant, the defect in K_{231} acetylation correlated with increased *ttss-1* expression, in particular in growth media harboring acetate, like LB from late logarithmic phase cultures or M9 with acetate. Based on these findings, we concluded that Acs can inhibit HilA by N_{ε}-acetylation of K_{231}. This represents a novel regulatory link between virulence factor expression and carbon metabolic pathways.

Earlier data had established that lysine N_{ε}-acetylation is a common post-translational protein modification in different bacterial species, including *Bacillus subtilis* (16, 17). In *S*. Tm a total of more than 150 proteins were found to be modified by lysine N_{ε}-acetylation and this modification was implicated in regulating central metabolic pathways. However, besides these metabolic enzymes, three SPI-1 encoded proteins were also identified as targets for this modification, i.e. SipC, SpaS and HilD (38). However, the biological relevance of these modifications had remained unclear. Our data extends this list of N_{ε}-acetylated SPI-1 encoded proteins and provides functional evidence for a role of this modification in *Salmonella* virulence.

HilD and HilA are subject to lysine N_{ε}-acetylation (41, this work). As both are important positive regulators of *ttss-1* (4, 12), this has raised the question whether the increased efficiency of *ttss-1* expression and host cell invasion displayed by the *acs* mutant was attributable to the HilD- or to the HilA modification. Earlier work has established that HilD is positioned upstream of HilA in the *ttss-1* regulatory nework. This, if Acs would inactivate HilD, *hilA* and *ttss-1* expression should be affected in a similar fashion. However, this was not the case, as indicated by our α-HilA Western blot data. Therefore, our data suggests that *acs* regulates *ttss-1* expression by inhibiting the transcription factor activity of HilA.

Former studies have elucidated the role of Acs in central carbon metabolism, i.e. its role in channeling acetate into the central carbon metabolism (33). Acs catalyzes the production of Acetyl-CoA from acetate,

coenzymeA and ATP, thus feeding new substrates into the tricarboxylic acid cycle. Interestingly, the catalytic mechanism involves the transient acetylation of certain lysine residues of Acs, itself (32-34). This may explain, how Acs transfers acetyl residues onto HilA. However, further work will be required to verify this hypothesis. Anyhow, these observations suggest that the load of acetate residues channeled through Acs might determine the degree of HilA modification.

Acetate is a well-established nutrient and an important environmental cue for *S*. Tm. This also pertains to the mammalian intestine. Commensals like *Bacteroides* spp. are known to produce high concentrations of acetate and butyrate in the mammalian gut (9, 18). Generally, this gut luminal acetate and butyrate are thought to inhibit *S*. Tm virulence and might explain why antibiotics-mediated elimination of the gut flora can enhance *Salmonella* enteropathy (7). The *acs*-mediated inhibition of *ttss-1* expression in the presence of acetate might explain the underlying mechanism of this phenomenon. However, the regulation is most likely more complicated than this. It should also be noted that addition of acetate to M9-glucose minimal media led to slightly enhanced *ttss-1* expression in wt SL1344. Thus, acetate might elicit inducing and repressing mechanisms in parallel and the response of *ttss-1* expression in response to acetate availability might depend on the exact environmental context.

The mechanism of HilA regulation by N_{ε}-acetylation of K_{231} might be explained by earlier findings for the homologous protein CheY of the chemotaxis system. N_{ε}-acetylation of K_{231} and K_{324} might have similar consequences as the Acs-mediated acetylation of K_{92} of CheY by Acs. In the latter case, this modification disturbs the interaction of CheY with CheA thus reducing the tumbling frequency (5, 6). In analogy, the N_{ε}-acetylation of HilA might reduce its efficiency as a transcription factor, e.g. by prohibiting tertiary or quartary structural changes or by reducing positively charged residues of HilA thus reducing the affinity for negatively charged DNA. Future work will have to address this issue.

In conclusion, our findings establish a novel type of *ttss-1* control by posttranslational N_{ε}-acetylation of HilA. This provides a link between virulence factor expression and central carbon metabolite availability in the environment. Clearly, this signaling input is integrated with multiple other inputs and virulence factor expression is determined by their concerted action. In vitro studies of this system are quite well advanced and

first modeling approaches can predict certain regulatory outputs of this system (3, 31). In the future it will be a great challenge to identify the relevant signals, the key signaling factors and their proper integration in vivo, which define site and timing of virulence factor expression during the course of an animal infection.

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Chapter V

Fever, an environmental cue for adjusting *Salmonella* Typhimurium virulence?

Fever, an environmental cue for adjusting *Salmonella* Typhimurium virulence?

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Abbreviations: *S*. Tm, *Salmonella enterica* subspecies 1 serovar Typhimurium; wt, wild type; SPI-1. *Salmonella* pathogenicity island 1; SPI-1+, SPI-1-, phenotype of *S*. Tm expressing SPI-1 genes or not, respectively; *ttss-1*, SPI-1 encoded type III secretion system; *gfp*, green fluorescent protein; MOI, multiplicity of infection; SCV, *Salmonella* containing vacuole

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Abstract

Salmonella Typhimurium (*S*. Tm) is a Gram-negative bacterium infecting warm-blooded animals and a frequent cause of self-limiting diarrhea in humans. Symptoms include nausea, abdominal pain and watery diarrhea manifesting 8-24 h after oral infection and ceasing after several days. Fever is only observed in severe cases when the pathogen spreads systemically. The mechanisms explaining the self-limiting nature of the infection are not well understood. We speculated that the host's body temperature might represent an important environmental cue tuning *S*. Tm virulence. Therefore, we have analyzed the temperature dependence of the type III secretion system 1 (TTSS-1) of *S*. Tm SL1344, a cardinal virulence factor for invading into the host tissue and for eliciting diarrhea. Using a transcriptional GFP-reporter plasmid and Western blot analyses, we found that TTSS-1 expression was low at 25 °C, increased at temperatures above 30 °C, was maximal at 37 °C and markedly reduced at 42 °C. The efficiency of host cell invasion showed the same temperature profile. Overexpression and deletion experiments revealed that the sharp decline of TTSS-1 expression between 37 °C and 42 °C was attributable at least in part to the heat shock sigma factor *rpoH*. This established temperature as a key environmental cue for TTSS-1 expression. This cue may allow specific expression within a typical host (i.e. 37 °C), but attenuates virulence at slightly higher temperatures (i.e. fever of > 40 °C). The attenuation at > 40 °C might serve to discontinue TTSS-1 mediated tissue invasion if fever occurs, thus enhancing host survival and thus prolonging pathogen spreading to other hosts. Our findings may have important implications for the use of anti-fever medication in acute *S*. Tm diarrhea.

Introduction

Salmonella enterica subspecies 1 serovar Typhimurium is a Gram-negative enteropathogenic bacterium causing a large number of diarrheal infections, worldwide. *S.* Tm has a very broad host spectrum infecting a wide range of warm-blooded animals. Generally, the infection is caused by consumption of contaminated food or water. In the digestive tract, the pathogen interacts with the intestinal mucosa, invades into the gut tissue and elicits disease symptoms, including nausea, diarrhea and abdominal pain by 8 h - 24 h post infection. In healthy human hosts, *S.* Tm remains restricted to the gut tissue and the pathogen only rarely disseminates into the bloodstream and systemic sites in a transient fashion. After 3-7 days, the symptoms do normally cease. If re-hydration therapy is available, mortality is only observed in very rare events, in particular in young children, immune-compromised patients and the elderly. The mechanisms explaining the self-limiting nature of the disease are not completely understood.

The *S.* Tm virulence factors required for eliciting diarrheal disease, have been a matter of intense research in the past decade (8, 30, 33). Clearly, the Type Three Secretion System (TTSS-1) that is encoded on the 40 kb *Salmonella* pathogenicity island-1 (SPI-1) is a key virulence factor facilitating gut tissue invasion (2, 6, 7, 31). TTSS-1 acts as a syringe for injecting bacterial virulence factors, called effector proteins, into the mucosal cells. Inside the host cell, the effector proteins trigger signaling cascades facilitating bacterial invasion and the release of pro-inflammatory cytokines (21, 32). Besides TTSS, the flagella of *S.* Tm are also important for efficient elicitation of intestinal disease (13, 23, 25). The importance of the diarrheal response has only been recognized recently. *S.* Tm and *Citrobacter rodentium* infection models revealed that mucosal inflammation allows enteropathogenic bacteria to out-compete the intestinal microflora (16, 27). This allows successful pathogen growth in an ecological niche occupied by a very dense bacterial community (24).

SPI-1, which encodes the TTSS-1 apparatus and some of the cognate effector proteins, also encodes several key regulators of *ttss-1* expression, e.g. *hilA*, *hilC*, *hilD*, i*nvF* (1, 4, 20, 29). Besides this, several global regulators are known to control *ttss-1* expression, e.g. regulators that are involved in

metabolism (Crp (28), Mlc (15)) and in stress responses (CpxRA (12), EnvZ/OmpR (4), fur (5)). Transcriptional silencing by proteins such as Hns and Hha (10, 18, 19) are also involved. All these regulatory factors act in concert to avoid expression in environmental niches (e.g. during food-borne transmission) and to ensure *ttss-1* expression in the intestine of the host. However, the regulatory mechanisms facilitating *ttss-1* expression in the gut or adjusting virulence to enhance the host's survival are not completely understood.

In this study we addressed whether the host's body temperature might represent a key environmental cue for *ttss-1* expression. A temperature of 37 °C would be encountered in the guts of most warm-blooded hosts. And we speculated that fever (body temperatures >40 °C), might serve as a cue for down-regulating *ttss-1* expression in order to attenuate further disease progression, to ensure the host's survival and to prolong pathogen excretion. We found that *ttss-1* expression is indeed low at 25 °C, optimal at 37 °C and declined sharply above 40 °C. This was attributable at least in part to the heat shock sigma factor *rpoH* and suggested that fever might indeed serve as an environmental cue for fine-tuning *S*. Tm virulence gene expression.

Materials and Methods

Bacterial strains and growth conditions. All strains were derivatives of *Salmonella* Typhimurium SL1344 (see Table 1). Deletion of *hilA* was performed by insertion of a kanamycin resistance cassette as previously described (3) . To obtain pM2859, *rpoH* were amplified from the genome of SL1344 by PCR with primers 5'-GAGAATTCATGACCAAAGAAATGCAAAATTTAG-3' and 5'-GATCTAGATTACGCTTCGATCGCAGCGCG-3' and cloned into pBAD24 using restriction sites of *Eco*RI and *Hin*dIII. The promoter of the SPI-2 *S.* Tm *ssaH* promoter region was also PCR amplified (primers: 5'-CAT TCT AGA CGG TAG ATT AGC CTT AAC CGC-3' and 5'-CAT GGA TCC AAT GCT TTT CCT TAA AAT AAA-3') and cloned into *Xba*I/*Bam*HI digested pM968 (B. Stecher), yielding pM975. Bacteria were inoculated (1:100 dilution in LB) from 12 h overnight cultures and grown under mild aeration as long as mentioned or for infection assays til OD_{600} of 1 at the indicated temperature. In the case of rpoH overexpression media were supplemented with 0.01% arabinose. Growth was analyzed via OD_{600} and *ttss-1* expression by FACS.

Western Blot. Bacterial LB cultures were grown to indicated densities, harvested by centrifugation and analyzed by Tricin gel electrophoresis and Western blot: For determination of HilA, SipA and SipC concentration, rabbit antisera were raised (Neosystems) and in the case of HilA purified by HilA-affinity chromoatography (AminoLink column, ThermoScientific). Flagellin antiserum was procured from difco. To guarantee equal amounts of bacteria OD600 was referenced.

FACS. SPI-1 expression was monitored using pM972 encoding for GFP driven by the promoter os *sicAsipBCDA*. For FACS analysis samples were withdrawn from a bacterial culture and fluorophore formation was completed by incubating at RT for 2h in the presence of chloramphenicol (30 µg/ml). FACS analysis was performed using a FACSCalibur 4-color cytometer (Becton Dickinson). Bacteria were identified by side scatter (SSC) and GFP emission was analyzed at 530 nm.

Table 1 strains and plasmids

Strains and plasmids	*genotype / charcteristics*	*resistance*	*reference*
strains			
SL1344	*S.* Tm *wt*	Sm	(11)
SB161	*wt ΔinvG*	Sm	(14)
M2080	*wt ΔhilA*	Sm, Kan	This study
plasmids			
pM972	pBR322ori with promoter of *sicA* dependent expression of *gfpmut2*	Amp	This study
pM975	pBR322ori with promoter of *ssaH* dependent expression of *gfpmut2*	Amp	(9)
pM2859	pBAD24, *araBAD* promoter driven expression of *rpoH*	Amp	This study

Gentamicin Protection Assay. HeLa cells were cultured in DMEM (Difco) at 37 °C. Cells were infected with late logarithmic phase *S.* Tm or its derivatives with an MOI of 125. After 20 min cells were washed with HBSS (Difco) three times. Afterwards they were incubated in DMEM supplemented with Gentamycin (400 μg/ml) to eliminate extracellular bacteria. Cells were lysed by Desoxycholate and the lysate were plated on LB agar and incubated at 37 °C. Intracellular survived bacteria grew to colonies and were counted.

Results

Temperature dependence of *ttss-1* expression

For analyzing *ttss-1* expression, we employed a *gfp* reporter plasmid using the promoter of the *sicAsipBCDA* operon, which encodes essential parts of the TTSS-1 injection machinery, i.e. the translocon proteins SipB and SipC as well as the effector protein SipA (pM972, Fig.1A). Earlier work had established that pM972 is an accurate reporter of *ttss-1* expression (Sturm and Hardt, unpublished). We inoculated wild type *S.* Tm pM972 in LB media at 37 °C and analyzed growth (OD_{600}) and *gfp*-expression via FACS (Fig.1BC; Materials and Methods). In line with earlier work, fast exponential growth was observed during the first two hours and *ttss-1* expression was initiated in the late logarithmic phase, approx. after 2.5 to 5 h (Fig. 1B).

In the first two hours we observed a decline of the *gfp* expression (Fig. 1B). Presumably, this was attributable to the stability of GFP, which had accumulated during the growth of the overnight culture (top flask; Fig. 1D) and which was out-diluted by bacterial growth (w/o *ttss-1* expression) in the early phase of the freshly inoculated culture.

Next, we have repeated the experiments at the indicated temperatures. Both the starter and the sub-culture were grown at the desired temperature (Fig.1D). At 25 °C, we did not observe *ttss-1* expression. This was not attributable to a kinetic defect in GFP-folding, as all aliquots were additionally incubated at 37 °C in the presence of 30 µg/ml chloramphenicol, to avoid any further reporter protein expression and to ensure complete maturation of the GFP fluorophore (also verified by Western blot; see, below). Increasing levels of *ttss-1* expression were observed at 30 °C, 35 °C and 36 °C and maximal expression was observed at 37 °C (Fig. 1D). At 39 °C, *ttss-1* expression was lower than at 37 °C and growth at 42 °C yielded virtually no *ttss-1* expression.

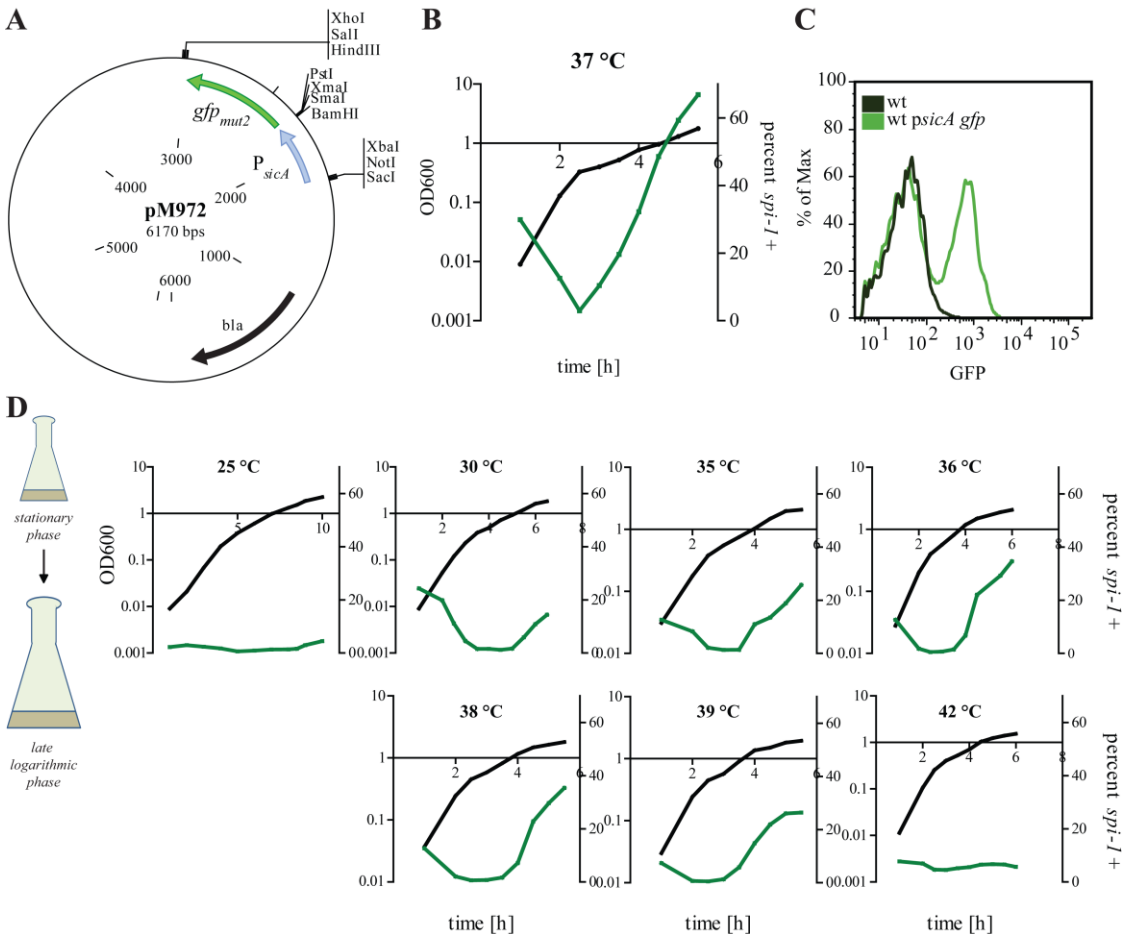

Figure 1, A) Construction of a plasmid carrying a *gfp* reporter showing the expression of SPI-1 at a single cell level. A gfpmut2 cassette were cloned downstream of the promoter of the operon sicAsipBCDA (PsicA). Both features were placed in a low copy vector (pWKS30). B) wt *S.* Tm (SB300) harbouring this plasmid were cultured in LB at 37 °C. Inoculated from stationary phase they show a growth phase dependent expression of SPI-1. At late logarithmic phase the fraction of SPI-1 expressing bacteria arises. Evaluation was done by FACS analysis. Wt *S.* Tm w/o pM972 was used for defining the cut off of non-fluorescing bacteria (C); D) SPI-1 expressing Salmonella at different temperatures. Cultures again were inoculated from a stationary phase culture (grown at the appropriate temperature from 25 to 42 °C) and followed for several hours till reaching the stationary phase again. The fraction of SPI-1

To support these results we performed Western blot analyses (Fig.2). Again cultures were grown as described for Fig. 1D at the indicated temperatures and aliquots were removed for Western blot analysis at the indicated culture densities. The expression levels of HilA, the master regulator of

ttss-1 expression, and of the effector protein SipA were determined. Again, the highest HilA- and SipA levels were observed at 37 °C. At temperatures below 35 °C and at/above 40 °C, SipA expression was much lower. Interestingly, this temperature dependence of HilA-expression was less pronounced as for SipA, suggesting that temperature-dependent regulation might override the master-regulator HilA, at least to some extent. Nevertheless, the Western blot data were in line with the hypothesis that temperature can fine tune *ttss-1* expression.

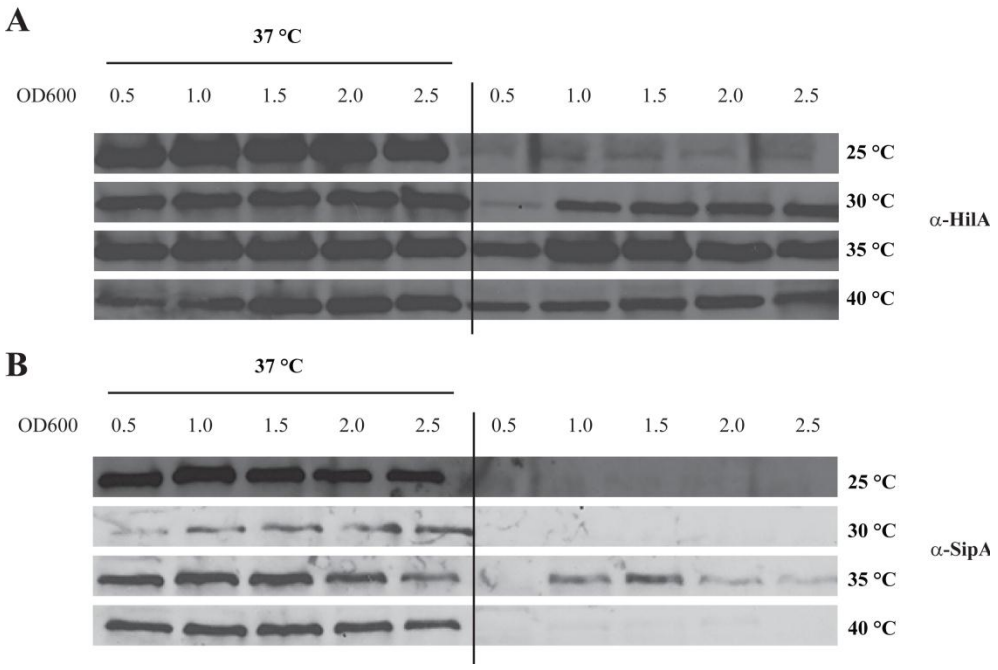

Figure 2, *S.* Tm wt were grown similar to the data shown in Fig. 1. Samples were withdrawn at different OD600 values and applied on SDS-Tricin Gel. Expression of HilA (A) and SipA (B) was biggest at 37 °C.

Temperature control of *ttss-1* expression is not attributable to the growth rate

The growth rate can have significant effects on gene expression. The maximal growth rate of S. Tm is observed at approx. 37 °C, while growth is slower at below 30 °C and above 42 °C. Thus, temperature-mediated effects on the growth rate could potentially explain the findings described, above. However, when we calculated the growth rates for the cultures shown in Fig. 1 and 2, we

observed equivalent growth rates at 30 °C to 42 °C (μ = 0.8-1.0 ± 0.1 h^{-1}). A reduced growth rate was only observed at 25 °C (μ = 0.67 ± 0.05 h^{-1}; Fig. 3). This suggested that the growth rate did not significantly affect *ttss-1* expression, at least in the range of 30 - 42 °C, and that other mechanisms might account for the strongly reduced *ttss-1* expression above 40 °C in our experiments.

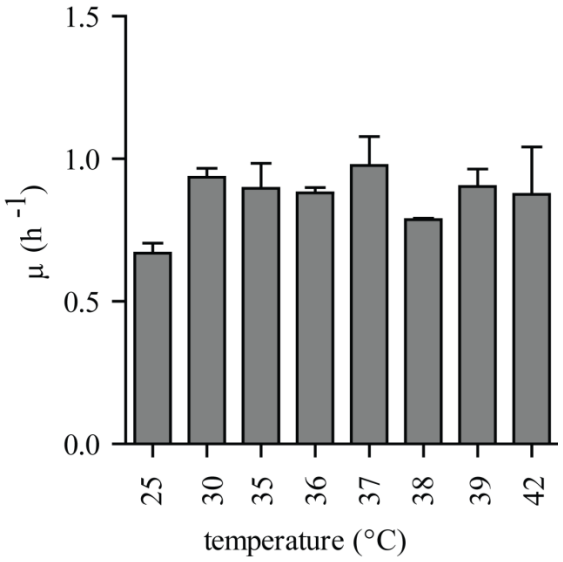

Figure 3, Growth rates of *S.* Tm in LB at different temperatures. The Growth rate is averaged over the whole period we measured in Fig. 1.

Host cell invasion efficiency is strongly affected by the growth temperature

Host cell invasion is a cardinal phenotype of *ttss-1* function. Therefore, we have employed a host cell invasion assay to verify the temperature-dependence of *ttss-1* expression. For this purpose, we have employed a modified gentamycin protection assay, described recently (22). In this assay, host cells are infected with *S.* Tm strains harboring pM975 which expresses *gfp* under control of the *ssaH* promoter. *ssaH* is part of the SPI-2 virulence system, which is expressed only after host cell invasion, when the pathogen resides in the *Salmonella* containing vacuole (SCV). Upon host cell invasion, pM975 yields a bright green fluorescence signal which allows quantifying host cell invasion by automated fluorescence microscopy (Materials and Methods).

We grew wild type *S*. Tm pM975 at the indicated temperatures. This induced *ttss-1* expression (or not) and the bacteria were used to infect HeLa tissue culture cells for 20 min. The infection assay itself was performed at 37 °C. This enabled us to focus on effects caused by the different growth temperatures of the inoculum. Host cell gene expression and pathogen expression within the host cell should be identical between all assays. Furthermore, we used an *invG* (pM975) mutant, rendered non-invasive by genetic disruption of the TTSS-1 apparatus (14), as a negative control.

Again, wt *S*. Tm grown at 37° showed the highest invasiveness (Fig. 4). Above 40 °C and below 30 °C, *S*. Tm was remarkably less invasive. This was attributable to reduced *ttss-1* expression as indicated by Western-blot analyses of the inoculum.

In addition, we also analyzed the expression of *fliC* and *fljB*, which encode the structural proteins forming the pathogen's flagella. Again, we observed maximal expression at approx. 37 °C and reduced expression at 42 °C and below 35 °C (Fig. 4B). However, this effect was not as pronounced as for SipA.

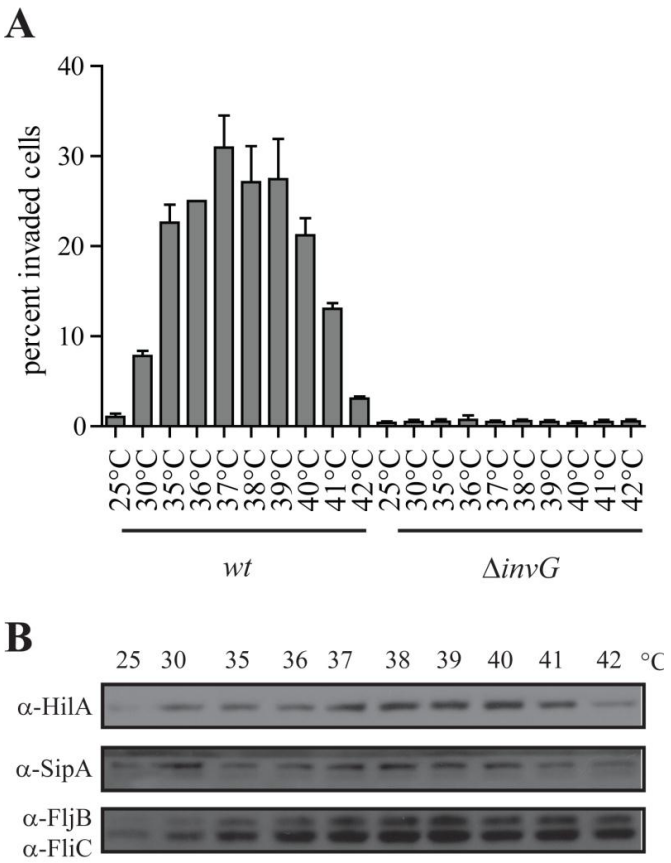

Figure 4, *S.* Tm is less invasive at higher or lower temperatures than 37 / 38 °C. *S.* Tm wt and Δ*invG* as a negative control, both possessing pM975, were grown in LB at the indicated temperature till stationary phase. Afterwards they were inoculated in fresh LB at the same temperatures (25-42 °C). After cells reached an OD_{600} of 1, HeLa Kyoto cells were infected with *S.* Tm pM975 or. *S.* Tm Δ*invG* pM975. Culturing of HeLa cells and the Invasion assay itself were performed at 37 °C in each case. The maximum of invasiveness *S.* Tm reaches at 37 °C degrees (A), according to expression of HilA and SipA (B). At 42 °C wt already behave as similar as the non-invasive strain Δ*invG*.

Overexpression of the heat shock sigma factor *rpoH* down-regulates *ttss-1* expression

The reduced *ttss-1* expression at temperatures above 40 °C suggested that the heat shock sigma factor might be involved. At 37 °C, RpoH (also called σ^{32}) is constantly degraded by DnaK. Above 40 °C, heat shock dampens this degradation, thus increasing RpoH levels and the expression of RpoH-dependent heat shock promoters (17).

We expressed *rpoH* from a plasmid (pM2859) at 37 °C to examine the consequences for *ttss-1* expression. Western blot analyses revealed that RpoH reduced the expression of the *ttss-1* master regulator HilA, and of the flagellins FliC and FljB (Fig. 5A). Similarly, host cell invasion by wt *S.* Tm was significantly reduced in cultures overexpressing *rpoH* (Fig. 5B). This suggested that *rpoH* contributes to the down-regulation of *ttss-1* expression at temperatures above 40 °C.

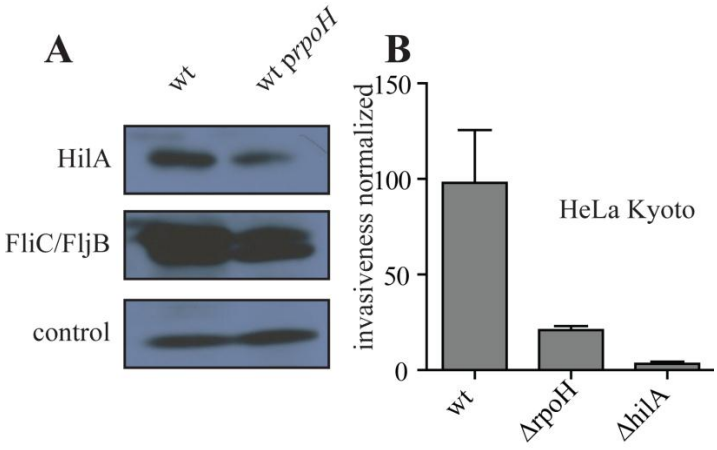

Figure 5, Heat shock sigma factor rpoH (σ^{32}) were cloned in a pBAD24 vector to allow arabinose induced expression of σ^{32} targets at lower temperatures. *S.* Tm wt and wt harboring p*rpoH*. Cultures were grown aerobically in LB at 37 °C supplemented with 0.01 % arabinose till an OD_{600} of 1.2. Western Blots were performed against HilA and FliC/FljB. Invasion were followed by applying Gentamycin Protection Assay.

Discussion

In the present study, we have analyzed how temperature affects the expression of cardinal virulence factors eliciting *S.* Tm diarrhea. We found that *ttss-1* expression was most pronounced at 37 °C but lower at temperatures below 30 °C and above 40 °C. The latter was attributable at least in part to σ^{32}, which controls the heat shock regulon. Based on these findings, we speculate that the temperature might represent a key environmental cue fine-tuning *S.* Tm virulence during its interaction with the host.

In non-host environments, *ttss-1* expression does not seem to be beneficial for *S.* Tm. This may explain, why *ttss-1* is not expressed efficiently at 25 °C. The *sicAsipBCDA* operon is encoded within SPI-1, which belongs to regions in the genome exhibiting an AT-rich region. Such genomic areas are considered to be preferred targets to silencing proteins, as Hns (10, 18). The Hns binding ensures that these regions are not transcribed at low temperatures. Weakening of the Hns-mediated silencing at 37 °C might explain in part why *ttss-1* is maximally expressed at this temperature. In terms of evolution those genomic regions have been taken up by horizontal gene transfer and incorporated into the genome.

In an otherwise healthy host, *S.* Tm diarrhea is not strictly associated with fever. Spread into the blood stream and episodes of fever are occasionally observed, but the pathogen generally remains restricted to the gut. Our results suggest that the temperature-control of the pathogen's virulence factor gene expression might contribute to this self-limiting nature of the diarrheal *S.* Tm infection. At the beginning of the infection, when the pathogen must compete with an intact microflora, the intestinal environment has a temperature of 37 °C. This allows maximal expression of *ttss-1* and the flagella which is essential for invading the gut mucosa and eliciting inflammation. On the one hand, this creates a beneficial environment fostering the pathogen's growth in the gut lumen. On the other hand, if unrestrained, this should result in a self-amplifying course of the infection with ever increasing pathogen densities, tissue invasion and inflammation. Potentially, this could compromise the host's viability and thereby limit the pathogen's spread. We hypothesize that this is avoided by a

negative feedback between the host (which mounts a feverish response to the overshooting gut infection) and the pathogen, which down-regulates virulence factor expression in response to the elevated body temperature. This temperature-responsive fine tuning of virulence factor expression may therefore establish an optimal compromise between the need to elicit gut inflammation and the need to ensure the host's survival. Indeed, mouse models have demonstrated that gut inflammation, efficient gut colonization and pathogen shedding can continue for several months, thus leading to the shedding of > 10^9 cfu/day of *S*. Tm for >6 weeks (26).

Due to the self-limiting nature of the infection, non-complicated cases of *Salmonella* diarrhea are generally not treated by pharmacological intervention. However, antibiotics are used if systemic spread and fever are observed. It had remained unclear, whether the antibiotics treatment should be accompanied by an antipyretic treatment or not. Our in vitro data suggest that lowering the fever might by-pass the temperature-control of *S*. Tm virulence factor expression and might therefore enhance the pathogen's tissue invasion. This would be counter-productive. It will be an important task for future research to determine how temperature affects *Salmonella* spp. virulence factor expression in the gut of an infected host and how antipyretics affect the disease progression.

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Chapter VI

Final Conclusions

Final Conclusions

The cost factor of SPI-1 expression and consequences for the current divison of labor model

TTSSs are potent tools of bacteria to establish communication with eukaryotic cells either in a symbiotic way (e.g. *Bradyrhizobium japonicum* (21)) or to establish infection as e.g. *S.* Tm. For *S.* Tm it could be shown that *ttss-1* genes are transcribed in a bistable manner (19, 31, 35). A similar expression pattern was found for *Pseudomonas aeruginosa* (29), but there are several other *ttss* expressing bacteria were bistability has not been observed (e.g. *Yersinia enterocolitica* (40), *Shigella flexneri*). We speculate that bistability of TTSS-1 might be explained by the costs associated with *ttss-1* expression. In our experiments we could observe that *S.* Tm suffers from expressing *ttss-1* genes. This manifests in a reduced growth rate of the TTSS-1$^+$ subpopulation compared to the TTSS-1$^-$ in LB media. Mutants like Δ*hilD* or Δ*hilA*, which generate exclusively TTSS-1$^-$ cells, do not display this burden and out-compete wt *S.* Tm in our in vitro experiments. In contrary, Δ*hilE* mutants, which yield a higher fraction of TTSS-1$^+$ individuals, grow more slowly than the wt. Thus, virulence in the absence of the host exhibits a burden for the individual and is without any advantage. Nevertheless, *S.* Tm expresses *ttss-1* genes in LB but might reduce the cost by expressing it only in a part of the population. The remaining TTSS-1$^-$ subpopulation is not affected in growth. This strategy might be of advantage even during the infection. Experiments with mice that were either infected by Δ*hilA* or Δ*hilE* mutants showing a complete TTSS-1$^-$ or TTSS-1$^+$ phenotype respectively are not able to efficiently colonize the gut in the long run (M. Diard, unpublished). Δ*hilE*, reflecting an enhanced *ttss-1* expression, even gather mutations that compromise virulence. Those mutants are no longer able to cause inflammation (M. Diard). Up to now, it has not been elucidated where mutations in the *S.* Tm genome occur, but Western blot analysis clearly show bacteria fail to express the translocon component and effector protein SipC. Taking this together, a regulation, which ensures that only a fraction of the population exhibits TTSS-1, seems to be necessary even during the infection process. In this context, our findings have impact on the *self-destructive cooperation* model, which was proposed in its original form by M.

Ackermann and W. Hardt (1). In that study, it had been shown, that only the TTSS-1$^+$ fraction is found in the tissue layer, while the TTSS-1$^-$ subpopulation and also a significant fraction of the TTSS-1$^+$ subpopulation remain in the gut lumen (1). This model describes an extreme form of division of labor. The inflammatory response provoked by the TTSS-1$^+$ fraction leads to the descent of commensals, which allows the remaining *S*. Tm in the lumen to occupy this space for further growth. Our new data indicate that TTSS-1$^+$ bacteria themselves would be weak competitors for the commensals and would probably fail to colonize the gut. As a result to not bearing the growth cost, TTSS-1$^-$ individuals are much more likely successful in competition. These findings are still largely in line with the hypothesis of M. Ackermann and W. Hardt claiming that this kind of division of labor is a possible scenario in vivo. The TTSS-1$^+$ bacteria suffer in two ways: (i) they are subject to killing by immune cells within the gut tissue and (ii) they undergo a growth defect in the gut lumen. Bistable expression can be regarded as a way to allocate the cost to a subpopulation. But besides *S*. Tm, there are pathogenic bacteria where expression of a TTSS does not happen in a bistable manner. This even includes enteropathogens with similar disease patterns such as *Y. enterocolytica* (40). This is of interest as *Y. enterocolitica* was also shown to grow at decreased rates upon *ttss* expression (40). One explanation for the difference might reside in the different regulation of *ttss* expression. In the case of *Y. enterocolitica ttss* expression is triggered only after host cell contact and entry into the tissue. This represents another way to circumvent the cost factor for the whole population and restricts it solely to a number of bacteria, which stay in direct contact with the host cell. Additionally, *Y. enterocolitica* as well as *P. aeruginosa* respond to Ca^{2+} levels within the media in vitro in an all or none fashion (40).

Considering other examples of bistability as e.g. (i) persistence of *E. coli*, where some individuals of a population are more resistant to antibiotics (4, 22) or (ii) alternative substrate preferences that are less energy yielding (the *lac* operon (30)). Both examples are accompanied by dormancy or at least reduced growth (4, 22). Here it is also not clear, why resistant bacteria (i) are dormant. It is not clear whether an increased MDR transporter (multiple drug resistance) production represents a sufficient explanation or if the metabolic state itself (dormancy) is adequate for resistance against antibiotics

(22). In the case of alternative nutrition, the lower energy release per substrate molecule and enzyme expression can argue for reduced growth, too. In most of these cases, bet hedging strategies are thought to explain the evolutionary advantage. Nevertheless, bistability allows populations to deal with unpredictable situations, where adaptation by classical sensing and reaction is not possible or less advantageous. But there is also evidence, that these "alternative" individuals are compromised under current conditions. Bistability is therefore vitally required to reduce costs for the entire population by restricting them to a small subpopulation.

Once again, the observations with respect to growth rates of TTSS-1$^+$, cells were made in vitro. We are still limited in our abilities to predict the impact on the in vivo situation during infection. Nevertheless, our reasoning is based on the description of a phenomenon that we (among others) called individualism, i.e. TTSS-1$^+$ and TTSS-1$^-$ individuals. The pervasive conception of an individual includes essential differences in the genome. Dissimilar to various philosophical interpretations of individuality by e.g. Hegel, Descartes or Kierkegaard, individuality in sciences should be decisive. Otherwise, at this point conflicts arise according to ambiguities in terminology (compare Introduction - Arguments for phenotypic variation – bet hedging; *multicellular-like strategy*). This point should be addressed in the future to avoid misconceptions and to distinguish terminologically phenotypic different but genetically uniform individuals from genetically different individuals.

TTSS-1 is embedded in a complex system of co-regulated genes

The question arises, if *ttss-1* expression is per se costly in terms of additional protein biosynthesis or by weakening the membrane integrity during insertion of the basal body (16). Alternatively, it could be that the expression of *ttss-1* is connected to additional genes not necessarily involved in virulence. Thus, a more complex kind of developmental program would be conceivable. For this purpose, we approached together with A. Schmidt (Aebershold laboratory, IMSB, ETH Zurich) a proteomic analysis based on FACS and Mass spectrometry (suppl. Fig. 1 and suppl. Table 1). Here

we analyzed proteins differentially expressed in the TTSS-1$^+$ subpopulation. Beside many hits of TTSS-1 proteins, we confirmed FliC that was enriched in the TTSS-1$^+$ fraction (suppl. Table hit no. 4) and was also subject to further investigations (12, 31, 36). The co-regulation of *ttss-1* and the flagella genes was also shown by reporter analysis (chapter 2, suppl. Figure 3). Besides this, members of the chemotaxis system, CheY and the Methyl-accepting protein at locus STM3216 have been found (suppl. Table hit no. 35 and 39). This observation is in line with the necessity of functional chemotaxis and flagella within the infection process, which was basically described by B. Stecher within our group (33, 34) and provides further evidence for coordination between *flagella* and *ttss-1*, i.e via HilA with CheA. Also proteins involved in LPS synthesis, are enriched (e.g. OafA, which acetylates the LPS chain, suppl. Table 1, hit No. 30). Obviously, multiple systems connected to virulence seem to be over-expressed in the TTSS-1$^+$ subpopulation. For this reason alone, we anticipate that *ttss-1* expression is embedded in a kind of differentiation program that modify the TTSS-1$^+$ subpopulation beyond simple *ttss-1* expression.

Beyond this, enzymes, which are connected to anaerobic metabolism like Ferredoxin (fdx, suppl. Table 1, hit No. 12) are predominantly found in TTSS-1$^+$ individuals. Those enzymes contain Fe-S clusters, which are less stable under aerobic conditions (27). All our in vitro experiments have been performed under aerobic conditions, but in fact, *ttss-1* expression arose in the late logarithmic phase when oxygen becomes limited (chapter 2, Fig. 3). Also stress-associated proteins like UspF and IbpB (suppl. Table 1, hit No. 40 and 41) are found. On the other hand, there are also proteins that are enriched in the TTSS-1$^-$ fraction e.g. a putative catalase and RelA as the strongest hits (suppl. Table, hit No. -1 and -2). Catalases might be useful during the inflammatory response and help the individual to withstand elevated levels of reactive oxygen species (ROS) that occur during the inflammatory response in the gut (32, 41). In a first attempt to resolve the proteome dedicated to the TTSS-1$^+$ fraction, the membrane stress sigma factor RpoE appeared in the hit list but could not be reproduced in a confirmatory analysis (suppl. Table 1 shows exclusively proteins that could be confirmed by a second evaluation of the data). Nevertheless, the appearance of stress proteins indicates that *ttss-1* expression represents a cost for the cell. But even if RpoE have not been found

to be enriched in the TTSS-1$^+$ subpopulation in a second evaluation, *rpoE* deficient *S.* Tm strains display lower levels of SipC and reduced invasion (suppl. Fig. 2). In the literature the role of *rpoE* in macrophage survival was already pointed out (37). Apparently, *rpoE* is involved in the regulation of *ttss-1* and as a future perspective it should be addressed if *rpoE* is responsible for *ttss-1* expression or if *rpoE* rather eradicate detriments caused by TTSS-1.

The accumulation of Ferredoxin and the down-regulation of catalases indicates that low aerobic conditions favor *ttss-1* expression. It has remained unclear why they are only enriched in the TTSS-1$^+$ fraction, since all bacteria are exposed to the identical environment. The same is true for motility, stress and LPS genes. All results suggest that *ttss-1* expression is accompanied by the coordinated transcription of multiple genes so that bacteria experience developmental differentiation, in which the TTSS-1$^+$ and TTSS-1$^-$ are only one aspect.

Development entails normally a process that is inherited from generation to generation and is associated with changes on the DNA level per definition. In the case of the observations we described here, changes happen on the transcriptional level but seem to be inherited over a few generations (bacteria which switched on *ttss-1* genes remain in this state for several generations, chapter 2, Fig. 2 and 3). Experiments, which addressed the back-switch, could show that TTSS-1$^+$ are indeed able to switch back and that a population constituted of different ratios between TTSS-1$^+$ and TTSS-1$^-$ again distribute in TTSS-1$^+$ and TTSS-1$^-$ subpopulations (suppl. Fig. 3). In conclusion, even if dramatic differences evolve during *ttss-1* expression between both subpopulations, the process is reversible. Otherwise, individuals deriving from different growth rates display varying preferences in respect to *ttss-1* expression. It is to question, wherein this manifests.

B. subtilis indiviuals that tend to sporulate, represent probably the best understood example for bistability among bacteria (9, 38). Here, differentiation is also exclusively mediated on the transcriptional level and is transferred to the following generation. The fact that *B. subtilis* endospores are able to switch from the dormant to the active state indicates also back-switching. We think, principally, both observations underlie similar mechanisms.

The occurrence of bistability is accompanied by reduced growth as described above. This is also supported by the proteomic analysis. We found decreased amounts of the cell division protein ZipA in the TTSS-1$^+$ fraction (suppl. Table 1, hit No. -27), which is an essential part of the septal ring that mediates bacterial cell division (24). This observation, of course, has to be validated by additional experiments. One might speculate if *ttss-1* expression determines the growth. An alternative explanation would be that slow growth favors TTSS-1+, while fast growth would prevent the expression of the TTSS. Since regulators have to pass a certain threshold to trigger a phenotype, fast growing bacteria would automatically dilute the concentration of regulators basically by faster division and would therefore fail to reach the threshold required to initiate the TTSS-1$^+$ phenotype. In contrast, slower bacteria would accumulate regulators and therefore display phenotypic variances. However, chemostate experiments performed by K. Kochanowski (Sauer laboratory, IMSB, ETH Zurich) and us could not confirm that a decreased growth rate triggers *ttss-1* expression (suppl. Fig. 4). Therefore, the reduced growth rate is rather interpreted as a cost that goes along with *ttss-1* than a prerequisite to express it.

Origin of *ttss-1* bistability

In line with our experiments, bistability of SPI-1 virulence genes is widely observed in many research groups working with different isolates of *S*. Tm (19, 31, 35). Fortunately, key genes involved in the regulatory network generating bistability have been unraveled. As a result, at least for the SPI-1 operons *prg-org*, *sicAsipBCDA* and *hilA* bistability is verified, mostly by utilization of *gfp* reporter plasmids (31, 35), but also in one case by chromosomal reporter insertions (19). Thus, SPI-1 regulators, TTSS-1 components and effector proteins appear exclusively in a TTSS-1$^+$ fraction of the population.

There is a discord about *hilD*, the transcription factor that takes the highest position in the hierarchy of SPI-1 transcription factors. K. Temme and coworkers demonstrated in 2007 that *hilD* is monostable (35). The generation of the *prg-org* operon should be generated by a split forward loop

constituted of the downstream regulators SicA and InvF (see also introduction). In contrast, three years later S. Saini and coworkers showed that bistability of *ttss-1* is entirely determined by the bistability of the *hilD* promoter (31). According to the author's interpretations, all other regulators in the cascade postulated by Ellermeier in 2005 are merely dedicated to amplify the expression of *hilA* and thus of *prg-org*, *sicAsipBCDA* and *inv-spa* (11).

However, our data in Chapter 3 and 4 showed additional contributions downstream of HilD like the chemotaxis sensory kinase CheA as well as the acetyl-coA synthetase Acs interact or modify HilA and thus manipulate the fraction of TTSS-1^+ and TTSS-1^- (see chapter 3 Fig. 4, chapter 4 Fig. 3 and Fig. 4). This argues that *hilD* is at least not the only factor generating bistability.

This is also supported by findings made for the BarA/SirA system. The two component system BarA/SirA is supposed to directly activate *hilA* and *hilC* (36) and thereby it bypasses the action of *hilD*. The *hilA* promoter region is silenced by Hns and mutants are characterized by elevated levels of *ttss-1* expression due to the vacant promoter of *hilA*, too. However, Hns also occupies the promoters of *hilC* and *hilD* (26). Further experiments that were performed in our lab hypothesize that the main origin of *ttss-1* bistability might originate from the interaction between HilD and HilE, which takes place on the protein level (6). Reporters utilizing a chromosomally tsr_{venus} accounting the expression of *hilD* and *hilE*, respectively, revealed variances in gene expression with a pronounced tailed distribution, which was reminiscent of observations made for phage burst sizes by M. Delbrueck in 1945 ((8), suppl. Fig. 5 and see also Introduction Fig. 8). This probably points to bistability of *hilD* and *hilE*, which would be in line with the interpretation of Saini and coworkers for the *hilD* promoter (31).

Beyond this, *hilE* expression has not been properly addressed yet; bistable expression of *hilE* as we would propose based on our data would broaden the origin of bistability (suppl. Fig. 5C). So far, *hilE* is thought to dampen the expression of *hilD*. In fact, in a *hilE* mutant transcription of *hilD* is enhanced ((6, 7) and suppl. Fig. 5AB). Obviously, some regulators within the regulatory cascade exhibit bistable expression and it is also known that their expression is highly dependent on each

other (11, 13, 14, 31). But beyond that, it remains still unclear to what extent the bistability of *ttss-1* genes can be attributed to asingle regulator. It might be that both theories proposed by Temme et al. (35) as well as by Saini et al. (31), describe contributions to the real scenario. But with the bistable expression of *hilE* the regulatory system would indeed gain even more complexity (suppl. Fig 5C). To address those questions, it is vital to follow expression of all *ttss-1* regulators using different fluorophores on the single cell level and thereby clarify which regulators appear simultaneously and which might exclude each other. A considerable difficulty is given by the poor folding efficiency of fluorescent proteins within *S.* Tm and the naturally weak transcription of *ttss-1* regulators. So far, GFP, Tsr_{venus} (both green) and mCherry (red) have proven to fold properly and enable expression analyses at the single cell level. By contrast, RFP leads to extreme retardations in growth. It is of future interest to create *S.* Tm reporter strains that monitor expression of several regulators by different fluorescent proteins in the same cell. This is possibly the most promising way to unravel the dynamics and the mechanism of the bistable output of this highly interesting and complex regulatory network.

However, a major issue is still not satisfactory elucidated: Bistability can be shown for SPI-1 operons. But is it generated here? As pointed out in the previous section, SPI-1 seems to be embedded in a developmental program. What is the hierarchy? Where is it decided? This makes the work by Temme and collaborators unlikely to describe a decision point of the bistability of *ttss-1*. It might embody a way to stabilize it. Temme and coworkers addressed this question by a split forward loop constituted of SicA and InvF the downstream in the regulatory cascade of HilA (35). But our findings as well as those by Saini et al. show already bistability of *hilA* and *hilD* and hence upstream of InvF and SicA (31). Interestingly, Temme et al. did not comment on the expression of *hilA*. As already indicated in the introduction, both groups worked with plasmids controlling *gfp* expression by different promoter lengths of *hilD* and therefore gathered different results (31, 35). An approximately 600 bp long upstream region of the promoter is missing in the experiments of Temme and coworkers. This might explain differences in the results. Again, this is a strong argument for utilizing chromosomal reporters in situ to avoid artifacts and misinterpretations.

However, it suggests a regulatory sequence upstream of *hilD* that has repressor function and diminishes transcription. Remarkably, this region encodes for the promoter and parts of the *prg-org* operon that is controlled by HilA; an indicator for a negative feedback? It might be that interfering transcription initiation points affect expression of both operons (see also the genomic island at Introduction Fig. 1). But again, even if we believe that *hilD* is bistable, it is not guaranteed that bistability is generated here. To elucidate this, further studies of factors upstream of *hilD* are required.

HilA connects chemotaxis and metabolism to virulence

A number of papers have shown that the expression of TTSS-1depends highly on the metabolic state of the cell, which is controlled by several carbon regulators as Crp, Cya, SirA, CsrA and Mlc (see also Chapter 1 Regulation). A common theme is the enhanced *ttss-1* expression during the absence of glucose. LB as an almost Glucose free system, is constituted mainly of peptides and oligosaccharides as carbon sources. Nevertheless, the composition of media determines whether and to which degree *ttss-1* genes are expressed. Since in vitro observations in LB-like media correlate with observations made in vivo regarding the distribution of TTSS-1^+ and TTSS-1^- subpopulations (1), we anticipate that similar regulatory processes are accountable for the observed phenotypes. The central role in SPI-1 regulation that is carried out by HilA is often regarded as a simple transmission module controlled by the transcription factor HilD (2). Indeed, the promoter region of *hilA* is normally silenced by Hns. At pos. -77 it contains a HilD specific activating sequence that allows HilD to displace Hns and thus activate *hilA* expression (25). Our data in Chapter 2 further suggest that HilA transcription correlates with the expression of the *sicAsipBCDA* operon (Chapter 2, Fig. 1E). Besides this, as we could show in Chapter 3 and 4, HilA is subject to posttranslational interactions, which modulate its activity and indeed, can manipulate the ratio between TTSS-1^+ and TTSS-1^- by acetylation, although the exact mechanism remains concealed. So far, only

transcriptional regulation has been considered in the generation of bistability (28). Our data suggest that posttranscriptional modifications might also be dedicated to generate bistability.

So far, the only known example for protein acetylation by Acs has been CheY (10). CheY also exhibits auto-acetylation activity (5). We found that HilA is acetylated and several residues are acetylated by Acs. However, we still found modified residues in a Δ*acs* mutant, which suggest that further enzymes are involved in the modification of HilA. Wang and collaborators found out that a number of proteins in *S*. Tm display acetylation in response to distinct nutrition supply by CobB and Pat (39). It is unclear, whether these enzymes also contribute to the acetylation of HilA and furthermore if auto-catalytical processes are involved like for CheY (5). But nevertheless, the changes in the acetylation pattern obtained by Acs seem to be sufficient to explain alterations in the ratio of subpopulations regarding *ttss-1* expression. In general, acetylation together with phosphorylation of proteins represents a potent counterbalance to transcription in signal sensing with regard to reaction time, specificity, economy and reversibility. But it represents a signaling system that rather responds to intracellular changes than to the environment as phosphorylation.

At first instance, CheA binding to HilA might be expected involving phosphorylation. This is suggested by the domain structure and the conserved D67 within the sequence of HilA and it would also be in line with the broad involvement of several two component systems that participate in the regulation of *ttss-1* genes (PhoPQ, PhoBR, EnvZ/OmpR, CpxAR (6, 13, 18)). Sensing the environment is thereby an essential requirement for the decent expression of SPI-1 virulence genes. It would not be astonishing if HilA features these characteristics by the interaction with CheA as well. However, we were not able to provide evidence for CheA mediated HilA phosphorylation.

In addition, the *Salmonella* pathogenicity island 2 (SPI-2) is under prominent control of a two component system, SsrAB (17). Here also, the sensed signal is not unraveled. In contrast to the SsrAB system, HilA and CheA belong to different operons. HilA represents hence an orphan response regulator as discussed in Chaper 3 (CheA normally interacts with CheY to manipulate the tumbling frequency of the flagella). It is not known, whether the interaction we observe, is based on

a process, which is actively propagated or if the interaction arose due to generic sequence similarities between sensory kinases and thus rather happens by accident (see also Chapter 3, Discussion). However, if there had been another sensory kinase (similar to *ssrAB*) within the *hilA* operon in previous times that must have been lost during evolution, i.e. before the divergence of *S. enterica* and *S. bongori*, is not known. In fact, this represents a minor possible scenario in light of the strong impact of HilD on *hilA* transcription and the direct correlation between HilA and SipA levels (Chapter 2, Fig. 1E). Anyhow, even if there are similarities between the regulation of SPI-1 and SPI-2, SPI-2 has not been reported to be bistably expressed. In analogy to the *Yersinia* TTSS, one might speculate that the differences are explained by the intracellular expression of SPI-2.

The Impact of Temperature

In Chapter 5 we investigated how SPI-1 virulence gene expression responds to varying temperatures. This might have potential implications in fever. However, we provided exclusively in vitro data. An apparently simple question to follow the effect of fever in vivo is, rather difficult to approach. The easiest access to *S.* Tm infection is represented by mice and well established. Unfortunately, mice do not develop fever. The inflammatory response is rather followed by a decrease in body temperature. Therefore, mice do not represent a suitable model organism to study the effect of fever on *Salmonella* virulence gene expression in vivo. An alternate approach is given by the infection of hogs, which we would like to perfomrm in collaboration with expert veterinary laboratories.

Further evidence, that temperature represents a control mechanism widely found in virulence gene expression, is given by *Listeria monocytogenes*, a pathogen that primarly affects immune-compromised and pregnant people (20). Johansson and coworkers of the group of P. Cossart demonstrated in 2002 that the key virulence regulator PrfA shows similar expression patterns with a maximum at 37 °C. The temperature control is thereby realized by a 5' UTR in front of the regulator

(20). Finally, also *Shigella flexneri* displays temperature depend expression according to transcription of *virF* (15).

Interestingly, the temperature might affect *S*. Tm *ttss-1* expression in two ways: (i) by induction of virulence genes by transcription factors as e.g. *hilD* and (ii) by repression of transcription factors like *hilD* due to *hilE*. Again, we applied *gfp* reporter assays in LB cultures similar to experiments described in Chapter 5, Fig. 1. In addition, we also monitored the expression of *ttss-1* genes in a Δ*hilE* mutant at 25 °C, 37 °C (body temperature) and 42 °C (severe fever) (suppl. Fig. 6). In all cases Δ*hilE* showed enhanced levels of *ttss-1* expression. At 37 °C *S*. Tm loses even bistability of *ttss-1* in the Δ*hilE* mutant. Remarkably, there seems to be only a small window where *ttss-1* is expressed in Δ*hilE* at 25 °C, which is not present in wt (suppl. Fig 6A). Apparently, the *hilD* promoter is active at different temperatures but to different extents. Why *ttss-1* expression drops again after 5 to 6 h at 25 °C (suppl. Fig. 6A) might be due to imperfect amplification loops that prevent a stable steady state. Besides this, *hilE* expression takes place at each temperature. Under any condition, *hilE* mutants display higher fractions of TTSS-1$^+$ bacteria than wt. Here again, tsr_{venus} reporter dedicated to the expression of *hilD* and *hilE* are probably the most promising approach, since RT-PCR fails to supply information about single cells and rather is applicable in cases where bistability is not an issue.

In conclusion, temperature represents an environmental signal that is able to manipulate the ratio between TTSS-1$^+$ and TTSS-1$^-$ *S*. Tm individuals.

The end is the beginning

Finally, we could show that bistability of *S*. Tm virulence factor expression have intriguing consequences for the single bacterium. We speculate that in contrast to bet hedging both phenotypes have to fulfill certain tasks during the infection. Future work will show, if our assumption will

pertain concerning the division of labor between TTSS-1$^+$ and TTSS-1$^-$. Nevertheless, this work could show a possible explanation for the emergence of bistability in *S.* Tm virulence.

In further investigations we could elucidate that HilA is post-translationally modified by the acetyl-coA synthetase Acs according to acetate. In addition, HilA interacts with the chemotaxis sensory kinase CheA. Both, Acs and CheA, represent prominent members of the central metabolism respectively determinants of chemotaxis mediated motility. Both affect *ttss-1* expression negatively. Here we described a mechanism that modulate the ratio between TTSS-1$^+$ and TTSS-1$^-$ bacteria apart from *hilD* and hence represents a novelty in the generation of TTSS-1 bistability. Together with the observation that temperature also affects the ratio between TTSS-1$^+$ and TTSS-1$^-$ bacteria, we could identify several environmental cues with impact on *ttss-1* expression. In view of the infection *S.* Tm induces in the gut, all of these regulators are involved in the appropriate response to changes within the intestinal environment. Thus, it is of importance that these regulatory systems can modulate *ttss-1* expression accordingly.

Since we provide exclusively in vitro data, we can only speculate with regard to implications to the real scenario within the infected gut. Furthermore, because of the pleiotropic effects of *cheA* and *acs* mutant studies in more complex environments like in the mice intestine, will probably not help to understand their implications in the regulatory system. However, the data could show that the virulence regulon of *ttss-1* genes harbors more determinants in addition to the already complex network, which has already been described (13).

Beyond that, several questions are unsolved. First, how do the components of the regulon contribute to the bistable distribution in TTSS-1$^+$ and TTSS-1$^-$ subpopulations. Many publications choose single regulators and describe their effects on the expression of *ttss-1* genes (e.g. (7, 18, 23)); other publications modulate the interaction of *ttss-1* specific regulators as e.g. *hilD* and *hilA* (3, 31, 35). For a deeper understanding, both kinds of approaches are less promising to encounter the switch that determines TTSS-1+ and TTSS-1- populations. For this purpose, most probably big data sets of mutants in combination with different fluorescence gene reporters have to be evaluated on a single

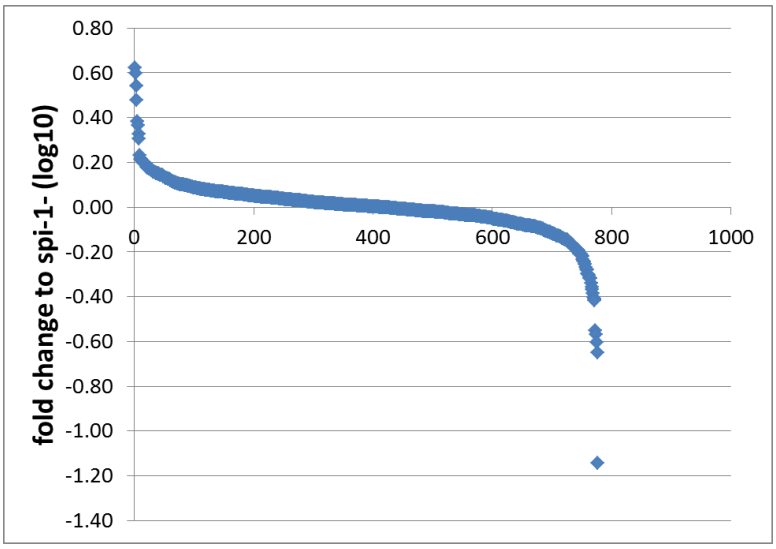
cell level. Afterwards a computational analysis might reveal the site of TTSS-1 yes or no decision. This hopefully gives conclusive insights, how from simple stochastics in transcription (which is subject to each gene), bistability of *ttss-1* genes can evolve.

A second question deals with proving bistability as a necessary mechanism. As already pointed out, several pathogenic species (e.g *Y. enterocolitica*) do not display bistable expression of virulence genes, also the *ttss-2* genes of *S*. Tm genes are monostable. As a monostable TTSS-1 mutant (Δ*hilE*) is not able to inflame successfully the gut, bistability of TTSS-1 is essential for propagation and transmission of *S*. Tm populations. It could be interesting to investigate differences in the infection of different pathogens and to correlate this with the occurrence of bistability in virulence gene expression. This also might give insights, which steps of the infection are determined by bistability. Together with the considerations of the previous paragraph this might point to the evolution of bistable virulence gene expression and how this has been maintained over several generations.

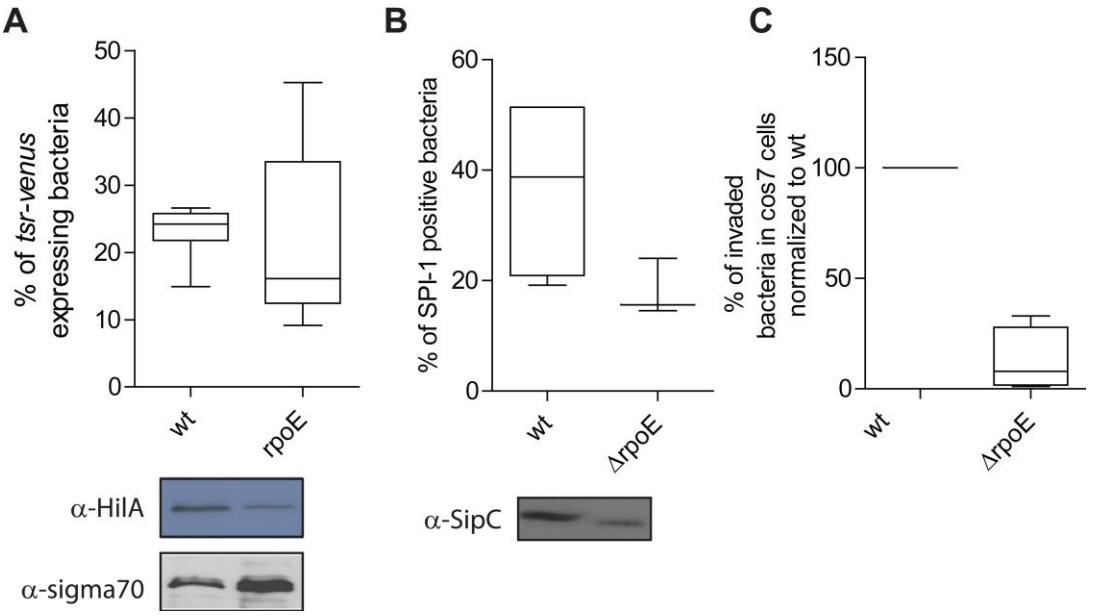
Finally, *ttss-1* expression has been shown to be involved in a dense network of global regulators. Therefore, it is not an uncoupled process and depends highly on the current state of the bacterial cell. Several publications that e.g. report flagella co-regulation or metabolic determinants, and our data (proteome analysis) highly suggest that *S*. Tm "differentiate" for some generations in TTSS-1^+ and TTSS-1^- subpopulations (comparable to *B. subtilis* sporulation). This exemplifies that primitive unicellular organisms like *S*. Tm possess already tools - based on stochastics and complex regulatory amplifications - that display developmental programs. Remarkably, these changes can be reverted.

To use stochastics in gene expression to generate different subpopulations might be common to a number of bacteria and might also be crucial for a variety of challenges bacteria encounter in their environment, beyond the reported cases of *B. subtilis* sporulation, persister formation and *S*. Tm virulence expression (9, 38).

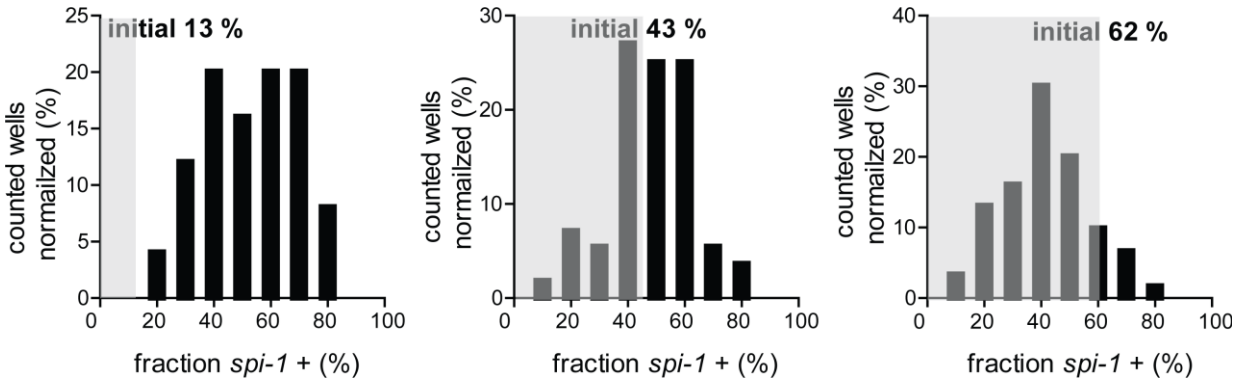
Supplementary Figures



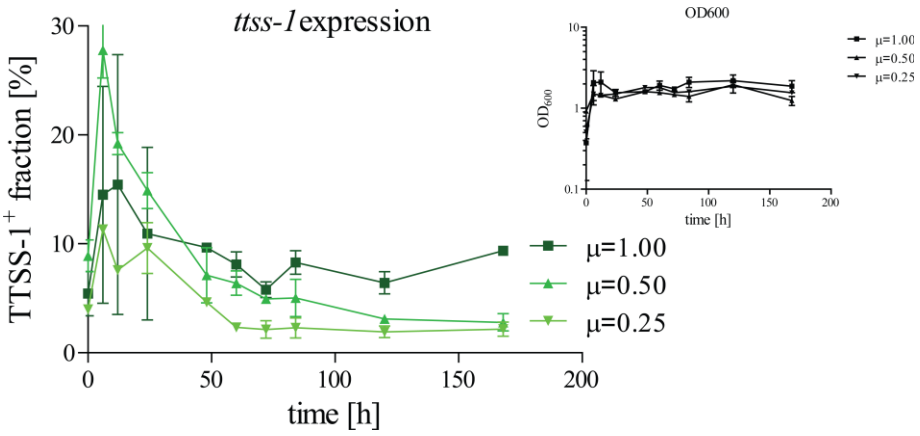
Suppl. Figure 1, Proteome analysis of *S.* Tm TTSS-1$^+$ and TTSS-1$^-$ subpopulations. A late logarithmic phase culture of *S.* Tm pM972 constituted of a mixture of TTSS-1$^+$ and TTSS-1$^-$ fractions expressed according to *ttss-1 gfp* and were separated by FACS analysis. Both fractions were analyzed by mass spectrometry and the peak intensities for each identified protein were related to each other. The graphic depicts the log10 fold change of single hits in the TTSS-1$^+$ compared to the TTSS-1$^-$ fraction. Approximately 1000 proteins could be identified. Suppl. Table 1 depicts the 50 most enriched and the 50 most depleted proteins in the TTSS-1$^+$ fraction.



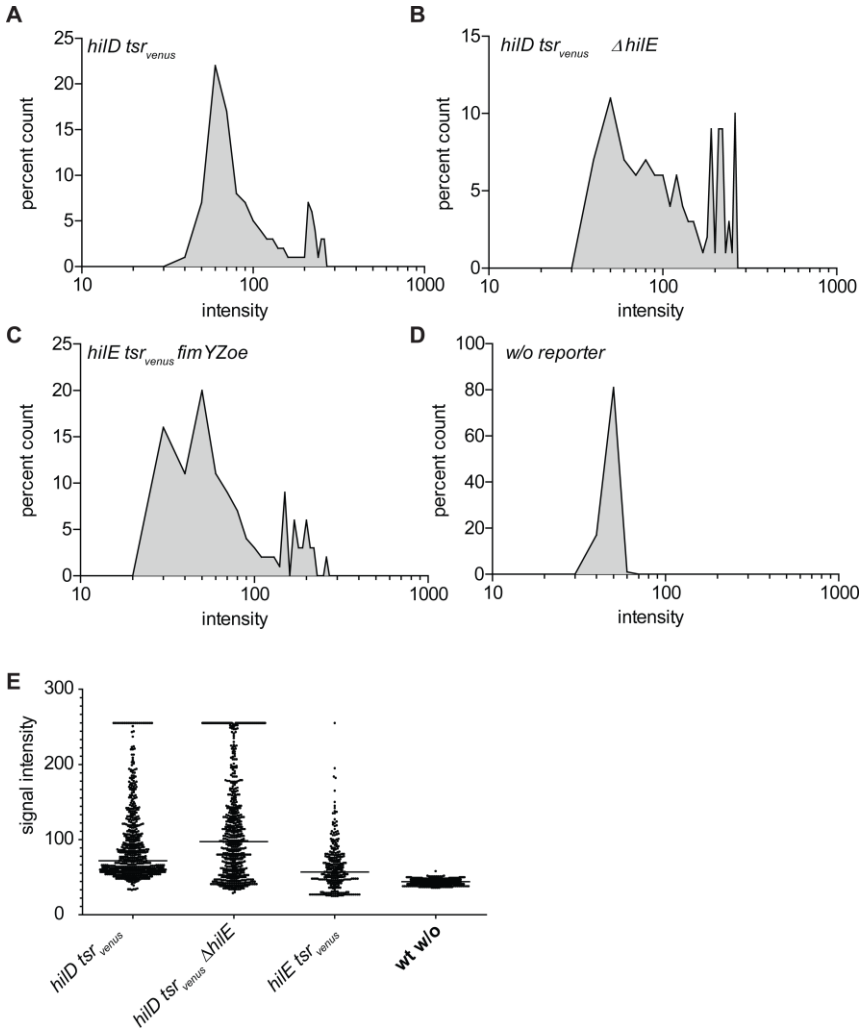
Suppl. Figure 2, Sigma factor RpoE affects expression of *ttss-1* genes. A) Overexpression of *rpoE* and B) deletion of the *rpoE* gene; both strains possessed the tsr_{venus} reporter as an indicator for *ttss-1* expression (see also chapter 2, suppl. Table 1). Western blots show the expression of HilA and SipC. C) Invasiveness of an *rpoE* mutant is decreased. All experiments show that *rpoE* is necessary for the proper expression of virulence genes. In A) overexpression of *rpoE* probably implies the overexpression of the anti-sigma factor leading to the high variance between single experiments. For the experiments the median is depicted. All experiments were performed at least 4 times.



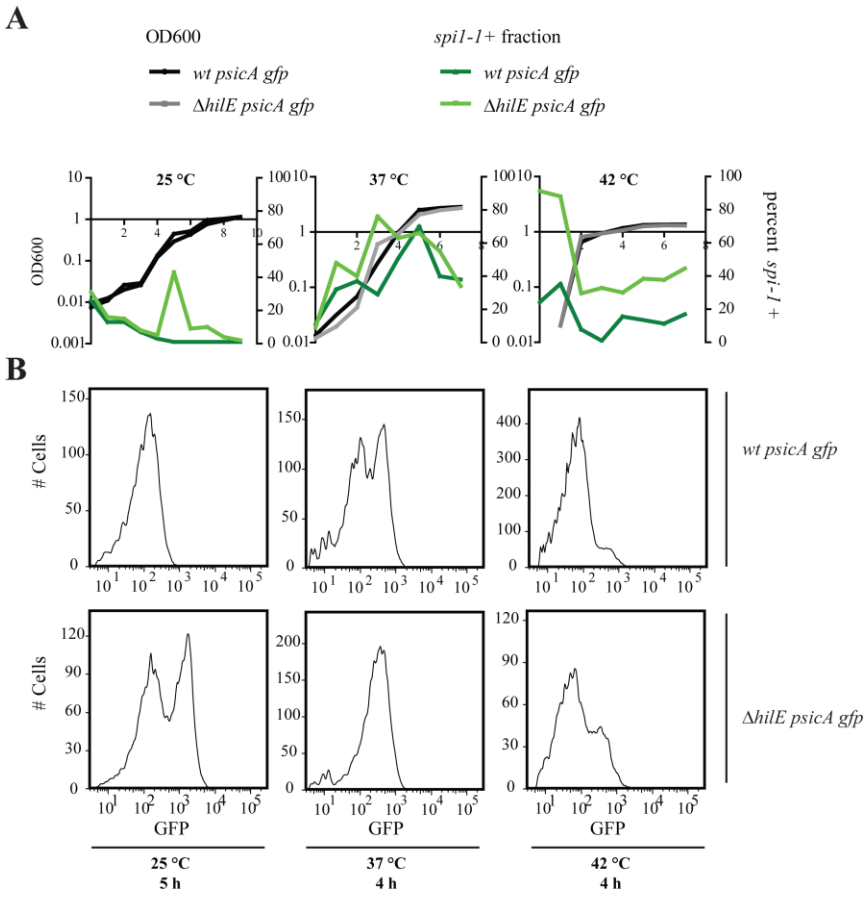
Suppl. Figure 3, *S*. Tm always splits in TTSS-1$^+$ and TTSS-1$^-$ subpopulations. Bacteria were cultured in LB and samples were withdrawn at different growth phases resulting in different fractions of TTSS-1$^+$ individuals (13 %, 43 % and 62 %), which has been determined by FACS. Samples were afterwards diluted and distributed into different wells of a 96 well plate so that each well harbored either one or no bacterium. I.e. in each inoculated well, the inoculum consisted either of TTSS-1$^+$ or TTSS-1$^-$ cells. A single bacterium served thereby as a starter for a new culture which was again analyzed by FACS after reaching the stationary phase. At least three 96 well plates were analyzed per initial ratio. In fact, all wells consisted of populations that displayed bistability of the TTSS-1 ranging from 10 % TTSS-1$^+$ to 80 % TTSS-1$^+$ bacteria per well. Interestingly, the distribution differs depending on the initial inoculum. Individuals of a population of initially 13 % TTSS-1$^+$ tend to evolve more individuals that are TTSS-1$^+$ compared to individuals of an initial 62 % TTSS-1$^+$ population.



Suppl. Figure 4, Slower growth does not favour *ttss-1* expression. *S.* Tm harbouring pM972 (see chapter 2 suppl. Table 2) were cultured in LB in a chemostate for several days. After approximately two days a steady state was reached (see OD_{600} and *ttss-1* expression) *S.* Tm were either grown with a generation time of μ = 1 h^{-1}, 0.5 h^{-1} or 0.25 h^{-1}. Different from our expectations, the slower growing culture (μ = 0.25 h^{-1}) were not constituted of a higher fraction of TTSS-1^+ cells. Instead, all chemostat conditions analyzed yielded reduced fractions of TTSS-1^+ bacteria compared to LB batch cultures (see chapter 2, Fig. 3).



Suppl. Figure 5, Getting closer to the origin of SPI-1 bistability. *S.* Tm were constituted with a chromosomal fusion of tsr_{venus} that either monitors expression of *hilD* in wt or in a Δ*hilE* mutant (A, B) or *hilE* expression (C). Microscopy images of *S*. Tm deriving from late logarithmic phase cultures were taken and analyzed by Volocity software to elucidate the intensity of the reporter. A wt control was included that did not harbor any reporter (D). Intensities and the median for single bacteria were plotted in E), histograms of intensity are depicted in A - D). Both reporters display a distribution of the expression state of the respective operons from either fully OFF to fully ON. It is intricate to extract bistability from these data for *hilD* and *hilE* but differences between single individuals become obvious. However, a Gaussian distribution as it would be expected for a uniformly expressing bacterial population cannot be observed. The lack of *hilE* enhances *hilD* transcription remarkably (compare A and B). This is line with the earlier observation that HilD is sequestered by HilE (6). In this case, our data suggest that there are three different subpopulations ON, OFF and intermediate.



Suppl. Figure 6, Temperature dependent expression of the *sicA* promoter in *S*. Tm wt and Δ*hilE* monitored by p*sicA gfp* (see Chapter 2 suppl. Table 2). *S*. Tm strains were cultured aerobically in LB at indicated temperatures. Samples were withdrawn at indicated time points and processed to measure reporter intensityby FACS. Representative histograms of *gfp* expression are depicted in B). Interestingly, wt displays no expression at 25 °C, whereas expression is observed in the absence of *hilE*. In general, all temperatures display higher TTSS-1^{+} fraction when *hilE* is deleted. At 37 °C bistability is no longer observed. The data suggest that temperature control is rather mediated by differing induction of activators than by the action of *hilE*. Reporter constructs for *hilE* and e.g. *hilD* should lead to further insights.

Supplementary Table 1: Proteomic Analysis of TTSS-1$^+$ and TTSS-1$^-$ subpopulations. The table displays the 50 proteins most strongly enriched (1 to 50) and the 50 nost depleted (-50 to -1) elucidated by the proteomic analysis by A. Schmidt (IMSB, ETH Zurich). Fold change depicts how more often the protein is found in the TTSS-1$^+$ than in the TTSS-1$^-$ fraction.

	Accession number	fold change	p-value	description
1	sp|O30916|SOPB_SALTY	4.18	0	Inositol phosphate phosphatase sopB OS=Salmonella typhimurium GN=sopB
2	sp|P41784|PRGI_SALTY	3.96	0	Protein prgI OS=Salmonella typhimurium GN=prgI
3	sp|Q56019|SIPB_SALTY	3.49	0	Cell invasion protein sipB OS=Salmonella typhimurium GN=sipB
4	sp|P06179|FLIC_SALTY	3.01	0	Flagellin OS=Salmonella typhimurium GN=fliC
5	sp|P37423|INVH_SALTY	2.41	0.02	Invasion lipoprotein invH OS=Salmonella typhimurium GN=invH
6	sp|P69066|SICA_SALTY	2.33	0.01	Chaperone protein sicA OS=Salmonella typhimurium GN=sicA
7	tr|Q8ZM72|Q8ZM72_SALTY	2.12	0.05	Proline aminopeptidase P II OS=Salmonella typhimurium GN=pepP
8	tr|Q8ZM22|Q8ZM22_SALTY	2.01	0	Putative methyl-accepting chemotaxis protein OS=Salmonella typhimurium GN=STM3138
9	sp|Q8ZRE7|YAIE_SALTY	1.7	0.03	UPF0345 protein yaiE OS=Salmonella typhimurium GN=yaiE
10	sp|P0A1N0|SPAK_SALTY	1.63	0.02	Surface presentation of antigens protein spaK OS=Salmonella typhimurium GN=spaK
11	sp|P66593|RS6_SALTY	1.63	0.16	30S ribosomal protein S6 OS=Salmonella typhimurium GN=rpsF
12	tr|Q7CQ13|Q7CQ13_SALTY	1.62	0.04	[2FE-2S] ferredoxin OS=Salmonella typhimurium GN=fdx
13	tr|Q8ZLT4|Q8ZLT4_SALTY	1.62	0.11	Cysteine sulfinate desulfinase OS=Salmonella typhimurium GN=deaD
14	tr|Q8ZLV8|Q8ZLV8_SALTY	1.6	0.14	Tartronate semialdehyde reductase (TSAR) OS=Salmonella typhimurium GN=garR
15	sp|Q56020|SIPC_SALTY	1.59	0.3	Cell invasion protein sipC OS=Salmonella typhimurium GN=sipC
16	sp|Q7CQN4|LPP1_SALTY	1.58	0.14	Major outer membrane lipoprotein 1 OS=Salmonella typhimurium GN=lpp1
17	sp|P0A7N6|RL32_SALTY	1.57	0.18	50S ribosomal protein L32 OS=Salmonella typhimurium GN=rpmF
18	sp|Q8ZJV7|DEOD_SALTY	1.56	0.03	Purine nucleoside phosphorylase deoD-type OS=Salmonella typhimurium GN=deoD
19	tr|Q8ZMW7|Q8ZMW7_SALTY	1.56	0.36	Chorismate mutase P OS=Salmonella typhimurium GN=pheA
20	tr|Q7CR26|Q7CR26_SALTY	1.55	0.02	Cytochrome o ubiquinol oxidase subunit II OS=Salmonella typhimurium GN=cyoA
21	sp|P0A1J7|FLGN_SALTY	1.5	0.16	Flagella synthesis protein flgN OS=Salmonella typhimurium GN=flgN
22	sp|P33901|NUOF_SALTY	1.5	0	NADH-quinone oxidoreductase subunit F OS=Salmonella typhimurium GN=nuoF
23	sp|O85139|FABH_SALTY	1.49	0.01	3-oxoacyl-[acyl-carrier-protein] synthase 3 OS=Salmonella typhimurium GN=fabH
24	tr|Q8ZRA5|Q8ZRA5_SALTY	1.49	0.07	Putative small-conductance mechanosensitive channel OS=Salmonella typhimurium GN=aefA
25	sp|P66451|RS17_SALTY	1.49	0.17	30S ribosomal protein S17 OS=Salmonella typhimurium GN=rpsQ
26	sp|P63601|AROK_SALTY	1.48	0.04	Shikimate kinase 1 OS=Salmonella typhimurium GN=aroK
27	tr|Q8ZLJ1|Q8ZLJ1_SALTY	1.48	0.04	Putative RNase R OS=Salmonella typhimurium GN=yhgF
28	sp|P0A1G5|DKSA_SALTY	1.47	0.07	DnaK suppressor protein OS=Salmonella typhimurium GN=dksA
29	sp|P08870|PYRE_SALTY	1.47	0.05	Orotate phosphoribosyltransferase OS=Salmonella typhimurium GN=pyrE
30	tr|Q8ZNJ3|Q8ZNJ3_SALTY	1.46	0.03	Acetylation of the O-antigen (LPS) OS=Salmonella typhimurium GN=oafA
31	sp|P66032|RIBB_SALTY	1.44	0.24	3,4-dihydroxy-2-butanone 4-phosphate synthase OS=Salmonella typhimurium GN=ribB
32	sp|P66409|RS14_SALTY	1.44	0.12	30S ribosomal protein S14 OS=Salmonella typhimurium GN=rpsN
33	sp|P0A9Y2|CSPA_SALTY	1.43	0	Cold shock protein cspA OS=Salmonella typhimurium GN=cspA
34	sp|P43015|HILA_SALTY	1.43	0.03	Transcriptional regulator hilA OS=Salmonella typhimurium GN=hilA
35	tr|Q8ZLX9|Q8ZLX9_SALTY	1.43	0.36	Putative methyl-accepting chemotaxis protein OS=Salmonella typhimurium GN=STM3216
36	sp|P0A2A9|RS16_SALTY	1.42	0.01	30S ribosomal protein S16 OS=Salmonella typhimurium GN=rpsP
37	tr|Q7CQ00|Q7CQ00_SALTY	1.42	0.17	Ribosome associated factor OS=Salmonella typhimurium GN=yfiA

38	tr|Q7CPU3|Q7CPU3_SALTY	1.41	0.71	Putative Zn-dependent proteases with possible chaperone function OS=Salmonella typhimurium GN=yggG
39	sp|P0A2D5|CHEY_SALTY	1.41	0.13	Chemotaxis protein cheY OS=Salmonella typhimurium GN=cheY
40	sp|P67091|USPF_SALTY	1.41	0.18	Universal stress protein F OS=Salmonella typhimurium GN=uspF
41	sp|Q8ZL03|IBPB_SALTY	1.41	0.14	Small heat shock protein ibpB OS=Salmonella typhimurium GN=ibpB
42	tr|Q7CPH6|Q7CPH6_SALTY	1.41	0.01	Putative rhodanese-related sulfurtransferases OS=Salmonella typhimurium GN=yibN
43	sp|Q8ZPL9|ADD_SALTY	1.39	0.01	Adenosine deaminase OS=Salmonella typhimurium GN=add
44	sp|P0A1Q2|LGUL_SALTY	1.39	0.28	Lactoylglutathione lyase OS=Salmonella typhimurium GN=gloA
45	tr|Q7CQW9|Q7CQW9_SALTY	1.39	0.1	Tol protein required for outer membrane integrity OS=Salmonella typhimurium GN=pal
46	sp|Q56078|BGLX_SALTY	1.39	0.4	Periplasmic beta-glucosidase OS=Salmonella typhimurium GN=bglX
47	sp|Q9L6L5|FADB_SALTY	1.39	0.01	Fatty acid oxidation complex subunit alpha OS=Salmonella typhimurium GN=fadB
48	sp|P15934|FLIH_SALTY	1.38	0.71	Flagellar assembly protein fliH OS=Salmonella typhimurium GN=fliH
49	tr|Q8ZM07|Q8ZM07_SALTY	1.37	0.04	Putative alcohol dehydrogenase OS=Salmonella typhimurium GN=yqhD
50	tr|Q8ZQD0|Q8ZQD0_SALTY	1.36	0.64	Pyruvate formate lyase activating enzyme 1 OS=Salmonella typhimurium GN=pflA
51	sp|P0A1H7|EXBB_SALTY	1.35	0.48	Biopolymer transport protein exbB OS=Salmonella typhimurium GN=exbB

-51	tr|Q93GL9|Q93GL9_SALTY	0.71	0.19	Conjugative transfer: surface exclusion OS=Salmonella typhimurium GN=traT
-50	sp|P0A1W4|LEPA_SALTY	0.7	0.15	GTP-binding protein lepA OS=Salmonella typhimurium GN=lepA
-49	sp|P74883|PUR5_SALTY	0.7	0.38	Phosphoribosylformylglycinamidine cyclo-ligase OS=Salmonella typhimurium GN=purM
-48	sp|O86090|PPK_SALTY	0.69	0.05	Polyphosphate kinase OS=Salmonella typhimurium GN=ppk
-47	sp|Q8ZPL7|FUMC_SALTY	0.69	0.56	Fumarate hydratase class II OS=Salmonella typhimurium GN=fumC
-46	tr|Q8ZP45|Q8ZP45_SALTY	0.69	0	Iron-dependent alcohol dehydrogenase of the multifunctional alcohol dehydrogenase AdhE OS=Salmonella typhimurium GN=adhE
-45	sp|Q8ZQP0|YBHG_SALTY	0.68	0.01	UPF0194 membrane protein ybhG OS=Salmonella typhimurium GN=ybhG
-44	sp|P05416|GLGA_SALTY	0.68	0.07	Glycogen synthase OS=Salmonella typhimurium GN=glgA
-43	sp|P26478|MALM_SALTY	0.67	0.04	Maltose operon periplasmic protein OS=Salmonella typhimurium GN=malM
-42	sp|P63223|GMHA_SALTY	0.67	0.06	Phosphoheptose isomerase OS=Salmonella typhimurium GN=gmhA
-41	sp|Q8ZPV2|ASTC_SALTY	0.66	0.05	Succinylornithine transaminase OS=Salmonella typhimurium GN=astC
-40	tr|Q7CPV8|Q7CPV8_SALTY	0.66	0.11	Putative lipoprotein OS=Salmonella typhimurium GN=ygdI
-39	tr|Q7CR49|Q7CR49_SALTY	0.65	0.04	Putative periplasmic protein OS=Salmonella typhimurium GN=yahO
-38	sp|Q7CQJ7|YNFB_SALTY	0.65	0.63	UPF0482 protein ynfB OS=Salmonella typhimurium GN=ynfB
-37	tr|Q7CQ14|Q7CQ14_SALTY	0.65	0.73	Putative uncharacterized protein yfhJ OS=Salmonella typhimurium GN=yfhJ
-36	tr|Q8ZP52|Q8ZP52_SALTY	0.65	0.01	Aconitate hydratase 1 OS=Salmonella typhimurium GN=acnA
-35	tr|Q8ZL72|Q8ZL72_SALTY	0.64	0.01	Aldehyde dehydrogenase B OS=Salmonella typhimurium GN=aldB
-34	tr|Q8ZP02|Q8ZP02_SALTY	0.64	0.01	Mannose-specific enzyme IID OS=Salmonella typhimurium GN=manZ
-33	sp|P36557|BASS_SALTY	0.64	0.43	Sensor protein basS\pmrB OS=Salmonella typhimurium GN=pmrB
-32	sp|Q7CPK0|UGPB_SALTY	0.62	0.14	sn-glycerol-3-phosphate-binding periplasmic protein ugpB OS=Salmonella typhimurium GN=ugpB
-31	sp|Q7CQJ0|SRA_SALTY	0.62	0.03	Stationary-phase-induced ribosome-associated protein OS=Salmonella typhimurium GN=sra
-30	sp|Q7CPB2|YJBJ_SALTY	0.62	0.02	UPF0337 protein yjbJ OS=Salmonella typhimurium GN=yjbJ
-29	tr|Q7CQP3|Q7CQP3_SALTY	0.62	0	Transcriptional activator of ntrL gene OS=Salmonella typhimurium GN=osmE
-28	tr|Q8ZQF0|Q8ZQF0_SALTY	0.61	0.01	Pyruvate dehydrogenase\oxidase FAD and thiamine PPi cofactors, cytoplasmic in absence of cofactors OS=Salmonella typhimurium GN=poxB
-27	sp|P0A2N6|ZIPA_SALTY	0.6	0.17	Cell division protein zipA homolog OS=Salmonella typhimurium GN=zipA
-26	tr|Q8ZLA9|Q8ZLA9_SALTY	0.58	0	Dipeptide transport protein OS=Salmonella typhimurium GN=dppA
-25	tr|Q8ZP03|Q8ZP03_SALTY	0.58	0	Mannose-specific enzyme IIAB OS=Salmonella typhimurium GN=manX
-24	sp|P69917|CSRA_SALTY	0.57	0.33	Carbon storage regulator OS=Salmonella typhimurium GN=csrA

-23	sp|P0A1D7|CLPP_SALTY	0.56	0.89	ATP-dependent Clp protease proteolytic subunit OS=Salmonella typhimurium GN=clpP
-22	sp|Q8ZQ40|WRBA_SALTY	0.55	0	Flavoprotein wrbA OS=Salmonella typhimurium GN=wrbA
-21	sp|P19576|MALE_SALTY	0.54	0	Maltose-binding periplasmic protein OS=Salmonella typhimurium GN=malE
-20	sp|P68684|RS21_SALTY	0.53	0.06	30S ribosomal protein S21 OS=Salmonella typhimurium GN=rpsU
-19	tr|Q7CQI9|Q7CQI9_SALTY	0.53	0	Alcohol dehydrogenase OS=Salmonella typhimurium GN=adhP
-18	sp|Q8ZMK6|SYA_SALTY	0.5	0.05	Alanyl-tRNA synthetase OS=Salmonella typhimurium GN=alaS
-17	tr|Q7CPR3|Q7CPR3_SALTY	0.5	0.19	Putative inner membrane protein OS=Salmonella typhimurium GN=yqjD
-16	sp|O68926|BFR_SALTY	0.5	0	Bacterioferritin OS=Salmonella typhimurium GN=bfr
-15	tr|Q8ZML9|Q8ZML9_SALTY	0.48	0.01	Putative LysM domain OS=Salmonella typhimurium GN=ygaU
-14	tr|Q8ZML4|Q8ZML4_SALTY	0.48	0.1	Putative inner membrane protein OS=Salmonella typhimurium GN=ygaM
-13	tr|Q8ZRB1|Q8ZRB1_SALTY	0.48	0.04	Glycoprotein\polysaccharide metabolism OS=Salmonella typhimurium GN=ybaY
-12	tr|Q8ZPD4|Q8ZPD4_SALTY	0.46	0	Putative NADP-dependent oxidoreductase OS=Salmonella typhimurium GN=yncB
-11	tr|Q8ZN82|Q8ZN82_SALTY	0.44	0	Transketolase 2 isozyme OS=Salmonella typhimurium GN=tktB
-10	sp|Q8ZP20|TREA_SALTY	0.43	0	Periplasmic trehalase OS=Salmonella typhimurium GN=treA
-9	tr|Q7CQF8|Q7CQF8_SALTY	0.41	0	Putative cytoplasmic protein (YciE protein) OS=Salmonella typhimurium GN=yciE
-8	sp|P0A2K5|UBIE_SALTY	0.4	0	Ubiquinone\menaquinone biosynthesis methyltransferase ubiE OS=Salmonella typhimurium GN=ubiE
-7	tr|Q8ZJZ3|Q8ZJZ3_SALTY	0.39	0	Putative NAD-dependent aldehyde dehydrogenase OS=Salmonella typhimurium GN=STM4519
-6	tr|Q8ZM09|Q8ZM09_SALTY	0.38	0	Putative oxidoreductase OS=Salmonella typhimurium GN=yghA
-5	tr|Q7CPH3|Q7CPH3_SALTY	0.28	0	Putative stress-induced protein OS=Salmonella typhimurium GN=yicC
-4	tr|Q8ZM41|Q8ZM41_SALTY	0.27	0.03	Putative cytoplasmic protein OS=Salmonella typhimurium GN=yggL
-3	tr|Q7CQF9|Q7CQF9_SALTY	0.25	0.02	Putative cytoplasmic protein (Putative uncharacterized protein putative yciF) OS=Salmonella typhimurium GN=yciF (ferritin related)
-2	tr|Q9KWV1|Q9KWV1_SALTY	0.22	0	Putative catalase OS=Salmonella typhimurium GN=STM1731
-1	tr|Q8ZME2|Q8ZME2_SALTY	0.07	0.03	(P)ppGpp synthetase I OS=Salmonella typhimurium GN=relA

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Curriculum vitae

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Publications

Bailly-Bechet, M., A. Benecke, W. D. Hardt, V. Lanza, A. Sturm, and R. Zecchina. 2010. An externally modulated, noise-driven switch for the regulation of SPI1 in Salmonella enterica serovar Typhimurium. J Math Biol.

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Educational Background

Since Sept. 2006	PhD Thesis	Institute of Immunology and Microbiology, ETH (Swiss Federal Institute of Technology) Zurich, Switzerland Supervision: Prof. W.D. Hardt
Okt. 2005 - Sept. 2006	Diploma Thesis	"The Tat dependent transport of YcdB", Institute of Microbiology, Martin-Luther-University Halle-Wittenberg, Germany Supervision: Prof. J.R. Andreesen and Dr. T. Brüser
Okt. 2001 - Sept. 2006	Studies in Biology	Martin-Luther University Halle-Wittenberg, Germany with emphasis in Microbiology, Genetics, Biochemistry and Biotechnology
May/June 2001	Abitur	Fritz Löffler Gymnasium, Dresden, Germany
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I am particularly grateful to my supervisor Prof. Wolf-Dietrich Hardt, who has given me the chance to work in his group for the last 55 months. He was always a source of inspiration and prudent providence. Literally spoken, he was constantly bubbling over with ideas that either contributed to a project or paved the way to a new one (which sometimes exceeded my schedule and led to several heaps of papers on my desk). I really enjoyed the discussions with unpredictable outcomes and appreciated that my previous convictions have more than once been disabused. However, I think, I learned a lot how to approach projects, how to solve problems and to better manage myself (even though I am convinced that a genius needs space and chaos to proliferate).

Of course, there are several other people of the group I have to be grateful to. First of all, I want to say thanks to the people that have already been here and gave me a warm welcome when I arrived: Andreas, Annette, Bärbel, Ben, Brit, Jay, Markus and Matthias. It was great to experience some of Zurich's most famous bars with Andreas right at the beginning of my PhD till his escape to Paris. He was moreover a good companion in terms of cultural discussion and to develop our intellectual snobbism that contrast us pleasantly from the general public. And here I would like to mention Patrick, who was also fond of cultural or political discussion dealing with politics, literature and music, which make the difference.

When I started, the group was in a - let's say - process of propagation. With me several other PhD students were continuing their scientific career at Wolf's lab: Kathrin, Sabrina, Pascal and some month later Rina and Bala. Also Claudia started as a Postdoc as well as Manja came back from her maternity leave. With the most of you I spend an awesome period of my life; even if there have been ups and downs with regard on e.g. how to discard glass and needles properly (and separately). I am especially thankful to Manja who made a great job in keeping the lab running and bringing sometimes good old Eastern German specialties back from home.

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Within four years remarkable changes can happen. So there was a huge turnover in our lab in 2009 and 2010, many people left but many others also decided to join the lab. I was very happy to get to know in the s*econd round* of PhD students, Postdocs and Practical students like Saskia, Lisa, Carmen, Pascale, Naomi, Sabine, Jette and Médéric who shares interest in probably the best scientific topic ever. I appreciated that our group became with this more international and enabled me to gain more and more insights in different aspects of people with different cultural background. I realized that we shared similar types of humor. That was a great experience as well as my personal discovery of the Swiss Alps within these years.

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