Expanding the targetome of *Salmonella* small RNA PinT using MS2 affinity purification and RNA-Seq (MAPS)

Erweiterung des Targetoms der kleinen RNA PinT von *Salmonella* mittels MS2-Affinitätsaufreinigung und RNA-seq (MAPS)

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Dedicated to Snigdhadip Dey.

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Summary

Bacterial small RNAs are key mediators of post-transcriptional gene regulation. An increasing number of sRNAs have been implicated in the regulation of virulence programs of pathogenic bacteria. Recently, in the enteric pathogen *Salmonella* Typhimurium, the PinT sRNA has gained increased importance as it is the most upregulated sRNA as *Salmonella* infects mammalian host cells (Westermann *et al.*, 2016). PinT acts as a temporal regulator of *Salmonella*'s two major pathogenicity islands, SPI-1 and SPI-2 (Kim *et al.*, 2019; Westermann *et al.*, 2016). However, the complete set of PinT targets, its role in *Salmonella* infection and host response is not yet fully understood. Building on the MS2 affinity purification and RNA-seq (MAPS) method (Lalaouna *et al.*, 2015), we here set out to globally identify direct RNA ligands of PinT, relevant to *Salmonella* infection. We transferred the classical MAPS technique, based on sRNA-bait overexpression, to more physiological conditions, using endogenous levels of the sRNA. Making the henceforth identified targets, less likely to represent artefacts of the overexpression. More importantly, we progressed the MAPS technique to *in vivo* settings and by doing so, we were able pull-down bacterial RNA transcripts bound by PinT during macrophage infection. While we validate previously known PinT targets, our integrated data revealed novel virulence relevant target. These included mRNAs for the SPI-2 effector SteC, the PhoQ activator UgtL and the 30S ribosomal protein S22 RpsV. Next, we follow up on SteC, the best characterized virulence relevant PinT target. Using genetic and biochemical assays, we demonstrate that PinT represses *steC* mRNA by direct base-pairing and translational interference. PinT-mediated regulation of SteC leads to alterations in the host response to *Salmonella* infection. This regulation impacts the cytokine response of infected macrophages, by altering IL10 production, and possibly driving the macrophages to an anti-inflammatory state, more permise to infection. SteC is responsible for F-actin meshwork rearrangements around the SCV (Poh *et al.*, 2008). Here we demonstrate that PinT-mediated regulation of SteC, impacts the formation of this actin meshwork in infected cells. Our results demonstrate that SteC expression is very tightly regulated by PinT in two layers; indirectly, by repressing *ssrB* and *crp*; and directly by binding to *steC* 5'UTR. PinT contributes to post-transcriptional cross-talk between invasion and intracellular replication programs of *Salmonella*, by controlling the expression of both SPI-1 and SPI-2 genes (directly and indirectly). Together, our collective data makes PinT the first

sRNA in Gram-negatives with a pervasive role in virulence, at the center of *Salmonella* virulence programs and provide molecular input that could help explain the attenuation of *pin*T-deficient *Salmonella* strains in whole animal models of infection.

Zusammenfassung

Kleine RNAs sind zentrale Stellschrauben der posttranskriptionellen Genregulation in Bakterien. Eine zunehmende Anzahl von sRNAs ist an der Regulation von Virulenzprogrammen pathogener Bakterien beteiligt. In jüngster Zeit hat beim enterischen Erreger *Salmonella* Typhimurium die PinT-sRNA an Bedeutung gewonnen, da sie die am stärksten hochregulierte sRNA während der Infektion von Säugetierwirtszellen ist (Westermann *et al.*, 2016). PinT fungiert als zeitlicher Regulator der beiden wichtigsten Pathogenitätsinseln von *Salmonella*, SPI-1 und SPI-2 (Kim *et al.*, 2019a; Westermann *et al.*, 2016). Die vollständige Liste der Targets von PinT und die Rolle von PinT bei der *Salmonella*-Infektion sowie der Wirstantwort sind jedoch noch nicht vollständig aufgeklärt. Mit Hilfe der MS2 affinity purification and RNA-seq (MAPS)-Methode (Lalaouna *et al.*, 2015) möchten wir hier direkte RNA-Liganden von PinT identifizieren, die für die *Salmonella*-Infektion relevant sind. Wir übertragen die klassische MAPS-Technik, die auf der Überexpression von sRNA-Baits basiert, auf physiologischere Bedingungen unter Verwendung endogener Mengen der sRNA. Dadurch wird die Wahrschienlichkeit, dass die identifizierten Targets Artefakte sind, verringert. Darüber hinaus sind wir in der Lage, die MAPS-Technik unter in vivo-Bedingungen durchzuführen. Auf diese Weise konnten wie bakterielle Transkripte, die während einer Makrophageninfektion an PinT gebunden wurden, isolieren. Während wir bereits bekannte PinT-Ziele validieren, identifizieren unsere integrierten Daten ein neues Target, das für Virulenz relevant ist. Dazu gehörten mRNAs für den SPI-2-Effektor SteC, den PhoQ-Aktivator UgtL und das ribosomale 30S-Protein S22 RpsV. Zunächst untersuchen wir SteC, das am besten charakterisierte virulenzrelevante PinT-Ziel. Anhand genetischer und biochemischer Assays zeigen wir, dass PinT die *steC*-mRNA durch direkte Basenpaarung und Translationsrepression reguliert. Die PinT-vermittelte Regulation von SteC führt zu einer veränderten Wirtsreaktion auf eine *Salmonella*-Infektion. Diese Regulation beeinflusst die Zytokinreaktion infizierter Makrophagen, indem sie die IL10-Produktion verändert und die Makrophagen möglicherweise in einen entzündungshemmenden Zustand versetzt, der sie anfälliger für eine Infektion macht. SteC ist verantwortlich für die Umlagerung von F-Actin-Netzen um die SCV (Poh *et al.*, 2008). Hier zeigen wir, dass die PinT-vermittelte Regulation von SteC die Bildung dieses Aktin-Netzwerks in infizierten Zellen beeinflusst. Unsere Ergebnisse zeigen, dass die Regulation der SteC-Expression durch PinT auf zwei

Ebenden stattfindet: indirekt durch Unterdrückung von *ssrB* und *crp*; und direkt durch Bindung an *steC* 5'UTR. PinT trägt zum posttranskriptionellen Crosstalk zwischen Invasions- und intrazellulären Replikationsprogrammen von *Salmonella* bei, indem die Expression von SPI-1- und SPI-2-Genen (direkt und indirekt) gesteuert wird. Insgesamt macht unterstreichen unsere Daten die zentrale Rolle von PinT in Virulenzprogrammen von *Salmonella*. PinT ist die erste sRNA in Gram-Negativen mit einer derart durchdringenden Rolle bei der Virulenz. Zudem liefern unsere Ergebnisse Einblick auf molekularer Ebene, die die Attenuation von PinT-defizienten *Salmonella*-Stämmen in Tiermodellen erklären könnte.

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Abbreviation index

CLIP-seq - Cross-linking immunoprecipitation-high-throughput sequencing

CLASH - Crosslinking, ligation, and sequencing of hybrids

EMSA - Electrophoretic mobility shift assays

GFP – Green fluorescent protein

GRIL-seq - Global sRNA target identification by ligation and sequencing

GREF - Guanine nucleotide exchange factors

IEC - Intestinal epithelial cells

IFN - Interferon

IL – Interleukin

K_D - Dissociation constant

LPS - Lipopolysaccharide

MAPK - Mitogen-activated protein kinase

MAPS - MS2-affinity purification coupled with RNA-seq

M cells - Microfold cells

NAIP - NLR family apoptosis inhibitory protein

NF-κB - Nuclear factor kappa B

NLRs - NOD-like receptors

NTS – Non-typhoidal *Salmonella*

ORF - Open reading frame

PAMP - Pathogen-associated molecular pattern

PRRs - Pattern recognition receptors

RBP - RNA binding protein

RBS – Ribosome binding site

RIL-seq - RNA interaction by ligation and sequencing

RIP-seq - RNA immunoprecipitation followed by RNA-seq

RNA - Ribonucleic acid

ROS – Reactive oxygen species

SCV - *Salmonella* containing vacuole

sRNA - small RNA

Socs3- Suppressor of cytokine signaling 3

SPI-1 - *Salmonella* pathogenicity island 1

SPI-2 - *Salmonella* pathogenicity island 2

T3SS - Type 3 secretion system

TLRs - Toll-like receptors

TSS – Transcriptional start site

UTRs - Untranslated regions

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1. Introduction

1.1. The model pathogen *Salmonella enterica* serovar Typhimurium

Salmonella are motile, Gram-negative enterobacteria that can invade a broad range of hosts causing both acute and chronic infections. There are two *Salmonella* species, *Salmonella bongori* and *Salmonella enterica*, with the later including over 2500 different serovars identified to date. These are classified on the basis of their flagellar and lipopolysaccharide (LPS) antigens, and include both typhoidal and non-typhoidal *Salmonella* (NTS) strains.

All *Salmonella* serotypes share the ability to invade the host by inducing their own uptake into cells of the intestinal epithelium (Ohl and Miller, 2001). Infection with *S. enterica* can lead to a broad range of clinical symptoms and can result in asymptomatic carriage, gastroenteritis, systemic disease such as typhoid fever and in severe cases, death; depending on the serovar (Hurley *et al.*, 2014). The different serovars have different abilities to cause disease. While all serotypes can cause disease in humans, a few are host-specific and can reside in only one or a few animal species. For example, the so called typhoidal *Salmonella*, which include *S. enterica* serovar Typhi (*S.* Typhi) or *S.* Paratyphi are restricted to human hosts and can lead to lethal typhoid fever. The NTS *S. enterica* subsp. enterica serovar Enteritidis and *S. enterica* subsp. enterica serovar Typhimurium, have a broader host range including poultry, swine and cattle, and infected humans, causing a self-limiting gastroenteritis. Typically, these serotypes cause gastroenteritis, which is often uncomplicated and does not need treatment, but can cause bacteraemia and systemic infection in immunosuppressed hosts, young children and the elderly. Children are the most likely group of individuals to present salmonellosis. The rate of diagnosed infections in children <5 years old is higher than the rate diagnosed in all other persons. Other groups of risk, such as the elderly and immunocompromised individuals are the most likely to present severe forms of the disease (Hurley *et al.*, 2014).

Salmonella spp. typically colonize orally through the ingestion of contaminated food or water and can survive gastric acidity to gain access to the intestinal epithelium. The infection is localized to the ileum, colon and mesenteric lymph nodes and commonly manifests within 12–72 h after ingestion. NTS can elicit inflammatory changes in the intestinal epithelium, including the infiltration of neutrophils and fluid into the intestinal lumen, resulting in inflammatory diarrhea. This inflammatory response is essential for the release of factors, such

as tetrathionate, that can be used as nutrient sources and help *Salmonella* outcompete local microbiota (LaRock *et al.*, 2015).

Salmonella Typhimurium is a facultative intracellular pathogen that has evolved several strategies to invade the gut epithelial barrier. It can invade intestinal epithelial cells (IEC), M cells (or microfold cells), by breaching the tight-junctions to reach the lamina propria or by being captured by dendritic cells (Watson and Holden, 2010). *Salmonella* promotes internalization by IEC (Finlay and Falkow, 1990) , by inducing extensive, although transient, modifications into the host cytoskeleton. After adhering to the cell surface, it can disrupt the epithelial border and induce membrane ruffling that results in engulfment (Ly and Casanova, 2007). Nevertheless, there is a preference for M cells, that overlay intestinal lymphoid tissues known as Peyer's patches. M cells are specialized epithelial cells that phagocytose molecules in the intestinal lumen for transepithelial transport to enable immunological sampling of antigens.

1.1.1. *Salmonella* virulence factors

Virulence genes often cluster together in the chromosome in regions designated as pathogenicity islands. These usually contain functionally related genes involved in a particular virulence phenotype. For example, the *Salmonella* pathogenicity island 1 (SPI-1) encodes for genes involved in invasion of epithelial cells and induction of intestinal secretory and inflammatory responses (Galán, 1996). On the other hand, SPI-2 encodes for genes essential for intracellular survival and replication. Both these pathogenicity island also encode a specialized machinery for the delivery of virulence proteins directly into the host cell, named Type Three Secretion System (T3SS) (Ohl and Miller, 2001; Shea *et al.*, 1996). *S.* Typhimurium pathogenesis is highly dependent on the two distinct T3SS encoded both in SPI-1 and SPI-2, and their cognate effector proteins, which are translocated directly into the host cytoplasm (LaRock *et al.*, 2015). Translocation of *Salmonella* effector proteins into the host cell, manipulate and induce a series of complex responses on the host's epithelial cells, that will benefit the pathogen (Haraga *et al.*, 2008).

After adhesion, *Salmonella* activates the expression of SPI-1 T3SS and effector proteins (Galán and Wolf-Watz, 2006), inducing cytoskeleton rearrangement, leading to membrane ruffling and consequent bacterial internalization (Zhou and Galán, 2001). This needle complex allows

the bacteria to inject directly into the host cytosol, more than 10 effector proteins, which will ultimately cause actin rearrangement, engulfment of the bacteria and induction of the NF-κB pathway leading to an inflammatory response (Coburn *et al.*, 2007; Haraga *et al.*, 2008a; LaRock *et al.*, 2015; McGhie *et al.*, 2009). Bacterial internalization is partially mediated by SopE and SopE2 effector proteins. These guanine nucleotide exchange factors (GEF), activate the small RHO GTPases RAC1 and CDC42, leading to local actin polymerization. The concerted activity of these effectors, together with the effector SopB leads to membrane ruffling and eventual bacterial uptake into vacuoles inside non-phagocytic epithelial cells. The effectors SipA and SipC are also involved in internalization of the bacterium. While SipA inhibits actin depolymerization, SipC, a component of the T3SS, nucleates actin polymerization and condensates actin filaments into bundles (Zhou and Galán, 2001). After internalization is completed, host cell cytoskeleton architecture, is restored by another SPI-1 effector, SptP (LaRock *et al.*, 2015). Once the epithelium barrier has been breached, *Salmonella* can reside inside macrophages, where they can multiply and disseminate to the spleen (Haraga *et al.*, 2008; LaRock *et al.*, 2015; Salcedo *et al.*, 2001).

Once the bacteria arrive at the ileum, environmental conditions (high osmolarity, high iron and magnesium among other) induce the expression of the SPI-1 T3SS and its effector proteins (Bajaj *et al.*, 1996). There are several regulatory inputs that make sure that SPI-1 is only expressed at the appropriate time and place within the host. This complex regulatory feedforward-loop is composed by three AraC-like regulators, HilD, HilC and RtsA, that together control the activation of HilA (Fig. 1.1a) (Ellermeier and Slauch, 2007; Ellermeier *et al.*, 2005; Golubeva *et al.*, 2012; Olekhnovich and Kadner, 2002; Saini *et al.*, 2010). Meanwhile HilA controls the expression of SPI1-1 T3SS apparatus genes and effector proteins (Bajaj *et al.*, 1996; Lostroh and Lee, 2001).

By contrast, the SPI-2 T3SS transports proteins important for intracellular survival, across the membrane of the *Salmonella*-containing vacuole (SCV). Once inside the host cell, the bacterium resides in a phagosomal compartment that rapidly matures into a SCV (Steele-Mortimer, 2008). Using the SPI-2 T3SS, *Salmonella* translocates at least 28 effector proteins into the host endomembrane system and cytoplasm (Figueira and Holden, 2012; Jennings *et al.*, 2017). Activation of genes encoding the secretion apparatus is mediated by two-component regulatory systems (TCS), OmpR/EnvZ and the SPI-2 encoded SsrA/B (Fig. 1.1 b)

(Feng *et al.*, 2004; Garmendia *et al.*, 2003; Lee *et al.*, 2000), in response to the vacuole acid pH and low nutrient availability (Löber *et al.*, 2006). During vacuole maturation, *Salmonella* alter the lipid content of the compartment. It also induces morphological changes, including cytosolic membrane-associated actin polymerization and endosomal tubulation of the vacuolar membrane. These modifications allow the SCV to be morphologically different from typical phagosomal compartment. Although the function of many SPI-2 effector proteins has not been characterized, several of these proteins are required for these steps. Mouse studies of deletion mutants for single SPI-2 effectors, point at an overlapping role or redundancy of certain effectors (Jennings *et al.*, 2017).

a)

b)
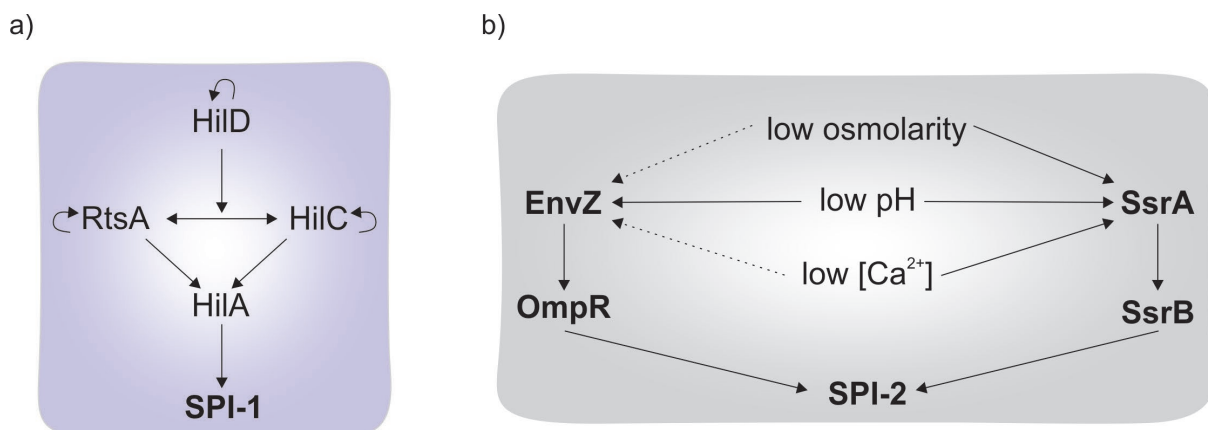
Figure 1.1.1 - Regulatory network of a) SPI-1 and b) SPI-2. *Adapted from (Ellermeier and Slauch, 2007; Fass and Groisman, 2009).*

The recognition of host innate immunity by *Salmonella* results in the transcriptional activation of genes that are important for remodeling of the bacterial cell surface, which promotes intracellular survival. Sensing of the intracellular environment and subsequent bacterial membrane remodeling are dependent on regulatory proteins, including the two- component systems PhoP–PhoQ, OmpR–EnvZ, PmrA– PmrB, RcsB–RcsC and Cya–Crp (LaRock *et al.*, 2015). PhoPQ is essential for intracellular survival of *Salmonella* (Miller and Mekalanos, 1990; Miller *et al.*, 1989). PhoQ is a membrane sensor kinase which senses environmental signals such as low Mg^{2+}, Ca^{2+} or Mn^{2+} (Shin and Groisman, 2005; Véscovi *et al.*, 1996), pH (phagosome acidification) (Alpuche Aranda *et al.*, 1992) and exposure to antimicrobial peptides. Phosphorylated PhoP binds to DNA to control expression of *phoP*-activated (*pag*) and *phoP*-

repressed (*prg*) genes, of which more than 200 have been identified (Kato and Groisman, 2008; Monsieurs *et al.*, 2005). Genes activated by PhoP include outer-membrane proteins, transcriptional and post-transcriptional regulators (including regulatory RNAs), components of the SPI-2 T3SS, inner-membrane transporters that buffer cytosolic pH; while repressed genes include flagellar components, SPI-1 T3SS and effector proteins (Dalebroux and Miller, 2014; Groisman, 2001; Prost and Miller, 2008).
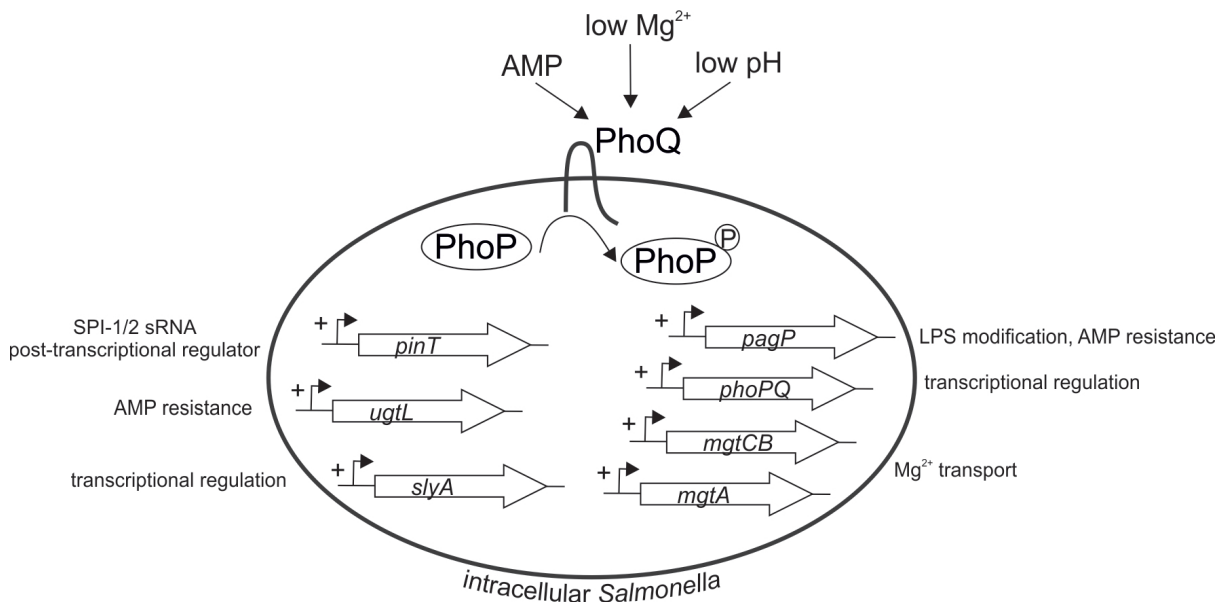
Figure 1.2 - Summary of PhoP activated genes. *Compiled from (Choi and Groisman, 2017; Groisman, 2001; Kato and Groisman, 2008; Kim et al., 2019a; Palmer et al., 2019; Prost and Miller, 2008; Westermann et al., 2016).*

1.1.1.1. Host immune response to *Salmonella*

Epithelial cells and phagocytic cells, such as dendritic cells, neutrophils and macrophages detect bacterial infection through pattern recognition receptors (PRRs) localized at their cell surface, in intracellular vesicles or in the cytosol (Abdullah and Knolle, 2014). PRRs are the first line of defense of mammalian host cells, as they recognize conserved pathogen-associated molecular patterns (PAMP). PRRs include NOD-like receptors (NLRs) and Toll-like receptors (TLRs), which can recruit and activate neutrophils and macrophages (Hurley *et al.*, 2014). PAMPs include LPS, flagella and bacterial DNA which activate TLR4, TLR5, and TLR9 signaling in the host, respectively (Kawai and Akira, 2006). These interactions are important to trigger the host inflammatory response (Hurley *et al.*, 2014).

After *Salmonella* detection by macrophages, autophagy and inflammasome activation can occur. Inflammasomes respond to pathogens by promoting cell death and tissue damage. Detection of PrgJ and flagellin by the NLR family apoptosis inhibitory protein 2 (NAIP2) and NAIP5, respectively, triggers NLRC4 (NOD-, LRR- and CARD-containing 4) mediated Caspase 1 activation. Caspase 1 can also be activated by *Salmonella* through NLRP3 (NOD-, LRR- and pyrin domain-containing 3). Coordinated activation of both NLRs leads to the induction of pyroptosis and consequently elicits the production of pro-inflammatory cytokine IL-1β (LaRock *et al.*, 2015).

Ligand binding and TLRs activation triggers intracellular signaling pathways via the adapter molecules MyD88 and/or TRIF to trigger the production of type I interferon (IFN) and inflammatory cytokines through the transcription factors NF-κB and interferon response factors (IRF) 3, 5, and 7 (Kawai and Akira, 2006). TLRs activated following the sensing of LPS, for example, promote macrophage activation and increased killing by the phagosomal compartment, as well as transcriptional activation of inflammatory caspase genes (LaRock *et al.*, 2015).

1.1.1.2.   *Salmonella* subversion of the host innate immune response

Salmonella can both promote and inhibit the host innate immune response. Localized inflammation of the intestinal tract is essential for NTS virulence. Many SPI-1 effectors, including SopB, SopE, SopE2, SipA, SipC and SopA, contribute to intestinal inflammation by stimulating production of the pro-inflammatory cytokine interleukin-8 (IL-8) via the mitogen-activated protein kinase (MAPK) and NF-κB pathways, which in turn destabilizes tight junctions and stimulates neutrophil transepithelial migration into the intestinal lumen (LaRock *et al.*, 2015). On the contrary, the SPI-1 effector AvrA and SPI-2 effector SspH can inhibit the NF-κB pathway and therefore, inflammation (Haraga and Miller, 2006; Jones *et al.*, 2008). SPI-2 effectors SseL, SspH and GogB, have also been described to inhibit the NF-κB pathway.

Another way, by which *Salmonella* can subvert the host innate immune response, is by using it to outcompete the gut microbiota. Invasion of cells by *Salmonella* triggers the release of reactive oxygen species (ROS) into the intestinal lumen. These ROS react with thiosulphate, a by-product of microbiota metabolism, resulting in the production of tetrathionate. This

compound can only be used as a terminal electron acceptor by *Salmonella* and not by the microbiota, resulting in metabolic advantage for the pathogen. The SPI-1 effector SopE can induce the production of nitrate by the host, which can be used exclusively by *S.* Typhimurium, as an alternative electron acceptor during anaerobic respiration. This leads to an enormous growth burst in the pathogen leading to dysbiosis. (Lopez *et al.*, 2012).

1.2. RNA-based control of gene expression in bacteria

sRNAs are key regulators of gene expression in pathogenic bacteria. They are involved in diverse biological processes, including response to environmental changes, oxidative stress and bacterial virulence.

1.2.1. Post transcriptional gene regulation in bacteria

Regulatory RNAs are key mediators of bacterial gene expression during adaptation to different environmental conditions such as environmental stress and virulence. Regulatory RNAs in bacteria comprise different groups of molecules with different regulation mechanisms, able to regulate targets both transcriptionally and post-transcriptionally. *Cis*-acting regulatory RNAs include riboswitches and RNA thermometers, which are elements that are part of the 5' untranslated regions (UTRs) of the mRNAs they regulate; as well as cis-encoded antisense RNAs located in the same region of their target gene.

The majority of enterobacterial sRNAs act as antisense RNAs on *trans*-encoded mRNAs (Gottesman and Storz, 2011; Waters and Storz, 2009). Small RNAs range from 50-250 nt and typically do not have an open reading frame (ORF). Most of the described sRNAs act by imperfect base-pairing with target RNAs, modulating the activity and stability of multiple targets. Base-pairing between the sRNA and its target mRNA usually leads to modification of translation, mRNA degradation by the RNases or both (Fig. 1.3). In Gram-negative bacteria most sRNAs also require interaction with RNA chaperone, the Host factor for bacteriophage Qß protein (Hfq) (Gottesman and Storz, 2011; Storz *et al.*, 2011; Wagner and Romby, 2015). Hfq not only facilitates target accessibility and interaction but also protects the sRNAs against degradation, and recruits nucleases, such as RNase E, that initiate mRNA degradation (Folichon *et al.*, 2003).
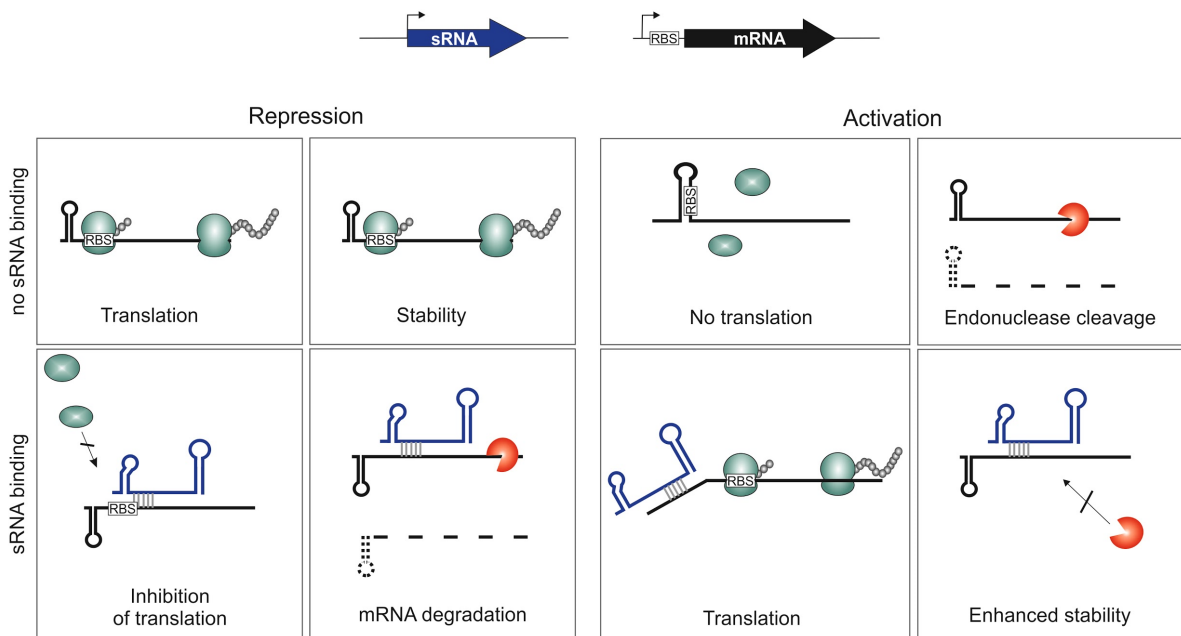
Figure 1.3 - sRNA regulatory mechanisms. *Base-pairing of a trans-encoded sRNA with its target mRNA close to the 5'UTR can block ribosome binding and translation inhibition or target the complex for degradation. It can also lead to translation activation by preventing the formation of secondary structures in the 5'UTR that block ribosome binding. Finally, sRNA can enhance mRNA target stability by binding to an endonuclease recognition site, preventing degradation. Ribosomal subunits are represented in green; ribonuclease in red; sRNA in dark blue and mRNA in black. "RBS" - ribosome binding site.*

In addition to acting by base pairing, sRNAs can also can modify protein activity by mimicking and thus competing with its targets (Gottesman and Storz, 2011; Storz *et al.*, 2011; Vogel and Luisi, 2011). Examples include, the first ever described case, the *E. coli* 6S RNA which mimics a DNA promoter open complex and interacts with RNA polymerase, leading to transcriptional reprogramming on stationary phase (Barrick *et al.*, 2005; Cavanagh and Wassarman, 2014; Chen *et al.*, 2017; Wassarman and Storz, 2000). Another well studied example is the CsrB family of sRNAs, that regulate the CsrA/RsmA family of translation regulatory proteins by competing with mRNA targets.

1.2.1.1.   Virulence related sRNAs in Salmonella

Salmonella is a very well characterized bacterium, for which there is a large data array of information regarding transcriptomics (Kröger *et al.*, 2013; Srikumar *et al.*, 2015; Westermann *et al.*, 2016). It encodes approximately 280 small RNAs, many of which are activated during infection, providing a rapid adaptation to environmental changes in the host. Examples of infection relevant sRNAs include SgrS. SgrS a 232 nt Hfq binding sRNA. It is encoded in the core genome of *Salmonella*. It is strongly induced under SPI-1 conditions (high salt and low oxygen) and regulates the expression of the SPI-1 effector protein SopD, inhibiting translation initiation and promoting *sopD* mRNA degradation (Papenfort *et al.*, 2012). A significant number of *Salmonella* infection relevant sRNAs are encoded in the pathogenicity islands. IsrM is a 329 nt sRNA, conserved in several *Salmonella* species, that does not associate with Hfq (Chao *et al.*, 2012). IsrM targets both HilE and SopA mRNAs independently, downregulating the expression of both proteins. Both targets represent major virulence factors, essential for bacterial invasion, SopA, a SPI-1 effector, and HilE, a global regulator of the expression of SPI-1 proteins. IsrM is important for *Salmonella* invasion of epithelial cells, intracellular replication inside macrophages, and virulence and colonization in mice (Gong *et al.*, 2011). IsrJ is a 72 nt sRNA, encoded in the gifsy prophage. IsrJ is part of the SPI-1 regulon and its expression is regulated (directly or indirectly) by HilA. It affects *Salmonella* invasion into non-phagocytic cells by altering translocation of SPI-1 effectors, namely of SptP. (Padalon-Brauch *et al.*, 2008). InvR, is 80 nt SPI-1-encoded sRNA. InvR transcription is activated by the SPI-1 master regulator, HilD. This sRNA targets the mRNA of the major outer cell membrane porin proteins in *Salmonella*, OmpD in an Hfq-dependent manner (Pfeiffer *et al.*, 2007). Downregulation of the outer membrane porin OmpD is thought to support *Salmonella* proliferation inside macrophages (Song *et al.*, 2017). In fact, disruption of the *invR* locus by transposon insertion assay leads to attenuation in pig, calves and chicken (Chaudhuri *et al.*, 2013).

1.2.1.1.1.   The sRNA PinT

PinT (PhoP-induced sRNA in intracellular *Salmonella*) is an 81 nucleotide long, Hfq-binding sRNA (Pfeiffer *et al.*, 2007). PinT and the associated promotor are conserved in the *Salmonella* genus, arguing for its importance for the bacteria. Previous transposon insertion studies

(Chaudhuri *et al.*, 2013) indicated a strong attenuation in intestinal colonization models (pig, cattle) for PinT deletions strains compared with the WT. Interestingly, the use of less complex cell-culture models for infection studies with PinT deletion strains, show no obvious effect on *Salmonella* replication compared with the WT (Westermann *et al.*, 2016).

The recently developed method, Dual RNA-seq (Westermann *et al.*, 2016), has allowed to study host and pathogen transcriptomic changes in response to infection. This technique has identified PinT as the most up-regulated sRNA in *Salmonella* infection (100 fold), in 14 different cell types. PinT expression is activated by the two-component system PhoP/Q, which is essential for intracellular survival. PinT temporally controls the transition from invasion to intracellular replication by simultaneously acting on SPI-1 and SPI-2 effector genes. Repression of SPI-1 genes is mediated through the SPI-1 regulators, *hilA* and *rtsA* mRNAs. PinT represses two mRNAS encoding for SPI-1 effectors, SopE and SopE2 (Westermann *et al.*, 2016), immediately upon host cell invasion, when the are no longer needed. SopE and SopE2 are guanine nucleotides exchange factors that activate host RHO GTPases to promote bacterial internalization by actin reorganization (Stender *et al.*, 2000). The sRNA base pairs near the start codon of both *sopE* and *sopE2*. Repression of SPI-2 genes occurs through the SPI-2 gene, *ssrB* mRNA and through the global regulator, *crp* mRNA. PinT also directly represses the mRNAs encoding for the proteins GrxA (glutathione/glutaredoxin system) and CRP (cyclic AMP receptor protein-cap), using the same seed region (Westermann *et al.*, 2016). GrxA and CRP are both involved in intracellular survival (Bjur *et al.*, 2006; Chen *et al.*, 2010). Interestingly, compared to the *sopE*/*sopE2* targets, PinT binding to *rtsA*, *hilA*, and *ssrB* mRNAs, requires an extended 5'-seed region for repression (Kim *et al.*, 2019a).
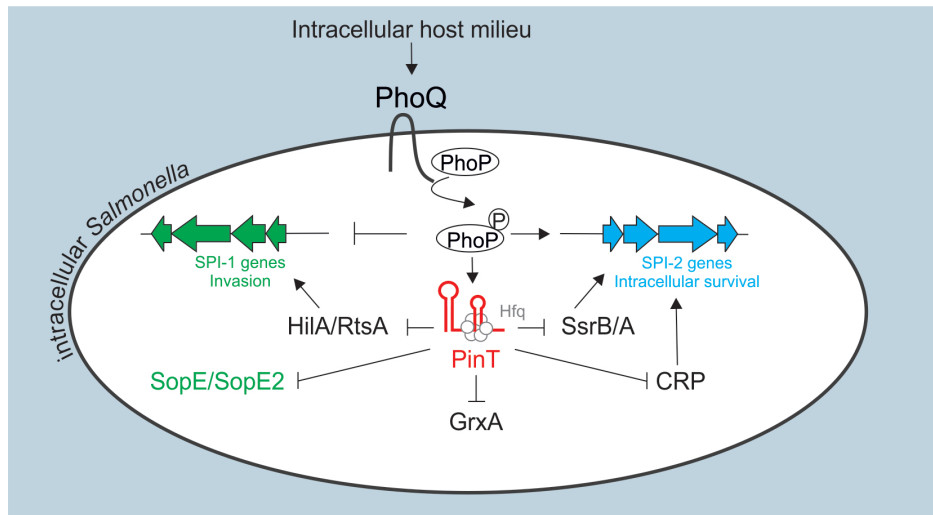
Figure 1.4 - Model for PinT target regulation. *Once Salmonella resides intracellularly, PhoPQ gets activated, leading to PinT upregulation. At this point, PinT post-transcriptionally inhibits the SPI-1 effectors directly by binding to sopE and sopE, which were previously important for internalization. It also indirectly regulates SPI-1 genes by repressing the SPI-1 regulators hilA and rtsA. At the same time, SPI-2 genes expression is delayed, by repression of the SPI-2 gene, ssrB. PinT repressed both crp and grxA, known activators of SPI-2 genes expression.*

Interestingly, during infection, the expression of this sRNA not only results in the differential expression of bacterial virulence genes but also of a tenth of all human transcripts from all classes of host RNA (Westermann *et al.*, 2016). The fact that PinT deletion or overexpression has such a pronounced effect both on *Salmonella* and host transcriptome, is not fully understood but could be mediated by downstream effects of its targets regulation.

1.3. Methods to study the role of bacterial sRNA

Discovery of a sRNA regulatory network is a key step to understanding its role. Here, we summarize the most commonly used approaches to describe new sRNA targets, validade newly described interactions and describe the regulatory mechanism.

1.3.1. Current techniques for the discovery of sRNA targets

1.3.1.1. Computational screens

Trans-encoded sRNAs regulate targets localized in distinct *locus* and therefore hold imperfect base pairing regions with their targets. This particular feature makes computational target prediction much more complicated for *trans*-encoded sRNAs, compared to cis-encoded. Nevertheless, computational approaches for the identification of sRNA-targets are a very useful tool for a first screen or even to crosscheck a larger experimental data-set, providing a list of most probable candidate targets. Initial tools included Blast searches, which successfully identified *micC* targets (Chen *et al.*, 2004), but were fairly simple and failed to take into account sRNA features into the search. The success of this application was facilitated by rather long interaction region with the target mRNA.

Since base pairing is the main regulatory mechanism of sRNA regulation (Vogel and Wagner, 2007), hybridization energy is a widely used criteria to predict RNA–RNA interactions. More general approaches, that rely on features such as hybridization energy and other, greatly improved the predictive power of such tools. The TargetRNA2 (Kery *et al.*, 2014) algorithm is a free web tool, that builds on the earlier version of TargetRNA (Tjaden, 2008) and other tools like IntaRNA (Busch *et al.*, 2008) and RNApredator (Eggenhofer *et al.*, 2011) and allows the prediction of mRNA targets in bacteria. It takes as an input the sequence of a particular sRNA and searches a specific genome. The output consists of a ranked list of candidate targets with individual predicted base-pairing interactions. For target search, this algorithm considers several features such as conservation of the sRNA (more conserved regions are more likely interacting or seed regions); secondary structure of both the sRNA and the target mRNA (more accessible regions are more likely to interact); and energy of hybridization between the two RNAs (low hybridization energy reflect a bigger probability of binding interaction between the two RNAs). However, it does not account for the role of Hfq and other RNA-chaperones for the formation of the interactions. Contrary to other computational prediction tools, it allows for the integration of RNA-seq data into the search, which considers genes that are co-differentially expressed with the sRNA.

RNApredator (Eggenhofer *et al.*, 2011) is another online tool for sRNA-target prediction. Similar to TargetRNA2, it screens a specific genome and provides a list of candidate targets for a specific sRNA. It further allows the selection of a particular region of interest in the

candidate target mRNA, and provides an enrichment analysis of GO terms in the set of selected targets.

IntaRNA (Busch *et al.*, 2008) has the particular feature of allowing to predict interaction between two RNA molecules, with a user-defined seed region. This webserver predicts the optimal interaction by calculating the minimum extended hybridization energy, which results from the sum of hybridization energy and the energy to make the binding sites accessible. Contrary to other algorithms, IntaRNA provides binding sites between the two RNA molecules and the energy of the hybridization, rather than classifying RNAs as interacting or not.

CopraRNA (Comparative prediction algorithm for small RNA targets) is one of the most widely used online tools for sRNA-target prediction (Wright *et al.*, 2014). CopraRNA usually outperforms other prediction tools because it takes into consideration conservation or co-variation for sRNA target prediction. Hence, the use of this tool is limited to sRNA conserved in several species, which excludes the *Salmonella* sRNA, PinT.

Therefore, current tools rely on different algorithms and sRNA features to predict target mRNAs. For a more comprehensive revision on computational prediction tools please read (Li *et al.*, 2012).

1.3.1.2. Pulse expression

Pulse expression of a sRNA for a short period of time, followed by total RNA extraction and microarray-based or RNA-seq based, global gene expression profiling, allows the detection of candidate mRNAs targets. Direct mRNA targets abundance would be affected earlier due to base pairing interactions. Typically, the sRNA is expressed from a plasmid with its expression tightly controlled by an arabinose inducible promoter (pBAD).

Several sRNAs of *E.coli*, *Salmonella* and other bacteria have been studied using this approach. The first reports of an experimental mRNA target prediction using sRNA pulse-expression in combination with whole-genome microarrays, were performed in *E.coli* and *Salmonella* sRNA RyhB, respectively (Massé *et al.*, 2005; Papenfort *et al.*, 2006) . The RyhB sRNA is controlled by Fur (iron-dependent repressor) and is responsible for the repression of several mRNA targets involved in the production of iron containing proteins. This approach confirmed known targets, such as *sodB* or *sdhCDAB* mRNAs, but also suggested several other potential targets. Comparative pulse expression of WT versus mutant sRNA has been used to identify

targets of *Salmonella* sRNA GcvB. Using this approach several new targets were identified, including the glycine transporter Cya, revealing a negative feedback loop that control GcvB synthesis (Sharma *et al.*, 2011).

1.3.1.3.   Comparative transcriptomics

The recently developed high-throughput sequencing based approach, Dual RNA-seq method, provides simultaneous transcriptome information of both pathogen and host (Westermann *et al.*, 2016). This technique allows a comprehensive understanding of host–pathogen interactions and the underlying gene expression changes in both the pathogen and the host. Comparison of Dual RNA-seq data from WT and *pinT*-deficient *Salmonella* strains, revealed PinT-mediated transcriptomic changes in *Salmonella*. More specifically, it revealed that SPI-2 genes were derepressed in the absence of the sRNA, although this effect was shown to be indirect, via PinT repression of *crp* (cyclic AMP receptor protein). Notably, this data also revealed hundreds of host transcripts that were affected by the presence or absence of PinT, which included several long non-coding RNAs, mitochondrial genes, and inflammatory response genes (IL8 and SOCS3).

1.3.1.4.   RBP-dependent global pull-down approaches for sRNA target identification

In Gram-negative bacteria, sRNA interaction typically depends on RNA chaperones, such as Hfq or ProQ. Several techniques take advantage of such sRNA-protein interactions to globally profile RNA-RNA interactions. The combined action of co-immunoprecipitation and RNA-seq technologies, allows the capture sRNA-target interactions *in vivo*.

Initial methodologies like RIP-seq (RNA immunoprecipitation followed by RNA-seq), are based on co-immunoprecipitation of RNA complexes interacting with a RBP (Hfq or other). These can be pulled, using either a tagged version of the RBP or by using a RBP specific antibody (Chao *et al.*, 2012; Vakulskas *et al.*, 2016). In *Salmonella*, RIP-seq revealed new class of Hfq dependent 3'UTR derived sRNAs. CLIP-seq (cross-linking immunoprecipitation followed by RNA sequencing), combines immunoprecipitation with UV crosslinking of the RNA-protein complexes, prior to RNA-seq. This additional step allows for more stringent denaturing conditions to reduce potential false-positive targets. More importantly, it provides

information regarding the interaction site. CLIP-seq was used to map Hfq and CsrA global pattern recognition motifs, in *Salmonella* (Holmqvist *et al.*, 2016).

Methods like RIL-seq (RNA interaction by ligation and sequencing) (Melamed *et al.*, 2016) and CLASH (crosslinking, ligation, and sequencing of hybrids) (Waters *et al.*, 2017) use central proteins such as Hfq or RNase E., respectively; combined with *in vivo* UV crosslink. After the RNA molecules are UV-crosslinked to the epitope tagged RBP *in vivo*, they are enzymatic trimmed via an RNase and ligated to RNA linkers before sequencing. The ligated RNA molecules can result from one single molecule or form chimeric fragments. The chimeric fragments most likely will arise from interacting RNAs. RIL-seq was used to study sRNA-RNA Hfq-dependent interactions in non-pathogenic *E. coli*, over three different growth conditions. By applying this method, authors where not only able to expand the repertoire of Hfq-dependent sRNA, but also to describe an extended targetome of several sRNAs including GcvB. RIL-seq captured known RNA sponges like ChiX and RyhB sRNAs but also lead to the discovery of a 3'UTR derived sponge for Spot42, named PspH. CLASH was applied to study enterohemorrhagic *E. coli* RNase E dependent interactions. This data-set allowed for the identification of new sRNA seed regions, as well as the identification of several targets of the EHEC specific sRNA Esr41, involved in iron transport and storage.

1.3.1.5. sRNA pull down approached

GRIL-seq (Global small non-coding RNA target identification by ligation and sequencing) method (Han *et al.*, 2017) is a technique that allows for the identification of individual sRNA targets. It employs the combination of *in vivo* overexpression of the sRNA and of T4 RNA ligase expression, followed by capture of candidate target mRNAs with a sRNA specific oligonucleotide. This additional ligation step locks and stabilize the sRNA-mRNA molecules that are in close proximity and in a complex prior to capture. By using GRIL-seq, new direct targets of PrrF1, a *Pseudomonas aeruginosa* iron-regulated sRNA, were identified.

Similarly to GRIL-seq, MAPS allows the identification of the targetome of an individual sRNA, independently of its mode of regulation. This system makes use of a strong naturally occurring RNA-protein interaction. MS2 is a small single-stranded RNA *E.coli* bacteriophage. The phage MS2 coat protein interacts with high specificity with a particular RNA aptamer, designated by MS2 aptamer. The protein dimer binds a unique hairpin in the MS2 phage genome. Binding

of the coat protein represses the translation of the phage replicase gene, since the hairpin contains the initiation codon of the replicase gene (Guzman *et al.*, 1998; Witherell *et al.*, 1991).

MS2 affinity purification and pull-down, consists of expressing the tagged sRNAs *in vivo* and running the cell lysates into a column with amylose resin, which allows to immobilize the MBP-MS2 protein (Maltose Binding Protein fused with the MS2 coat protein). Next, elution is achieved by addition of a competitor for binding to the MBP (maltose). This allows to wash off RNAs that are not bound to the MBPMS2/MS2-tagged-sRNA complex and specifically elute those which are. Then the eluate samples can be analysed by RNA-seq (Lalaouna and Massé, 2015). Previously, MS2 pull-down assays were used to identify targets of the RyhB and RybB sRNA in *E.coli* (Lalaouna and Massé, 2015). Using this method, additionally to the previously identified targets, the 3' external transcribed spacer of leuZ (3'ETS^{leuZ}), was strongly enriched with both sRNAs. Revealing a previously unknown mechanism whereby a tRNA fragment serves as sponge for both sRNAs. Ever since, several publications have used this approach to identify targets of different sRNAs and even in different bacteria, including CyaR, RprA (Lalaouna *et al.*, 2018) and GcvB in *E.coli* (Lalaouna *et al.*, 2019a), RsaC in *S.aureus* (Lalaouna *et al.*, 2019b), and SarL in *Salmonella* Typhimurium (Silva *et al.*, 2019).
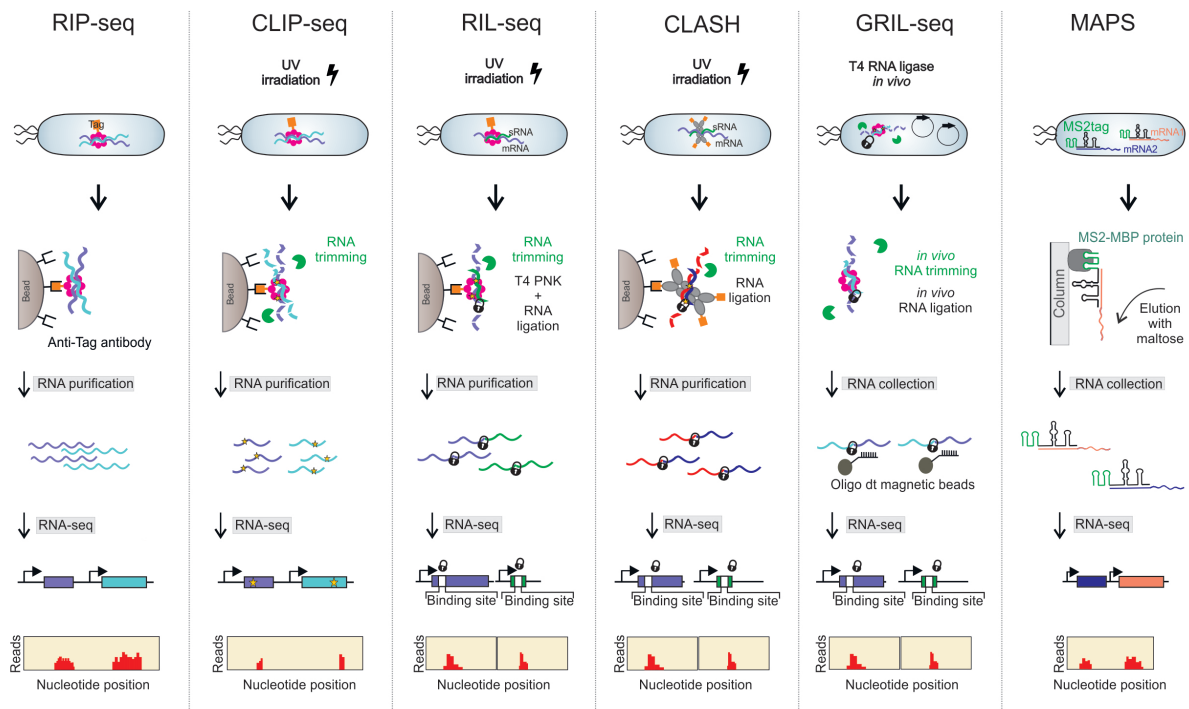
Figure 1.5 - Current techniques for the discovery of sRNA targets based on physical interactions.
RIP-seq (native RNA immunoprecipitation followed by RNA-seq): target transcripts are immuno-precipitated using a tagged protein of interest (Hfq) and analysed by RNA-seq. **CLIP-seq** *(cross-linking immunoprecipitation-high-throughput sequencing): interacting RNAs and proteins are in vivo covalently crosslinked using UV treatment before co-purification and RNAseq. RNAs are protected from trimming by ribonucleases by the protein. This allows to map the binding region at single-nucleotide resolution.* **CLASH** *(crosslinking, ligation, and sequencing of hybrids): interacting RNA molecules are UV-crosslinked to the flagged RNaseE in vivo, followed by RNA trimming and ligation to RNA linkers. The interacting RNA molecules are ligated into one single molecule or chimeric fragments.* **RIL-seq** *(RNA interaction by ligation and sequencing): interacting RNAs are crosslinked to Hfq. After trimming, RNAs are ligated and analysed by RNA-seq. Interacting RNAs will originate chimeric reads.* **GRIL-seq** *(Global sRNA target identification by ligation and sequencing): direct targets of sRNAs are identified by in vivo proximity ligation before capture with a sRNA-specific oligonucleotide.* **MAPS** *(MS2-affinity purification coupled with RNA-seq) - an MS2-tagged sRNA of interest is co-purified with its interacting RNAs and sequenced. Adapted from (Saliba et al., 2017).*

1.3.2. sRNA-Target validation

In this section, we focus on sRNA-target validation. After a candidate target has been identified, regardless of the way it has been identified, assessment of target regulation

mechanism is critical. Typically, a sRNA-target interaction will lead to changes in either the RNA or the protein levels, which in turn will have consequences for the bacteria. There are several biochemical and genomic tools that allow to us to validate and characterize sRNA-target interaction. Usually, a combination of several of these methods provides a more complete picture of the interaction. Some of the most relevant methods will be described in this section.

1.3.2.1. sRNA overexpression and deletion

As mentioned above, sRNA interaction with a target typically alters either its RNA or protein levels. For this reason, one of the first steps of target validation, includes measuring the steady-state levels of the candidate target in the presence or absence of the sRNA. It has often been observed that deletion of a sRNA, might lead to subtle changes and this may only occur under specific conditions (Storz *et al.*, 2011). For this reason, overexpression of the sRNA is frequently used to screen for more dramatic changes.

Measurement of the target RNA steady-state levels can be done by Northern Blot or by quantitative RT-PCR. The target levels should be compared between wild type, sRNA depleted strain and/or sRNA overexpression strain. In case an inducible overexpression system is being used, before and after induction of the sRNA should be compared. The effect on direct targets should be visible soon after sRNA overexpression.

In case the sRNA regulates the target at the protein level, the same experimental setting could be used, but in this case, the target protein levels can be measured via Western Blot. Antibodies specific for every target are typically not available, and the production of a new antibody may be a very long and cumbersome process. For this reason, usually the candidate target protein can be fused to a tag, typically FLAG tag, in order to allow its detection by Western Blot.

1.3.2.2. Reporter systems

Some sRNAs are known to post-transcriptionally activate translation of mRNAs while others repress translation. There are several bacterial reporters for gene expression, one of the most common tools to study target gene regulation by sRNAs are the translational *lac* reporters, where the target 5'UTR of a candidate target mRNA is fused to *E.coli lacZ,* encoding

β-galactosidase. If the fusion is driven by the target gene promoter, effects on translation (rather then transcription), has to be determined separately.

Another alternative reporter systems that is very frequently used is the green fluorescent protein (GFP) translational reporter. The GFP reporter system enables the study of sRNA-mediated translational control *in vivo*, using a two compatible plasmids system. The target plasmid is expressed from a low-copy vector, the 5' UTR plus the early coding sequence of the target, as a translational fusion to the N-terminus of GFP. Transcription of the *gfp* fusion gene is driven by a constitutive promoter. The sRNA plasmid is expressed from a high-copy vector, with its expression controlled either by a constitutive promoter, like mentioned above, or from an inducible promoter, like the P_{BAD} promoter. This approach allows to measure changes in GFP fluorescence using different methods such as LB agar plates, standard culture curves or microtiter plates and by flow cytometry (Corcoran *et al.*, 2012; Urban and Vogel, 2007). The method allows quick and reliable verification of putative sRNA- mediated target regulation and can be adapted to high throughput screens.

1.3.2.3. In vitro approaches

Electrophoretic mobility shift assays (EMSA) are widely used to assess complex formation between different RNAs (or between RNA and proteins). The molar ratio of bound to unbound sRNA to the mRNA in question can be used to study kinetics and calculate binding affinity, which is quantified through the dissociation constant (K_D) (Bak *et al.*, 2015).

Mapping the site of interaction between a sRNA and its target can be done through *in vitro* footprinting. The site of interaction is protected from enzymatic, spontaneous or chemical cleavage and the comparison of cleavage patterns in the presence or absence of the sRNA allows to determine the nucleotides involved in duplex formation (Ellis *et al.*, 2015; Regulski and Breaker, 2008; Ross *et al.*, 2013; Yi *et al.*, 2012).

Translational inhibition can also be assessed *in vitro* by toe-printing assay (Hartz *et al.*, 1988), where a mRNA fragment, comprising of the TSS, RBS and translation initiation site of the gene of interest, is incubated with 30S ribosomal subunits and the initiator tRNA so they can form a ternary complex at the Shine-Dalgarno sequence. Reverse transcription of the mRNA using a radiolabeled primer stops at the position of this complex, giving a length-specific toeprint that can be detected upon gel-electrophoresis on a sequencing gel. Absence of this signal in

the presence of a sRNA indicates impaired formation of the ternary complex, *i.e* translation inhibition.

Finally, another way to determine whether a sRNA interferes with a target translation efficiency is to compare its *in vitro* translation efficiency in presence or absence of the sRNA. The use of commercially available pure translation systems enables the study of sRNA regulation in an easily altered minimal system (Sharma *et al.*, 2011).

1.4. Aim of this study

The recently identified sRNA PinT is the the most upregulated sRNA in intracellular *Salmonella*. It acts as a temporal regulator of *Salmonell*a's two major pathogenicity islands, SPI-1 and SPI-2 (Westermann *et al.*, 2016). However, the complete set of PinT targets, its role for *Salmonella* virulence and host infection, has not yet been systematically defined.

Our knowledge on PinT targets, is based on educated guesses followed by targeted validation (Kim *et al.*, 2019) or assessed the expression changes upon deletion or over-expression of PinT on transcriptome level (comparative transcriptomics and pulse-expression (Westermann *et al.*, 2016), bearing the risk that certain PinT targets may have escaped detection; especially targets that would be primarily regulated at the level of translation rather than transcript stability. This possibility may further be supported by the fact that, while PinT is highly conserved within the *Salmonella* genus, several of its currently known targets (*sopE*, *sopE2*, *ssrB*) are not.

In the present study, we therefore set out to expand the target repertoire of PinT using an approach based on physical sRNA-RNA interaction by applying MAPS (Lalaouna and Massé, 2015) to *Salmonella* PinT. MAPS has successfully revealed sRNA-targets in *Salmonella* and other bacteria (Lalaouna and Massé, 2015; Lalaouna *et al.*, 2018, 2019a, 2019b; Silva *et al.*, 2019). Using MS2 affinity purification with RNA-seq we aim to uncover other PinT targets relevant for *Salmonella* infection.

Additionally, we set out to describe an advanced toolkit of RNA affinity purification methods, including the pull-down not only off ectopically expressed, but also chromosomally MS2-tagged sRNAs, and the transfer of MAPS from bacterial *in vitro* cultures to intracellular bacteria after host cell invasion. This toolkit offers a route to uncover molecular functions for other infection-relevant sRNAs in *Salmonella* and beyond.

2. Expanding *Salmonella* sRNA PinT interactome

In this chapter, the targetome of the *Salmonella* sRNA PinT has been studied. Using MAPS, new candidate targets have been identified. Here we describe the implementation of the classical protocol to *Salmonella* Typhimurium SL1344. Moreover, we describe additional variations of the MAPS protocol, including expressing the tagged sRNA from its native promoter (whether from a plasmid or from the chromosome), showing that it is possible to adapt this protocol to more physiological conditions. By applying MAPS to *Salmonella* grown in infection mimicking condition and to *Salmonella* infected cells, we were able to uncover new PinT targets whose regulation may be particularly relevant during infection. Overall, the PinT expanded targetome indicates that this sRNA provides an additional layer of crosstalk regulation between SPI-1 and SPI-2 virulence programs.

2.1. Establishing MAPS for *Salmonella* PinT sRNA

In order to establish conventional MAPS (Lalaouna and Massé, 2015) for *Salmonella* PinT, we fused the MS2 aptamer to the 5' end of PinT. *In silico* prediction of the secondary PinT structure suggested that 5'-addition of MS2 does not interfere with PinT folding (Fig. 2.1).
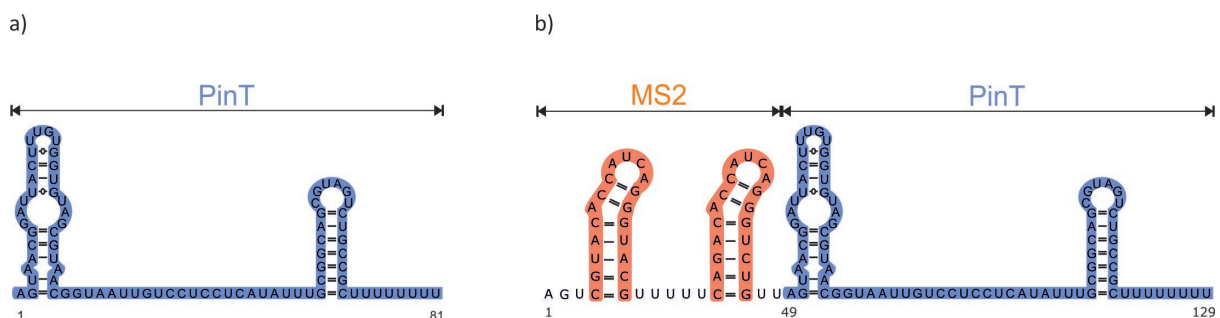
Figure 2.1- Secondary structure prediction of a) Pint and b) MS2-PinT. Secondary structure prediction using mfold web server and VARNA applet for visualization of RNA secondary structure.

The tagged sRNA was expressed from an arabinose-inducible promoter on the high-copy plasmid pBAD. The resulting strains were grown in LB until OD_{600nm}2 prior to the addition of the inducer for 10 min. The 5'-addition of MS2 did not interfere with steady-state levels of PinT as judged from Northern blot (Fig. 2.2a). We also monitored the expression levels of the tagged PinT under the control of the native promoter on a pZE12-luc derived plasmid (Urban and Vogel, 2007), during growth in LB at OD_{600nm}2. Again, sRNA levels were highly similar

between MS2-tagged and untagged PinT (Fig. 2.2b). Despite being expressed from its endogenous promoter and pZE-luc being a low-copy plasmid, PinT levels are still elevated in this strain as compared to the endogenous PinT levels (Fig. 2.2b, compare lanes 2 and 3 with lane 1). Therefore, we constructed also a chromosomally tagged version of PinT, by integrating the sequence for MS2 immediately downstream of the transcription start site of *pinT* in its native genomic locus (Fig. 2.2c).
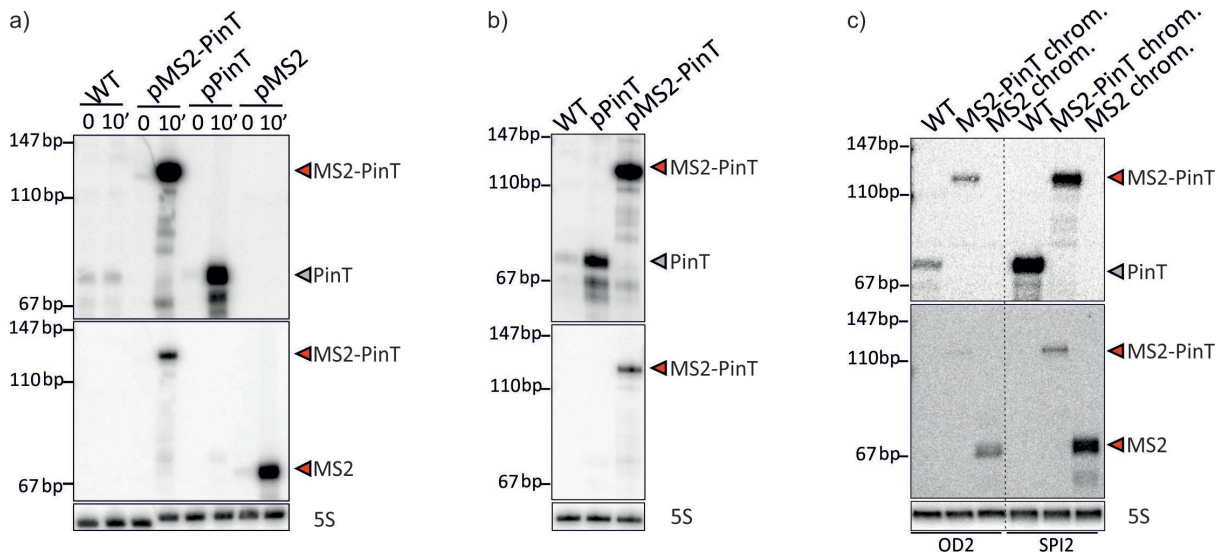
Figure 2.2 - Expression of the different construct for MAPS pull-downs. a) *Northern Blot analysis of Salmonella Typhimurium SL1344 WT carrying empty vector control (lanes 1-2 - pKP8-35), or the pinT deletion strain carrying a plasmid expressing the WT PinT (lanes 5-6 - pYC5-34); the aptamer tagged PinT, i.e. MS2-PinT (lanes 3-4 – pYC310); or the tags alone, MS2 (lanes 7-8 – pYC310) under an arabinose inducible promoter; before or after 10 minutes of induction.* *b)* *Northern Blot analysis of Salmonella SL1344 WT carrying empty vector control (lane1 - pJV300), or the PinT deletion strain carrying a plasmid constitutively expressing either the WT PinT (lane 2 – pYC55), the MS2-PinT (lane 3 – pSS31) or the MS2 aptamer (lane 4 – pSS32), under the control of the PinT native promoter.* *c)* *Northern Blot analysis of Salmonella Typhimurium SL1344 WT (lanes 1,4) or Salmonella carrying a chromosomal copy of either MS2-PinT (lanes 2, 5) or of MS2 (lanes 3, 6); grown under SPI-1 or SPI-2 inducing conditions. Includes 5S as a loading control.*

To confirm functionality of MS2-PinT, we selected the well-characterized PinT target SopE (Westermann *et al.*, 2016) as a read-out and monitored its expression in presence or absence of tagged and untagged PinT by the established fluorescence reporter assay (Urban and Vogel,

2007), using previously described constructs (Westermann *et al.*, 2016). To this end, a plasmid containing a translational *sopE::gfp* gene fusion was co-expressed with the pBAD plasmid containing either PinT alone or MS2-PinT or the empty control plasmid, respectively, in LB until $OD_{600nm}2$ in the presence of the sRNA inducer arabinose, followed by the quantification of the GFP signal emitted by different bacterial cells. In accordance with previous results (Westermann *et al.*, 2016), ectopic expression of untagged PinT reduced the levels of SopE::GFP by ~2-fold (Fig. 2.3). Of note, overexpression of the tagged variant of PinT led to a similar reduction in relative fluorescence, indicating that aptamer fusion to PinT does not disrupt *sopE* repression.
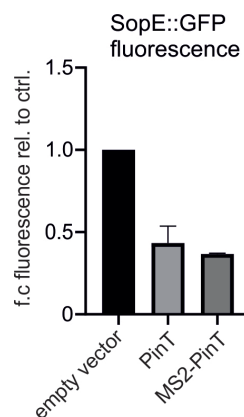
Figure 2.3 - GFP reporter assay. *sopE::gfp reporter gene fusion (Westermann et al., 2016) was used to measure the repression by the pulse expressed wild-type (PinT) or the tagged PinT (MS2-PinT). Bars correspond to the mean fluorescence relative to the empty vector ± s.d. from three biological replicates.*

Having demonstrated the ability of MS2-PinT to execute mRNA repression, we set out to identify the optimal time-point upon its induction to perform the pull down. Ideally, by the time the cells are lysed, the tagged sRNA would be highly expressed and interacting with its cellular ligands, while target degradation would not yet be maximal. We therefore performed a time-course pulse expression of MS2-PinT in *Salmonella* growing in LB, adding arabinose once the cultures reached $OD_{600nm}2$ (a condition at which endogenous PinT is expressed at intermediate levels (Westermann *et al.*, 2016)), and followed MS2-PinT induction and target degradation kinetics. RNA samples were collected before induction and at 1, 2, 5 and 10 min after the pulse. Transcript levels of MS2-PinT and the two known PinT targets *sopE* and *sopE2*

mRNA (Westermann *et al.*, 2016) were measured by qRT–PCR (Fig. 2.4). PinT induction occurred immediately, with a >10-fold increase already 1 min after arabinose addition, and saturated after 5 min. *sopE/sopE2* mRNA levels responded rather rapidly, with reduced transcript levels being detected after two minutes of sRNA overexpression (4- or 1.5-fold repression, respectively, for *sopE* and *sopE2*). Maximal repression levels were observed at 10 min of sRNA induction (7- and 4-fold for *sopE* or *sopE2*, respectively). Based on these findings, we selected the 2 min time-point for MAPS analysis, i.e. when MS2-PinT is already highly expressed but full target degradation is not yet reached.
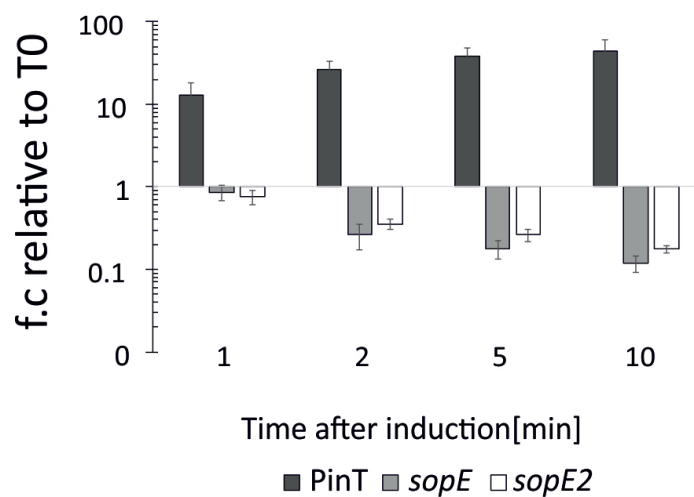
Figure 2.4- Time-course pulse expression of MS2-PinT. *qRT-PCR measurements of PinT, sopE and sopE2 over a time-course of 20 minute of MS2-PinT overexpression. Bars correspond to the mean fold change relative to before induction ± s.d. from three biological replicates and normalized to the control gene 5S.*

2.2. MAPS in OD2 and SPI-2 inducing conditions

Having determined the experimental conditions for the pull-downs, we set out to apply the classical MAPS approach to *Salmonella* grown in LB $OD_{600\,nm}$ 2, a condition known to induce expression of SPI-1 related genes (Ibarra *et al.*, 2010). For this, we used strains harboring the same plasmids as described above (pBAD-PinT, pBAD-MS2-PinT and pMS2). As described before, bacterial cells were harvested and snap frozen 2 min after the arabinose-induced pulse and the cleared lysate was used for MAPS.

The purified RNA was then converted in cDNA libraries and sequenced to ~20 million reads/library. Reads were mapped to the *Salmonella* genome and normalized against rRNA-derived reads. We pulled down different classes of RNA together with MS2-PinT, including mRNAs, other sRNAs, and rRNAs. Read distribution of the different RNA classes varied between different samples, with the majority mapping to rRNA (68% for MS2 alone; 73% for MS2-PinT; 96% for untagged PinT). Fold enrichment was calculated by comparing the ratio of normalized read counts between the MS2-PinT and untagged PinT samples, from two independent replicate experiments (Table 2.1). Reassuringly, known PinT targets (*sopE*, *grxA* and *crp* mRNAs) were contained in the list of most enriched transcripts with MS2-PinT. Interestingly, the most enriched transcript was *steC* mRNA, encoding for a virulence effector translocated via the secretion machinery of the *Salmonella* Pathogenicity Island (SPI) 2 (Fig. 2.5). Besides, several further virulence-associated transcripts were co-purified, including the transcripts of several SPI-1 and SPI-2 effectors. Other enriched transcripts included *ugtL*, encoding for a membrane protein involved PhoQ activation in mildly acidic pH, and *rpsV*, encoding for 30S ribosomal protein S22 (Table 2.1).

Table 2-1|Summary table of some of the most enriched transcripts in MAPS in Salmonella in LB OD$_{600nm}$2. Enrichment was calculated by the ratio of normalized reads between MS2-PinT and PinT pulled down transcripts.

Rank #	Candidate target	Product	Reference
1	*steC*	SPI-2 effector protein	This study
5	*grxA*	Glutaredoxin 1	(Westermann *et al.*, 2016)
6	*ugtL*	PhoQ activator	This study
7	*sopE*	SPI-1 effector protein	(Westermann *et al.*, 2016)
10	*rpsV*	30S ribosomal protein S22	This study
22	*crp*	Global transcriptional regulator	(Westermann *et al.*, 2016)
105	PinT	bait sRNA	
106	*sopE2*	SPI-1 effector	(Westermann *et al.*, 2016)
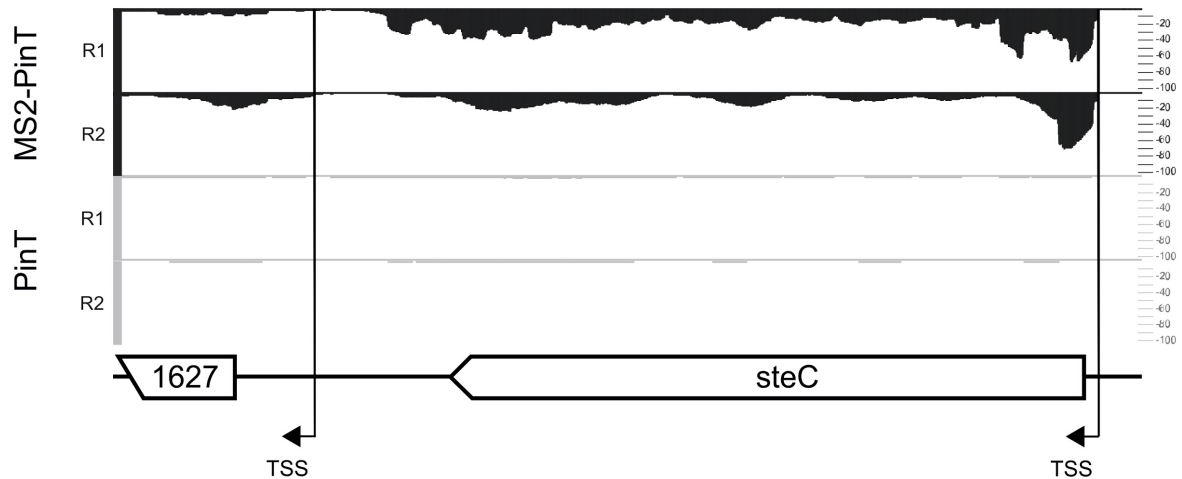
Figure 2.5 - Read coverage plot for steC mRNA in the MS2-pull down. *Read coverage for steC mRNA from MS2-PinT (black), and PinT (grey) pull-down samples at OD$_{600nm}$ 2. Results from two biological replicates are shown.*

Next, to further support our *in vitro* findings, we set out to apply the original MAPS protocol for PinT in *Salmonella in vitro* conditions that mimic infection. Previous MAPS studies (Lalaouna and Massé, 2015; Lalaouna *et al.*, 2017, 2018, 2019a; Silva *et al.*, 2019) were based on overexpressed sRNAs, accounting for the fact that high levels of a given sRNA are needed for efficient pull-down. Under infection conditions, PinT is highly abundant (e.g. ~100-fold induced after host cell invasion compared to growth in LB (Westermann *et al.*, 2016)), potentially allowing for MAPS under native conditions. To test if the above identified PinT targets are also bound under physiological conditions (that mimic infection) and do not represent artefacts of the overexpression, we performed MAPS on *Salmonella* with chromosomally MS2-tagged PinT by integrating the sequence for MS2 immediately downstream of the transcription start site of *pinT* in its native genomic locus (Fig. 2.1c). In order to identify infection-relevant PinT targets, this experiment was performed in a minimal medium mimicking the intracellular environment (Löber *et al.*, 2006) and thus induces PinT expression *in vitro*. Using this approach, we sequenced ~30 million reads/library. Although most of the reads mapped to rRNA (98% WT; 97% cMS2-PinT; 93% cMS2), it was possible to map reads to the different classes of RNAs, including mRNAs and other sRNAs. The enrichment score was once more calculated by the ratio of read counts of transcripts co-purified with chromosomal MS2-PinT to the WT PinT control. Subjecting the thus grown

bacteria to MAPS resulted once more in the detection of validated PinT targets (*sopE*, *sopE2*, *crp,* and *grxA*) as well as the newly identified targets (including *steC, ugtL and rpsV*) (Table 2.2). We therefore conclude that the here identified PinT candidate targets might be physiologically relevant.

Table 2-2|Summary table of the most enriched transcripts in MAPS in Salmonella in SPI-2 inducing conditions. Enrichment was calculated by the ratio of normalized reads between MS2-PinT and PinT pulled down transcripts.

Rank #	Candidate target	Product	Reference
3	PinT	sRNA (bait)	
5	*ugtL*	PhoQ activator	This study
22	*steA*	Effector protein	This study
29	*rpsV*	30S ribosomal protein S22	This study
37	*steC*	SPI-2 effector protein	This study
49	*sopE*	SPI-1 effector protein	(Westermann *et al.*, 2016)
58	*ssrB*	TCS|SPI-2 transcriptional regulator	(Kim *et al.*, 2019b)
70	*crp*	Global transcriptional regulator	(Westermann *et al.*, 2016)
78	*ecnB*	Enteredicin B	This study
83	*grxA*	Glutaredoxin 1	(Westermann *et al.*, 2016)
139	*sopE2*	SPI-1 effector protein	(Westermann *et al.*, 2016)

2.3. MAPS on *Salmonella* inside macrophage cells

To identify PinT target candidates relevant in a natural set-up and describe regulations that are actually executed during infection, we set out to apply the MAPS method to infection settings. To this end, we introduced MS2-PinT, untagged PinT, and the MS2 tag alone into a pZE12-luc backbone plasmid, keeping the native *pinT* promotor (Fig. 2.1c). The resulting *Salmonella* strains were used to infect macrophages (iBMMs), the infected host cells were collected after 4 h and lysed, and the thus released bacteria subjected to the MAPS procedure. Of the ~30 million reads per library obtained 5-46% mapped to the eukaryotic host genome. Despite the majority of the remaining *Salmonella*-specific reads being derived from rRNA, 3-

18% of the non-rRNA reads allowed for the identification of potential interacting transcripts (Fig. 2.6).
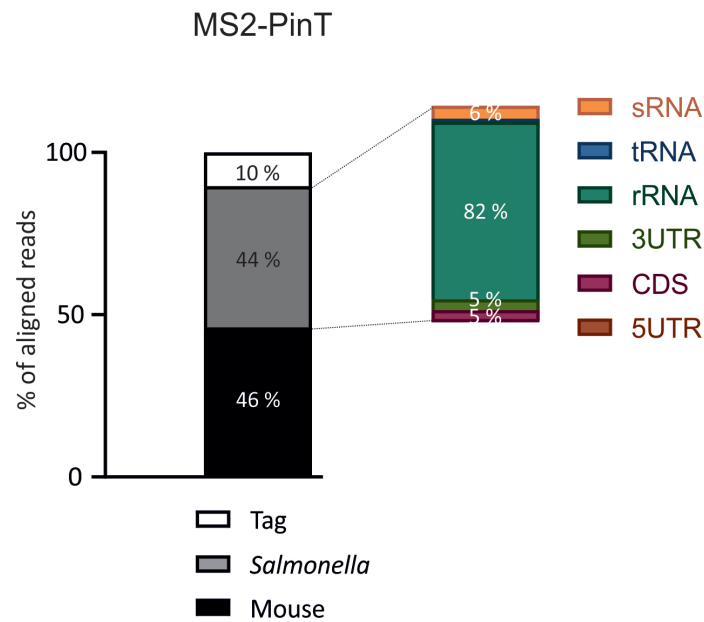
Figure 2.6 - Read distribution per organism and per class of RNA in the in vivo MAPS MS2-PinT pull down. *"Tag": MS2 aptamer; "Salmonella": Salmonella Typhimurium SL1344, "Mouse": iBMMs.*

By calculating enrichment of transcripts co-purified with MS2-PinT compared with the untagged PinT control, we identified putative RNA ligands of PinT (Table 2.3). In this way, we were able to detect the known PinT targets *grxA* and *crp* (Westermann *et al.*, 2016). Other targets, including *sopE* and *sopE2*, are expressed earlier during infection and were thus not expected to interact with PinT at 4 h after host cell invasion. On the other hand, several new PinT candidate targets, e.g. the CsrA-sponging sRNAs CsrB and -C as well as the SPI-1-associated InvR sRNA, were enriched in the *in vivo* MAPS data. Other enriched transcripts included *ugtL*, *rpsV*, OxyS, STnc630 and ChiX sRNAs; *fliC, hilC* and *ssrA* (Table 2.3).

Interestingly, the *steC* mRNA, encoding a SPI-2 virulence effector, was also enriched in the MS2-PinT pull-down from intra-macrophage *Salmonella*. SteC was previously used as a readout for the effect of the PinT target CRP, on SPI-2 activity (Westermann *et al.*, 2016). However, the combined MAPS data suggested that PinT-mediated SteC repression might occur not only indirectly, through CRP, but also by direct interaction of this sRNA with the *steC* mRNA.

Table 2-3|Summary table of the most enriched transcripts in MAPS in intra-macrophage Salmonella. Enrichment was calculated by the ratio of normalized reads between MS2-PinT and PinT pulled down transcripts.

Rank #	Candidate target	Product	Reference
2	CsrC	sRNA	This study
6	CsrB	sRNA	This study
8	InvR	sRNA	This study
12	*rpsV*	30S ribosomal protein S22	This study
13	OxyS	sRNA	This study
15	PinT	sRNA (bait)	
16	STnc630	sRNA	This study
34	ChiX	sRNA	This study
66	*ugtL*	PhoQ activator	This study
76	*hilC*	SPI-1 regulator	This study
113	*fliC*	Flagellin	This study
125	*ssrA*	TCS|SPI-2 regulator	This study
244	*grxA*	Glutaredoxin 1	(Westermann *et al.*, 2016)
264	*steC*	SPI-2 effector protein	This study
448	*crp*	Global transcriptional regulator	(Westermann *et al.*, 2016)

2.4. Validation of newly identified PinT target candidates

To *in silico* predict interaction sites between PinT and these putative RNA ligands, we used the RNA-RNA interaction program IntaRNA (Busch *et al.*, 2008). To this end, the sequence of the respective target candidate including approximately 50-100 nt flanking sequences on either side of the TSS and terminator and the full-length PinT sequence were used as an input for RNA:RNA hybrid prediction. In most of the cases, the same seed region of PinT was predicted to interact with the putative targets. This interaction region included an extension of the previously described PinT seed region (Westermann *et al.*, 2016). With respect to the

PinT target candidates, interaction was predicted to occur close to the respective start codons (Fig. 2.7), reminiscent of the classical target control mechanism employed by Hfq-dependent sRNAs, namely interference with translation initiation.

a)

```
           -14              +1                    18
            |                |                     |
steC 5'-UUUCA    U A    U A                   U  GAUCG-3'
            GAGGA G GACA  UGCCGUU    UACAUU CA
            ||||| | ||||  |:||||||   ||||:: ||
            CUCCU C CUGU  AUGGCAA    AUGUGG GU
PinT 3'-UUAUA           UA         UGCG     U  UUCAU-5'
            |                                |
           48                               15
```

b)

```
           -13             -1
            |               |
ugtL 5'-CCUGC      CU    A AUGA -3'
           GAGGAGG   CAA
           |||||||   |||
           CUCCUCC   GUU
PinT 3'-UUAUA       U     AAUGG-5'
           |               |
          48              36
```

c)

```
           -14             +1                       29
            |               |                        |
rpsV 5'- AGGAU     UUA   UGAAAUCGA       C G           UACUU-3'
            UGAGGAG    AC A          ACCGU A GCU CGUCAUA
            |||||||    |||           ||||| | ||| |::||:|
            ACUCCUC    UGU           UGGCA U CGA GUGGUGU
PinT 3'-UUUAU        C      UAA        A G   U      UUCAU -5'
            |                                        |
           49                                       15
```
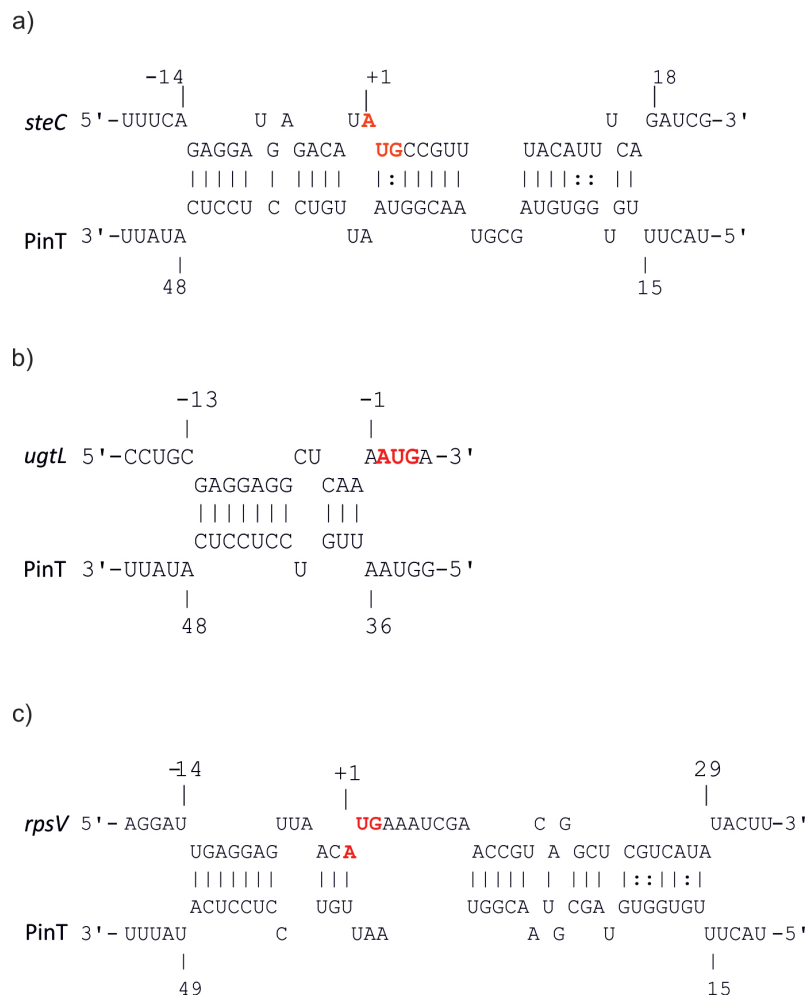
***Figure 2.7 - a-c) In silico interaction prediction between PinT sRNa and stec (a) ugtL (b) and rpsV (c)
mRNA, respectively.*** *This interaction was predicted using the IntaRNA - RNA-RNA interaction
prediction tool. The start codon is marked in red. Nucleotide positions labeled relative to the start
codon (+1) in the mRNAs; and from PinT's 5' (+1) for the sRNA. Predicted hybridization energy
a) -26.6 kcal/mol, **b)** -14.5 kcal/mol, **c)** -25 kcal/mol.*

To study the outcome of PinT interaction with its newly predicted targets, we monitored the consequence of PinT pulse-expression on the steady-state levels of these mRNAs over time. To this end, the WT PinT was pulse-expressed at $OD_{600nm}2$ in LB. Total RNA samples were taken, at defined time points, and analyzed by qRT-PCR. Expression of the PinT target candidates was compared to their expression level in a strain carrying the empty vector. Three

candidate targets, *steC*, *rpsV* and *ugtL* mRNAs, were consistently differentially expressed in the presence of PinT compared to its absence. These were all downregulated, in a time-dependent manner, upon PinT expression relative to the empty vector control (Fig.2.8).
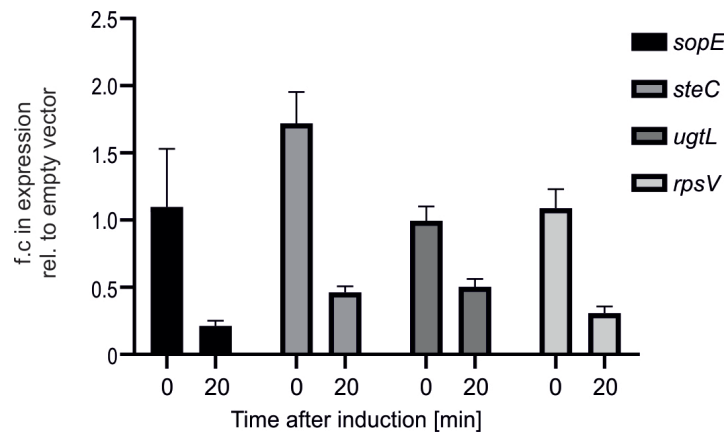
Figure 2.8 - Steady state levels of mRNA candidate targets after PinT overexpression. *qRT-PCR measurements of sopE (known target), steC, ugtL and rpsV mRNAs before and after 20 minutes of pulse expression of PinT. Transcript fold change expression was compared with empty vector before and after induction and normalized to the control gene 5S. Bars represent the mean fold change ± s.d. from three biological replicates.*

This PinT-dependent repression was also observed at the protein level by detection of levels of the FLAG-tagged SteC, UgtL or RpsV by Western blotting (Fig. 2.9). While *pinT* deletion had no significant effect on SteC, UgtL or RpsV protein abundance – an observation often made for sRNA deletion mutants and their targets (Storz *et al.*, 2011) – ectopic PinT expression from a plasmid under the control of a constitutive promoter or the native *pinT* promoter significantly reduced the levels of all three proteins (Fig. 2.9).
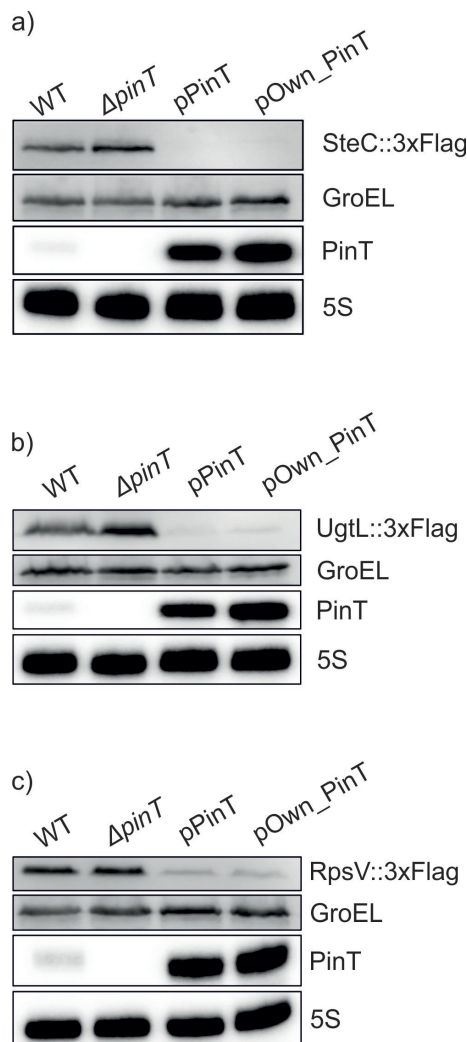
Figure 2.9 - PinT regulates protein level of candidate targets. *Western-blot and Northern blot analysis of Flag tagged SteC (a), UgtL (b) and RpsV (c) in a pinT deletion strain, or a complemented strain where PinT expressed from a plasmid under the control of a constitutive promoter or of its own promoter. Includes GroEL and 5S as a loading control for protein and RNA, respectively. Results from one out of the two biological replicates are shown. Protein and RNA samples were collected in Salmonella grown in SPI-2 media at $OD_{600nm}0.3$.*

To assess if PinT regulates the candidate target genes at the translational level, GFP translational reporter systems (Urban and Vogel, 2007) were constructed for *steC* and *rpsV* (Fig. 2.10). For this, the complete 5'UTR plus 21 codons of the coding region of each of the two mRNAs, were fused in frame with the *gfp* gene in a low copy plasmid, under the control of a constitutive promoter. Using this reporter system, we were able to observe that PinT overexpression reduces the production of SteC::GFP but not of RpsV::GFP. In the case of *ugtL*,

unfortunately we were not able to construct an efficient reporter system (possibly due to the presence of the small protein UgtS, in the 5' region of *ugtL*).
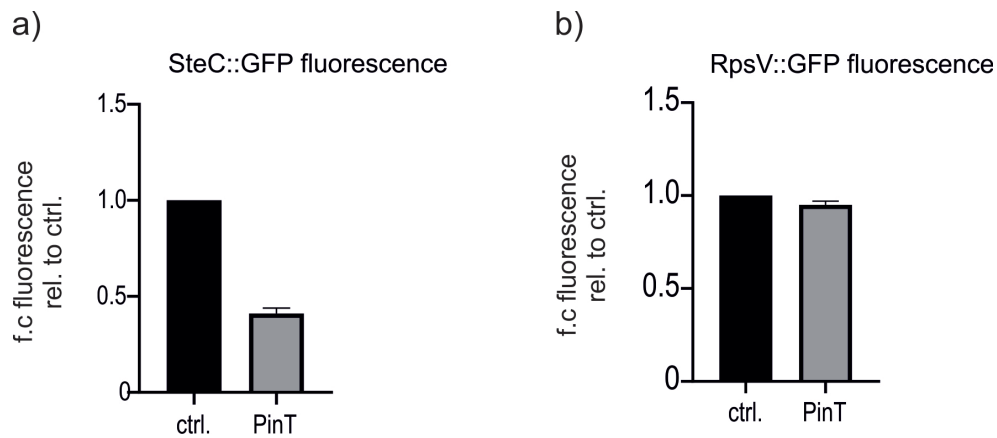
Figure 2.10 - GFP reporter assay. *steC::gfp or rpsV::gfp translational reporter gene fusion was used to measure of the interaction between the pulse expressed wild-type (PinT) and mRNA fusion, on a pinT deletion background. Salmonella strains were grown until $OD_{600nm}2$ in LB, prior to sampling. Bars represent the mean fold change ± s.d. from three biological replicates.*

The *in vivo* MAPS revealed several candidate sRNA targets. To test if PinT could affect the expression of the new sRNAs candidates, we monitored their steady-state levels in a PinT deletion strain, or the same strain complemented with PinT expressed from a plasmid, under the control of an inducible promoter (pBAD). *Salmonella* strains were grown both in LB until $OD_{600nm}2$ and SPI-2 media until $OD_{600nm}0.3$. At this point, total RNA samples were taken before and after 10 minutes of induction, and analyzed by Northern Blot. Overall, overexpression of PinT seemed to have a slight effect on the stability of the tested sRNAs (Fig. 2.11). This effect was most evident for the InvR sRNA, specially under SPI-2 conditions. *In silico* predictions, using the IntaRNA-RNA tool, identified putative binding site between PinT and the candidate sRNA-targets (Fig. 2.12). Nevertheless, these results do not exclude indirect effects caused by, for example, titration of Hfq.
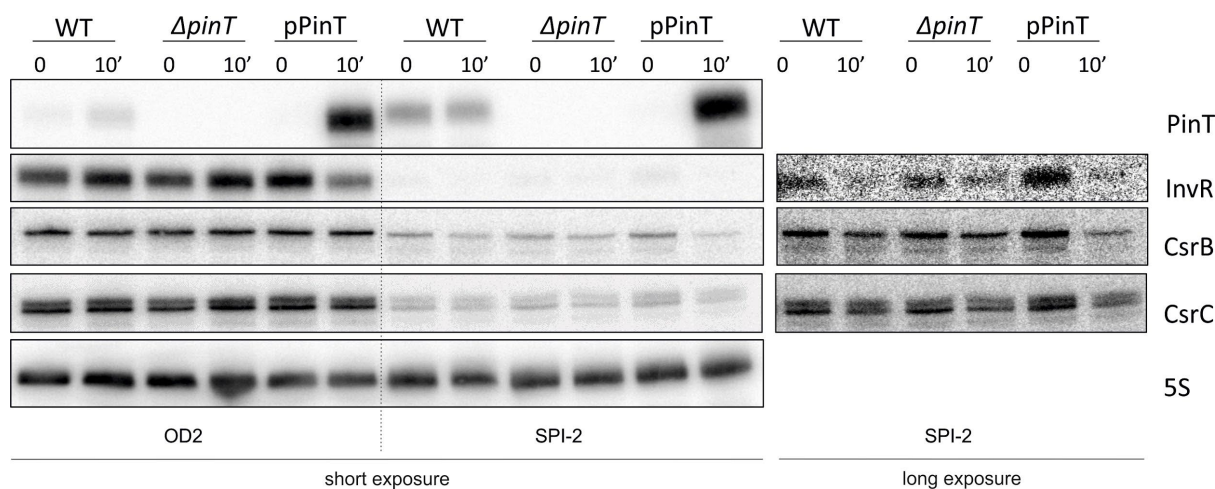
Figure 2.11 - Steady state levels of sRNA candidate targets before and after PinT overexpression. *Northern Blot analysis of PinT, InvR, CsrB, and CsrC sRNAs in WT Salmonella carrying an empty plasmid, in a ΔpinT strain carrying an empty plasmid or a PinT complementation strain growth in LB to an OD$_{600nm}$ of 2.0 (OD2) or in the minimal SPI-2-inducing medium to an OD$_{600nm}$ of 0.3 (SPI-2), respectively. RNA samples collected before and after 10 minutes of arabinose induction. Since both RNAs were expressed at low levels in SPI-2 at an OD$_{600nm}$0.3, the respective membrane was re-exposed for an extended time period (shown to the right). Includes 5S rRNA as a loading control.*
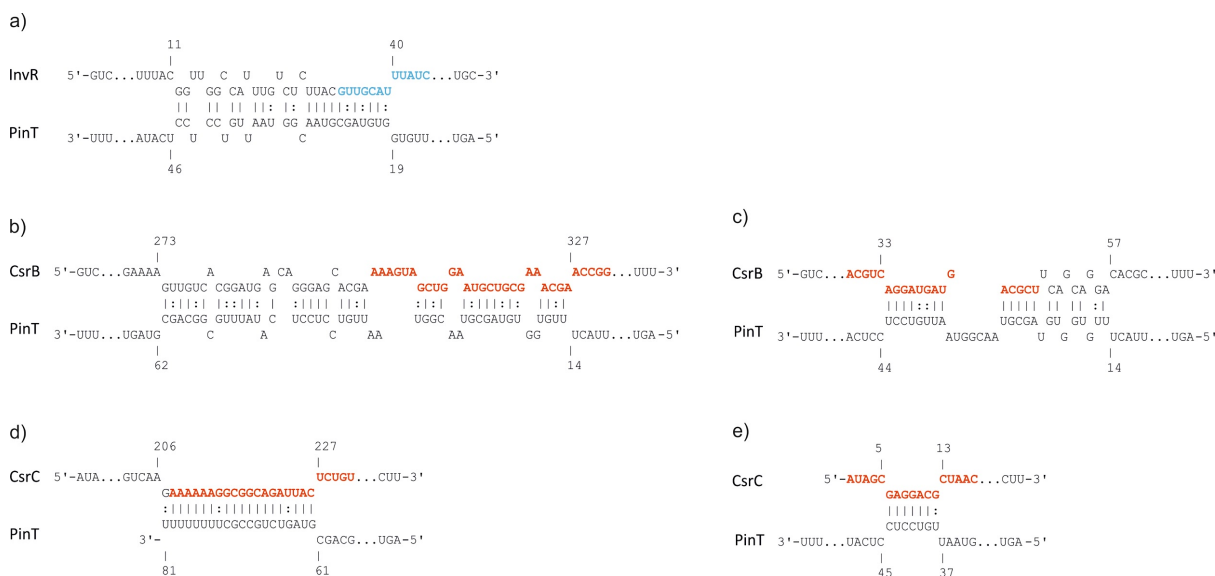
Figure 2.12 - a-c) In silico interaction prediction between PinT sRNA and InvR (a) CsrB (b-c) and CsrC (d-e) sRNAs, respectively. *This interaction was predicted using the IntaRNA - RNA-RNA interaction prediction tool. Positions labeled relative to the 5´of the sRNA (+1).* **a)** *InvR seed region used to interact with ompD mRNA highlighted in light blue (Pfeiffer et al., 2007) .* **b-e)** *CLIP-seq peaks for CsrA highlighted in red. Predicted hybridization energy* **a)** *-20.6 kcal/mol,* **b)** *-29.4kcal/mol,* **c)** *-10.3kcal/mol,*

d) *-37.1 kcal/mol,* *e)* *-16.6 kcal/mol. PinT terminator starts at position 53. Interactions with the terminator loop are very unlikely, even though they have a very low energy of hybridization (b and d).*

CsrB and CsrC sRNA are known to bind multiple copies of the CsrA protein. This protein is a post-transcriptional regulator that controls the expression of genes involved in carbon metabolism and biofilm formation. CsrA is also implicated in SPI-1/SPI-2 regulation by direct repression of *hilD* translation initiation (Altier *et al.*, 2000; Lawhon *et al.*, 2003; Martínez *et al.*, 2011). CsrB/C sRNA levels have been described to be greatly reduced under *in vitro* conditions, that mimic intracellular conditions (Potts *et al.*, 2019), when PinT expression is highest. As indicated by *in silico* binding predictions, PinT might compete with CsrA for binding to CsrB and CsrC sRNAs, titrating them away from the protein. This could facilitate CsrA repression of HilD and consequently, contribute for SPI-1 genes repression. InvR sRNA expression is activated by the SPI-1 regulator, HilD. Until now, only one target has been described for this sRNA, the *ompD* mRNA (Ipinza *et al.*, 2014; Pfeiffer *et al.*, 2007), encoding for the most abundant outer membrane protein. Altough this porin has been implicated in reducing intra-macrophage survival (Ipinza *et al.*, 2014) its role in infection is still poorly understood. Nevertheless, further experiments are necessary to validate PinT targeting of these sRNAs and dissect potential functional implications.

2.5. Concluding Remarks

In this chapter, the classical MAPS technique has been established for the *Salmonella* sRNA PinT. MAPS pull-downs had typically been applied to optimized lab conditions, in order to allow for a high RNA yield. In addition, variations to the classical approach, that resorted to more physiological set-up for the pull-downs were implemented. Moving from an artificial system based on overexpression of the bait sRNA, to a sRNA expressed from its native promoter, allowed to identify targets that are bound under physiological conditions and less likely to represent artefacts of the overexpression. Here, we implemented the MAPS pull-downs in infection relevant conditions, either *in vitro* (SPI-2 inducing conditions) or *in vivo* (intra macrophage *Salmonella*). By doing this, we were able to identify new physiologically relevant targets of PinT. Overall, the list of new candidate targets includes mRNA targets such

as the SPI-2 effector SteC, the PhoQ activator UgtL and the ribosomal protein Rpsv, and several sRNAs like CsrB and C or InvR. To further characterize the role of newly uncovered PinT-mediated regulations for the infection process, we focused on the the SPI-2 effector SteC.

3. PinT-mediated regulation of the *Salmonella* SPI-2 effector, SteC

This chapter will discuss the mechanism of PinT-mediated regulation of *steC* mRNA. We will detail the *in vitro* studies that demonstrate how PinT binds to the 5'UTR of the *steC* mRNA. By doing so, PinT prevents ribosome binding and translation initiation. This chapter also describes that PinT regulation of *steC* depends on the RNA chaperone Hfq.

3.1. PinT binds the 5'UTR of *steC*, blocking translation in an Hfq-dependent manner

In-silico prediction of interaction sites between PinT and the candidate target *steC* proposed a 5'-extended seed region as compared to the previously determined PinT seed (Westermann *et al.*, 2016) to base-pair with a region around the *steC* start codon (Fig. 2.6, Fig. 3.1). This predicted region also shows a strong conservation (Fig. 3.1). Such an interaction implies the classical control mechanism employed by Hfq-dependent sRNAs, namely an interference with target translation initiation.
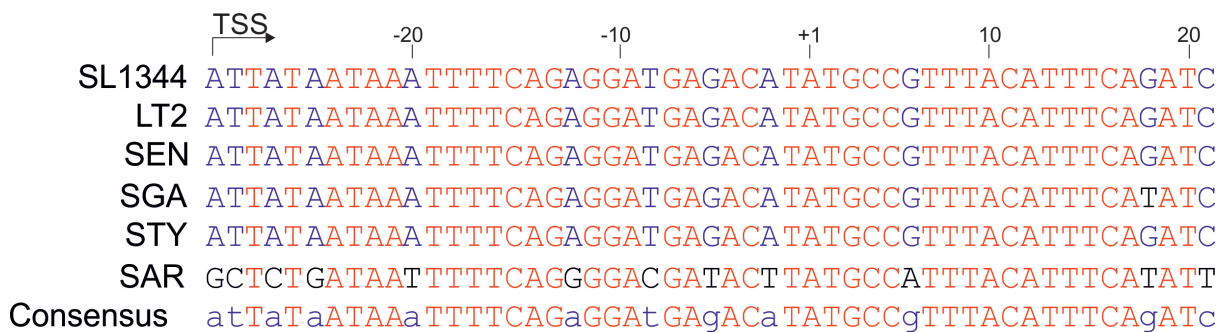
Figure 3.1 - Sequence alignment showing the conservation of steC mRNA within the genus Salmonella. "STY": S. Typhi, "SEN": S. Enteritidis, "SGA": S. Gallinarum, "SAR": S. arizonae, "SBG": S. bongori. Conserved ribonucleobases are labelled in red, less conserved bases are shown in blue. The numbers indicate the position relative to the start codon of steC (+1 position). Lines denotes the PinT target region, as determined if Fig. 2.7a.

The data to this point indicated that PinT physically interacts with *steC*, resulting in the reduced mRNA levels (Fig. 2.6, Fig. 2.7) and SteC protein (Fig. 2.8a), in line with the predicted base-pairing around the Shine-Dalgarno (SD) sequence and translation start codon of *steC* (Fig. 3.1). To validate the predicted interaction *in vitro*, we performed electrophoretic mobility shift assays (EMSAs). Gel-shift assays were performed with a fixed amount of 5'end-labeled PinT and increasing amounts of unlabeled *steC* mRNA (truncated version including the complete 5'UTR and 21 codons of the coding region) (Fig. 3.2a), or vice versa (Fig. 3.2c). Indeed, there was a complex formed between *steC* mRNA and PinT with a dissociation constant of 230 nM (Fig. 3.2b). Since PinT is an Hfq-dependent sRNA (Chao *et al.*, 2012; Sittka *et al.*, 2008), we assessed the role of Hfq in the stabilization of this interaction and incubated 5'end-labeled PinT in the presence or absence of a fixed amount of Hfq and increasing concentrations of *steC* mRNA (Fig. 3.2d). Indeed, a super-complex formed between PinT, *steC* mRNA and Hfq.
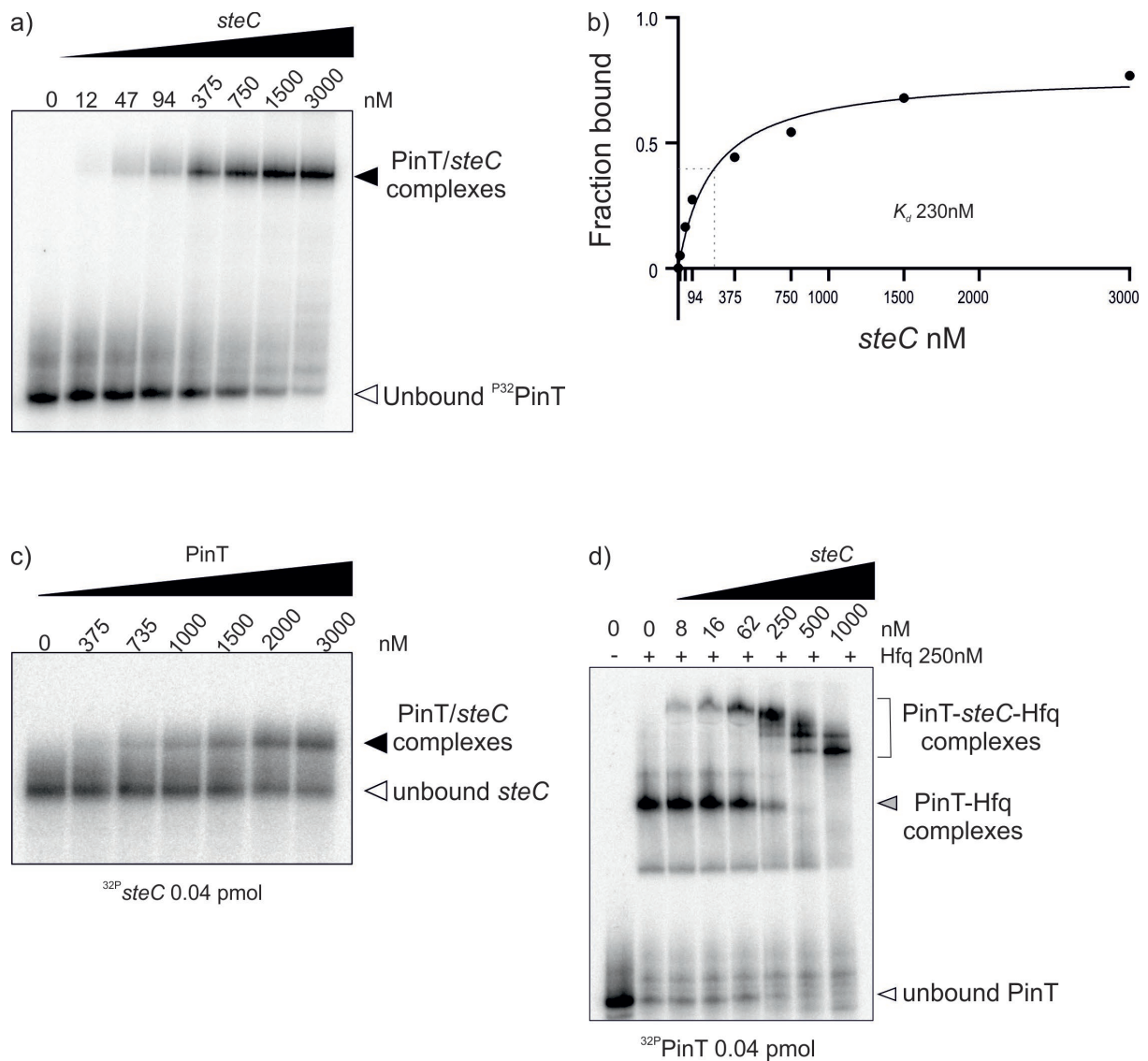
Figure 3.2 - Electrophoretic mobility shift assay (EMSA). a) *Approximately 0.04 pmol ^{32}P-labeled PinT incubated with increasing concentrations of unlabeled steC. Full arrows indicate PinT/steC complex formation and empty arrows indicate unbound PinT.* **b)** *Quantitation of dissociation constant (K_d) values based on EMSA analysis from a). Apparent K_d=230nM.* **c)** *Electrophoretic mobility shift assay (EMSA) of approximately 0.04 pmol ^{32}P-labeled steC incubated with increasing concentrations of unlabeled PinT. Full arrows indicate PinT-steC complex formation and empty arrows indicate unbound steC.* **d)** *Electrophoretic mobility shift assay (EMSA) of approximately 0.04 pmol ^{32}P-labeled PinT incubated with a fixed amount of Hfq and increasing concentrations of unlabeled steC. Full arrows indicate PinT/steC complex formation, grey arrows indicate PinT-Hfq complexes and empty arrows indicate unbound PinT.*

In order to map the precise interaction site between PinT and *steC* mRNA, we performed in-line probing. A fixed amount of *in vitro* transcribed and radioactively labeled truncated *steC* mRNA was incubated at room temperature for 40 h in the presence or absence of increasing concentrations of PinT. Visualization of the resulting cleavage products on a denaturing gel validated the in-silico-predicted interaction site close to the start codon of *steC* (Fig. 3.3).
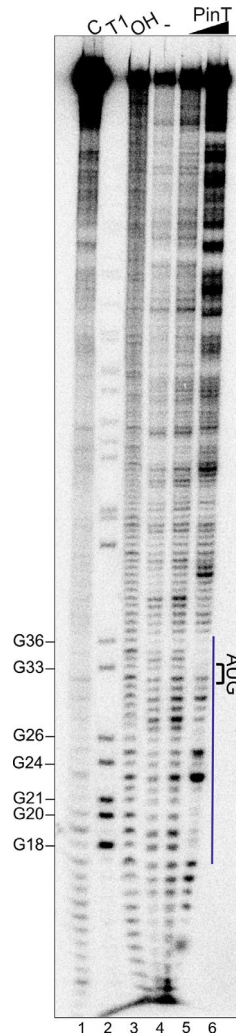
Figure 3.3 - In-line probing. *Approximately 0.2 pmol ^{32}P-labeled steC mRNA (truncated version including the complete 5'UTR and 21 codons from the coding region) in the absence (lane 4) or presence of either 30 (lane 5) or 3000 nM (lane 6) of PinT. Spontaneous cleavages of single-stranded regions were analyzed on a 10% PAA gel under denaturing conditions. A blue line indicates protection region. Untreated RNA (lane 1), RNase T1 (lane 2) or partially alkali (lane 3) digested steC served as ladders.*

Introduction of point mutations into the *steC* 5' UTR overlapping the PinT interaction site (*steC*# mutant) abolished regulation while the introduction of compensatory mutations in the PinT sequence (PinT# mutant) restored repression of the SteC::GFP reporter (Fig. 3.4).
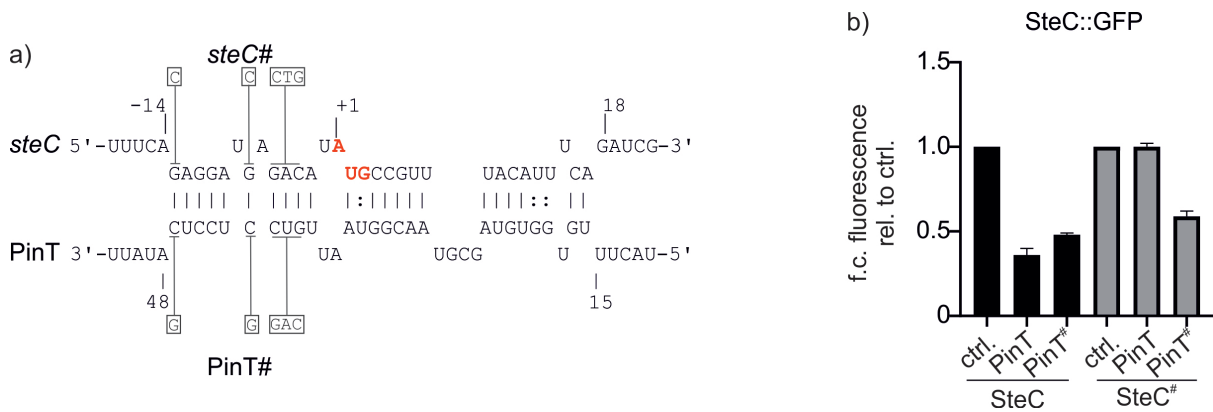
***Figure 3.4 - PinT directly binds steC mRNA 5'UTR.* a)** In silico prediction of interaction between steC mRNA and PinT. PinT# and steC# mutations are show in boxes; the start codon of steC is highlighted in red. **b)** GFP reporter assay. Validation of the base-pair interactions as shown in panel a) using translational steC::gfp reporter gene fusions by compensatory base-pair exchanges. The bars indicate the mean value ± s.d. from three biological replicates.

The finding that PinT binds near the SD sequence and start codon of *steC* indicates that the sRNA may inhibit ribosome binding and translation initiation of *steC*. To test this assumption, we performed *in vitro* translation assays (Fig.3.5). In the absence of Hfq, increasing concentrations of PinT interfered only slightly with translation of SteC::GFP (Fig. 3.5, Lanes 2-3). However, in the presence of both Hfq and PinT, *steC* translation was markedly repressed (Fig. 3.5, lanes 6-8). This effect depended on PinT, since addition of Hfq alone did not affect SteC::GFP levels (Fig. 3.5, lane 5).
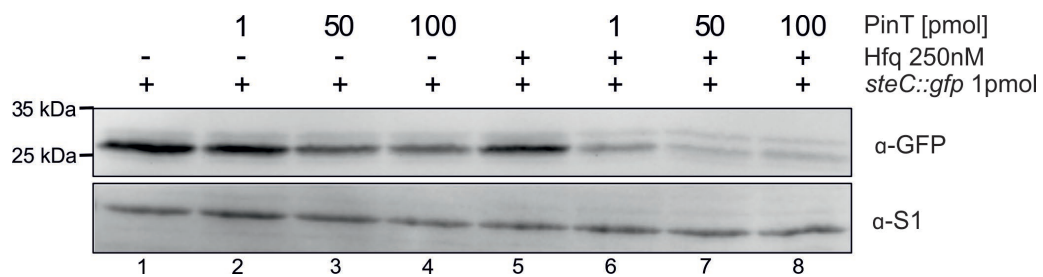
Figure 3.5 - In vitro translation assay. *In vitro synthesized, full-length steC::gfp mRNA fusion was in vitro translated with reconstituted 70S ribosomes in the presence or absence of PinT and/or Hfq. "+/-" indicate the presence or absence of PinT or Hfq.*

To corroborate these findings, we performed 30S ribosome toeprinting assays (Hartz *et al.*, 1988). The *steC::gfp* mRNA was annealed to an end-labeled primer complementary to the GFP coding region, and incubated with 30S ribosomal subunits in the presence or absence of uncharged tRNAfMet, followed by reverse transcription. Analysis of the extension products revealed one ribosome-induced, tRNAfMet-dependent toeprint at the characteristic +15 nt position (lane 3 in Fig. 3.6). This toeprint signal strongly decreased in the presence of both PinT and Hfq (lanes 6, 7). Loss of the toeprint was specific and required the presence of all interaction partners (PinT, *steC* and Hfq), whereas in absence of PinT, Hfq alone did not lead to the loss of the toeprint (lane 8). These results are in agreement with the *in vitro* translation experiment and together demonstrate that PinT binding to the *steC* 5'UTR blocks translation initiation of this SPI-2 effector.
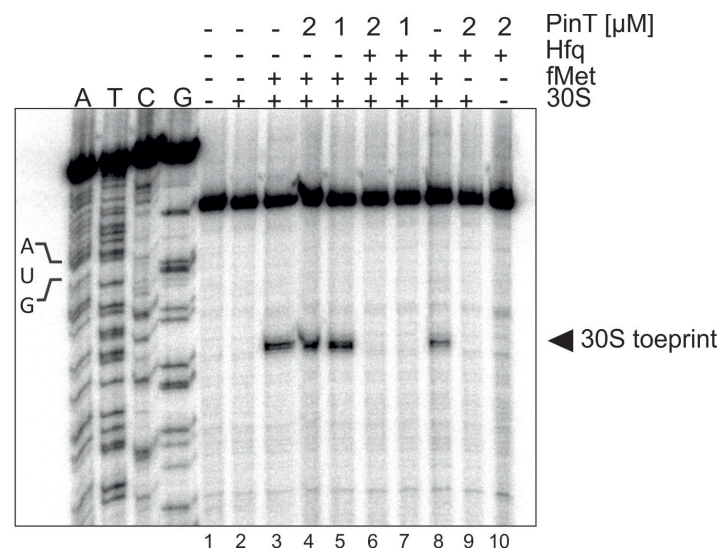
Figure 3.6 - Toeprinting assay. *Ribosome toeprinting of steC mRNA in the presence or absence of PinT and/or Hfq. "+/-" indicate the presence or absence of 30S subunit, fMet initiator tRNA, PinT or Hfq. The steC AUG start codon position is shown. The arrow indicates the 30S toeprint.*

3.2. Concluding Remarks

Making use of the several *in vitro* experiments, here we describe the regulatory mechanism of PinT-SteC interaction. Overexpression of PinT affects the overall mRNA and protein levels of *steC*. Initial *in silico* studies, predicted the PinT interaction site with the *steC* mRNA to overlap with the RBS and the start codon, reminiscent of the canonical mechanism of translational inhibition by sRNAs. Introduction of mutations in the binding region of either *steC* mRNA of PinT, abolished this interaction, while introduction of compensatory mutations restored repression. Using several independent methods (GFP reporter assays, *in vitro* 70S translational pure system, toe-printing), we could show that PinT directly binds the 5'UTR of the *steC* mRNA, establishing a stable interaction that is mediated by the extended seed region and the RNA chaperone, Hfq. In this way, PinT is able to efficiently repress translation of SteC.

4. Relevance of PinT-mediated regulation of SteC in the context of infection

This chapter will discuss the known functions of the effector protein SteC and detail how PinT-mediated regulation of *steC* affects *Salmonella* during intracellular growth. Furthermore, we will explore how this regulation affects the host response to *Salmonella* infection.

4.1. The SPI-2 effector protein SteC

Following uptake, *Salmonella* resides in the SCV. Several SPI-2 genes are involved in intracellular replication, survival and vacuole maturation. Using SPI-2 T3SS, *Salmonella* translocates several SPI-2 effector proteins, across the vacuole membrane into the host cytosol (Fass and Groisman, 2009). SteC is a SPI-2 T3SS dependent effector. It encodes for a 457 amino acid serine/threonine kinase, conserved in *Salmonella* (Fig 3.1) (Geddes *et al.*, 2005; Poh *et al.*, 2008). SteC is mostly present in in gastrointestinal serovars, which presumably indicates a role in intestinal infection. It promotes actin cytoskeleton reorganization, through the accumulation of a dense meshwork of F-actin close to the SCV. SteC has greater similarity to eukaryotic kinases, specifically Raf-1 (Fig. 4.1) (Poh *et al.*, 2008), than to known bacterial kinases. Raf-1 is involved, among other cellular processes, in actin cytoskeleton dynamics through the Rho/ROCK signaling pathway (Ehrenreiter *et al.*, 2005). The similarity between these two proteins suggests that SteC mimics Raf-1. Nevertheless, SteC-dependent F-actin rearrangement does not require activation of signaling pathways through Rho-associated protein kinase. Once in the host cytosol, SteC auto-phosphorylates and phosphorylates MEK1 directly, activating a pathway involving ERK, MLCK, and Myosin IIB, leading to the formation of F-actin cables (Odendall *et al.*, 2012). Other targets include the the cytoskeletal regulators HSP27 (Imami *et al.*, 2013) and FMNL1 (personal communication N. Typas, EMBL).
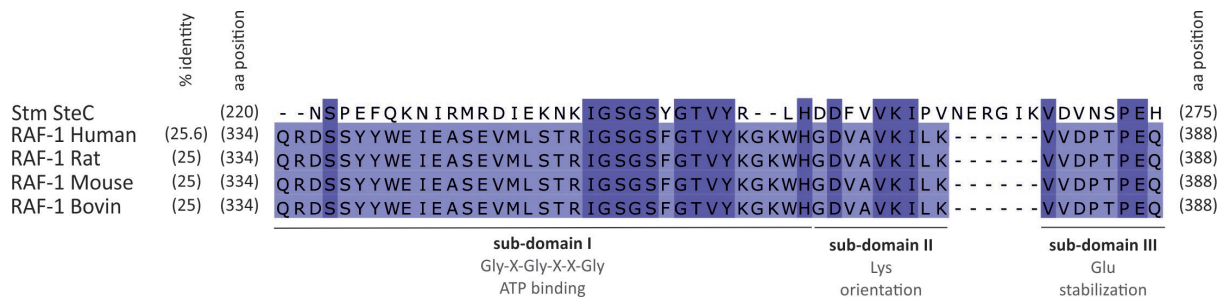
Figure 4.1 - Amino acid alignment of SteC with closely related Raf-1 kinases in several mammalian organisms. *Alignment of SteC region from aa 220 to 275. Percentage of identity of the complete protein sequence is shown. Highly conserved kinase domains I-III are shown. Adapted from (Poh et al., 2008). "Stm": Salmonella Typhimurium, "Human" : Homo sapiens, "Rat": Rattus norvegicus, "Mouse": Mus musculus, "Bovin": Bos taurus.*

This effector protein controls intracellular growth of *Salmonella,* both in epithelial cells (HeLa) and mouse bone marrow derived macrophages, as well as in mice (Odendall *et al.*, 2012). The *steC* defective *Salmonella* shows a competitive advantage compared to the WT, suggesting that the F-actin meshwork might constrain bacterial replication. This effect is dependent on the kinase activity of SteC (Odendall *et al.*, 2012). A role in colonization of the chick intestine has also been described for SteC (Morgan *et al.*, 2004).

4.2. PinT temporally controls SteC expression in intracellular *Salmonella*

Having shown that PinT represses *steC* translation *in vitro*, we set out to determine how PinT affects SteC protein expression during infection. To this end, the protein levels of the chromosomally Flag-tagged SteC protein, were measured during a time-course of infection. Here the well established *in vitro Salmonella* infection model based on human cervical carcinoma HeLa-S3 (Kihlström, 1977), was used. This infection model has been extensively used to study *Salmonella* infection, including to study the role of the sRNA PinT (Westermann *et al.*, 2016) Infections were carried out with *Salmonella* WT or *Salmonella* devoid of *pinT*. Only a weak signal for SteC was detected at 4 h p.i., in the cells infected with WT *Salmonella* (Fig. 4.2). Deletion of *pinT*, not only lead to earlier detection of SteC but also to an accumulation of higher protein levels starting from 4 h after infection.
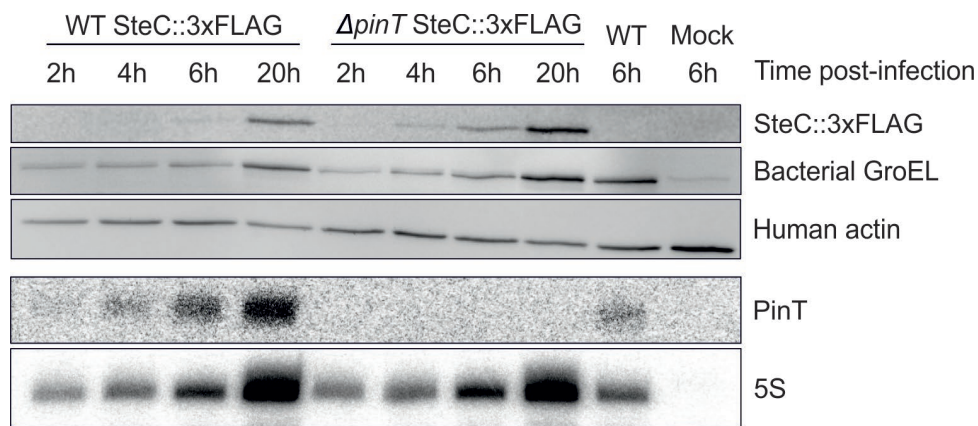
Figure 4.2 - Time course expression of SteC. *Western blot and Northern Blot analysis of intracellular Salmonella expressing SteC::3xFlag in the WT or a pinT deletion strains. Includes a WT Salmonella strain expressing WT SteC (non-tagged SteC) and a Mock control. Hela cells were infected with an M.O.I of 50. Protein and RNA samples were collected at 2h, 4h, 6h and 20h post infection. Includes GroEL and Human α-actin as a loading control for protein and RNA, respectively. Results from one out of two biological replicates are shown.*

SteC expression is not detected before 4 h upon host cell invasion (Poh *et al.*, 2008) and increases over time up until 20 h p.i., while PinT levels peak already at 8 h (Westermann *et al.*, 2016). PinT-mediated repression thus likely creates a time window of delayed SteC secretion, potentially allowing *Salmonella* to first adapt its metabolism to the SCV environment without prematurely signaling to the host. Transposon insertion studies (Chaudhuri *et al.*, 2013) failed to disrupt the *steC* locus in intestinal colonization models (chick, pig, cattle), show no attenuation for the mouse model.

4.3. PinT-mediated regulation of SteC impacts host response to *Salmonella* infection in macrophages

In order to determine the effect of PinT-mediated regulation of SteC during infection, we conducted infections in immortalized bone marrow murine macrophages (iBMMs). *Salmonella* is known to reside inside macrophages, and murine macrophages are frequently used as infection models. Here, murine macrophages were infected with either a constitutively GFP-expressing *Salmonella* WT (Papenfort *et al.*, 2009), or depleted for either

pinT or *steC*. The *steC* defective mutant showed higher infection rates when compared to WT *Salmonella*, at 4 h p.i. (Fig. 4.3a). This trend was still observed at later times, although the change was not significant. On the other hand, the *pinT* deletion strain, showed a significantly higher infection rate at 20h p.i., compared to the WT (Fig. 4.3a). The fluorescence signal intensity emitted by *Salmonella* in infected cells, measured by flow cytometry, increased over time, reflecting intracellular replication. This confirmed that the *steC* mutant has a growth advantage in intracellular bacteria at 20 h post-infection, reflected by a significant increase in GFP signal over time compared to the WT. Cell death was monitored over time, by measuring the levels of lactate dehydrogenase (LDH) release as a proxy for necrosis in infected macrophages. Neither deleting *pinT* nor *steC* had a significant effect on the cytotoxicity of infected macrophages.
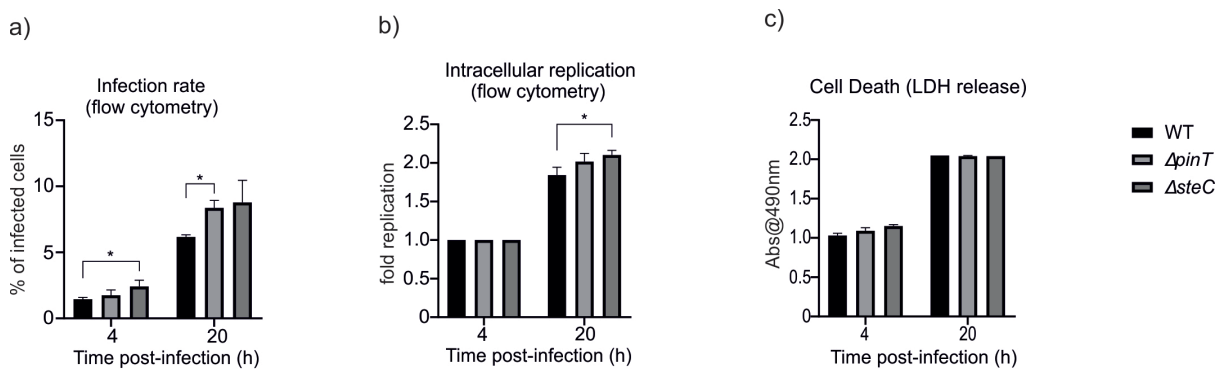
***Figure 4.3 - iBMM infections with Salmonella WT, pinT mutant or steC mutant. a)** Rate of infected macrophages during infection with a constitutively GFP-expressing WT Salmonella, a Δpint or a ΔsteC strain. Quantification of infectivity was achieved via flow cytometry (FACSCalibur, BD Biosciences) and the Cyflogic software (CyFlo) by gating for the GFP+ and GFP– populations. **b)** Intracellular replication rate of Salmonella. Flow cytometry-based quantification of the increase in GFP intensity per infected host cell over time **c)** Cell death measurement by lactate dehydrogenase (LDH) release assay in Salmonella. The colorimetric product was quantified by measuring the absorbance at 490 nm. infections were carried out using an M.O.I of 20. Results indicate the mean value ± s.d. from three biological replicates.*

To uncover the consequences of PinT-mediated SteC repression in the host response to *Salmonella*, we compared changes in the transcriptome of bulk macrophage cultures infected with either wild-type *Salmonella* or a mutant strain devoid of *steC*, at different times of

infection (2h, 4h, 6h and 20h). Our results indicated that during macrophage infection, deletion of *steC* leads to only subtle changes in the host transcriptome (Fig. 4.4), potentially due to functional redundancy among SPI-2 effectors (Figueira *et al.*, 2013). Murine pathway enrichment analysis of the host genes most upregulated in Δ*steC* compared to wild-type-infected macrophages, revealed cytokine-cytokine receptor interaction and chemokine signaling pathways as the most differentially affected host processes (Figure 4.4, Table 4.1). The majority of remaining genes were classified as putative or as non-coding RNAs, with unknown function.

a)
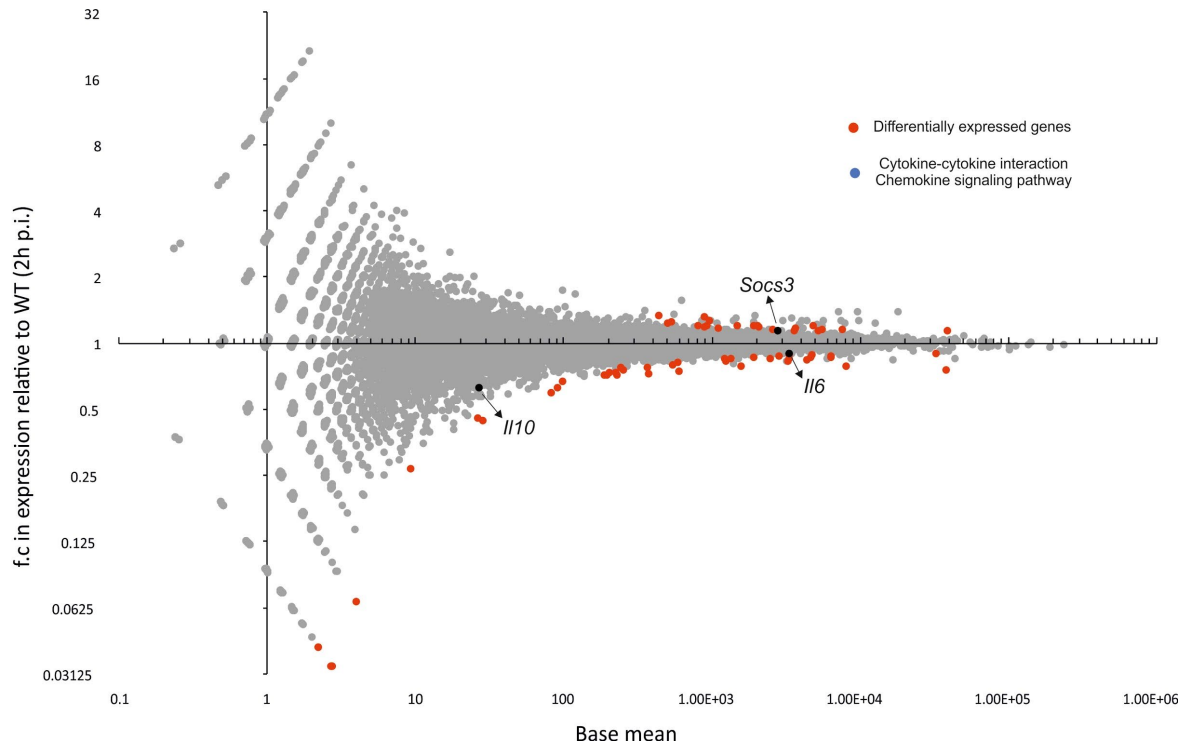
b)
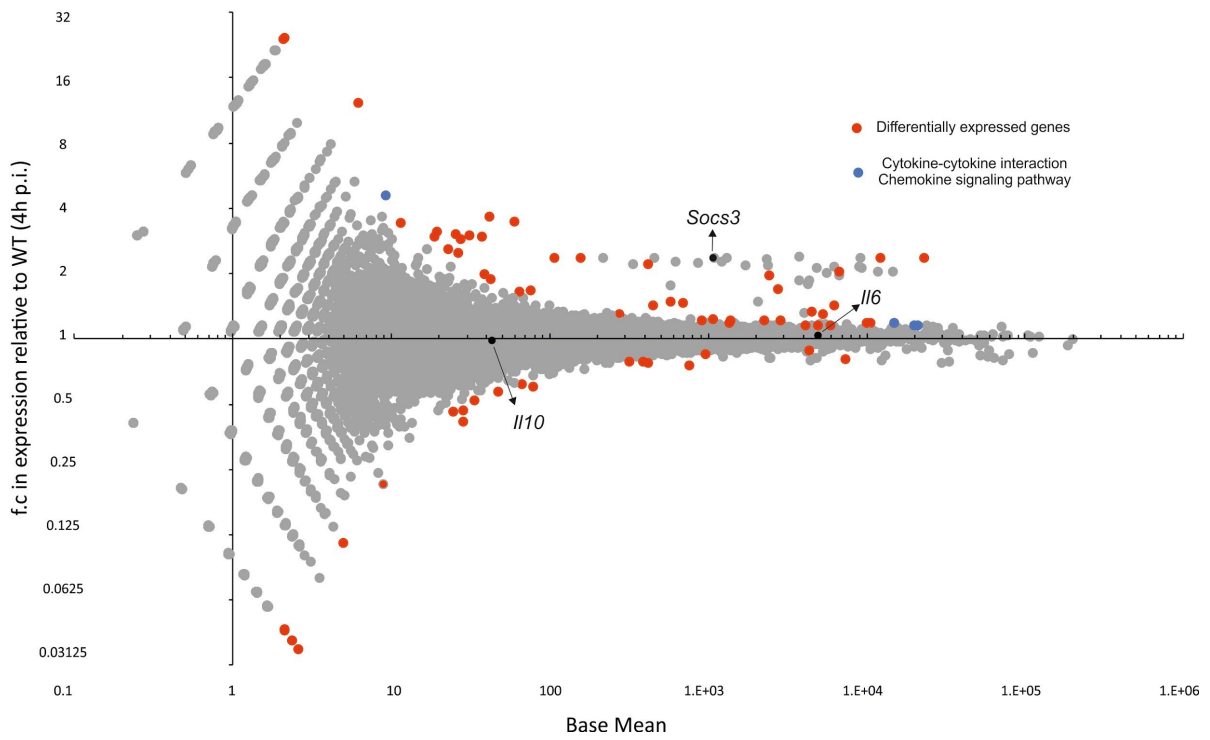
c)
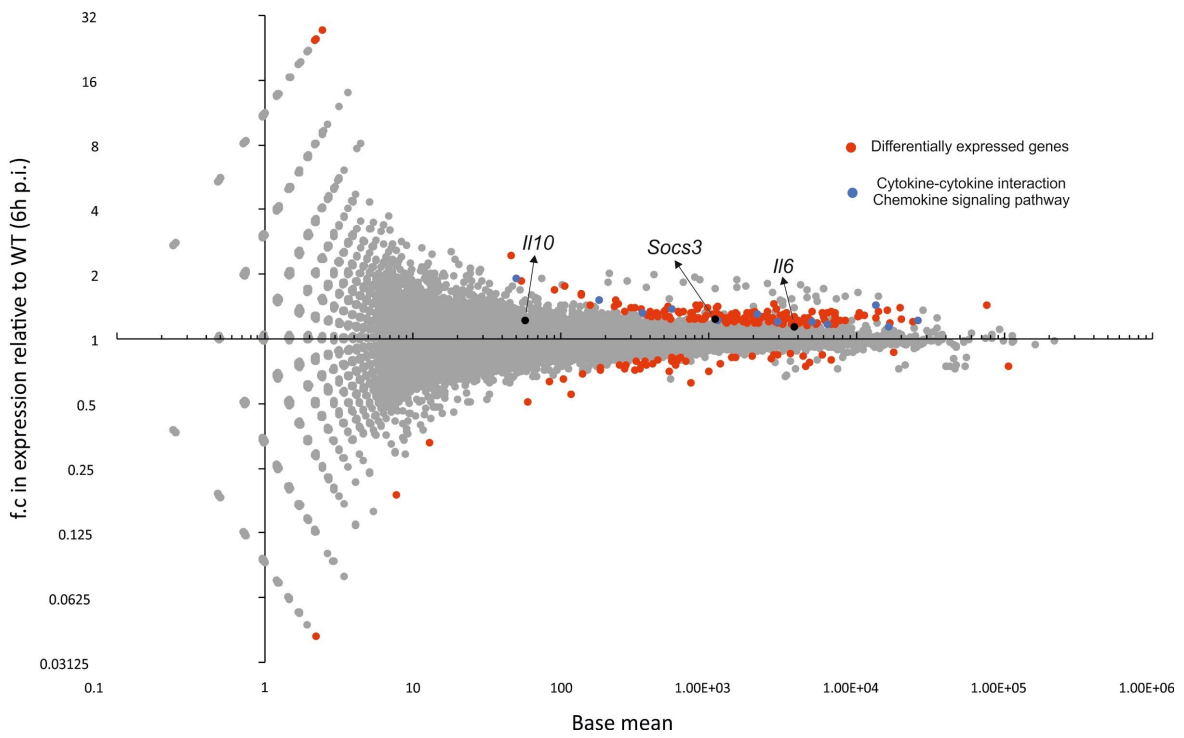
d)
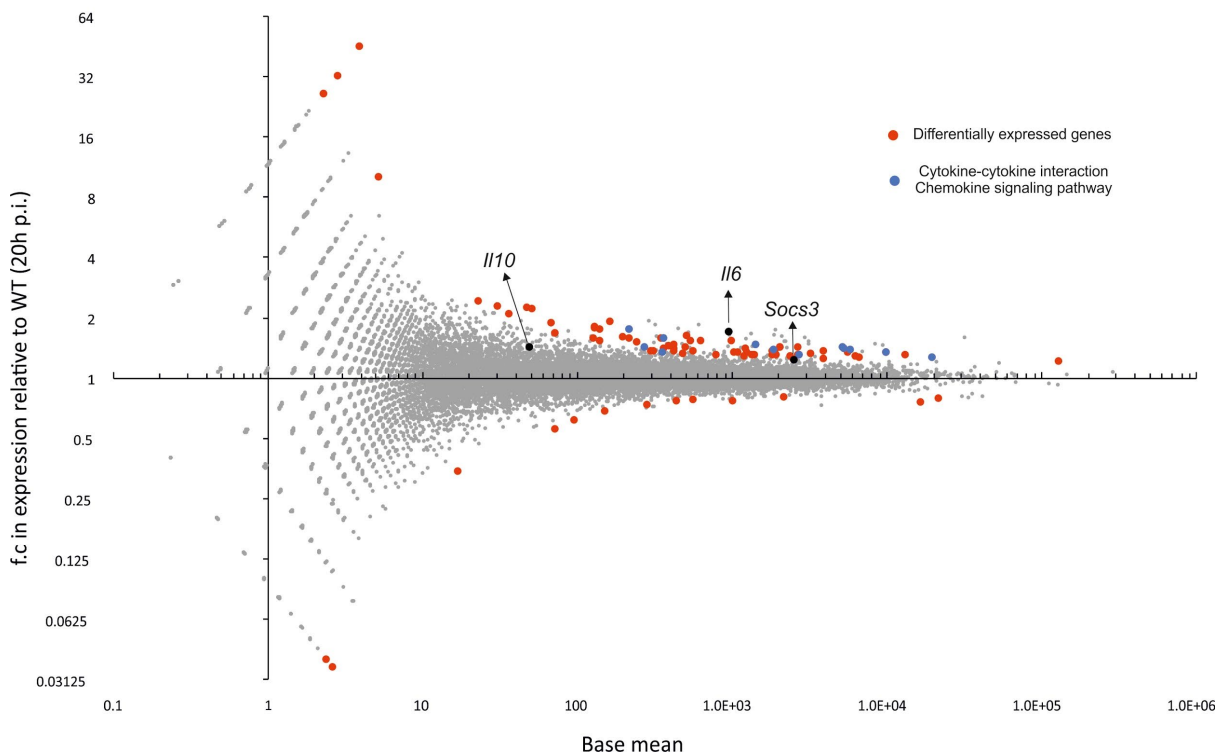
Figure 4.4 - RNA-seq profile during the time-course of infection of iBMMs. *MA plots show fold change in expression in iBMM infection with a ΔsteC mutant Salmonella strain relative to infection with wild-type Salmonella (M.O.I 50). Red filled dots identify the significantly differentially expressed genes (p-value <0.05), blue dots identify genes enriched in the KEGG pathways "Cytokine-cytokine receptor*

*interaction" and "Chemokine signaling pathway". Il6, Socs3 and Il10 are indicated with yellow, green and black, respectively. Normalized fold changes for all detected mouse genes at **a)** 2h, **b)** 4 h, **c)** 6h and **d)** 20h p.i. in the ΔsteC Salmonella strain relative to WT are plotted. Results correspond to two biological replicate experiments.*

Table 4-1 | Murine pathway enrichment analysis of the host genes most upregulated in ΔsteC compared to wild-type-infected macrophages at 20h post-infection.

KEGG Term up-regulated genes	Gene #	*p-value*
Cytokine-cytokine receptor interaction	14	5.8E-11
Chemokine signaling pathway	8	4E-5
Toll-like receptor signaling pathway	6	1.3E-4
NOD-like receptor signaling pathway	5	1.7E-4
TNF signaling pathway	6	1.8E-4
Salmonella infection	5	5.6E-4
Inflammatory bowel disease (IBD)	4	3.0E-3
Cytosolic DNA-sensing pathway	4	3.8E-3
Jak-STAT signaling pathway	5	5.4E-3
Intestinal immune network for IgA production	3	1.8E-2

Next, we proceeded with qRT-PCR-based validation of the differential expression of some of the corresponding host genes. Yet, this proved difficult, likely reflecting the high intra-experimental variation in these bulk experiments that may overlay potentially subtle effects. We therefore enriched infected macrophages by fluorescence-activated cell sorting (FACS), taking advantage of a constitutively GFP-expressing *Salmonella* strain , prior to RNA isolation and qRT-PCR measurement. As previously reported (Westermann *et al.*, 2016), *Socs3* (S*uppressor of cytokine signaling 3*) expression was elevated after infection with a *pinT* deletion mutant compared to wild-type *Salmonella* infection (Fig. 4.5a). After infection with the *ΔsteC* mutant, expression of *Socs3* showed a similar trend than after Δ*pinT* infection, although in both cases, changes were not statistically significant. Conversely, upon *steC* overexpression from a plasmid under the control of a constitutive promoter, *Socs3* expression

returned to levels seen after wild-type infection (Fig. 4.5a). Implying that, although SteC has an effect on Socs3 expression, this is independent of PinT-mediated regulation.

The pro-inflammatory cytokine interleukin 6 (IL-6) and the anti-inflammatory cytokine IL-10 activate SOCS3, paradoxically resulting in opposing cellular responses (Bode *et al.*, 1999; Ito *et al.*, 1999; Sommer *et al.*, 2005; Starr *et al.*, 1997). Since both *Il6* and *Il10* mRNAs were higher expressed in Δ*steC*- compared to wild-type-infected macrophages in our RNA-seq screen at 20h p.i. (Fig. 4.4), we included them in our qRT-PCR analysis. While *Il6* levels were not substantially altered in the absence of either *pinT* or *steC*, overexpression of *steC* again significantly reduced *Il6* mRNA levels (Fig. 4.5b). Compared to wild-type infection, *Il10* expression was reduced after infection of macrophages with Δ*pinT Salmonella*, while deleting *steC* had the opposite effect with *Il10* expression increasing 1.5-fold compared to wild-type infection. Infection with the *steC* overexpression strain significantly reduced the levels of *Il10* mRNA (3.3-fold lower than after wild-type infection) (Fig.4.5c). These results suggest that during macrophage infection, the PinT-mediated repression of SteC may alter the activation kinetics of host cytokine signaling, particularly, the production of anti-inflammatory IL-10.
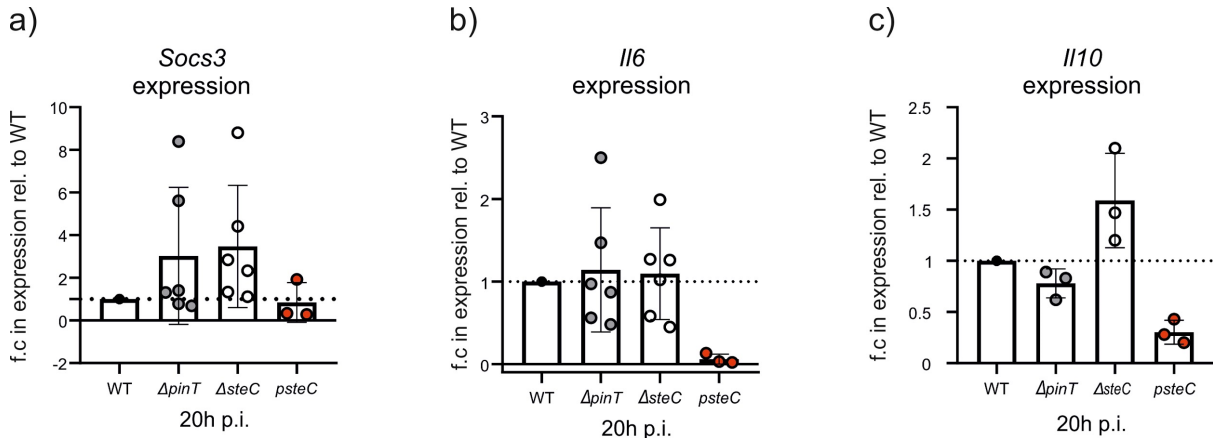
***Figure 4.5 - qRT-PCR measurements of Socs3, Il6, and Il10 host mRNAs after 20h of infection with either Salmonella** pinT or steC deletion mutants, or steC complementation strain. Transcript fold change expression was compared cells infected with WT Salmonella and normalized to the control gene U6.*

4.4. SteC and its role in regulating the host actin cytoskeleton in non-phagocytic cells

The role of SteC is best understood in non-phagocytic cells. After secretion into the host cytosol, SteC functions primarily as a kinase, phosphorylating a specific set of target proteins involved in host immune signaling cascades (Imami *et al.*, 2013; Odendall *et al.*, 2012; Poh *et al.*, 2008). All SteC target proteins in the host cell identified so far are involved in actin rearrangement, and their phosphorylation by SteC is thought to trigger formation of actin bundles in the vicinity of *Salmonella* micro-colonies inside host cell. We investigated PinT-mediated regulation of SteC impacts on actin rearrangement during infection, using Swiss T3 fibroblasts. These cell lines are a well-studied cytoskeleton model actin rearrangement by *Salmonella* in the host. e (Méresse *et al.*, 2001; Odendall *et al.*, 2012; Poh *et al.*, 2008) They allow for a particularly well defined actin phenotype, especially compared to other cell types. The Swiss 3T3 fibroblasts were infected with constitutively GFP-expressing wild-type *Salmonella*, Δ*steC* or Δ*pinT* mutants, or the corresponding complementation strains expressing either *steC* or *PinT* under a constitutive promoter. Infected cells were fixed at 10 h post-invasion and actin filaments labelled with Alexa Fluor phalloidin conjugate (Fig. 4.6). *Salmonella* inside the infected cells was visualized by detecting the GFP signal.

Uninfected cells contained very few organized actin filaments. In agreement to previous reports (Poh *et al.*, 2008), 97.5% of WT *Salmonella* infected cells showed large clusters of highly condensed F-actin associated with the bacterial microcolonies, compared to 2% in cells infected with the Δ*steC* mutant (Fig. 4.6). Similarly to WT infected cells, 97.5% of cells infected with the Δ*pinT* presented actin rearrangement. Complementation of SteC from a plasmid, resulted in the same phenotype observed in cells infected with WT bacteria, with 90% of the bacterial microsoloponies associated with the actin meshwork. However, complementation of PinT, reduced the numbers of microcolonies associated with F-actin to 7%. These results show that PinT-mediated regulation affects SteC ability to induce the formation of the F-actin meshwork.
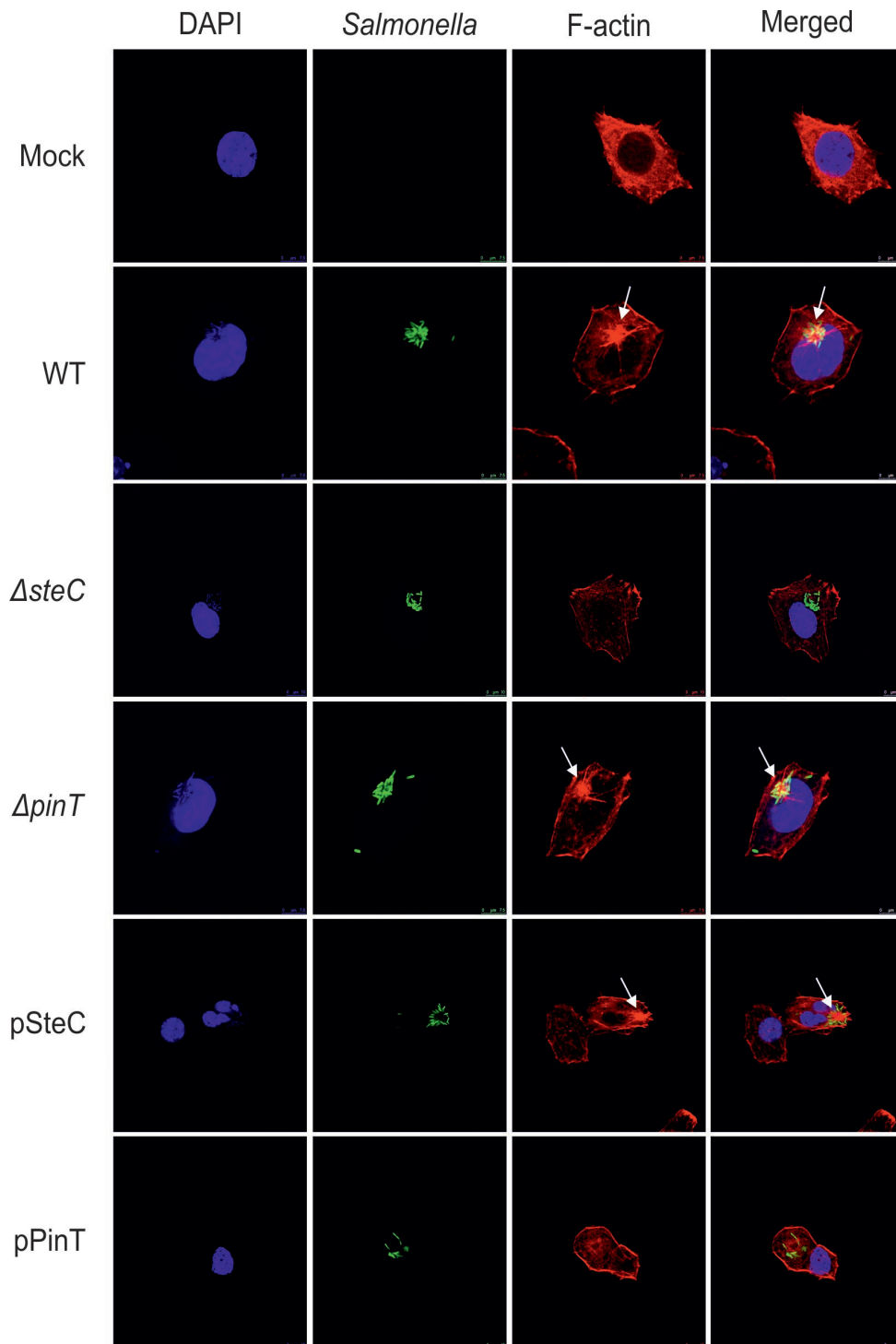
Figure 4.6 - PinT-dependent effects on F-actin rearrangement in infected cells. *Swiss 3T3 cells were infected for 10 h (M.O.I 100) with a constitutively GFP-expressing WT Salmonella, steC mutant, Pint mutant, SteC complementation or PinT complementation (under a constitutive promoter). Representative confocal images of cells infected with GFP expressing Salmonella (green in merged image). F-actin was visualized by immuno-fluorescent labelling with Alexa Fluor 594 Phalloidin (red in merged image). Chromosomal DNA, shown in blue, was stained with DAPI.*
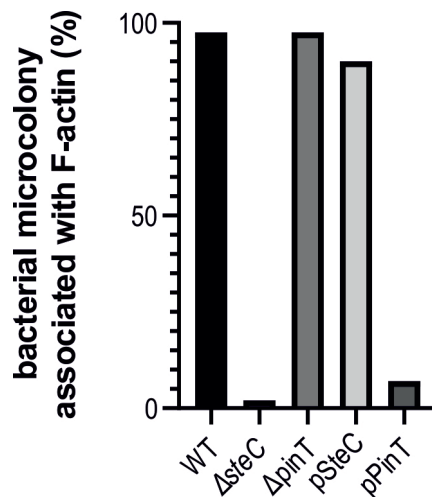
Figure 4.7 - Quantification of F-actin remodeling by Salmonella strains. *Values correspond to the percentage of infected cells where F-actin was associated with the bacterial microcolony, at 10h post-infection. 40 infected cells for each strain were analyzed.*

4.5. Concluding Remarks

Our combined results demonstrate that PinT regulates *steC* in a dual manner: directly, by blocking translation initiation of SteC, and indirectly, through repression of CRP. Once *Salmonella* resides inside of the host cell, PinT expression is upregulated 100 fold (Westermann *et al.*, 2016). At the same time, expression of SteC is delayed until at least 4 h post-infection (Poh *et al.*, 2008). Our results, demonstrate that PinT times SteC expression and, in this way, modulates the temporal dynamics of the host cellular responses. More specifically, PinT-mediated regulation of SteC has implications in terms of host F-actin rearrangements. SteC is a kinase, with similarity to human (and other mammalians) Raf-1. It is known to interact with host proteins, ultimately leading to the formation of F-actin bundles around the bacterial microcolony. PinT regulation of *steC*, allows to delay the formation of these structures until later in infection (8-10h p.i.). Our results also demonstrate that SteC alters the activation kinetics of host cytokine signaling. While regulation of SocS3 and Il6 expression is be independent of PinT; Il10 production, on the other hand, is mediated by PinT-regulation of SteC. Moreover, SteC has a mild suppressive effect on bacterial growth, therefore, delayed expression of this effector could facilitate bacterial replication intracellularly and adaption to the host environment. This assigns a specific importance to the

SteC effector as compared to the remaining SPI-2 factors, and could explain why there is a need for such a complex regulatory network, with PinT-mediated regulation targeting this effector both directly and indirectly.

5. Discussion

5.1 RNA affinity purification for bacterial sRNAs

We provide an improved MAPS protocol, which allowed to expand the targetome of the sRNA PinT, in different growth conditions. Importantly, this is the first demonstration that MAPS can be performed under physiological conditions, using endogenous levels of the bait sRNA (Table 5.1). Here, we took advantage of the fact that PinT is highly upregulated in the conditions tested, to make use of native expression of the tagged sRNA, from a low copy plasmid or from the chromosome. These results further support our observations made by the classical MAPS approach (based on overexpression of sRNAs) and allowed to confirm these were bound under physiological conditions and less likely to represent artefacts of the overexpression. Moreover, this is the first time RNA-affinity purification was used to identify RNA-RNA complexes in intracellular bacteria.

*Table 5-1|RNA affinity based purification of bacterial sRNAs. * Truncated version of the sRNA*

Organism	Background	sRNA bait	Expression	Experimental details	Growth conditions	Ref.
E. coli K12	*Δrne131 ΔryhB* *Δrne131 ΔrybB*	RyhB, RybB	pBAD promoter	0.1% arabinose 10 min	-exponential phase	(Lalaouna and Massé, 2015)
E. coli MG1655	*Δrne131 ΔcyaR* *Δrne131 ΔrprA*	CyaR, RprA	pBAD promoter	0.1% arabinose 10 min	-exponential phase - early stationary phase	(Lalaouna *et al.*, 2018)
E. coli MG1655	*ΔgcvB Δrne131*	GcvB	pBAD promoter	0.1% arabinose 10 min	-exponential phase - early stationary phase	(Lalaouna *et al.*, 2019a)
S. aureus HG001	*ΔrsaC*	RsaC*	quorum sensing dependent promoter P3		6h, 37°C, BHI	(Lalaouna *et al.*, 2019b)
Salmonella Typhimurium SL1344	*ΔsraL*	SraL	Plac promoter	1 mM IPTG 10 min	-exponential phase after anaerobic shock - late stationary phase	(Silva *et al.*, 2019)
Salmonella Typhimurium SL1344	*ΔpinT*	PinT	pBAD promoter	0.1% arabinose 2 min	early stationary phase	This study
Salmonella Typhimurium SL1344	*ΔpinT*	PinT	native promoter on a plasmid		SPI-2 inducing conditions	This study
Salmonella Typhimurium SL1344	*ΔpinT*	PinT	native promoter on the chromosome	M.O.I 50 4h p.i.	intra macrophage *Salmonella*	This study

In the future, these new MAPS approaches can be used to study the function of virulence-related sRNAs in *Salmonella* and other pathogenic bacteria. Nevertheless, the present study failed to identify RNA-RNA complexes in different types of *Salmonella* infected cells. Even though we were successful in identifying new PinT targets in *Salmonella* infected macrophages, we failed to reproduce this experiment using HeLa cells for infection. The infection rate in epithelial cells is lesser when compared to macrophages, which might explain why we failed to recover sufficient amounts of bacterial RNA in the pull-downs. Several strategies can be used in the future, to overcome these limitations. The use of new aptamers,

like the PP7 and Csy4 aptamers, could improve signal to noise ratio and allow to recover a higher yield of RNA (see Appendix). Other strategies could include enrichment of infected cells. For this, RNA stabilization strategies that preserve the RNA-RNA complexes would need to be applied.

5.2 PinT directly represses three novel, virulence related targets

In addition to the known targets, we were able to identify three new targets, of which the mRNAs for the SPI-2 effector SteC, the PhoQ activator UgtL and the 30S ribosomal protein S22 RpsV (Figure 5.1). PinT binds these targets at the 5'UTR, close to the start-codon, using a more extended region than previously described for *sopE* mRNA. PinT affects both the RNA and protein levels of these targets. Reinspection of existing PinT pulse-expression data under SPI-1 or SPI-2 conditions, or in intracellular bacteria (Westermann *et al.*, 2016), respectively, indicated *steC*, *rpsV* and *ugtL* to be down-regulated upon PinT induction, despite not reaching the significance cutoffs applied in this former study.

Both *rpsV* and *ugtL* are highly upregulated in SPI-2 inducing conditions (Kröger *et al.*, 2013) as well as inside macrophages (Srikumar *et al.*, 2015). However, their putative role in infection is only poorly understood. UgtL is a *Salmonella*-specific inner membrane protein that mediates resistance to antimicrobial peptides by modifying lipid A (Shi *et al.*, 2004) and is required for gut colonization in streptomycin-treated mice (Goto *et al.*, 2017). Recently, UgtL was described as activating the two-component system PhoP/Q under mildly acidic conditions by directly binding to PhoQ and promoting its autophosphorylation (Choi and Groisman, 2017). These authors also showed the importance of UgtL for *Salmonella* virulence in BALB/c and C3H/HeN mice infection models. The role of the ribosomal protein RpsV is currently not understood.

Having set out to find infection relevant PinT targets, we next focused on describing in more detail its interaction with *steC* mRNA. SteC SPI-2 effector protein is present in most gastrointestinal strains of *Salmonella*. SteC is a kinase described to be involved in F-actin meshwork rearrangement around the SCV. By applying several independent methods, we were able to show that PinT binds the 5'UTR of the *steC* mRNA *in vitro*. We mapped the binding interaction to the SD and the start codon of *steC*, in what corresponds to a very extended binding region. Further characterization, showed that this binding is facilitated by the RNA chaperone Hfq, resulting in translational inhibition.

The emerging PinT regulon reinforces the importance of this sRNA for the regulation of *Salmonella* virulence. PinT sRNA functions as a timer, regulating the transition between *Salmonella*'s two major virulence programs (Fig. 5.1). This regulation occurs at several layers, with PinT repressing both SPI-1 and SPI-2 regulators, as well as SPI-1 and SPI-2 effector proteins. This makes PinT the first sRNA in Gram-negatives with a pervasive role in virulence, something only previously known for some Gram-positive sRNAs involved in quorum-sensing and biofilm formation (Durand *et al.*, 2015).
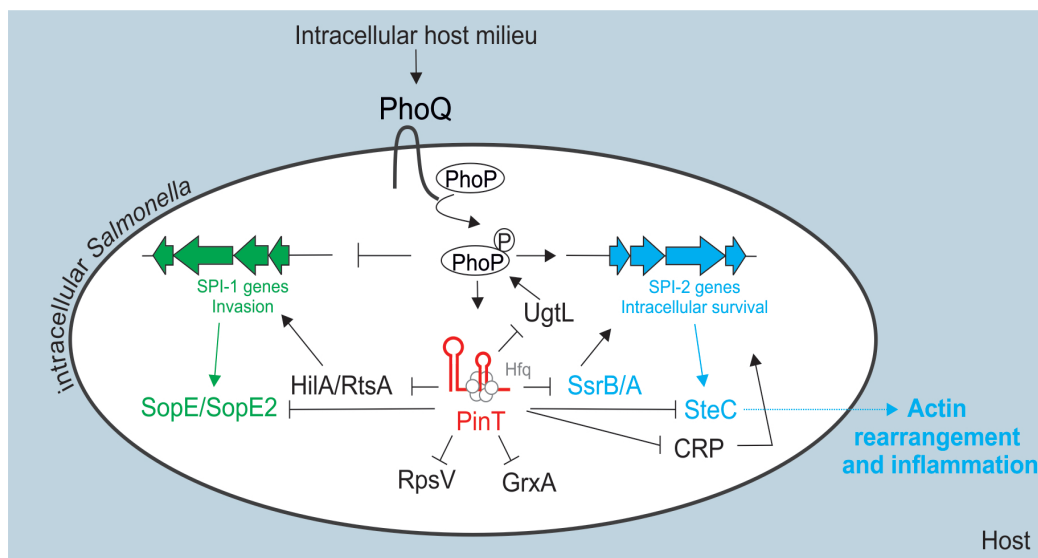
Figure 5.1 - The expanded targetome of Salmonella sRNA PinT. *The PinT sRNA temporally controls both the invasion and intracellular replication virulence programs by repressing both SPI-1 and SPI-2 expression. It directly targets some of the major regulators of SPI-1 (hilA and rtsA) and of SPI-2 (ssrB, crp, grxA). At the same time, PinT directly inhibits the expression of both SPI-1 and SPI-2 effector proteins (sopE/E2 and steC). PinT forms a negative feedback loop with ugtL and PhoQ. Repression of ugtL by PinT, reduces induction of PhoQ phosphorylation and PinT activation itself.*

5.2   Impact of PinT-mediated SteC regulation on the host response to *Salmonella* infection

When inspecting SteC protein production during HeLa infection, we observed the accumulation of this effector earlier in cells infected with *Salmonella* devoid of *pinT*, compared to WT *Salmonella*. To narrow in on the consequences of PinT-mediated SteC regulation on the host response, we first investigated RNA-seq data from a time course of iBMMs infected with *Salmonella* WT or the *steC* mutant. The number of differentially expressed genes in the Δ*steC*-infected cells, compared to the WT-infected cells, increased over-time. Nevertheless, only a small number of differentially expressed genes were identified and with fold changes that were not greater than 2 fold. At 20h p.i., most of the differentially expressed genes were involved in cytokine-cytokine receptor interaction pathway. Socs3 had previously been reported to be upregulated in cells infected with *Salmonella* devoid of *pinT* (Westermann *et al.*, 2016), here RNA-seq data of infection with the Δ*steC* mutant, showed a similar trend for *Socs3*. Following up on this gene and some of its known activators (IL6 and IL10), we were able to show that *steC* affects expression of *Socs3*, *Il6* and *Il10*, but only in the case of *Il10* this regulation seems to be mediated by PinT. SteC is known to affect F-actin remodeling in the host. To determine if this phenotype could be affected by PinT-mediated regulation of SteC, we determined the consequences of deleting or overexpressing both *pinT* and *steC* individually for actin rearrangement. F-actin polymerisation around the SCV, was affected by PinT regulation *in vivo*. Deletion of *steC* or overexpression of PinT had similar effects, that is, a significant reduction in the numbers of microcolonies associated with F-actin.

PinT sRNA is important for colonization of the pig and cattle model (Chaudhuri *et al.*, 2013). Together with its strong conservation in *Salmonella*, and strong induction after internalization, this argues for its importance during infection. Indeed, several infection relevant targets have recently been described (Kim *et al.*, 2019; Westermann *et al.*, 2016). In this work, we identified several new targets that are relevant for infection, including *steC*. The *steC* mRNA is, as well, conserved in the *Salmonella* genus, and only absent from *S. bongori*. This SPI-2 effector, shows a repressive effect on *Salmonella* intracellular replication in epithelial cells and macrophages. PinT represses *steC* on two layers, indirectly, by repressing *ssrB* and *crp*; and directly by binding to *steC* 5'UTR.

IL10 is essential for systemic infection of *Salmonella* in mice and to survive intracellularly and prevent clearance (Salazar *et al.*, 2017). IL10, a well-characterized anti-inflammatory cytokine, can inhibit the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in activated macrophages, which are a major source of IL-10 production. For this reason, SteC repression of IL10 production in macrophages, could help explain the repressive effect of this effector, on intracellular replication. *Salmonella* residing inside of macrophages are able to drive polarization (Saliba *et al.*, 2017b). PinT-mediated regulation of SteC, could allow the bacteria to drive macrophage polarization to an anti-inflammatory M2 state. Yet, the current data is not sufficient to prove this theory, and more studies need to be performed.

PinT contributes post-transcriptional cross-talk between invasion and intracellular replication programs of *Salmonella*, by controlling the expression of both SPI-1 and SPI-2 genes (directly and indirectly). PhoQ, the PinT activator, is a primary mediator of these systems by transcriptional repression of *hilA* and activation of *ssrB* (Kim, 2016). Together, PhoPQ and PinT represent multiple layers of regulation occurring at the transition from invasion to intracellular survival that allows fine-tuning the expression of several genes in response to a variety of signals. This might explain why deleting *pinT* does not produce a robust replication phenotype in cell culture assays, since PhoP regulation will still be in place.
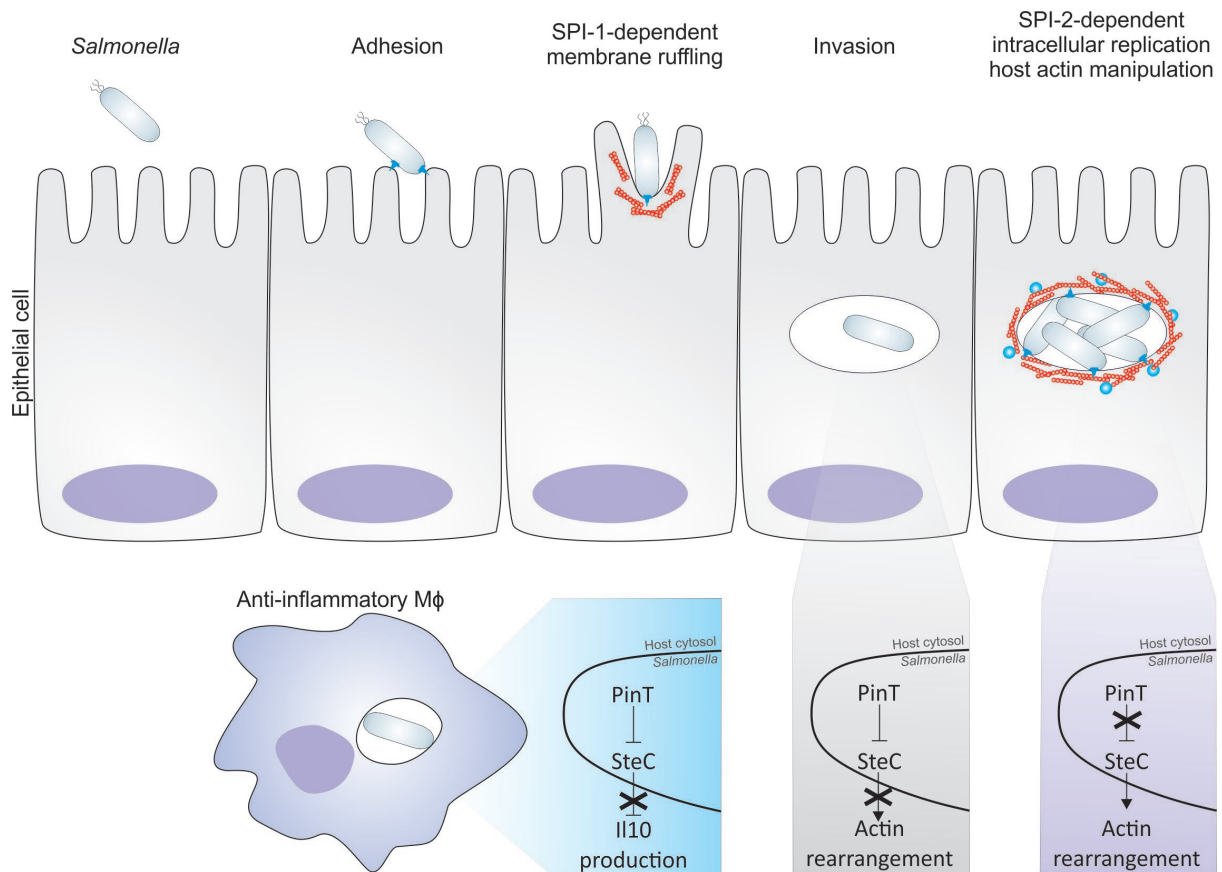
Figure 5.2 - PinT-mediated regulation of SteC impacts host actin rearrangement and inflammatory response. *Immediately after epithelial cell invasion, PinT expression is highly upregulated. At this time, expression of SteC is delayed by PinT binding to the stec and ssrB mRNAs. Later in infection, repression by PinT is alleviated and SteC is expressed. SteC production induces assembly of F-actin meshwork around the SCV. When Salmonella resides inside of macrophages, PinT-mediated repression of SteC, might facilitate immune evasion by allowing production of the anti-inflammatory cytokine, Il10. Dashed lines indicate indirect effects, while full lines indicate direct effects.*

6. Material and methods

6.1. Material

The following tables list all labware (Table 6.1), instruments (Table 6.2), enzymes, proteins, and size markers (Table 6.3), and commercially available systems (Table 6.4) that were used throughout this study.

6.1.1 Technical instruments

Table 6-1|List of instruments and devices used.

Instrument/device	Manufacturer
Bio-Link BLX 254 UV-Crosslinker	PeqLab
Centrifuge Eppendorf 5415R	Eppendorf
Centrifuge Eppendorf 5424	Eppendorf
Eraser for imaging screens	Molecular Dynamics
FACSAria III	BD Biosciences
FACSCalibur	BD Biosciences
Gel documentation system Gel iX Imager	INTAS UV Systeme
Heat block Thermomixer comfort	Eppendorf
Horizontal electrophoresis system Perfect Blue Mini S, M, L	PeqLab
Hybridization oven HP-1000	UVP
Imaging system ImageQuant LAS 4000	GE
Incubator for bacterial plates	Memmert
Incubator for cell culture flasks HERAcell 150i	Thermo Scientific
Innova 44 shaker	New Brunswick Scientific
LEICA SP5 confocal microscope	Leica
Light microscope Eclipse TS100	Nikon
MicroPulser electroporator	BioRad
Multiskan Ascent	Thermo Electron Corporation
PCR cycler MJ Mini	BioRad
Phospho-imager Typhoon FLA 7000	GE

Photometer Ultraspec 10 Cell Density Meter	Amersham Biosciences
Power supplies peqPOWER E300	PeqLab
Real time PCR cycler CFX96 Real-Time System	BioRad
Refrigerated Incubator Shaker C24KC	PeqLab
Safe 2020 cell culture hood	Thermo Scientific
Semi-dry electro-blotter Perfect Blue SEDEC M	PeqLab
Shaking water bath incubator 1092	GFL
Spectrophotometer NanoDrop 2000	Thermo Scientific
VacuSafe pump	IBS Integra Biosciences
Vertical electrophoresis system Perfect Blue Twin S, ExW S, L	PeqLab
Victor3 1420 Multilabel Counter	PerkinElmer
Vortex-Genie 2	Scientific Industries
Water bath HAAKE A10	Thermo Scientific

6.1.2 Glass/plastic ware and consumables

Table 6-2|List of lab ware used.

Labware	Manufacturer
12-well plates	Corning
24-well plates	Corning
50 mL syringe	BD Plastipak
6-well plates	Corning
96-well microtiter plates	Nunc
cell scraper 25 cm	Sarstedt
Cellstar serological pipettes 5 mL, 10 mL, 25 mL (plastic)	Greiner bio-one
Cover slips	Hartenstein
Bio-Spin® Chromatography Columns	BioRad
Dynabeads TM M-270 Streptavidin	Thermo Scientific
Electroporation cuvettes (2 mm)	Cell projects
G-25, G-50 MicroSpin columns	GE Healthcare

Gilson pipettes 10 µL, 20 µL, 200 µL, 1000 µL	Gilson
Glass beads (0.1 mm)	Roth
Glass bottles	Schott
Glass test tubes and lids	Roth
Glass tips (for pump)	Geyer
Hard-shell PCR plates 96-well WHT/WHT	BioRad
Hybond-XL Membrane for nucleic acid transfer	GE Healthcare
Imaging cassettes	Fujifilm
Imaging screens	Fujifilm
Inoculation loops (10 µL)	VWR
L-shape bacteriology loops	VWR
MACS pre-separation filters (30 µm pore size)	Miltenyi Biotec
Multidispenser combitips (5 mL)	Eppendorf
Multidispenser Repeater Plus	Eppendorf
Neubauer counting chamber	HBG Henneberg-Sander
Object slides	Hartenstein
PCR tubes (8 x 0.5 mL stripes)	Thermo Scientific
Petri dishes	Corning
Phase-lock gel (PLG) tubes 1.5 mL, 2 mL	5 PRIME
Pipetboy accu-jet pro	BRAND
Pipetman P10, P20, P200, P1000 pipet tips	Sarstedt
PolyScreen PVDF transfer membrane	PerkinElmer
Reaction tubes 15 mL, 50 mL	Sarstedt
Safe-lock tubes 1.5 mL, 2 mL	Eppendorf
Spectrophotometer cuvettes	Sarstedt
Sterile filters (0.2 µm pore size)	Sarstedt
T-150 flasks	Corning
T-25 flasks	Corning
T-75 flasks	Corning
TC Microwell 96F	Thermo Scientific

6.1.3 Chemicals, reagents, proteins and size markers

Table 6-3|List of chemicals, reagents and proteins used.

Chemicals/reagents/proteins/size markers	Manufacturer
6x DNA loading buffer	Fermentas
Albumin Fraction V	Roth
Amylose resin	New England Biolabs
Ampicillin sodium salt	Roth
Chloramphenicol	Roth
D(+)-glucose	Merck
Dimethyl sulfoxide (DMSO)	Roth
DMEM	Roth
EDTA	Gibco
Ethanol	Roth
Ethanol (absolute)	Merck
Gel loading buffer II	Ambion
GeneRuler 1 kb+ DNA Ladder	Fermentas
Gentamicin sulfate salt	Sigma
GlycoBlue	Ambion
Isopropanol	Roth
Kanamycin sulfate	Roth
L(+)-arabinose	Roth
Methanol	Roth
Milk powder	Roth
PBS	Gibco
Prestained Protein Marker Broad Range	New England Biolabs
pUC Mix Marker, 8	Fermentas
RedSafe	ChemBio
RNA Ladder High Range	Fermentas

RNA Ladder Low Range	Fermentas
RNAlater	Qiagen
Roti-Aqua P/C/I	Roth
Roti-Free	Roth
Roti-Hybri-Quick	Roth
Rotiphorese gel 40 (19:1)	Roth
Rotiphorese gel 40 (37.5:1)	Roth
SUPERaseIN RNase Inhibitor	Ambion
Triton X-100	Sigma
TRIzol reagent	Invitrogen
Western Lightning chemiluminescence reagent	GE

6.1.4 Commercial kits

Table 6-4|Commercial kits used.

Kit	Manufacturer
CytoTox 96(R) Non-Radioactive Cytotoxicity Assay	Promega
MEGAscript T7 Kit	Ambion
NucleoSpin Extract II	Macherey-Nagel
NucleoSpin Plasmid QuickPure	Macherey-Nagel
Power SYBR Green RNA-to-CT 1-Step Kit	Life technologies

6.1.5 Enzymes

Table 6-5|List of enzymes used.

Enzyme	Manufacturer
Calf Intestine Alkaline Phosphatase (CIP, 10 U/μL)	New England Biolabs
Deoxyribonuclease I (DNase I, 1 U/μL)	Fermentas
DpnI (20 U/μL)	New England Biolabs
Lysozyme	Roth, Karlsruhe
Phusion High-Fidelity DNA Polymerase (2 U/μL)	New England Biolabs

Shrimp Alkaline Phosphatase (SAP, 1 U/μL)	Fermentas
SuperScript II Reverse Transcriptase	Invitrogen
T4 DNA Ligase (5 U/μL)	Fermentas
Taq DNA polymerase (5 U/μL)	New England Biolabs
Trypsin-EDTA (0.05%), phenol red	Gibco
Various restriction enzymes from New England Biolabs or Fermentas	

6.1.6 Antibodies

Table 6-6|List of primary and secondary antibodies and -sera used.

Antibody/antiserum	Source	Dilution	Provider
anti-FLAG	mouse	1:1 000 in 3% BSA	Sigma
anti-GroEL	rabbit	1:10 000 in 3% BSA	Sigma
anti-GFP	mouse	1:1 000 in 3% BSA	Roche Applied Science
anti-human β-Actin	mouse	1:5000 in 3% BSA	Sigma
anti-mouse	goat	1:10 000 in 3% BSA	Thermo Scientific
anti-rabbit	goat	1:10 000 in 3% BSA	Thermo Scientific

6.1.7 Synthetic oligonucleotides

Table 6-7|List of synthetic oligonucleotides used.

ID	Target	Purpose	Sequence
Salmonella-directed oligonucleotides			
JVO 14657	*5S*	qRT-PCR sense primer	ACTAGCGCGGTGGTCCC
JVO 14658		qRT-PCR antisense primer	GCAGTTCCCTACTCTCGCATG
JVO 15033	*ugtL*	sense oligo to add 3xFLAG to *ugtL* in SL1344	GTCCAAAAAATTTGGGGCACAGGATGTTTCTTCACGCCCGGACTACAAAGACCATGACGG
JVO 15034		antisense oligo to add 3xFLAG to *ugtL* in SL1344	ATAGCCATTATTCAGTAAGACCGCAGGTTGCAGCGGCGGACCATATGAATATCCTCCTTA

JVO 15035		Sense for _ugtL_:3xFLAG verification PCR	TATGCCTATGGCATTACGGTATCATC
JVO 15036		Antiense for _ugtL_:3xFLAG verification PCR	GACCACTATATAGTCAGGAAGGCAAT
JVO 15037	_rspV_	sense oligo to add 3xFLAG to _rpsV_ in SL1344	TAACCCAACCGGCAGAAAGCGCCGCGCCGACAGCCAAAAGGACTACAAAGACCATGACGG
JVO 15038		antisense oligo to add 3xFLAG to _rpsV_ in SL1344	CAGATGCAATGGTGTTTAACGTCATTTCAGGACTGTACGACCATATGAATATCCTCCTTA
JVO 15039		Sense for _rpsV_:3xFLAG verification PCR	CTCGTCATATACTTGGACTGGATTAC
JVO 15040		Antiense for _rpsV_:3xFLAG verification PCR	AGTATAAAGCATTGTGAGACGTTAAG
JVO 15049	_PinT_	Sense oligo for amplification of pinT and insertion into pZE12-luc	P-AGTAACGGATTACTTTGTGGTGTAG
JVO 15050		Antisense oligo for amplification of pinT and insertion into pZE12-luc (with XbaI site)	TTTTTTCTAGAAAAAAAAAAGCGGCAGA
JVO 15401	_rpsV_	Forward oligo to make rpsV-20aa-GFP translational fusion, start from +1 in SL1344, carries BfrBI (Mph1103I) site.	gtttttttATGCATGCAACCTTAGGGGTTACGTT
JVO 15402		Reverse oligo to make rpsV-20aa-GFP translational fusion, start from +1 in SL1344, carries NheI site.	GTTTTTTGCTAGCACGCTGGTTGGAAATTCTGT
JVO 15406	MS2	Reverse oligo for MS2 fusion in pZE12Luc	AACGTACCCTGATGGTGTACGACTCTAGAGTCATTACAATTTGG
JVO 15477	_steC_	Forward oligo to make steC-21aa or 261aa-GFP translational fusion, start from +1 in SL1344, carries BfrBI (Mph1103I) site.	gtttttttATGCATATTATAATAAATTTTCAGAG
JVO 15478		Reverse oligo to make steC-261aa-GFP translational fusion, start from +1 in SL1344, carries NsiI site.	GTTTTTTGCTAGCCTCATTAACTGGAATCTTA
JVO 15494	MS2	Forward oligo to fuse MS2 to PinT in pYC55.	TTTCAGACACCATCAGGGTCTGTTACTGTCACAGTATGCTAAAA
JVO 15495		Reverse oligo to fuse MS2 to PinT in pYC55.	AACGTACCCTGATGGTGTACGACTCTAGGGTGAAGACGAAAGGG
JVO 15723	_steC_	Forward oligo for transcription with T7 promoter.	GTTTTTTTTTAATACGACTCACTATAGGATTATAATAAATTTTCAGAG
JVO		Reverse oligo for transcription.	CATCTCGCTTCGAAAATGGTCC

15724			
JVO 15730	PP7	Sense oligo to fuse PP7 aptamer to PinT in pYC5-34	CTCTCTACTGTTTCTCCGGAGCAGACGAT ATGGCGTCGCTCCTTTTTT
JVO 15731		Antisense oligo to fuse PP7 aptamer to PinT in pYC5-34	CCACAAAGTAATCCGTTACTAAAAAAGG AGCGACGCCATATCGTCTGCTCC
JVO 15732	Csy4	Sense oligo to fuse Csy4 aptamer to PinT in pYC5-34	GTTCACTGCCGTATAGGCAGAGTAACGG ATTACTTTGTGG
JVO 15733		Anti-sense oligo to fuse Csy4 aptamer to PinT in pYC5-34	CTGCCTATACGGCAGTGAACGGAGAAAC AGTAGAGAGTTG
JVO 16022	PP7	Antisense oligo for PP7 aptamer Northern Blot	GCGACGCCATATCGTCTGC
JVO 16023	Csy4	Antisense oligo for Csy4 aptamer Northern Blot	CCTATACGGCAGTG
JVO 16044	*rpsV*	Forward oligo for transcription with T7 promoter.	gtttttttTAATACGACTCACTATAGGGCAA CCTTAGGGGTTACGTT
JVO 16045		Reverse oligo for transcription.	TTACTTTTGGCTGTCGGC
JVO 16063	*ugtL*	Forward oligo for transcription with T7 promoter.	gtttttttTAATACGACTCACTATAGGAACA ACAATGAGATGTTTAG
JVO 16064		Reverse oligo for transcription.	CACGATCATGGCAAGTA
JVO 16440	PinT	sense oligo to amplify MS2-PinT from pYC362	AGTCGTACACCATCAGGGTA
JVO 16441		reverse oligo to amplify MS2-PinT from pYC362	AAAAAAGCGGCAGACTACGC
JVO 16442		sense oligo downstream of *Salmonella* PinT with p1 site (template pKD4)	GCGTAGTCTGCCGCTTTTTTgtgtaggctgga gctgcttc
JVO 16443		sense oligo for chromosomal insertion of MS2-PinT instead of PinT (binds from -40 to +20 of MS2-PinT) (2 step pcr for chromosomal insertion).	CGTTGATTCATTGTTGGGGATATTTATGT TTTACTTACCTCAGTCGTACACCATCAGG GT
JVO 16444		reverse oligo for P2 Binding site at PinT 3' end for insertion of MS2-PinT in the chromosome (keeps terminator) (2 step pcr for chromosomal insertion).	TTCATTGTCTGTTAATTATTACAGAGAGA GTTAATTTATAAggtccatatgaatatcctcctta g
JVO 16445		gtttttttttaatacgactcactataggGTTCACTGCCG TATAGGCAG	gtttttttttaatacgactcactataggGTTCACTGC CGTATAGGCAG
JVO 16509		frw oligo to check insertion of MS2-PinT in the chromosome	GTGATATACAGAATGACTAA

JVO 16510		rev oligo to check insertion of MS2-PinT in the chromosome	GCACATCGCCGACGAAAAAC
JVO 16519	*Csy4*	Sense oligo to fuse Csy4 and 4U linker to PinT in pYC5-34	GTTCACTGCCGTATAGGCAGTTTTAGTAACGGATTACTTTG
JVO 16522		Reverse oligo to fuse Csy4 to 5' of Pint in pYC5-34	GGAGAAACAGTAGAGAGTTG
JVO 16925	*steC#*	Reverse oligo to mutate steC in pSS06	ATGCATGTGCTCAGTATCTC
JVO 16952		Forward oligo for stec mutant using pSS06 as a template	ATTATAATAAATTTTCACAGGATCACTGATATGCCGTTT
JVO 16954	PinT#	Reverse oligo for PinT#	TTACGCTACACCACAAAGT
JVO 16955		Forward oligo for PinT#	CGGTAATACAGGTCCTCATATTTG
JVO 17177	*gfp*	qRT-PCR sense primer	ATGCTTTTCCCGTTATCCGG
JVO 17178		qRT-PCR reverse primer	-GCGTCTTGTAGTTCCCGTCATC
JVO 17179	*steC*	Forward oligo to introduce steC into pZE12-luc (from +1)	ATTATAATAAATTTTCAGAG
JVO 17180		Reverse oligo to introduce steC into pZE12-luc. Carries XbaI site and includes 58 nt after stop codon	gttttttctagaCGGTAAATCTGTAGCGAAT
mouse-directed oligonucleotides			
JVO 17053	*IL6*	Forwad oligo for qPCR detection of Mus musculus Il6	TTCCATCCAGTTGCCTTC
JVO 17054		Reverse oligo for qPCR detection of Mus musculus Il6	CTGTTGGGAGTGGTATCC
JVO 17091	*Socs3*	Reverse oligo for qPCR detection of Mus musculus Socs3	GTCACCCACAGCAAGTTT
JVO 17092		Forward oligo for qPCR detection of Mus musculus Socs3	TTCACCACCAGCTGGTA
JVO 17272	*Il10*	Frw oligo for qPCR amplification of Mus musculus Il10.	GCTATGCTGCCTGCTCTTACT
JVO 17273		Rev oligo for qPCR amplification of Mus musculus Il10	GGTGCAGTTATTGTCTTCCCG
human-directed oligonucleotides			

JVO-16511	*premiR-21*	Forward oligo for transcription with T7 promoter.	GTTTTTTTTAATACGACTCACTATAGGTAGCTTATCAGACTGATG
JVO-16513		Reverse oligo for transcription.	GACAGCCCATCGACTGGTGT
JVO-16512	*Csy4-premir-21*	Forward oligo for transcription with T7 promoter.	GTTTTTTTTAATACGACTCACTATAGGGTTCACTGCCGTATAGGCAGTAGCTTATCAGACTGATG

6.1.8 Plasmids

Table 6-8|List of plasmids used.

Trivial name	Stock number	Relevant insert	Description	Parental plasmid	Resistance marker	Reference
pBAD-ctrl.	pKP8-35		empty control plasmid		Amp	(Westermann *et al.*, 2016)
pBAD-PinT	pYC5-34	PinT	wild-type STnc440 under arabinose-inducible promoter	pBAD	Amp	(Westermann *et al.*, 2016)
FLP helper plasmid	pCP20		used to heal genetically modified SL1344 derivatives		Amp	(Datsenko and Wanner, 2000)
pOWN-ctrl.	pJV300		empty control plasmid	pZE12	Amp	(Westermann *et al.*, 2016)
	pXG-10		empty control plasmid		Cm	(Urban and Vogel, 2007)
	pXG-1	*gfp*	positive control vector		Cm	(Urban and Vogel, 2007)
pOWN-PinT	pYC55	PinT	wild-type PinT under native promoter	pZE12	Amp	(Westermann *et al.*, 2016)
pPinT	pYC11	PinT	wild-type PinT under constitutive promoter	pZE12	Amp	(Westermann *et al.*, 2016)
pBAD-MS2PinT	pYCS362					
rpsV::gfp	pSS05	*rpsV::gfp*	*rpsV21aa* translational GFP fusion	pXG-10	Cm	This study
steC::gfp	pSS06	*steC::gfp*	*steC21aa* translational GFP fusion	pXG-10	Cm	This study
pBAD-Csy4-PinT	pSS10	Csy4-PinT	Csy4-PinT under arabinose-inducible promoter	pBAD	Amp	This study

pBAD-PP7-PinT	pSS13	PP7-PinT	PP7-PinT under arabinose-inducible promoter	pBAD	Amp	This study
pOwn-MS2-PinT	pSS031	MS2-PinT	MS2-PinT under native promoter	pZE12	Amp	This study
pOwn-MS2	pSS032	MS2	MS2 (keeps PinT terminator) under PinT native promoter	pZE12	Amp	This study
pBAD-Csy4-4U-PinT	pSS033	Csy4-4U-PinT	Csy4-4U-PinT under arabinose-inducible promoter	pBAD	Amp	This study
pBAD-Csy4	pSS038	Csy	Csy4 (keeps PinT terminator) under arabinose-inducible promoter	pBAD	Amp	This study
pBAD-PP7	pSS039	PP7	PP7 with PinT terminator under arabinose-inducible promoter	pBAD	Amp	This study
pBAD-PinT#	pSS048	PinT#	wild-type STnc440 under arabinose-inducible promoter	pBAD	Amp	This study
pSteC#::GFP	pSS054	*steC#::gfp*	*steC#21aa* translational GFP fusion	pXG-10	Cm	This study
pSteC	pSS64	*steC*	wild-type SteC under constitutive promoter	pZE12	Amp	This study

6.1.9 Bacterial strains

Table 6-9|List of Salmonella strains used.

Trivial name	Stock name	Resistance marker	Comments	Reference
wild-type	JVS-1574	Str	*Salmonella* Typhimurium SL1344	Laboratory stock
wild-type lambda red	JVS-3013	Str, Amp	*Salmonella* WT carrying temperature sensitive plasmid pKD46, expressing lambda Red genes for recombination or chromosomal deletions	(Datsenko and Wanner, 2000)
wild-type GFP	JVS-3858	Str, Cm	constitutively GFP-expressing SL1344 strain (GFP integrated in the chromosome; ptet-GFP)	(Papenfort *et al.*, 2009)
Δ*pinT*	JVS-10854	Str	*pinT* deletion (terminator was kept)	(Westermann *et al.*, 2016)

ΔpinT GFP	JVS-10038	Str, Cm	*pinT* deletion (terminator was kept) in the ptet-GFP background background	(Westermann *et al.*, 2016)
ΔsteC GFP	JVS-11355		constitutively GFP-expressing SL1344 strain (GFP integrated in the chromosome; ptet-GFP) in a *steC* deletion background	Vogel lab strain
ΔpinT, steC::3xFlag PinT	JVS-11635	Str, Amp	epitope-tagged endogenous *stec* on the chromosome in the *pinT* deletion background with PinT complementation in a plasmid under a constitutive promoter	This study
ΔpinT, RpsV::3xFlag PinT	JVS-11636	Str, Amp	epitope-tagged endogenous *rpsV* on the chromosome in the *pinT* deletion background with PinT complementation in a plasmid under a constitutive promoter	This study
ΔpinT, UgtL::3xFlag PinT	JVS-11637	Str, Amp	epitope-tagged endogenous *ugtL* on the chromosome in the *pinT* deletion background with PinT complementation in a plasmid under a constitutive promoter	This study
ΔpinT, steC::3xFlag PinT	JVS-11638	Str, Amp	epitope-tagged endogenous *stec* on the chromosome in the *pinT* deletion background with PinT complementation in a plasmid under the native promoter	This study
ΔpinT, rpsV::3xFlag PinT	JVS-11639	Str, Amp	epitope-tagged endogenous *rpsV* on the chromosome in the *pinT* deletion background with PinT complementation in a plasmid under the native promoter	This study
ΔpinT, ugtL::3xFlag PinT	JVS-11640	Str, Amp	epitope-tagged endogenous *ugtL* on the chromosome in the *pinT* deletion background with PinT complementation in a plasmid under the native promoter	This study
ΔpinT, pBAD-pinT	JVS-11716	Str, Amp	complementation of PinT from a pBAD plasmid in a *pinT* deletion background	This study
ΔpinT, pBAD	JVS-11717	Str, Amp	empty pBAD plasmid in a *pinT* deletion background	This study
ΔpinT, pBAD-PinT, GFP control	JVS-11719	Str, Amp, Cm	complementation of PinT from a pBAD plasmid in a *pinT* deletion background and GFP control in a pXG-10 plasmid (for GFP translational reporter assay)	This study
ΔpinT, pBAD + GFP control	JVS-11721	Str, Amp, Cm	empty pBAD plasmid in a deletion of *pinT* background and GFP control in a pXG-10 plasmid (for GFP translational reporter assay)	This study
ΔpinT, pBAD , *steC::gfp*	JVS-11727	Str, Amp, Cm	empty pBAD plasmid in a deletion of *pinT* background and GFP tagged *steC* in a pXG-10 plasmid (for GFP translational reporter assay)	This study

ΔpinT, pBAD-PinT, steC::gfp	JVS-11728	Str, Amp, Cm	complementation of PinT from a pBAD plasmid in a deletion of *pinT* background and GFP tagged *steC* in a pXG-10 plasmid (for GFP translational reporter assay	This study
ΔpinT, PP7-PinT	JVS-11866	Str, Amp	*pinT* deletion strain with overexpression of PPP-PinT from a pBAD plasmid	This study
ΔpinT, pown_PinT	JVS-11979	Str, Amp	complementation of PinT from a pZE12-luc with the native promoter in a *pinT* deletion background	This study
ΔpinT, pOWN MS2-PinT	JVS-12092	Str, Amp	deletion of *pinT* expressing MSPinT2 from a plasmid under the control of the PinT promotor	This study
ΔpinT, pOWN_MS2	JVS-12093	Str, Amp	deletion of *pinT* expressing MS2 (with PinT terminator) from a plasmid under the control of the PinT promotor	This study
ΔpinT, pBAD-PP7	JVS-12098	Str, Amp	*pinT* deletion strain with overexpression of PP7 (keeping PinT terminator) from a pBAD plasmid	This study
ΔpinT, SteC3xFLAG	JVS-12401	Str, Amp	epitope-tagged endogenous *stec* on the chromosome in the *pinT* deletion background with empty pJV300 control plasmid	This study
ΔpinT, RpsV3xFLAG	JVS-12402	Str, Amp	epitope-tagged endogenous *rspV* on the chromosome in the *pinT* deletion background with empty pJV300 control plasmid	This study
ΔpinT, UgtL3xFLAG	JVS-12403	Str, Amp	epitope-tagged endogenous *ugtL* on the chromosome in the *pinT* deletion background with empty pJV300 control plasmid	This study
SteC3xFLAG	JVS-12407	Str, Amp	epitope-tagged endogenous *steC* on the chromosome in the wild-type background with empty pJV300 control plasmid	This study
RpsV3xFLAG	JVS-12408	Str, Amp	epitope-tagged endogenous *rpsV* on the chromosome in the wild-type background with empty pJV300 control plasmid	This study
UgtL3xFLAG	JVS-12409	Str, Amp	epitope-tagged endogenous *ugtL* on the chromosome in the wild-type background with empty pJV300 control plasmid	This study
Chromosomal MS2-PinT	JVS-12103	Str	*pinT* locus replaced by MS2-PinT	This study
ΔpinT, Csy4-4U-PinT	JVS-12113	Str, Amp	*pinT* deletion strain with overexpression of Csy4-4U-PinT from a pBAD plasmid	This study

ΔpinT, Csy4-4U-PinT, *sopE::gfp*	JVS-12116	Str, Amp	*pinT* deletion strain with overexpression of Csy4-4U-PinT from a pBAD plasmid and GFP tagged *sopE* in a pXG-10 plasmid (for GFP translational reporter assay)	This study
c.pinT>MS2-Term	JVS-12247	Str,	*pinT* locus replaced by the MS2 aptamer (keeping the PinT terminator)	This study
ΔpinT + MS2-PinT	SCS001	Str, Amp	*pinT* deletion strain with overexpression of MS2-PinT from a pBAD plasmid	This study
ΔpinT + MS2PinT + sopE::GFP	SCS003	Str, Amp, Cm	*pinT* deletion strain with overexpression of MS2-PinT from a pBAD plasmid and GFP tagged *sopE* in a pXG-10 plasmid (for GFP translational reporter assay)	This study
ΔpinT + MS2PinT + *sopE2::gfp*	SCS004	Str, Amp, Cm	*pinT* deletion strain with overexpression of MS2-PinT from a pBAD plasmid and GFP tagged *sopE2* in a pXG-10 plasmid (for GFP translational reporter assay)	This study
ΔpinT + PinT + *sopE2::gfp*	SCS005	Str, Amp, Cm	*pinT* deletion strain with overexpression of PinT from a pBAD plasmid and epitope GFP *sopE2* in a pXG-10 plasmid (for GFP translational reporter assay)	This study
ΔpinT, MS2PinT, GFP control	SCS007	Str, Amp, Cm	*pinT* deletion strain with overexpression of MS2PinT from a pBAD plasmid and GFP control in a pXG-10 plasmid (for GFP translational reporter assay)	This study
ΔpinT, PinT, GFP control	SCS008	Str, Amp, Cm	*pinT* deletion strain with overexpression of PinT from a pBAD plasmid and GFP control in a pXG-10 plasmid (for GFP translational reporter assay)	This study
ΔpinT, PinT, s*opE::gfp*	SCS017	Str, Amp, Cm	*pinT* deletion strain with overexpression of PinT from a pBAD plasmid and GFP *sopE* in a pXG-10 plasmid (for GFP translational reporter assay)	This study
ΔpinT, MS2	SCS039	Str, Am	*pinT* deletion strain with overexpression of MS2 from a pBAD plasmid	This study
ΔpinT, PP7PinT, GFP control	SCS054	Str, Amp, Cm	*pinT* deletion strain with overexpression of PP7PinT from a pBAD plasmid and GFP control in a pXG-10 plasmid (for GFP translational reporter assay)	This study
ΔpinT, PP7-PinT, *sopE::gfp*	SCS055	Str, Amp, Cm	*pinT* deletion strain with overexpression of PP7PinT from a pBAD plasmid and GFP *sopE* in a pXG-10 plasmid (for GFP translational reporter assay)	This study
ΔpinT, Csy4-	SCS082	Str, Amp	*pinT* deletion strain with overexpression of	This study

term			Csy4 (keeping PinT terminator) from a pBAD plasmid	
ΔpinT, PinT#, GFP control	SCS104	Str, Amp, Cm	*pinT* deletion strain with overexpression of Pin# from a pBAD plasmid and GFP control in a pXG-10 plasmid (for GFP translational reporter assay)	This study
ΔpinT, PinT#, steC::gfp	SCS106	Str, Amp, Cm	complementation of PinT# from a pBAD plasmid in a deletion of *pinT* background and GFP tagged *steC* in a pXG-10 plasmid (for GFP translational reporter assay	This study
ΔpinT, pBAD, steC#::gfp	SCS108	Str, Amp, Cm	empty pBAD plasmid in a deletion of *pinT* background and GFP tagged *steC#* in a pXG-10 plasmid (for GFP translational reporter assay	This study
ΔpinT, PinT, steC#::gfp	SCS109	Str, Amp, Cm	complementation of PinT from a pBAD plasmid in a deletion of *pinT* background and GFP tagged *steC#* in a pXG-10 plasmid (for GFP translational reporter assay	This study
ΔpinT, PinT#, steC#::gfp	SCS110	Str, Amp, Cm	complementation of PinT# from a pBAD plasmid in a deletion of *pinT* background and GFP tagged *steC#* in a pXG-10 plasmid (for GFP translational reporter assay	This study
ΔpinT, Csy4-4U-PinT	SCS155	Str, Amp	*pinT* deletion strain with overexpression of Csy4-4U-PinT (keeping PinT terminator) from a pBAD plasmid	This study

6.1.10 Media and supplements

SPI-2 MES liquid medium (pH 5.8):

1x MES buffer

1x phosphate buffer

0.4% (w/v) glucose

15 mM NH4Cl

1 mM MgSO4

10 µM CaCl2

0.04% (w/v) L-histidine

0.001% (w/v) thiamine

10x Micronutrients

DMEM complete:

DMEM (Gibco)

10% fetal calf serum (FCS; Biochrom)

2 mM L-glutamine (Gibco)

1 mM sodium pyruvate (Gibco)

DMEM complete + Pen/Strep:

DMEM complete

1% penicillin/streptomycin

DMEM complete + gentamicin (low):

DMEM complete

10 µg/mL gentamicin

DMEM complete + gentamicin (high):

DMEM complete

50 µg/mL gentamicin

6.1.11 Buffers and solutions

30:1 ethanol/sodium acetate:

30 parts of 100% ethanol

(for RNA precipitation) 1 part of 3M sodium acetate (pH 6.5)

Agarose gel electrophoresis solution:

X% (w/v) agarose in 1x TAE buffer

Chemiluminescence solution A:

0.1 M Tris-HCl (pH 8.6)

0.025% (w/v) luminol

Chemiluminescence solution B:

0.11% (w/v) p-coumaric acid (in DMSO)

DNA loading dye (5x stock):

10 mM Tris-HCl (pH 7.6)

60% (v/v) glycerol

60 mM EDTA (pH 8.0)

0.025% (w/v) bromphenol blue

PAA gel electrophoresis solution (6%):

100 mL 10x TBE

(for Northern blots) 420 g 7M Urea

150 mL Rotiphorese Gel 40 (19:1)

H2O ad 1 L

PAA stacking gel solution (4%):

1.25 mL Tris solution ("upper buffer")

(for Western blots) 1 mL Rotiphorese Gel 40 (37.5:1)

7.5 mL H2O

75 µL 10% (w/v) APS

7.5 µL TEMED

PAA separation gel solution (12%):

3.75 mL Tris solution ("lower buffer")

(for Western blots) 3 mL Rotiphorese Gel 40 (37.5:1)

3.25 mL H2O

150 µL 10% (w/v) APS

15 µL TEMED

Protein loading dye RPA (2x stock):
98% (v/v) formamid
2 mM EDTA (pH 8.0)
0.02% (w/v) bromphenol blue
0.02% (w/v) xylene cyanol

RNA loading dye GL-II (2x stock):
0.13% (w/v) SDS
18 µM EDTA (pH 8.0)
95% formamid
0.025% (w/v) bromphenol blue
0.025% (w/v) xylene cyanol

SDS running buffer (10x stock):
30.275 g Tris base
144 g glycin
10 g SDS
H2O ad 1 L

SSC buffer (20x stock):
3 M NaCl
0.3 M sodium citrate
titrate to pH 7.0 (using HCl)

TAE buffer (50x stock):
242 g Tris base
51.7 mL acetic acid
10 mM EDTA (pH 8.0)
H_2O ad 1 L

TBE buffer (10x stock):
108 g Tris base
55 g boric acid
20 mM EDTA (pH 8.0)
H_2O ad 1 L

TBS buffer (10x stock):
24.11 g Tris base
72.6 g NaCl
titrate to pH 7.4 (using HCl)
H_2O ad 1 L

TBST buffer (10x stock):
1x TBS
0.1% (v/v) Tween-20

Transfer buffer (10x stock):
30 g Tris base
144 g glycin
H_2O ad 1 L

Tris "lower buffer" solution:
1.5 M Tris-HCl (pH 8.8)
0.4% (w/v) SDS

Tris "upper buffer" solution:
0.5 M Tris-HCl (pH 6.8)
0.4% (w/v) SDS

Western development solution:
2 mL chemiluminescence solution A
200 chemiluminescence solution B
5 µL 3% (v/v) H_2O_2

6.2. Methods

6.2.1   Microbiological methods

All materials used throughout this study were autoclaved for 20 minutes at 121°C and 1 bar before use. Where necessary, solutions were sterilized by filtration and glassware by heating to 180°C for a minimum of three hours (h), respectively.

6.2.1.1   Bacterial culture

<u>*Standard growth conditions*</u>: *Salmonella enterica* serovar Typhimurium strain SL1344 (JVS-1574) is referred to as wild-type in this study. While *Salmonella enterica* serovar Typhimurium strain SL1344 constitutively expressing GFP from a chromosomal locus (strain JVS-3858) previously described (Papenfort *et al.*, 2009) is referred to as GFP wild-type throughout this study. The complete list of bacterial strains used in this study is provided in Table 6.9. Bacteria were grown in Lennox broth (LB) medium at 37°C with shaking at 220 rpm. When appropriate, 100 μg/mL ampicillin (Amp), 50 μg/mL kanamycin (Kan) or 20 μg/mL chloramphenicol (Cm) (final concentrations) were added to the liquid medium or agar plates.

<u>*Growth under SPI-2 conditions:*</u> To grow *Salmonella* under SPI-2-inducing conditions, 1 ml of *Salmonella* grown in LB to OD_{600nm}2 was washed 2x in PBS and 1x in synthetic SPI-2 medium (Löber *et al.*, 2006). The resulting pellet was resuspended in 1 mL of SPI-2 medium and diluted 1:50 in fresh, pre-warmed SPI-2 medium (10 mL total culture volume) in 100 mL Erlenmeyer flasks. The culture was grown at 37°C, 220 rpm until it reached an OD_{600nm} of 0.3 (takes approximately 3 h).

<u>*Transformation of chemically competent E. coli:*</u> 10 μl of chemically competent *E. coli* TOP10 cells (Invitrogen) were mixed with 2 μL of a ligation reaction or 10-50 ng of plasmid DNA. The mixture was pre-incubated for 10 min on ice. Subsequently cells were heat-shocked for 1 minute at 42°C and chilled for 1 min on ice. Then 900 μl of LB medium was added and cells were recovered for 1 h at 37°C, 220 rpm. 100 μL of the culture and the residual volume were plated on LB agar supplemented with the appropriate selection antibiotics.

Preparation and transformation of electro-competent Salmonella: In order to prepare electro-competent *Salmonella* cells, LB cultures were inoculated with bacteria from an overnight culture (1:100 dilution) and grown at 37°C, 220 rpm to an OD_{600nm} of 0.5. Subsequently, the cells were chilled for 30 min on ice and centrifuged (20 min; 4,000 rpm; 4°C). The cell pellets were next washed once with ice-cold H_2O and twice with 10% (v/v) glycerol. The pellets were resuspended in 1 OD_{600nm}/100 µL of ice-cold H_2O and each 100 µL were distributed in pre-cooled electroporation cuvettes (2 mm gap size) and mixed with ~10 ng of the respective plasmid DNA. Transformation was achieved by electroporation (200 Ω; 25 µF; 2.5 kV). Transformed bacteria were resuspended in 900 µL of LB medium and recovered by shaking at 220 rpm and 37°C for 1 h.

One-step modification of genes in the chromosome: Chromosomal mutagenesis of *Salmonella* SL1344 was performed as previously described (Datsenko and Wanner, 2000). The respective resistance cassette was eliminated using the FLP helper plasmid pCP20 at 42°C (Datsenko and Wanner, 2000). All mutations were transduced into the wild-type background using phage P22 (Sternberg and Maurer, 1991).

Phage P22 transduction: For the transfer of chromosomal mutations to different strain backgrounds, P22 phage lysates were prepared using soft agar plates as previously described (Sternberg and Maurer, 1991). LB cultures were inoculated the donor strain grown over-night, were incubated with 5-30 µL of WT P22 phage. Next day, cultures were keep for 20 minutes at 4°C. The bacteria are pelleted and the supernatant collected into a glass tube. The remaining bacteria are killed by addition of chloroform. For transduction, 100 µL of a culture of the recipient strain (OD_{600nm} of 1.0) were mixed with 1-50 µL of the phage lysate and incubated for 20 min at room temperature. Addition of EGTA to a final concentration of 10 mM was used to stop the transduction. The sample was then plated on pre-warmed LB agar plates supplemented with appropriate antibiotics to select the positive transductants, which were subsequently verified by PCR.

6.1.10 Cell culture methods

Passaging and seeding: The following cell lines were used in this study: Human cervix carcinoma (HeLa-S3; ATCC CCL-2.2), murine macrophage cell line (NR-9456) from wild-type (WT) C57BL6/J mice (iBMMs), and NIH/3T3 fibroblasts. Cells were cultured in DMEM (Gibco) supplemented with 10% fetal calf serum (FCS; Biochrom), 2 mM L-glutamine (Gibco) and 1 mM sodium pyruvate (Gibco) for 3 days at $3x10^6$ cells per 10 mL in a T-75 flask (Corning) in a 5% CO2, humidified atmosphere at 37°C. HeLa-S3 cells were kindly by Thomas Rudel (Biocenter Würzburg) The NIH/3T3 were kindly provided by David Holden (Imperial College, London). The following reagent was obtained through BEI Resources, NIAID, NIH: Macrophage Cell Line Derived from Wild Type Mice, NR-9456."

LDH release assay: Necrosis was evaluated by quantifying released lactate dehydrogenase (LDH) via the Cytotox96 assay (Promega) according to the manufacturer's instructions. The absorbance at 490 nm was measured using a Multiskan Ascent instrument (Thermo Fisher). In order to convert the measured absorbance values into the relative proportion of dead cells, the maximal absorbance was determined by using 1x Lysis Solution (Promega) following the manufacturer's instructions and referred to as 100% cytotoxicity. Measurements were repeated at least three biological replicates were analyzed, each of which comprised three technical replicates.

6.1.11 Infection assays

In vitro Salmonella infection assay: Infection of HeLa-S3 cells and murine macrophages were carried out following the protocol of (Schulte _et al._, 2011) with slight modifications. Two days prior to infection $2x10^5$ cells were seeded in 2 mL complete DMEM (six-well format). For the NIH/3T3 cells 1 day prior to infection $5x10^4$ cells were seeded in 2 mL complete DMEM (six-well format). Overnight cultures of _Salmonella_ were diluted 1:100 in fresh LB medium and grown aerobically to an $OD_{600nm}2$. Bacterial cells were harvested by centrifugation (2 min at 12,000 rpm, room temperature) and resuspended in complete DMEM medium. Infection were carried out by adding the bacterial suspension directly to each well. In the case of murine macrophage infection, bacterial cells were incubated with 10% fetal calf serum (final concentration), for 20 min at room temperature, before addition to the cells.. Immediately

after addition of bacteria, the plates were centrifuged for 10 min at 250 g, room temperature followed by 30 min incubation in 5% CO_2, humidified atmosphere, at 37°C. Medium was then replaced for gentamicin-containing complete DMEM (final concentration: 50 mg/mL) to kill extracellular bacteria. After a further 30 min incubation step, medium was again replaced by 10 mg/mL gentamicin/DMEM and incubated for the remainder of the experiment. Time point 0 h was defined as the time when gentamicin was first added to the cells.

Quantification of intracellular replication: Infected murine macrophages cells were washed twice with PBS, detached from the bottom of the plate by trypsinization and resuspended in complete DMEM. Upon pelleting the cells (5 min at 250 g, room temperature) they were resuspended in PBS and analyzed by flow cytometry using a FACSCalibur instrument (BD Biosciences) and the Cyflogic software (CyFlo Ltd.). Selection of intact cells was achieved by gating based on cell diameter (forward-scatter) and granularity (side-scatter) (linear scale). Of those, infected (GFP-positive) and non-infected (GFP-negative) sub-fractions were defined based on GFP signal intensity (FITC channel) vs. auto-fluorescence (PE channel) (logarithmic scale). To quantify the relative amount of intracellular pathogens per host cell over time, the increase in GFP intensity (geometric mean) was measured in the GFP-positive sub-population and normalized to that of the non-infected population from the same sample as described above. Alternatively, infected cultures were solubilized with PBS containing 0.1% Triton X-100 (Gibco) at the respective time points. Cell lysates were serially diluted in PBS, plated onto LB plates and incubated at 37°C overnight. The number of colony forming units (c.f.u.) recovered was compared to that obtained from the bacterial input solution used for infection. In all cases, at least three biological replicates were analyzed, each of which comprised three technical replicates.

Cell sorting: RNAlater-fixed cells were first passed through MACS Pre-Separation Filters (30 μm exclusion size; Miltenyi Biotec.) and then analysed and sorted using the FACSAria III device (BD Biosciences) under continuous cooling to 4°C (both the input tube holder and the collection tube rack) and at a medium flow rate (~4) using the same gating strategy as described above, except that the gates for GFP-positive and -negative fractions were more conservative in order to prevent cross contamination. Typically, 10 000 to 100 000 cells were collected in each fraction and subjected to RNA isolation.

Immunofluorescence and confocal microscopy: immunofluorescence of *Salmonella* infected 3T3 cells was performed as previously described (Poh *et al.*, 2008). In summary, one day prior to infection, 3T3 cells were seeded on 10 mm coverslips at a density of $5x10^4$ cell per well. The next day, infections were carried out as mentioned above, using an M.O.I of 100. At 10 h p.i. the coverslips were washed with PBS (Gibco) and fixed in 4% (w/v) PFA for 15 min in the dark. After three additional PBS washing steps, cells were stained with Alexa Fluor 594 Phalloidin (ThermoFisher; 1:250 diluted in PBS) for 15 min in the dark and again washed twice with PBS. After coverslips had been air-dried, they were embedded in VECTASHIELD(R) Mounting Medium (Biozol) with Dapi and analyzed using the LEICA SP5 confocal microscope (Leica) and the LAS AF Lite software (Leica).

6.1.12 Basic molecular biological methods

Preparation of genomic DNA: 200µl of an overnight culture of *Salmonella* was resuspended in 100µl water and boiled for 5 minutes. Cells were briefly vortexed to ensure disruption of the cell wall. Cell debris were pelleted at 13000 rpm for 5 min. The supernatant was recovered, mixed with one volume of chloroform and vortexed for 30 seconds to ensure mixing of the aqueous and chloroform phase. Next, the aqueous phase was separated by centrifugation at 13000 rpm for 10 min. This aqueous phase was recovered and gDNA concentration measured using the NanoDrop. gDNA was used as template for PCR reactions.

Preparation of plasmid DNA: Plasmid DNA was extracted from transformed TOP10 cells using the NucleoSpin Plasmid QuickPure kit (Macherey-Nagel) following the manufacturer's instructions.

Polymerase chain reaction (PCR) :

Mix	PCR 1 reaction
Taq 10X Master Mix	1x
dNTPs	100 μM each
Sense primer	1 μM
Anti-sense primer	1 μM
Taq DNA polymerase	1.25 U
nuclease-free water	Variable volume up to 50 μL
template DNA	10-50 ng (in 1-2 μL) or single colony

To amplify DNA fragments of interest different protocols were performed. For simple PCR-based validation screens a Taq-based protocol was applied:

PCR Program		
95º C	1 min	
95º C	30 sec	
55-60º C	30 sec	30-35 cycles
72º C	1 min per KB	
72º C	5 min	
4ºC	∞	

For cloning purposes or analysis by Sanger sequencing a Phusion-based protocol was applied:

Mix	PCR 1 reaction
10 μL Phusion reaction buffer (5x)	1x
dNTPs)	100 μM each
Sense primer	1 μM
Anti-sense primer	1 μM
Phusion DNA polymerase	0.5 U
nuclease-free water	Variable volume up to 50 μL
template DNA	10-50 ng (in 1-2 μL) or single colony

PCR Program		
98º C	1 min	
98º C	30 sec	30-35
55-60º C	30 sec	cycle
72º C	30 sec per KB	s
72º C	5 min	
4ºC	∞	

Resulting PCR products were either purified directly using the QIAquick PCR Purification kit (Qiagen) following the manufacturer's instructions or separated by AGE, excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen).

Restriction digestion, DpnI digestion and DNA ligation: Restriction digestions were performed using restriction enzymes and buffers from NEB and Fermentas according to the instructions given by the manufacturers. Plasmid products from mutagenesis PCR were treated for 1 h at 37°C with DpnI (NEB) to remove remnants of the template plasmid. Blunt and sticky end ligation of DNA fragments was achieved using the T4 DNA ligase (NEB) according to the manufacturer's recommendations

Agarose gel electrophoresis: After PCR or restriction digestion, individual DNA fragments were separated by AGE. Gels were prepared by dissolving pure agarose in 1×TAE buffer to the desired concentration (typically 1-2% (w/v)). The solution was boiled until all agarose was dissolved, supplemented with 0.02% (v/v) of the RedSafe dye (ABC Scientific) and casted. Samples were supplemented with 1⁄4 volume of 5x DNA loading buffer, mixed and loaded on the gel. The GeneRuler 1 kb plus DNA Ladder (Fermentas) was loaded as a size marker. Separation was at 100-150 V.

Fusion plasmid construction: Translational GFP fusions of PinT target mRNAs were constructed as described (Urban and Vogel, 2007). in brief, BseRI/NheI digested DNA products were cloned into the BsgI/NheI-digested fusion plasmid pXG-10.

6.2.1 RNA techniques

RNA isolation: For qRT-PCR or Northern blot experiments as well as for RNA-seq-based analysis of pulse- expression experiments, total RNA was extracted using the TRIzol LS reagent (Invitrogen) according to the manufacturer's recommendations.

DNase I treatment: To get rid of contaminating genomic DNA, RNA samples for RNA-seq or qRT-PCR were treated with 1 U of DNase I (Fermentas) per 4 µg of RNA for 45 min at 37°C. When applicable, RNA quality was checked on the Agilent 2100 Bioanalyzer (Agilent Technologies).

Denaturing polyacrylamide gel electrophoresis (PAGE): To size-separate individual RNA species, denaturing polyacrylamide (PAA) gels were used. Prior to casting the gels, all glassware and equipment was cleaned with 70% ethanol. The 40% PAA solution (19:1 acrylamide/bis- acrylamide) was diluted in water to the desired concentration and supplemented with urea to a final concentration of 8.3M. The addition of 0.01 volumes of 10% APS and 0.001 volumes of TEMED polymerized the PAA solution. RNA samples were supplemented with 1 volume of 2x GL-II loading dye, denatured by boiling for 5 min at 100°C and chilled on ice for 5 min. 1x TBE was used as a running buffer. PAGE was performed at 300 V and room temperature.

qRT-PCR analysis: qRT-PCR experiments were performed with the Power SYBR Green RNA-to-CT 1-Step kit (Applied Biosystems) according to the manufacturer's instructions. Fold-changes were determined using the $2^{(-\Delta\Delta Ct)}$-method (Livak and Schmittgen, 2001). Primer sequences are given in Table 6.7 and their specificity had been confirmed using Primer-BLAST (NCBI).

Northern blotting: Total RNA was prepared using the TRIzol LS reagent and separated in denaturing PAA gels as described above. 5-10 µg of RNA per lane were loaded. Northern blotting was as described (Sittka *et al.*, 2007). For detection, Hybond XL membranes (Amersham) were hybridized at 42°C with gene-specific [32P] end-labeled DNA oligonucleotides (see Table 6.7 for sequences) in Rapid-hyb buffer (GE Healthcare).

Generation of radioactively labelled DNA oligo nucleotides for RNA detection: For labelling of DNA oligonucleotides, 10 pmol of the oligonucleotide was incubated in a 10 µl reaction volume with 25 µCi of 32P-γ-ATP in the presence of 1 u T4 polynucleotide kinase (PNK, New England Biolabs) for 1 h at 37°C. Unincorporated nucleotides were removed using MicroSpinTM G-25 columns.

in vitro transcription and 5' end labelling of RNA: DNA templates that contain the T7 promoter sequence were generated by PCR using oligos listed in Table 6.7. *in vitro* transcription was performed using the MEGAscript T7 kit, followed by DNase I digestion (1 unit) for 15 min at 37°C. Followed by Gel Extraction on a denaturing PAA gel and visualized with Ethidium Bromide. 20 pmol RNA was dephosphorylated with 10 ul of calf intestine alkaline phosphatase (CIP) in a 20 µl reaction at 37°C for 1 h. Following phenol extraction, the RNA was precipitated overnight with ethanol:sodium acetate (30:1 v/v) and 20 µg GlycoBlue. The dephosphorylated RNA was 5' end-labelled with 32P-γ-ATP (20 µCi), using 1 u of T4 polynucleotide kinase (PNK) for 30 min at 37°C in a 20 µl reaction. Unincorporated nucleotides were removed by purification of the labelled RNA on a denaturing gel (6%PAA/ 8.3 M urea). Upon visualization of the labelled RNA by exposure on a phosphorimager, the RNA was cut from the gel and eluted with RNA elution buffer at 4°C overnight, followed by P:C:I extraction and ethanol precipitation.

RNA elution buffer:

0.1 M sodium acetate

0.1% SDS

10 mM EDTA, pH 8.0

Electrophoretic mobility shift assay (EMSA): Gel-shift assays were performed with ~0.04 pmol 5'-end labeled PinT (4 nM final concentration) and increasing amounts of unlabeled RNA in 10 µl reactions. After denaturation (1 min at 95 °C), labeled RNAs were cooled for 5 min on ice and 1 µg yeast RNA and 10 x RNA structure buffer (Ambion) were added. Increasing concentrations of unlabeled RNA were added to final concentrations of 12 nM, 47nM, 94 nM, 375nM, 750 nM, 1500 nM, and 3000 nM. For gelshift assays with ^{32}P-labeled *steC* mRNA truncated variant (from TSS to +250nt in the CDS), ~0.04 pmol ^{32}P-labeled *steC* was incubated

increasing concentrations of unlabeled PinT to final concentrations of 375 nM, 750 nM, 1500 nM, 2000 nM and 3000 nM. After incubation for 1h at 37 °C, samples were immediately loaded after addition of 3 µl 5x native loading dye (0.5 x TBE, 50% (vol/vol) glycerol, 0.2% (wt/vol) xylene cyanol and 0.2% (wt/vol) bromophenol blue) to a native 6% (vol/vol) PAA gel. For gelshifts with Hfq, ~0.04 pmol 5'-end labeled PinT was incubated increasing concentrations of unlabeled *steC* to final concentrations of 8nM, 16nM, 62nM, 250 nM, 500 nM and 1000 nM. Upon addition of purified Hfq 250nM) or Hfq dilution buffer (control), samples were incubated at 37°C for 30 min. Gel electrophoresis was done in 0.5 x TBE buffer at 300 V. Afterwards, gels were dried and analyzed using a PhosphoImager (FLA-3000 Series, Fuji) and AIDA software (Raytest, Germany).

in-line probing assay: ~0.2 pmol labeled *steC* truncated mRNA (20 nM final concentration) was incubated in absence or presence of 30 nM or 3000 nM unlabeled PinT for 40 hrs at room temperature in 1x in-line probing buffer (50 mM Tris-HCl, pH 8.3 at 20 °C, 20 mM MgCl2, and 100 mM KCl). For RNase T1 ladders, ~ 0.2 pmol labeled *steC* truncated mRNA was incubated in 0.25 M sodium citrate buffer (pH 5.0 at 23 °C) with 1 U/µl RNase T1 for 5 min at 55 °C. For alkaline ladders, ~0.2 pmol labeled *steC* truncated mRNA was denatured for 5 min at 95 °C in Na2CO3 buffer (0.5 M Na2CO3, pH 9.0 at 23 °C and 10 mM EDTA). All reactions were stopped by adding 10 µl colorless gel-loading solution (10 M urea, 1.5 mM EDTA, pH 8.0 at 23 °C) on ice. Cleavage products were analyzed on 8 or 10% (vol/vol) PAA gels under denaturing conditions and visualized as described above.

30S ribosome toeprints: Toeprinting reactions were carried out as described (Hartz *et al.*, 1988) with a few modifications. 0.2 pmol of an unlabelled *stec* mRNA fragment (845 nt, T7 template amplified with JVO- 15723/PZE-XbaI), and 0.5 pmol of 5'end labelled primer JVO-1976 complementary to the *gfp* coding region were annealed. For inhibition analysis, 1 and 2 pmol of PinT sRNA were added. Nucleic acids were denatured in annealing buffer (10 mM Tris-acetate pH 7.6, 1 mM DTT, 100 mM potassium acetate) for 1 min at 95 °C and chilled on ice for 5 min, upon which Mg^{2+} acetate and all dNTPs were added to final concentrations of 0.5 mM. All subsequent incubation steps were at 37 °C. After 5 min incubation, 2 pmol of 30S ribosomal subunit (provided by Knud Nierhaus, Max Planck Institute for Molecular Genetics,

Berlin, Germany; pre-activated for 20 min prior to the assay) were added. Following incubation for 5 min, uncharged tRNAfMet (10 pmol) was added, and incubations continued for 15 min. Reverse transcription was carried out by addition of 100 units of Superscript II and incubation for 20 min. cDNA synthesis was terminated with 100 µl stop buffer. Following phenol-chloroform extraction, alkaline hydrolysis of template RNA at 90°C, and ethanol precipitation, cDNA was dissolved in 10 µl of loading buffer II (Ambion). Sequencing ladders were generated with CycleReader^{TM} DNA Sequencing Kit according to the manufacturer's protocol on the same DNA template as used for T7 transcription and the same 5'- end-labelled primer as in the toeprinting reactions. cDNAs and sequence ladders were separated on a 6% polyacrylamide/ 7M urea gel. Autoradiograms of dried gels were obtained as above.

Stop buffer:

50mM Tris-HCl pH 7.5

0.1% SDS

10 mM EDTA

RNA-sequencing: Total RNA samples or MAPS elution samples were converted into cDNA libraries for Illumina sequencing by Vertis Biotechnologie AG, Freising-Weihenstephan, Germany (http://www.vertisbiotech.com). DNase I-treated total RNA samples were first sheared via ultrasound sonication (4 pulses of 30 s at 4°C each) to generate on average ~200-400 bp fragmentation products. Then, fragments <20 nt were removed using the Agencourt RNAClean XP kit (Beckman Coulter Genomics).

6.2.2 Protein techniques

Western blotting: Immunoblotting of *Salmonella* proteins was done as previously described (Urban and Vogel, 2007). Briefly, samples from *Salmonella in vitro* cultures were taken according to 0.4 OD$_{600nm}$, centrifuged for 4 min at 16,100 g at 4°C, and pellets resuspended in protein loading buffer to a final concentration of 0.01 OD/µL. After denaturation for 5 min at 95°C, 0.05-OD equivalents of the sample were separated via SDS-PAGE. Gel-fractionated proteins were blotted for 90 min (0.2 mA/cm2; 4°C) in a semi-dry blotter (Peqlab) onto a PVDF membrane (PerkinElmer) in transfer buffer (25 mM Tris base, 190 mM glycin, 20% methanol). Blocking was for 1 h at room temperature in 10% dry milk/TBST. Appropriate primary

antibodies were hybridized at 4°C overnight and – following 3x 5 min washing in TBST – secondary antibodies for 1 h at room temperature. Primary and secondary antibodies used and the respective dilution are listed in Table 6.6. For Western blotting of human proteins, infected cells were harvested in protein loading buffer (500 µL/well; six-well format), transferred to 1.5 mL reaction tubes, boiled for 5 min at 95°C and 20 µL/lane were loaded onto a 10% PAA gel for SDS-PAGE as above. After blotting and blocking, the membrane was probed with the respective primary antibody at 4°C overnight and for 1 h at room temperature with the corresponding secondary antibodies. Blots were developed using Western Lightning solution (PerkinElmer) in a Fuji LAS-4000. Where indicated, intensities of bacterial protein bands were quantified using the AIDA software (Raytest, Germany) and normalized to GroEL levels.

in vitro translation assays: Translation reactions were carried out with PureSystem (Cosmo Bio, PGM-PURE2048C) according to the manufacturer's instructions. In brief, 1 pmol _in vitro_ transcribed mRNA (_steC::3xFLAG, steC::gfp_) was denatured in absence or presence of 1 or 50 or 100 pmol of PinT RNA for 1 min at 95°C and chilled for 5 min on ice. Hfq (250 pmol) was mixed with mRNA (and sRNA) and preincubated for 10 min at 37°C before addition of PureSystem mix. Translation was performed in a 10 µl reaction for 30 min to 1h at 37°C, and stopped with four volumes of ice-cold acetone and chilled on ice for 15 min. Reactions were stopped by addition of 60 µl acetone, chilled for 15 min on ice and proteins were collected by centrifugation for 10 min at 10,000 g and 4 °C. In vitro translated TlpB or CagA was quantified by Western blot analysis using monoclonal anti-FLAG or anti-GFP and anti-mouse IgG (GE-Healthcare) antibodies. The ribosomal protein S1 served as a loading control and was detected by an S1 antibody, (1:10,000, kindly provided by M. Springer, IBPC Paris, France) and anti-rabbit secondary antibody (GE-Healthcare).

6.6.5 MS2-PinT overexpression

To test the expression levels of MS2-PinT and PinT, the strains SCS001, JVS-11716 and JVS-1574 were grown in LB with 50 µg/mL ampicillin (diluted 1:100 from an overnight culture). At $OD_{600nm} = 2$, expression of the plasmid was induced by adding 0.1% of arabinose to the media. After 10 minutes, 4 OD_{600nm} of cells were collected and mixed with StopMix. Total RNA was

extracted using Trizol. 5 µg of total RNA were analyzed by Northern blot to verify expression of the MS2-PinT construct compared to the untagged PinT construct.

6.6.7 Time course overexpression of MS2-PinT

To measure *sopE* and *sopE2* turnover after PinT overexpression, a strain with arabinose-induced overexpression of MS2-PinT (SCS001) was grown overnight and diluted 1:100 in LB with 50 µg/mL ampicillin at 37°C with shaking. At OD_{600nm} = 2, $4OD_{600nm}$ of cells were harvested. Next, 0.1% of arabinose was added to the media in order to induce MS2-PinT induction. Samples were collected at 1, 2, 5 and 10 minutes after induction, mixed with StopMix and immediately frozen in liquid nitrogen. Total RNA was extracted using Trizol extraction. RNA samples were treated with DNase I (Fermentas) for 45 min at 37°C. DNase I was then removed using phenol-chloroform extraction and RNA was again precipitated. DNase treatment was controlled by PCR using the oligos JVO-1224/1225 (Table 5). qRT–PCR was performed with the Power SYBR Green RNA-to-CT 1-Step kit (Applied Biosystems) according to the manufacturer's instructions. Fold changes for pinT, sopE and sopE2 were determined using the 2(−ΔΔCt) method relative to time before induction and using 5S as reference gene (see Table 5 for oligos).

6.6.8 MAPS in *Salmonella* $OD_{600nm}2$ and SPI2

Three different *Salmonella* strains, expressing either MS2-PinT, PinT or MS2 under the control of pBAD inducible plasmid were used. Cells were grown in LB supplemented with 50 µg/mL ampicillin (diluted 1:100 from an overnight culture grown). At $OD_{600nm}2$, overexpression of the different constructs was induced by the addition of 0.1% arabinose. After two minutes, a volume of $60OD_{600nm}$ of cell was harvested and chilled on ice for 5 minutes. For MAPS in SPI-2 conditions, three different *Salmonella* strains, expressing either MS2-PinT, PinT or MS2 under the control of a constitutive promoter on a plasmid. $60OD_{600nm}$ of cell was harvested at $OD_{600nm}0.3$ in SPI-2 media. Cells were centrifuged at 10000 g for 10 minutes and the pellets frozen in liquid nitrogen. After thawing on ice, the pellets were resuspended in 600 µl of chilled Buffer A (20 mM Tris pH8.0, 150 mM KCL, 1 mM of MgCl2, 1 mM DTT). A volume of 750 µl of glass beads was added to the cells and lysed using Retsch (10 min, 30 Hz) (adaptors were pre-chilled at -20°C). Next, lysate was cleared by centrifugation 10 min at 16000g at 4°C

and the clear lysate was collected into a new tube. While the lysate is being prepared, affinity purification columns were prepared at the 4°C room. ~70 μl of amylose (New England Biolabs #E8021S) were added to 2mL Bio-Spin disposable chromatography columns (BioRad #732-6008). Amylose beads were washed three times with 2ml of Buffer A. 1ml of Buffer A with 250 pmol of MS2-MBP coat protein was added to the closed column followed incubation with rotation. After 5 minutes, the column was open and the MS2-coat protein was to run through the column and collected in a tube. This incubation step was repeated one more time until the lysate was ready. At this point, the solution was allowed to run and the column was washed once with 1ml of Buffer A. The clear lysate was subjected to affinity chromatography (all the following steps were performed at 4 °C). Lysate was added to the closed column and incubated for 5 minutes with rotation. After the incubation, the lysate run was collected and the incubation step was repeated. Next, the column is washed 8 times with 2ml of Buffer A. Bound RNA was eluted using 300μl of Elution Buffer (Buffer A + 15 mM maltose). This step was repeated one more time. Eluted RNA was extracted with phenol-chloroform (V/V) and precipitated by the addition of ethanol (2 vol) and ~15μg of glycogen (1μl of Glycoblue 15mg/ml). RNa samples were treated with DNase I (Fermentas) for 45 min at 37°C. DNase I was then removed using phenol-chloroform extraction and RNA was again precipitated.

6.6.9 MAPS in Salmonella infected Hela cells and mouse macrophage

Three different *Salmonella* strains, expressing either MS2-PinT, PinT or MS2 from the *pint* chromosomal *locus* were used to infect murine macrophages with an M.O.I of 50. Infections were carried as described above. After 4h of infection, cells were washed twice with ice cold PBS. Next, cells were scraped, collected and centrifuged at 250g for 10 min. Next the cell pellet was resuspended in 0.1% of Triton for 5 min, followed by a 5 minute centrifugation at 200g and snap freezing. Next the cells were thawed on ice, washed with PBS and pelleted at 200g for 5 min. The supernatant containing the released intracellular bacteria was collected and centrifuged at maxi. speed for 5 min. Finally we proceeded with the MAPS protocol as described above.

6.6.1 cDNA library preparation

For MAPS, the RNA samples were first fragmented using ultrasound (4 pulses of 30 s each at 4°C). Then, an oligonucleotide adapter was ligated to the 3' end of the RNA molecules. First-strand cDNA synthesis was performed using M-MLV reverse transcriptase and the 3' adapter as primer. The first-strand cDNA was purified and the 5' Illumina TruSeq sequencing adapter was ligated to the 3' end of the antisense cDNA. The resulting cDNA was PCR-amplified to about 10-20 ng/µl using a high-fidelity DNA polymerase (11 cycles). The TruSeq barcode sequences which are part of the 5' TruSeq sequencing adapter are included in Table 2. The cDNA was purified using the Agencourt AMPure XP kit (Beckman Coulter Genomics) and was analyzed by capillary electrophoresis. For Illumina NextSeq sequencing, the samples were pooled in approximately equimolar amounts. The cDNA pool in the size range of 200-550 bp was eluted from a preparative agarose gel. The cDNA pool was paired-end sequenced on an Illumina NextSeq 500 system using 2x75 bp read length.

6.6.2 MAPS data processing

Read counts were normalized by total read count for rRNA, using a scaling factor for each library. Next, we compared the ratio between the normalized read of the MS2-PinT sample and the PinT sample. Potential base-pairing with PinT was analyzed for the top hits of the list by using RNA-RNA interaction prediction tools, like IntaRNA software and RNA-up web server. Between the list of most enriched transcripts in the MS2-PinT sample and the PinT sample, nine candidate targets were selected based on their calculated free binding energy to PinT, infection relevance and SalComMac (Kröger *et al.*, 2013; Srikumar *et al.*, 2015) expression profile.

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8. Appendix

8.1 New RNA-based affinity purification and RNA-Seq to studying RNA-RNA interactions

In an attempt to improve the signal to noise ratio and increase sensitivity of RNA affinity purification, we evaluated the performance of additional RNA aptamer/coat protein combinations that are well-characterized, yet have not yet been harnessed for global RNA-RNA interaction screens in bacteria. Particularly, we used the *Pseudomonas* phage 7 (PP7) aptamer and corresponding coat protein (PP7) (Lim and Peabody, 2002) as well as CRISPR subtype Ypest protein 4 (Csy4) (Haurwitz *et al.*, 2010). The PP7 aptamer has previously been used to identify RNA interacting partners of the sRNA GsrN in *Caulobacter crescentus* (Tien *et al.*, 2018). Csy4 has been used to uncover RNA interaction partners for cell type-specific human pre-microRNA-binding proteins (Lee *et al.*, 2013). The coat protein in this system is an inactive version of the Csy4 endoribonuclease, whose activity can be induced by the addition imidazole. Once the enzyme is activated, it cuts 3' of the aptamer sequence, releasing the RNA and its binding partners.

8.1.1 Implementation of PAPS and CAPS

To establish new RNA affinity pull-downs for *Salmonella* sRNAs, and similar to the MS2 constructs, we first fused the PP7 and Csy4 aptamers, comprising 27 or 20 nt, respectively, to the 5'-end of PinT and the resulting tagged sRNA variants cloned under the arabinose-inducible promoter in the pBAD plasmid. In order to preserve secondary structure of the PP7-tagged PinT, a 6 nt U-stretch was inserted between the tag and the first nucleotide of PinT (Fig. 8.1). Likewise, to ensure that Csy4 recognizes its cognate aptamer, linkers of 4 U's was inserted between the Csy4 aptamer and PinT. In this way, according to *in silico* prediction using *mfold*, the overall secondary structure should be preserved. Similarly, as with MAPS, two other control plasmids were constructed, the Tag-alone and the untagged sRNA, using the same plasmid background. Importantly, neither addition of the PP7 nor the Csy4 tag interfered with PinT steady-state expression or target regulation (Fig. 8.2). We refer to the use of PP7- or Csy4- tagged bait sRNA for affinity purification as PAPS and CAPS.
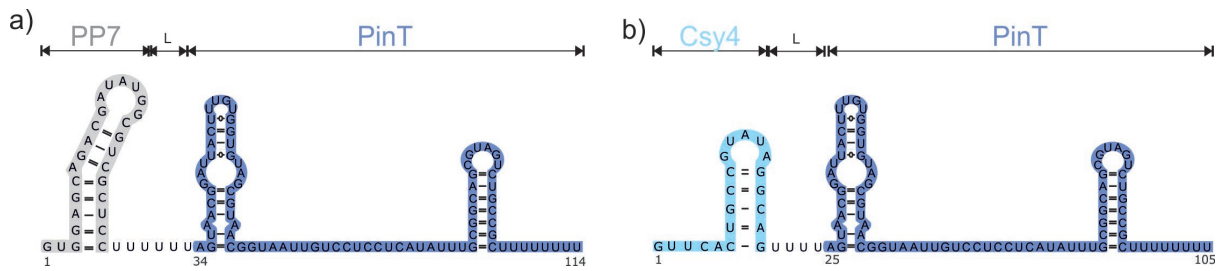
Figure 8.1 - Secondary structure prediction of a) PP7-PinT and b) Csy4-4U-PinT. *Secondary structure prediction using mfold web server and VARNA applet for visualization of RNA secondary structure. "L": linker.*
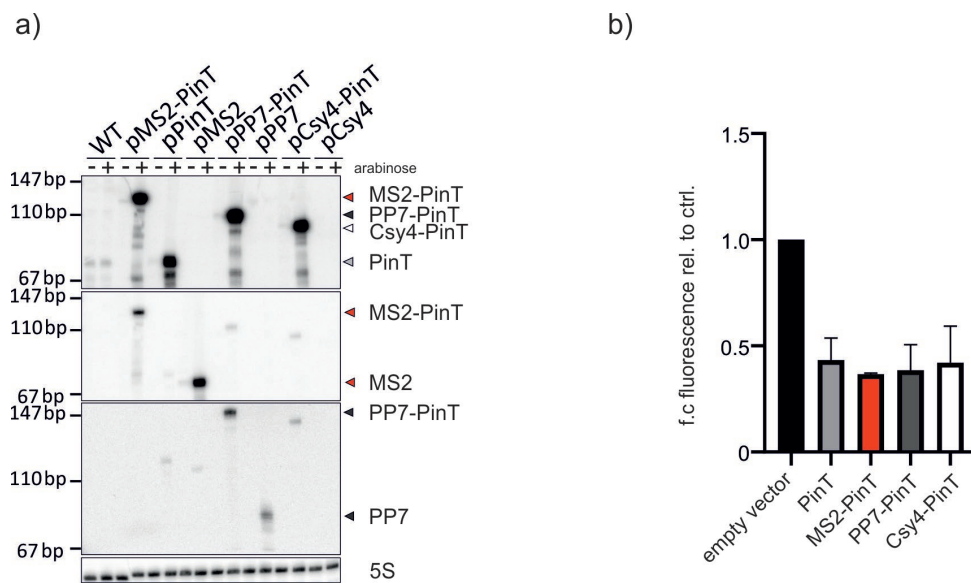
Figure 8.2 - Expression of the different constructs for RNA--based affinity purification. a) *Northern Blot analysis of Salmonella SL1344 WT carrying empty vector control (lanes 1-2 - pKP8-35), or the pinT deletion strain carrying a plasmid containing the WT PinT (lanes 5-6 - pYC5-34); the aptamer tagged PinT, i.e. MS2-PinT (lanes 3-4 – pYC310), PP7-PinT (lanes 9-10 – pSS13), Csy4-PinT (lanes 13-14 – pSS33); or the tags alone, MS2 (lanes 7-8 – pYC310), PP7 (lanes11-12 – pSS39) and Csy4 (lanes 15-16 – pSS38); under the control of an arabinose inducible promoter. Includes 5S rRNA as a loading control* **b)** *GFP reporter assay. sopE::gfp reporter gene fusion was used to measure the interaction between the pulse expressed wild-type (PinT) or the tagged PinT (MS2-PinT, PP7-PinT or Csy4-PinT). Bars correspond to the mean fluorescence relative to the empty vector ± s.d. from three biological replicates.*
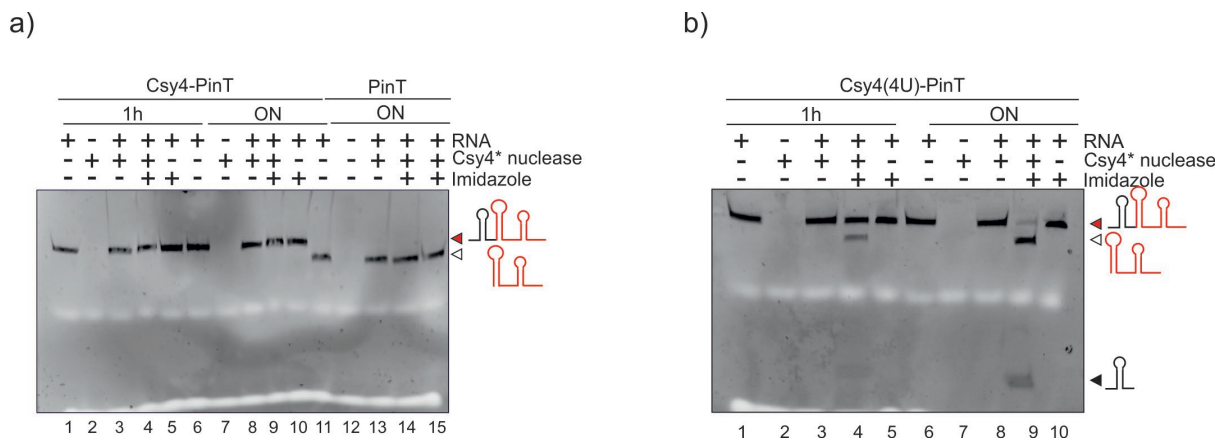
8.1.2 Csy4* nuclease activity

Previous studies have shown the habibily of the inducible Csy4* nuclease cut Csy4-pre-mir21 (Lee *et al.*, 2013). To test our Csy4* nuclease activity, *in vitro*-synthesized pre-mir21 and Csy4-pre-mir21 were used as negative or positive control, respectively, and incubated for 1 h or over-night at 4°C, in the presence or absence of the inactive or imidazole-activated nuclease (Lee *et al.*, 2013). As expected, only in the presence of both the nuclease and imidazole the tagged RNA was recognized and cut (Fig. 8.3). Enzymatic activity increased with incubation time, reflected by the complete loss of the full–length, tagged transcript upon overnight incubation. Conversely, the untagged RNA remained stable, even after overnight incubation in the presence of both imidazole and nuclease.
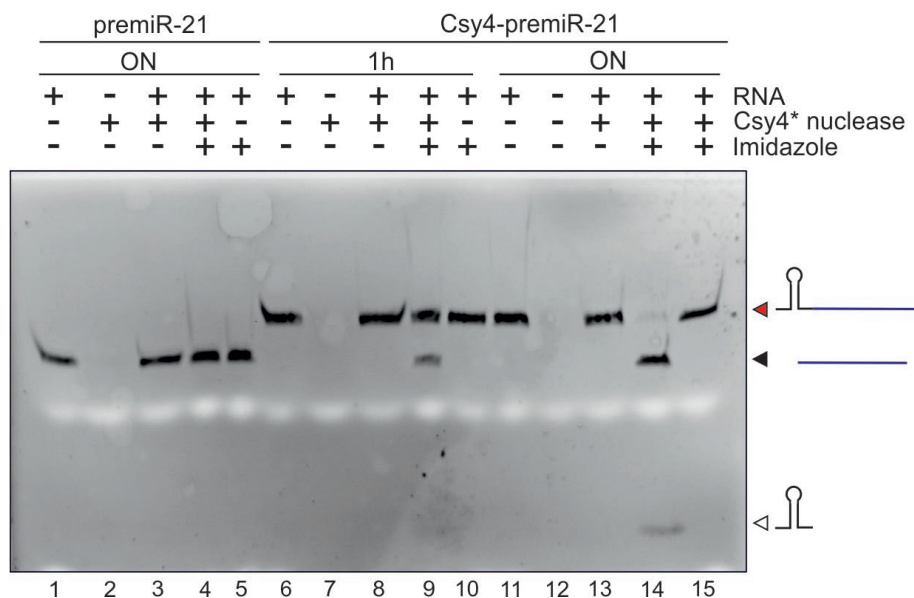
Figure 8.3 - Csy4* nuclease activity assay. *The in vitro transcribed Csy4 tagged premiR-21 or untagged premiR-21 were incubated at 4°C in the presence or absence or the Csy4* nuclease and or imidazole. Products were separated by 12% denaturing PAGE and visualized with ethidium bromide staining. Red arrow corresponds to the uncut full-length sRNA, empty arrow corresponds to the Csy4 aptamer and black arrow corresponds to the cleaved tagged RNA.*

Next, we tested weather the Csy4* nuclease could recognize and cut the tagged-PinT. For this, a similar experiment as detailed above was performed, but this time by incubating the tagged or untagged PinT in the presence or absence of the nuclease and imidazole. The nuclease efficiently cut all Csy4-PinT variants with an additional U-stretch, but did not cleave the variant without a linker (Fig. 8.4), suggesting that the U-stretch allows the nuclease to dock on to the aptamer and cut the RNA. Based on these findings, we used the Csy4-4U-PinT construct for all downstream experiments.

a)   b)

Figure 8.4 - Csy4* nuclease activity assay with Csy4-PinT. *The in vitro transcribed Csy4 tagged PinT without linker (a) or with a 4U linker (b) and untagged PinT (a) were incubated at 4°C in the presence or absence or the Csy4* nuclease and or imidazole. Products were separated by 12% denaturing PAGE and visualized with ethidium bromide staining. Red arrow corresponds to the uncut full-length sRNA, empty arrow corresponds to the Csy4 aptamer and black arrow corresponds to the cleaved tagged RNA.*

8.2 Establishing PAPS in *Salmonella*

In analogy to the above described MAPS experiment, PAPS and CAPS were performed on *Salmonella* strains growing in LB to OD_{600nm} 2 prior to the induced expression of tagged PinT for 2 min, and included the respective control strains harboring either untagged PinT or the respective aptamer alone.

Using PAPS, we again pulled-down all classes of RNA, with most of the reads mapping to rRNA loci. To directly compare the performance of PAPS with the MAPS results, PinT target candidates were again called by calculating their enrichment between the fractions co-purifying with aptamer-tagged PinT and the untagged sRNA control. The known PinT targets

(*sopE*, *sopE2*, *grxA* and *crp* mRNAs) as well as the newly validated targets (*steC*, *ugtL* and *rpsV*) were contained among the transcripts enriched with PP7-tagged PinT variant (Table 8.1). This notwithstanding, the ranked list of enriched targets differed between MAPS and PAPS. For instance, the PAPS approach identified the *rtsA* mRNA, recently demonstrated to be a bona fide PinT target (Kim *et al.*, 2019b), which was missed in the MAPS results. This implies that combination of multiple aptamer affinity pull-downs increases the likelihood of charting the complete targetome of a given sRNA.

Table 8-1|Summary table of the most enriched transcripts in PAPS in Salmonella in LB OD$_{600nm}$2.

Rank #	Candidate target	Product	Reference
1	STnc4130	candidate sRNA	This study
9	*ecnB*	Enteredicin B	This study
25	*ugtL*	PhoQ activator	MAPS
46	*rmf*	ribosome modulation factor (protein E)	This study
50	*rtsA*	Regulator of SPI-1	(Kim *et al.*, 2019b)
83	*sopE*	SPI-1 effector	(Westermann *et al.*, 2016)
119	*grxA*	Glutaredoxin 1	(Westermann *et al.*, 2016)
383	*ssrB*	Two component system Regulator of SPI-2	(Kim *et al.*, 2019b)
436	*crp*	Global transcriptional regulator	(Westermann *et al.*, 2016)
528	*rpsV*	30S ribosomal protein S22	(Westermann *et al.*, 2016)
609	*steC*	SPI-2 effector	This study

8.3 Future applications

Further analysis is necessary in order to compare the traditional MAPS method with the newly developed PAPS and CAPS. Specifically, RNA-seq analysis of CAPS is still pending, but it will allow to determine if known and new targets can be identified using this approach. Finally, taking all these newly created data-sets into account, will allow for an integrated data analysis, that could reveal high-confidence clusters of PinT candidate targets. If one of these new approaches provides less signal to noise ratio, while maintaining or increasing yield, it might replace the classical MAPS protocol. Future applications of PAPS and CAPS, should include its application in *Salmonella* infected cells, in order to identify new *in vivo* RNA-RNA interactions.

9. Curriculum vitae

Personal details

Born: March 16th, 1987 (Sintra, Portugal)

Nationality: Portuguese

Education

September 2009 to November 2011:

Masters in Applied Microbiology

Faculdade de Ciências da Universidade de Lisboa

Thesis entitled "Dual role of enterococci in food technology: bacteriocin

production versus pathogenicity potential".

September 2006 to August 2009:

Bachelor in Biology

Instituto Superior de Agronomia, Universidade Técnica de Lisboa

Thesis entitled "Phenotypic and molecular characterization of *E.coli* O157 and

E.coli O157:H7 strains".

Practical courses apart from/prior to the studies

May 2013 to March 2014:

Trainee at the MICALIS INRA (Jouy-en-Josas, France) with Dr. Marie-Françoise

Noirot-Gross.

January 2008 to January 2009:

"Bolsa de Iniciação na Investigação" Scholarship C2008-CBAA/ISA–UTL (Initial

training program for bachelor students at the Instituto Superior de

Agronomia at the Microbiology Lab).

Würzburg, 09 of December 2019

10. List of publications

Publications co-authored during the PhD:

- A. E. Saliba, **S. C. Santos**, J. Vogel (2017). *New RNA-seq approaches for the study of bacterial pathogens.* Current Opinion in Microbiology 35, 78–87.
- N. L. Schulte , M. Schweinlin , A. J. Westermann, H. Janga, **S. C. Santos** , S. Appenzeller, H. Walles, J. Vogel, M. Metzger *An immunocompetent human intestinal co-culture model reveals compartmentalized host and pathogen strategies during Salmonella infection. (submitted)*

The following manuscripts related to the PhD work are in preparation:

- **S. C. Santos**, T. Bischler, S. Dietrich, L. Barquist, A. J. Westermann, J. Vogel *The extended targetome of PinT, a small RNA timer of Salmonella virulence programs.*
- **S. C. Santos**, A. J. Westermann, J. Vogel *Optimization of RNA affinity pull-downs for Salmonella sRNAs.*

Previous publications

- García García, T., Ventroux, M., Derouiche, A., Bidnenko, V., **S. C. Santos**, Henry, C., Mijakovic, I., Noirot-Gros, M.-F., and Poncet, S. (2018). *Phosphorylation of the Bacillus subtilis Replication Controller YabA Plays a Role in Regulation of Sporulation and Biofilm Formation.* Front. Microbiol. 9.
- **S. C. Santos**, M.J. Fraqueza, M. Elias, A. Salvador Barreto, T. Semedo-Lemsaddek (2017). *Traditional dry smoked fermented meat sausages: Characterization of autochthonous enterococci.* LWT - Food Science and Technology 79, 410–415.
- **Santos, S.C.**, and Lemsaddek, M.O. and T.S. (2015).The challenges of antibiotic resistance in the development of new therapeutics. Book Series: Frontiers in Antimicrobial Agents. Bentham Science Publishers Ltd; 2015. pp. 178–207.
- A. Barata, **S. C. Santos**, M. Malfeito-Ferreira, V. Loureiro (2012). *New Insights into the Ecological Interaction Between Grape Berry Microorganisms and Drosophila Flies During the Development of Sour Rot.* Microb Ecol 64, 416–430.

11. Attended conferences and courses

1) RNA Biochemistry Meeting 2016 & Workshop 'Extracellular RNA'
   06 - 09. 10. 2016
   Bonn, Germany
   Poster presentation

2) Microbiology and Infection 2017, 5th joint conference DGHM and VAAM
   05 - 08 .02. 2017
   Würzburg, Germany
   Poster presentation

3) 22nd Annual meeting of the RNA Society 2017
   30. 05. 2017 - 04. 06. 2017
   Prague, Czech Republic
   Poster presentation

4) Eureka 2017
   11 - 12. 10. 2017
   Poster presentation

5) 5th Meeting of Regulating with RNA in Bacteria and Archaea
   19 - 22. 03. 2018
   Seville, Spain

   Poster presentation

6) RNA Biochemistry Meeting 2018 & Workshop 'Synthetic RNA Biology'
   04 - 07. 10. 2018
   Bonn, Germany

7) RNA-seq Seminar, Workshop Hands-on-training, IMIB
   13 - 14. 12. 2018
   Würzburg, Germany

8) 3D Tissue infection symposium
   05 - 07. 04. 2019
   Organization and poster presentation

9) Infection and Immunology course, HIRI
   08 - 10. 05. 2019
   Würzburg, Germany

10) *Salmonella* Biology and Pathogenesis, Gordon Research Conference
    02 - 07. 06. 2019
    Easton, MA, USA
    Poster presentation

12. Contribution by others

The work described in this doctoral thesis was conducted under the supervision of Prof. Dr. Jörg Vogel and Prof. Dr. Alexander J. Westermann at the Institute for Molecular Infection Biology (IMIB) at the University of Würzburg, Würzburg, Germany. Several parts of the work described in this dissertation have been contributed by others and are indicated below.

- Processing of MAPS, PAPS, CAPS RNA-seq and bioinformatics analyses were mainly performed by Dr. Thorsten Bischler (Core Unit Systems Medicine, Uni. Würzburg) with assistance of Prof. Dr. Lars Barquist (HIRI, Würzburg).

- Processing of the RNA-seq data from murine macrophage infections and bioinformatics analyses were mainly performed by Dr. Sacha Dietrich (Core Unit Systems Medicine, Uni. Würzburg).

- Several synthetic oligonucleotides (Tab. 6.7), plasmids (Tab. 6.8) and *Salmonella* strains (Tab. 5.9) used in this study were designed or constructed by other group members. Especially plasmids and strains used in the second and third chapter were mostly cloned by Dr. Yanjie Chao with the assistance of Barbara Plaschke and published (Westermann *et al.*, 2016).

- Some wet lab experiments were conducted with the help of the technical assistants from Barbara Plaschke, Sarah Reichardt and Verena Herbst (cell culture, cloning, etc).

- MAPS, PAPS and CAPS cDNA libraries were constructed by the vertis Biotechnologie AG in Freising-Weihenstephan, Germany (http://www.vertis-biotech.com).

- cDNA libraries and sequencing were performed by the Core Unit Systems Medicine, Uni. Würzburg.

13. Affidavit

I hereby confirm that my thesis entitled "Expanding the targetome of Salmonella small RNA PinT using MS2 affinity purification and RNA-Seq (MAPS)" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, Date   Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation "Erweiterung des Targetoms der kleinen RNA PinT von Salmonella durch MS2-Affinitätsreinigung und RNA-seq (MAPS)" eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum   Unterschrift