CRP-cAMP mediated silencing of virulence expression in *Salmonella enterica* serovar Typhimurium

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CRP-cAMP mediated silencing of virulence expression in *Salmonella enterica* serovar Typhimurium.

Memoria presentada por Youssef El Mouali Benomar para optar al Grado de Doctor por la Universidad de Barcelona

Programa de Doctorado: Microbiología Ambiental y Biotecnología

Memoria presentada por:

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VºBº del director y tutor de la Tesis

Carlos Balsalobre Parra

Acknowledgments

La verdad es que no tengo muy claro como se debe escribir esto. Y haciendo caso omiso a las recomendaciones de dejar esto escrito antes de ponerse con la tesis, es lo último que hago antes de enviar a imprimir a esta pequeña tortura. Y me sabe mal dedicarle tan poco tiempo, porque como todos sabemos, la tesis se la van a leer tres personas y esto de aquí algunas más. Aviso a navegantes, ya sabéis como soy, esto no van a ser unos agradecimientos de esos que se agradece hasta al apuntador, os quiero mucho a todos pero hay gente que un pelín más.

Primero de todo agradecer estos tres años a Carlos mi tutor. Ha sido una tesis corta en el tiempo pero nos ha dado tiempo a todo. Sobre todo gracias por dejarme ir haciendo, por discutir sin filtro lo próximo a hacer, por guiar más que mandar durante la tesis. Por siempre estar abierto a discutir cualquier paja mental. Mira que empezar con CRP-cAMP y acabar haciendo una tesis con tanto RNA no codificante… Con la broma hace ya unos años que nos conocemos, desde que vine el verano aquél a aprender a hacer una β-gal, menuda idea, no sabía que iban a tocar cientos. Ahora cuando explico la β-gal en prácticas de BMCM lo vendo como lo más. Ni tú ni yo somos muy fans de toda la parafernalia esta de los agradecimientos así que vamos a hacerlo corto. Hacer la tesis en el Lab 4 ha sido divertido, se me ha hecho corto y nos ha salido de puta madre, poco más se puede pedir.

Donar les gràcies a la Cristina per les paraules d'ànims aquests dies, quan ja venia vestit de vagabund amb la tesi a la esquena. Per fer que el laboratori funcioni i posar-se molt nerviosa quan el desordre se'ns anava de les mans.

Agradecer al laboratorio de Pepe Casadesús lo bien que me trataron cuando fui a visitarles un mes a Sevilla a meterme en el mundo RNA. A Maritoñi por introducirme a la radioactividad y decirme que siempre intente evitarla, lo intenté, pero ha salido regular...

I would also like to thank Jörg Vogel for hosting me at his lab for three months. It was priceless the intensive RNA course, it is always great to be taught by the best. The acknowledgement is extensive to all people in Vogel lab, especially to Alex Westermann, always available for discussion.

A el Lab 4 pasado y presente. A Llorenç, Marta, Pedro, Carla… A Tania por cantarme el "welcome to my lifeee" cada vez que me quejaba de algo de la tesis. Però sobretot a la Paytu, ella no ho sap prou, però es un amor. Gràcies per ser com jo tan poc tolerant amb depèn de quines opinions, trobo que em canalitzat bastant d'odi… no reproduiré res que ja saps que pel meu nom soc mes fàcilment empresonable, però trobo que em perdut bastants companys de dinar, y no em sembla gens malament. Gulag de fachas ya! Gràcies per les invitacions a Prospes y Sant xibecos, quan les ulleres fúcsia de sol apareixien es que la cosa anava be… continuaré venint! Veus, ja m'estic allargant Paytu, hauria d'haver escrit els agraïments que et vaig dir, "gracias a mi! Que la tesis la he escrito yo hijos de puta!"A la Sonia del Parc, tercer i últim integrant dels dinars de la intolerància, no se que faràs sense nosaltres ee. Et vindrem a veure!

Al Lab 3 east coast, a Ale que es la bondad personificada y no le digo nada mas que se nos pone rojo. Y a Manu, que míralo, es nuevo y ya en agradecimientos de tesis y todo, gracias por hacer las ultimas practicas algo más llevaderas con tremenda fauna. Vuestro mérito es ser del Madrid...

A los del furbol! Pablo, Kike... Cuesta menos ir un jueves a trabajar cuando a las 13h hay un partidito de futbol, ojo, que estamos en la final! Que una banda como nosotros este en la final dice mucho del nivel...

A la mafia de genética que aceptaron a un microbiólogo en sus filas. Madre mía, organizamos un congreso y todo. No hay campo sin grillo ni hortera sin amarillo! Espero que me invitéis de speaker el año que viene, prometo no turra. A Jose F, Núria (aka lávate la boca antes de hablar de mi), a Miquel y Mariona, que ja serem casi tots doctors! Y especialment al Victor i l'Eudald. Que marxem a Canadà! FUAAA! LA VIRGEN!

Eudald! Amb la conya fa mil anys que ens coneixem! I ahir aniversari i va caure una arracada i tot... Estic segur que compartirem molts mes aniversaris. Gràcies per estar sempre! Que duri! 'La dedicació' Sus quiero!

A los integrantes de Mallorca 61, sobre todo por ser casa, que como la tesis también se acaba... Especialmente a Samu y Leo. Cuando se vive con amigos todo es más fácil. A Samu por preocuparse por mí más que mi madre, por estar para lo que fuera siempre, por preguntarme todos los días "¿cómo estás corazón?" por prepararme esas "cosas" para los días estresantes guiño guiño. A Leo por el primer año de locura (lo necesitaba también) y luego todo calma, los dos Leo son de puta madre. Por aguantar mis turras de rojo y por compartir el psicólogo que llevas dentro, tiene tarjeta de visita y todo el tío.

A los biotec, que nos juntamos 7 y 7 nos quedamos, socializando en clase y tal… Alguns estàn mes o menys lluny, la vida tu… pero que nos quiten lo bailao, van ser anys molt feliços i això queda per sempre. Dani perra déjate ver, mi compañero del Maresme con horas de rodalies a las espaldas. Cuando entregue esto prometo aparece por la periferia! A Nieves, que si la llamo porque estoy en la mierda viene, y no sé que es la amistad, pero tiene que ser algo así. Gracias por traerme cena casera el otro día, según ella, escribiendo la tesis seguro que no estaba comiendo muy bien, pues eso que sus quiero!

Ya casi estamos llegando a Premià. Año que viene de nuevo Viñarock! A que nos caiga otra chuza tres días, a comer tapas en el bar franquista, a pasarlo fatal y luego endulzar el recuerdo. A Andy, Jose, German, Anthony… Gracias a Alberto todos estos años, por arreglar el mundo mil veces durante horas en terrazas, por tener el gaznate seco muy a menudo. Cuando conoces a alguien de hace más de 20 años pues todo es más fácil. Salud y República!

Y ahora los más importantes. Mi familia. Primero a mis hermanas. A las cuatro si! A Fati, Samira, Habiba y Nadia. No sabéis la suerte que es crecer con tanto amor! Así les han salido los hijos, que son lo mejor. Tengo mil dibujitos de sobrinas y seguiré trabajando duro para ser el tío enrollado al que siempre llamaran Youssef porque no me quiero sentir mayor. Gracias por ser mi centro de operaciones en época universitaria! Por dejar el terreno allanado en casa, ni Angela Davis ni Virginie Despentes, mis feministas preferidas son mis hermanas.

A mis padres, que se partieron la espalda para que pudiéramos estudiar. Mi padre se dejó la espalda trabajando en el campo durante 40 años y mi madre limpiando casas sin contrato, y yo no estoy nada más que orgulloso por eso, orgullo de clase lo llaman ahora. Lo dieron todo para que a mí me fuera mejor que a ellos, es una pena que no puedan leer esto porque jamás fueron a la escuela, pero siempre quisieron que yo estudiara. No les podía fallar, esto va por ellos. Por una educación universal y gratuita para todos, porque solo con la educación cambiaremos el mundo.

Salut!

'Perhaps the most important principle that emerges out of the study of regulation of gene expression is that general principles do not exist'

Jon Beckwith and Tom J. Silhavy

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1.INTRODUCTION

1. INTRODUCTION

1.1 *Salmonella enterica* serovar Typhimurium pathogenicity.

Salmonella enterica serovar Typhimurium (from now referred as *Salmonella*) is a gastrointestinal pathogen that is found worldwide. Ingestion of contaminated foods or water is the primary cause of *Salmonella* infection. *Salmonella* genus is of particular clinical relevance in both developed and developing countries, since it is is one of the most common causes of food-borne illneses. According to Centers of Disease Control and Prevention (CDC, http://www.cdc.gov/salmonella/general/index.html, consulted May 2017), it is estimated that *Salmonella* causes every year one million foodborne illnesses in the United States, with 19,000 hospitalizations and 380 deaths. Most pacients infected with *Salmonella* develop diarrhea, fever, and abdominal cramps 12 to 72 hours after infection. The illness usually lasts 4 to 7 days, and most patients recover without treatment. However, in some cases, the diarrhea may be so severe that the patient needs to be hospitalized (1).

After ingestion of contaminated food or water, the first obstacle to overcome is the acidic pH at the stomach; *Salmonella* is able to tolerate this stress conditions by activating the acid tolerance response (2). After entering the small bowel, *Salmonella* must reach and traverse the intestinal mucus layer to encounter and adhere to epithelial cells (Fig. 1).

Salmonella cells attach to the intestinal epithelium by the use of adhesins. Adhesion is followed by invasion of bacteria. In mice, *Salmonella* cells appear to preferentially adhere to and enter the M cells of the Peyer's patches in the intestinal epithelium; however, invasion of non-phagocytic enterocytes also occurs (3,4). Alternatively, bacterial cells can also be taken up from the submucosa (Fig. 1). After adhesion, *Salmonella* interferes with the host cell signaling pathways leading to profound cytoskeletal rearrangements (5–7).

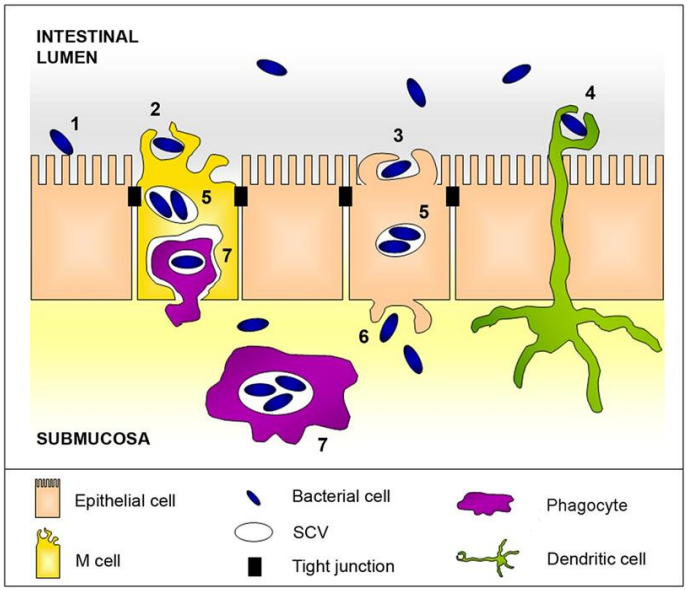

Figure 1. Pathogenesis model of *Salmonella enterica* serovar Typhimurium. 1. *Salmonella* cells attach to the intestinal epithelium by means of adhesins. **2.** and **3.** Invasion of bacteria follows and engulfment of bacteria occurs. **4.** Alternatively, bacterial cells can also directly taken up by dendritic cells from the submucosa. **5.** Inside the cytoplasm, *Salmonella* localize within the SCV where it replicates. **6.** SCVs transit to the basolateral membrane and release of bacteria to the submucosa. **7.** Bacteria internalized within fagocytes, locates within SCV. Infected fagocytes can disseminate through the lymph and the bloodstream. Adapted from (1).

The internal cytoskeleton modifications allow the engulfment of the adhered bacterial cells and the formation of endocytic vesicles, so called *Salmonella*-containing vacuoles (SCVs) (Fig. 2). The only described scenario where *Salmonella* can survive and replicate within a host cell (8,9). Upon arrival to the intestinal lumen, *Salmonella* cells are able to both invade epithelial cells and survive within phagocytes. *Salmonella* is a facultative intracellular pathogen, able to survive and replicate both outside and inside a host cell.

In order to interact with the host and to overcome the infectious process, *Salmonella* possesses many virulence strategies. The majority of the genes encoding the most important virulence factors are located within highly conserved *Salmonella* pathogenicity islands (SPIs) (1).

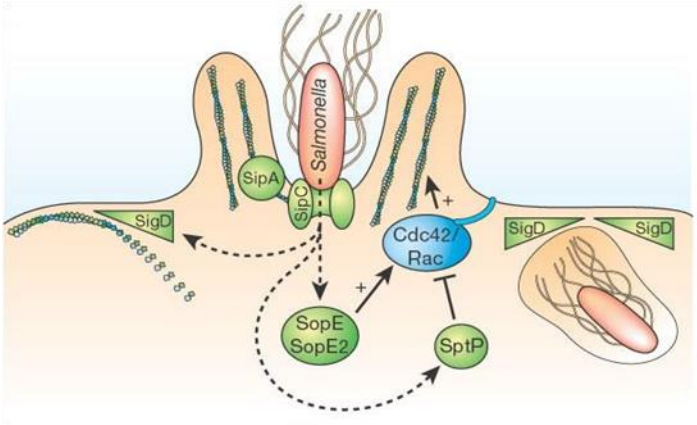

Figure 2. ***Salmonella enterica*** **serovar Typhimurium invasion.** *Salmonella* cells can invade epithelial cells of the intestinal epithelium. Secretion of effector proteins encoded in SPI-1 (SipA, SipC) or encoded outside the island (SopE) are secreted by T3SS-1. The effector proteins modulate the host cytoskeleton and promote bacterial engulfment by the host. Adapted from (7).

1.1.1 *Salmonella* pathogenicity islands.

Pathogenicity islands are clusters of genes encoding for virulence related functions that were horizontally transferred to *Salmonella* chromosome having a different GC content when compared to the core genome. Genomic studies have revealed the presence of at least five *Salmonella* pathogenicity islands (SPIs) in the *Salmonella* chromosome clearly involved in *Salmonella* virulence (Fig. 3). *Salmonella* encodes for further virulence components in either the chromosome such as several types of adhesins, flagella and essential components for biofilm formation or plasmids, as exemplified by the plasmid pSLT that carries de virulence related *spv* operon (10–12).

The role of SPI-1 and SPI-2 in *Salmonella* physiology has been extensively studied whereas the role of the genes encoded in SPI-3, SPI-4 and SPI-5 are poorly understood. The involvement of these islands in *Salmonella* infection it seems to be secondary compared to the importance of SPI-1 and SPI-2.

SPI-1 encodes for several effector proteins which mostly trigger invasion of epithelial cells by mediating cytoskeletal rearrangements and subsequent internalization of the bacteria. These effectors are translocated into the host cell by means of a type III secretion system (T3SS), encoded within the SPI-1 and termed T3SS-1.

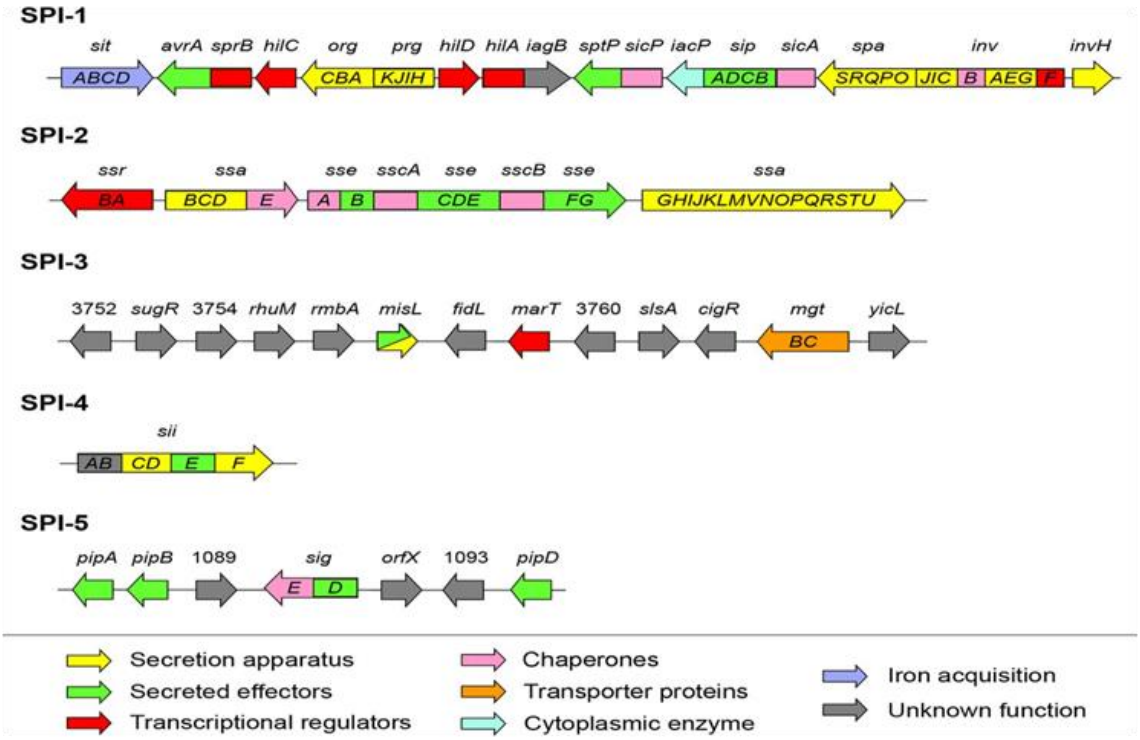

Figure 3. *Salmonella* pathogenicity islands (SPIs). Schematic representation of the genes encoded in the most ubiquitous found pathogenicity islands in the genus *Salmonella*. Adapted from (1).

The approximately 40 genes present in the SPI-1 encodes for: i) the *prg*/*org* and *inv*/*spa* operons that encode for the needle complex, ii) the *sic*/*sip* operons that encode for the effector proteins and for the translocon (SipBCD), the latest is a pore-forming structure that embeds in the host cell membrane to deliver the effector proteins within the host cytosol, and iii) regulatory proteins that control the expression of SPI-1 genes and that will be described in the next section. Interestingly, it exists effector proteins, such as SopE, that are encoded in the *Salmonella* genome outside the SPI-1 although they are secreted by the T3SS-1 (13–15).

SPI-2 encodes for up to 31 genes that are required for the survival and replication of *Salmonella* within SCVs in the host cells, both epithelial cells and macrophages (16,17). SPI-2 events are triggered by the injection of SPI-2 encoded effector proteins through another T3SS, encoded within SPI-2 (T3SS-2). Genes encoded within SPI-2 can be divided in 4 groups i) *ssa* encoding for T3SS-2 ii) *ssr* encoding for the regulators iii) *ssc*

encoding for the secreted effector proteins chaperones and iv) *sse* encoding for the translocon machinery (SseBCD) and for the secreted effector proteins (11,18).

Expression of virulence properties often requires the expression of a high number of proteins, with significant energetic cost for the bacterial cell. A paradigm for this is the expression of the genes present in SPI-1. It has been shown that induction of SPI-1 expression has a negative impact on cell physiology, resulting in an overall deleterious effect on *Salmonella*'s growth (19). Consequently, the expression of virulence functions is generally tightly regulated being expressed only under specific conditions, defined by the integration of a variety of environmental and physiological signals. When it comes to laboratory conditions, *Salmonella* shows a clear growth dependent pattern of expression of SPI-1 when grown in LB medium, being poorly expressed in exponentially growing cells and induced upon entry to early stationary phase (20) (Fig. 4). LB medium with high osmolarity, which resembles the environmental conditions in the intestine, it also induces the expression of SPI-1 genes (21).

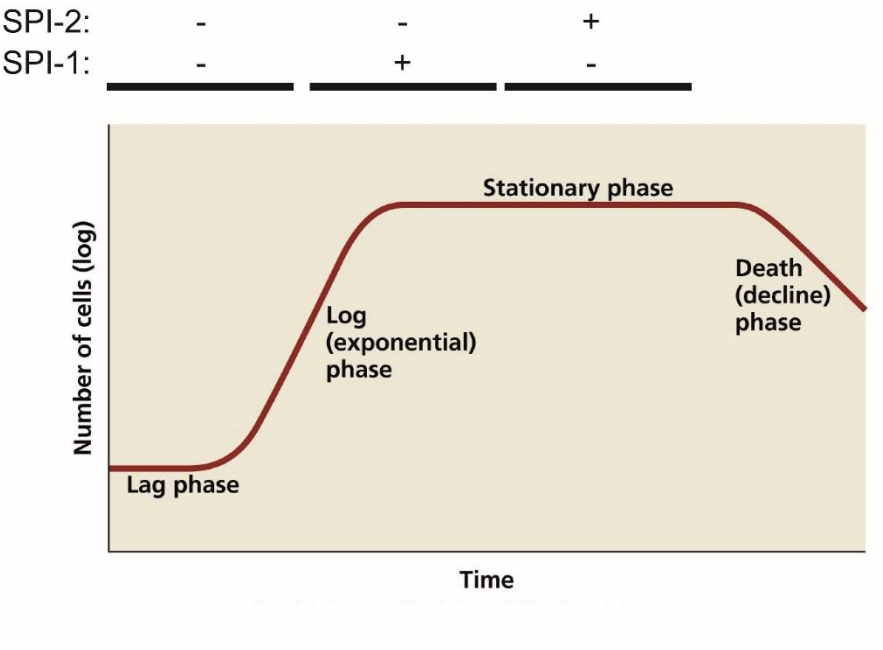

Figure 4. Growth phase dependent expression of SPI-1. Low levels of SPI-1 expression are reported at exponential growth phase. The induction of SPI-1 encoded genes is detected upon entry into stationary phase in LB grown cultures.

1.1.2 Regulation of SPI-1 encoded genes.

The expression of genes encoded within the SPI-1 is tightly regulated. Within SPI-1 many transcriptional regulators are encoded, HilD, HilC, HilA and InvF. InvF is a transcriptional regulator encoded in the SPI-1 *inv* operon (22,23). It can be directly or indirectly activated by HilA. InvF activates the expression of downstream virulence genes, mainly the effector proteins encoded within SPI-1, the *sic*/*sip* operons.

HilA is a transcriptional activator encoded within SPI-1. It plays a central role in SPI-1 mediated invasion, its deletion is phenotypically equivalent to the deletion of the entire SPI-1 locus (24). The majority of the multiple signal transduction systems that modulate SPI-1 expression converge in the control of *hilA* expression (15,21,25). HilA activates all the operons encoding for a functional T3SS-1. The *prg*/*org* and *inv*/*spa* operons are directly activated by HilA through binding to its promoters. The *sic*/*sip* operons are indirectly induced by HilA through the activation of InvF (23,25,26) (Fig. 5).

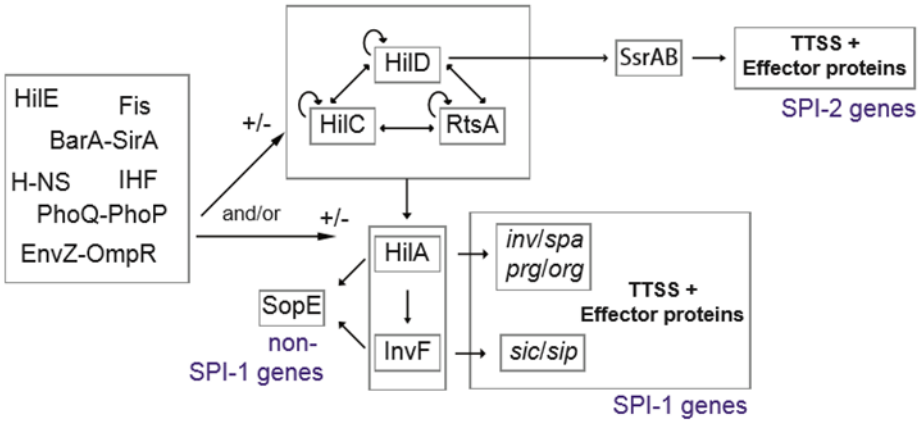

Figure 5. SPI-1 regulation. Schematic representation of the regulatory network controlling SPI-1 expression. Adapted from (27).

hilA expression is under the control, at the transcriptional level, of three AraC-like activators: HilD, HilC and RtsA (24). The first two transcriptional activators are encoded within SPI-1 while RtsA is encoded outside this locus (28). HilD, HilC and RtsA form a feed-forward regulatory loop, whereby each activator autoregulates its expression and

there is cross activation among them (24). In other words, HilD HilC and RtsA bind to *hilA* promoter region to activate its expression, and each of these activators binds to the promoter region of the other two and to its own to activate its expression. This regulatory triad responds to a wide range of physiological and environmental stimuli that are sensed by a variety of cellular factors, including both global and specific regulators (1). Within this triad, a prominent role has been attributed to HilD, the main target for signaling pathways controlling SPI-1 expression (24,29) (Fig. 5).

Accordingly with the mentioned growth phase dependent control of SPI-1 expression, *Salmonella* does not express HilA when it is growing exponentially in discontinuous LB cultures. Those conditions are stated in this thesis as non-permissive conditions for SPI-1 expression. On the other hand, HilA expression is induced during entry into stationary phase, when growth conditions become harsh. In this thesis those conditions are indicated as permissive conditions for SPI-1 expression (20).

There is a crosstalk between SPI-1 and SPI-2 (20). SPI-2 genes are required for the survival of *Salmonella* within the host cells, both epithelial cells and phagocytes such as macrophages. Consequently, its expression will be essential after the *Salmonella* invasion process encoded in SPI-1. SPI-2 encoded genes are also regulated hierarchically being the transcriptional factor SsrA the major regulator (16). Interestingly under laboratory conditions, there is a sequential activation of virulence genes in *Salmonella* through the growth curve (20). At exponential phase there is low expression of SPI-1 and SPI-2 genes. Upon entry into stationary phase SPI-1 encoded genes are induced, whereas in late stationary phase SPI-1 expression is downregulated and SPI-2 expression is induced. Remarkably, the master regulator of the SPI-1, the transcriptional factor HilD, is responsible of this sequential activation. HilD activates the transcriptional expression of *ssrA*, encoding for the main regulator of the SPI-2 encoded genes. Of note, HilD is only able to activate SPI-2 expression when the bacterial cells are growing in LB medium, a media that resembles the conditions in the intestinal lumen. When *Salmonella* cells are grown in low magnesium conditions to resemble the conditions within the SCVs, HilD expression has no effect on SPI-2 expression (20).

1.1.3 HilD as a master regulator of *Salmonella* virulence.

HilD is an AraC-like transcriptional regulator. It activates the expression of the transcriptional factor HilA which activates the expression of both the type III secretion system TTSS-1 and several effector proteins that are translocated into the host cell during the invasion process (15). HilD is the master regulator of SPI-1 encoded genes and, as earlier mentioned, it seems to act as a regulatory hub, being the target of many signaling pathways that control virulence in *Salmonella*. Expression of *hilD* is very tightly regulated and regulatory mechanisms acting at all levels of *hilD* gene expression - transcriptional, post-transcriptional, translational and post-translational - have been described (30–33). Many regulatory factors have been described to regulate the transcriptional expression of *hilD*, however, to our knowledge there is no examples of direct transcriptional regulation on *hilD* (1).

Recently, it has been described that *hilD* mRNA carries a long 3' untranslated region (UTR). While 5'UTR regions have been extensively studied in bacteria, little is known about 3'UTR and its role in gene expression. The 3'UTR of *hilD* is 310 nt long and it has a negative effect on *hilD* expression, since the presence of the 3'UTR promotes the degradation of the *hilD* transcript. Accordingly, when the 3'UTR is removed, the levels of *hilD* expression are remarkably induced compared to the wild type (WT) strain carrying the 3'UTR (Fig. 6) (31). Therefore, *hilD* 3'UTR is a regulatory motif involved in post-transcriptional *hilD* regulation. In our group we have recently described that the transcription elongation factors GreA and GreB are required for proper expression of *hilD.* Gre factors prevent backtracking of paused complexes to avoid arrest during transcription elongation. We have shown that the 3'UTR of *hilD* is required for the Gre-mediated regulation of *hilD* (27).

At the translational level, it was shown that the RNA binding protein CsrA represses *hilD* expression. CsrA binds to *hilD* mRNA, in a region overlapping the Shine-Dalgarno and the translation initiation codon leading to a downregulation of HilD expression (30). The role of CsrA as a regulator is further described in section 1.4.

At the post-translational level, several regulators have been described to affect the expression of HilD. HilD protein is targeted for degradation by the protease Lon (34).

The flagellar protein FliZ seems to post-translationally regulate HilD activity (35). Another regulator of HilD is the HilE protein which directly interacts with HilD to promote its degradation by the protease Lon. *hilE* locus is located outside of the SPI-1, however, its chromosomal location seem to resemble a pathogenicity island (36). It has been reported that the global regulator Mlc and the LeuO transcriptional factor may modulate *hilD* expression by promoting *hilE* expression (33,37).

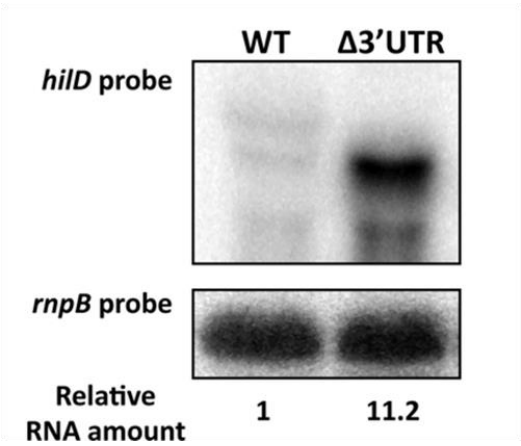

Figure 6. *hilD* mRNA accumulation upon *hilD* 3'UTR deletion. Direct detection of *hilD* mRNA levels by northern blot. Adapted from (31).

Metabolites commonly present in the intestine such as fatty acids and L-arabinose also affect SPI-1 expression through the modulation of HilD. It has been proposed that propionyl-CoA, a product of the fatty acid propionate metabolism, causes a post-translational modification in HilD that promotes its degradation (34). It has been also shown that L-arabinose seems to exert a negative effect on SPI-1 expression, presumably by a post-translational mechanism that targets HilD (38).

1.2 CRP-cAMP as a global regulator of metabolism in enterobacteria.

Most bacteria can use various compounds as source of carbon. In the presence of a mixture of different carbon sources, bacteria have the ability to preferentially use the carbon source that is more accessible and allow faster growth. One widely studied example of selective carbon-source usage is the preference of glucose over lactose in

Escherichia coli, described by Jacques Monod and that served as a model for the demonstration that gene expression can be regulated. Further studies on other bacteria and higher organisms has revealed that selective carbon source utilization is a common phenomenon and that glucose is the preferred carbon source in most of the models tested (39), highlighting the famous Monod statement "Anything found to be true of *E. coli* must also be true of elephants".

The preference of glucose over other carbon sources has been generally termed as carbon catabolite repression (CCR) (40). In general terms, CCR is defined as a phenomenon by which the expression of genes required for the use of secondary carbon sources and the activity of the corresponding enzymes are reduced in the presence of the preferred carbon source. CCR-mediated regulation is of key importance in bacteria as 5 to 10% of bacterial genes are subjected to catabolite repression (41). CCR is important for competition in natural environments, as selection of preferred carbon source is a major factor determining growth rate and therefore competitive success in front to other microorganisms. Moreover, CCR has a crucial role in the expression of virulence genes, which often enable bacteria to access new sources of nutrients. The ability to select the carbon source that allows fastest growth is the driving force for the evolution of CCR both in free-living and pathogenic bacteria. In *E. coli* and *S. enterica* there are three major components of CCR. The transcriptional regulator CRP, for cAMP receptor protein, that is active upon binding to the second messenger cAMP. The adenylate cyclase enzyme that produces cAMP and that is encoded in the *cyaA* locus. In addition, the IIA component of the glucose-specific phosphoenlopyruvate-carbohydrate phosphotransferase system ($EIIA^{Glc}$; also called catabolite repression resistance Crr) that regulates the adenylate cyclase activity.

Phosphorylation state of $EIIA^{Glc}$ is a crucial intracellular signal to modulate CCR. Shortly, $EIIA^{Glc}$ is preferentially dephosphorylated when bacterial cells are growing with glucose since the phosphate group is transferred to the sugar to be uptaken as glucose-6-phosphate (42,43). Accordingly, in absence of glucose, the levels of $EIIA^{Glc}$ phosphorylated raises. The phosphorylated form of $EIIA^{Glc}$ activates the adenylate cyclase CyaA causing the concomitant production of cAMP. Then, CRP binds cAMP and the complex acts as a transcriptional activator of metabolic pathways involved in the

use of alternative sugars. CRP-cAMP in addition to regulate genes required for catabolism of sugars other than glucose, it also regulates a large number of other genes not always directly related with the central metabolism. *In vitro* and *in vivo* transcriptional profiling of the CRP regulon in *E. coli* has been performed, revealing up to 152 genes previously unknown to be regulated by CRP (44).

1.2.1 CRP-cAMP role in virulence.

Rapid response to environmental changes is of key importance in unicellular organisms. The signal transduction pathways that allow bacteria to respond to a stimulus at the cellular level and to regulate processes such as chemotaxis, motility and virulence are essential. Second messengers, as diffusible molecules, are of key importance in the rapid adaptation of bacteria to novel environmental niches. The cAMP was the first second messenger to be described. Although its primary role has been attributed to metabolism, upon binding to CRP and homologues, cAMP seems to play myriad roles in bacterial pathogens (45).

Highlighting the relevance of the crosstalk between central metabolism and accessory cellular processes such as virulence, in many pathogenic bacteria, elements of CCR are crucial for expression of virulence genes. It is important to keep in mind that the primary aim of pathogenic bacteria is to gain access to nutrients rather than to cause damage to the host. The proteins that are encoded by virulence genes are often involved in the use of alternative nutrients, and therefore it would make sense if the expression of virulence genes is linked to the bacterial signaling pathways that sense and respond to the nutrient supply (39).

Vfr, an homologue of CRP in *Pseudomonas aeruginosa*, controls the expression of many virulence-associated genes, such as genes encoding for the exotoxin A, a type IV pili, a T3SS and a quorum sensing system (46). In *Vibrio cholerae*, cAMP signaling is important for carbon source availability but also for quorum sensing, biofilm formation, resistance to bacteriophages and virulence gene expression (47). In *Yersinia pestis*, CRP-cAMP negatively regulates the *sycO-ypkA-ypoJ* operon, laterally acquired

and involved in virulence. CRP-cAMP seems to act by directly binding to the promoter region of *sycO* (48). Additionally, the expression of the plasminogen activator gene requires activation by the CRP-cAMP complex. The plasminogen activator is needed for dissemination of *Y. pestis* to cause systemic infection (49).

In uropathogenic *Escherichia coli*, the colonization factor type I fimbriae is controlled by the metabolic sensor CRP-cAMP. Through the regulation of Lrp and possibly the DNA Gyrase, CRP-cAMP affect the phase variation that governs type I *fimbriae* expression in *E. coli* (50). In uropathogenic *E. coli* strains expression of other virulence-related fimbriae such as S- and P- *fimbriae* is activated by CRP-cAMP (51). Additionally, several virulence factors have been shown to respond to the expression of the presence of glucose in the media, such as hemolysin and adhesion in the Vero cytotoxin producing *E. coli* (52,53).

In bacteria of the genus *Streptococcus*, CcpA (homolog of CRP) and CCR are important for virulence. In *S. pneumoniae*, CcpA is required for colonization of the nasopharynx and for survival and multiplication in the lung (54). In *S. pyrogenes*, the *ccpA* mutant seems to be less virulent than the WT strain (55).

In *Listeria monocytogenes*, expression of genes that are required for entering the host cell, release from the phagosome into the cytosol and intracellular motility are controlled by the transcription activator PrfA (56). The activity of PrfA is strongly inhibited in the presence of glucose (57).

In *Clostridium perfringens*, the causative agent of severe histolytic diseases, glucose represses several virulence-associated processes, such as motility and toxin production (58).

Many other examples of CCR mediated regulation of virulence are left out of the introduction. It is evident that CCR plays a role on the regulation of virulence in bacteria; further evidence of the role of CRP-cAMP in the regulation of virulence in *Salmonella* will be addressed in this thesis.

1.2.2 CRP-cAMP in *Salmonella* virulence.

Interestingly, in *Salmonella*, CRP-cAMP seems to be also playing a role in virulence. A key regulatory system of virulence, the BarA-SirA two-component system is subjected to CRP-cAMP-mediated CCR. SirA controls the expression of SPI-1 encoded genes through the modulation of the levels of the SPI-1 repressor CsrA, which will be more extensively introduced in following sections. Accordingly, in *Salmonella enterica* serovar Typhimurium, in absence of CRP, genes encoded in the SPI-1 are downregulated in permissive conditions (59,60). The plasmidic component of virulence in *Salmonella* is also subjected to CCR as the pSLT-encoded *spv* virulence operon it seems to be negatively regulated by CRP-cAMP at the transcriptional level (61).

As early mentioned, fatty acids as propionate has been shown to regulate SPI-1 in *Salmonella*. Remarkably, the metabolic pathway involved in the metabolism of the precursors of propionate is also under CCR control. Moreover, CRP-cAMP is involved in the regulation of cobolamin and propanediol metabolism. The genes for cobolamin biosynthesis (*cob*), also known as vitamin B12, are coregulated with the genes required for the degradation of propanediol (*pdu*). The *pdu* and *cob* operon are regulated by the transcriptional factor PocR. Interestingly, PocR expression is positively regulated at the transcriptional level by CRP-cAMP (62).

Virulence features of other serovars of *Salmonella* are also subjected to CCR. In *Salmonella enterica* serovar Choleraesuis, CRP-cAMP seems to be also activating the expression of the SPI-1 genes (63). *Salmonella enterica* serovar Typhi is the causing agent of typhoid fever in humans. The bacterium is also able to persist in its host, causing a chronic disease by colonizing the spleen, liver and gallbladder. In the gallbladder is able to form biofilm which makes the bacteria resistance to bile. The *yihU-yshA* operon, among other genetic elements, is involved in the resistance of *S*.Typhi to bile and CRP-cAMP is positively regulating this operon (64).

1.3 Regulatory non-coding RNA in Bacterial pathogens.

Bacteria need to adapt rapidly to changing environmental conditions. The adaptive ability is crucial for the pathogenic bacteria during the infection process. Gene regulation studies have been mainly focused on changes in the protein profile expression. Moreover, for decades it was believed that transcriptional regulation depends only on proteinaceous regulatory factors. However, now we know that non-coding RNAs play a crucial role in gene expression and present a plethora of mechanisms by which affect gene expression. Regulatory non-coding RNAs extend the central dogma of gene expression (65). They can be divided in two major groups. The first group defined as non-coding RNA, including the house-keeping RNAs such as ribosomal RNA, the regulatory elements in 5'UTRs of mRNA such RNA thermometers and the CRISPR-derived RNAs. The second group defined as small non-coding RNAs (sRNA), which are regulators involved in the regulation of gene expression at the transcriptional, post-transcriptional, translational and even protein level.

1.3.1 Small non-coding RNA.

In this thesis we will mainly focus on the role of sRNAs in gene regulation. When it comes to structure, sRNAs are a highly heterogenic group, ranging in size from 50 to 500 nucleotides long single strand RNAs. First sRNAs were discovered in the early 70's, followed by the first discovery of antisense RNAs in the 80's encoded in plasmids. The first regulatory sRNAs from chromosomes were identified during 90's and in 2001 the first systematic screen to identify sRNAs was performed (66). More than 60 sRNAs in *E. coli* were identified by then, a tiny number compared to the thousands of sRNA predicted in bacteria by now. With the first bacterial genomes sequenced, biocomputational prediction based on the sRNA transcriptional features was the first approach for sRNA identification. Basically, looking for promoter sequence consensus followed by a Rho-independent terminator at chromosomal intergenic regions (67). New techonologies allowed improving sRNAs identification. Microarrays based identification, shot-gun cloning or co-purification with proteins were the following approaches that led to further identification of sRNAs (68). However, the game changer in the sRNA field was the application of the high-throughput sequencing

technology developed from 2005, the so-called RNA-seq allowed the simultaneously sequencing of millions of RNA molecules. Data analysis of the RNA-seq output let to the identification of novel transcripts, among them sRNAs.

As mentioned previously, sRNAs are a heterogeneous group. Based on its genomic location sRNAs are classified in cis-encoded sRNAs and trans-encoded sRNAs.

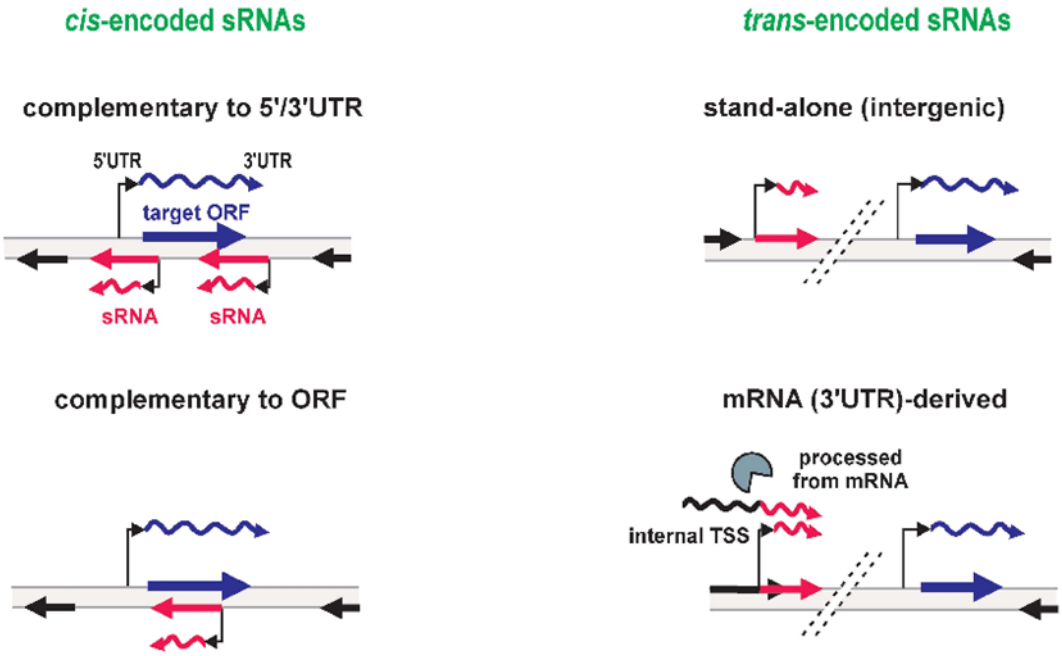

Figure 7. Small non-coding RNA. Cis-encoded RNA are encoded in the complementary sequence of a given gene. Trans-encoded RNA are encoded in intergenic regions or can be the results of the processing of a given mRNA. sRNAs are indicated in red, target gene is indicated in blue. Adapted from (69)

Cis-encoded sRNAs have a full complementarity with the target mRNA, as are transcribed from the complementary DNA strand to the one used as template for the target mRNA transcription. This antisense sRNA based gene control is typically used on plasmids, transposons and phages, in general terms, on mobile genetic elements. Cis-encoded sRNA in addition to complementarity to the ORF can also be transcribed from the complementary region to the untranslated regions of the target gene (5'UTR or 3'UTR), as exemplified by GadY sRNA (70). On the other hand, trans-encoded sRNAs are usually short, are transcribed from sequences unrelated with the target mRNAs and act by an imperfect base-pairing with the target mRNA. Trans-encoded sRNAs are

encoded in intergenic regions and are monocistronic. Alternatively, it has been recently shown that trans-encoded sRNA can also be derived from processing of 3'UTR of unrelated mRNAs (71). Trans-encoded sRNAs are the most common found sRNA encoded in chromosomes (Fig. 7) (69).

1.3.2 Mechanism of action of sRNA in gene regulation.

The most studied cis-encoded sRNAs are i) encoded in plasmids and control the regulation of this genetic element (72) or ii) part of a toxin-antitoxin systems present in plasmids or in the chromosome (73). Cis-encoded antisense sRNAs are relevant factors involved in maintaining stability of genetic elements in bacteria. There are several molecular mechanism of antisense-mediated regulation at both transcriptional and post-transcriptional level. As they are encoded in the complementary strand of the target region, transcriptional expression of the cis-encoded sRNA can regulate the target mRNA by interference with the target mRNA transcription. Alternatively, they can also attenuate transcription of the target mRNA by base pairing with the nascent target mRNA molecule that is being transcribed (Fig. 8) (74).

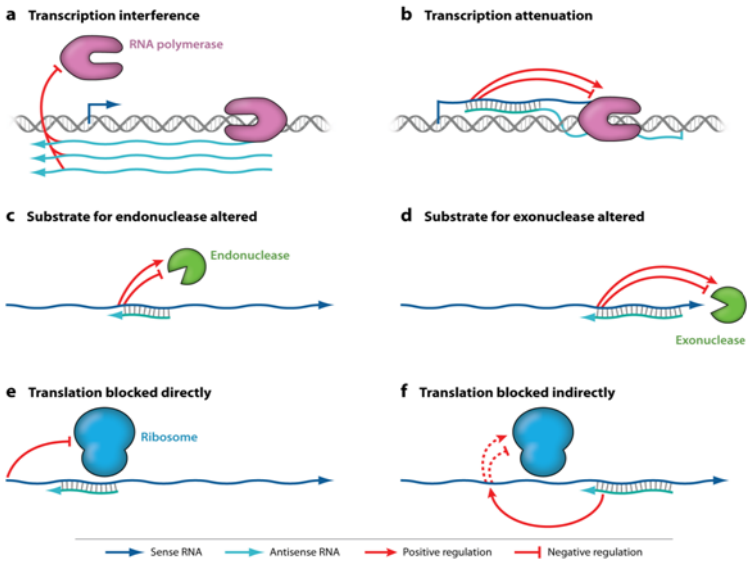

Figure 8. Mechanism of action of sRNA. A. Transcription interference. **B.** Transcription attenuation. **C.** Substrate for endonuclease altered. **D.** Substrate for exonuclease altered. **E.** Translation blocked directly. **F.** Translation blocked indirectly. Adapted from (74).

When it comes to trans-encoded sRNA, typically, within its short length contain one or several seed region involved in the imperfect base pairing with the target mRNA(s), a region of interaction with the RNA binding protein Hfq and a stem loop terminator at the 3' end (75).

Overall sRNA can i) regulate at the transcriptional level by interfering or attenuating transcription (Fig. 8A and B), ii) alter the affinity of the target mRNA to endonucleases and exonucleases (Fig. 8C and D) and iii) affect the target mRNA by either directly or indirectly blocking translation (Fig. 8E and F) (74).

1.3.3 Hfq, a major sRNA binding protein.

In many bacteria, *Salmonella enterica* serovar Typhimurium among them, sRNAs interact with the RNA binding protein Hfq, a key player in the sRNA-mediated gene regulation. Hfq is a protein firstly identified in the 60s as host factor for replication of RNA bacteriophage Qβ (Hfq) in *E. coli*. It is a conserved Sm-like protein, homologous to eukaryotic splicing-related proteins, with a homohexameric ring-like structure (76).

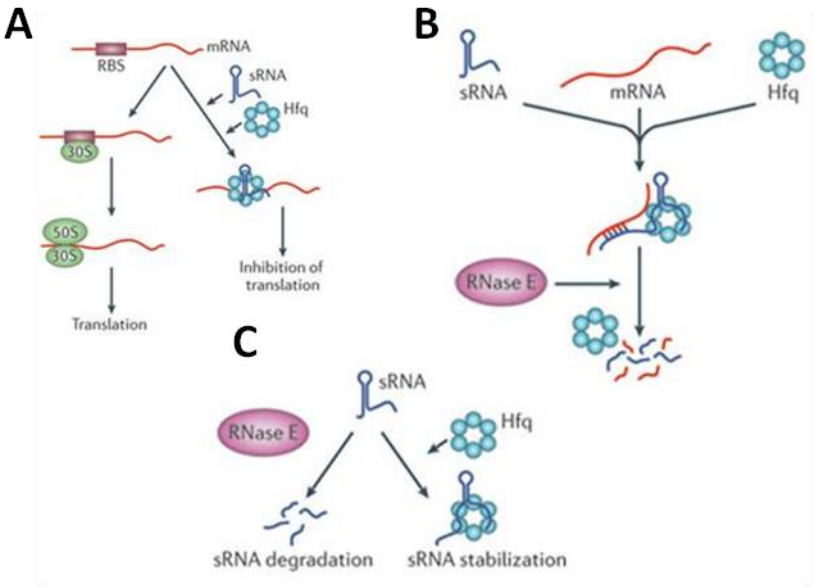

Figure 9. Hfq in sRNA regulation. A. Hfq binds the sRNA and promotes the mRNA target repression by blocking translation through binding near the ribosome binding site. **B.** Hfq recruits RNAse E to degrade the sRNA and the target mRNA. **C.** Hfq mediated stabilization of the sRNA. Adapted from (76).

Hfq can affect sRNA mediated regulation through different mechanisms: i) Hfq facilitates the interaction between the sRNA and the target mRNA, interaction of the complex Hfq-sRNA with the ribosome binding site (RBS) of the target mRNA can let to inhibition of translation (Fig. 9A), ii) Hfq can affect the RNA stability of the sRNA, it can protect the sRNA from degradation by ribonucleases such RNAase E (Fig. 9C), iii) alternatively, the formation of the complex target mRNA-sRNA-Hfq can recruit the presence of the RNAase E and degrade both the sRNA and the target mRNA which in turn downregulate the target mRNA expression (Fig. 9B) (76).

Hfq is widespread, but not ubiquitous. Besides Hfq seem to be playing a major role in sRNA-mediated regulation, is only present in 50 % of all bacteria and is not required for sRNA-mediated regulation in Gram-positive bacteria. It cannot be rule out that other major sRNA binding proteins with similar functions to Hfq in bacteria, where Hfq was not found, exist. Recently, it has been described a new major sRNA binding protein in *Salmonella*, ProQ, as Hfq, binds to a specific pool of sRNAs to regulate gene expression (77).

1.3.4 The role of sRNA in *Salmonella*.

Salmonella enterica serovar Typhimurium has been exceptionally well investigated with respect to virulence mechanisms, genome evolution and many fundamental pathways of gene expression and metabolism. Most of the studies were previously focused on protein functions but *Salmonella* has also become a model organism for RNA-mediated regulation. sRNAs participate in global regulatory networks, it has to be highlighted that *Salmonella* encodes for more than 200 structurally diverse sRNAs with up to 1250 potential mRNA targets.

Since Hfq regulates gene expression by modulating sRNA activities, deletion of Hfq allow us to infere molecular pathways were sRNAs are involved. In *Salmonella*, *hfq* mutation affect directly or indirectly up to 20 % of all *Salmonella* genes, having a pleiotropic effect on its physiology. Among the phenotypes observed we found: i)

reduced host cell adhesion and invasion, ii) chronic envelope stress, iii) a decrease in secreted effector proteins, iv) loss of motility, v) defect on SPI-1 expression under aerobic growth and vi) reduced replication in macrophages. All these features led to the fact that in absence of Hfq *Salmonella enterica* serovar Typhimurium has attenuated virulence in mice, suggesting that Hfq is a key regulator of virulence in *Salmonella* (78).

As previously introduced, *Salmonella* encodes for several pathogenecity islands (SPIs) that were acquired through horizontal gene transfer. Interestingly, sRNAs are involved in the cross-talk between the core genome and the horizontal transferred genes (HGT) in *Salmonella* (79). The sRNA InvR encoded in SPI-1 regulates the chromosome encoded *ompD* mRNA while the chromosome encoded sRNA SgrS regulates the SPI-1 encoded gene *sopD* mRNA (80). Both InvR and SgrS are Hfq-dependent sRNA.

The molecular mechanisms involved in the regulation mediated by sRNA are complex. As mentioned previously, trans-encoded sRNA interact with its target mRNA by an imperfect base-pairing allowing that these sRNAs may regulate multiple target mRNA. Similar to transcription factors that act on the DNA level, sRNAs recognize short consensus sites to regulate multiple targets. Additionally, a given target mRNA can be regulated by several different sRNA. As examples, the sRNA RybB regulates many membrane protein encoding genes (65). Furthermore, the biofilm formation regulator CsgD it is regulated by several sRNAs in *E. coli* and *Salmonella* (81).

The role of sRNAs is particularly relevant for the bacterial stress response and for virulence. sRNAs regulation potentiates the fast response in gene expression under changing environmental conditions. Bacterial pathogens face many stress conditions as they need to adapt to the new niches that the pathogen face during the transit within the host. Examples of stress responses in which sRNAs are involved are: MicA in envelope stress or SgrS in phosphosugar stress (82,83). Interestingly, although sRNA play an important role as virulence regulators either by regulating the metabolism of the cell or by directly regulating virulence factor expression, deletion of a single sRNA often does not present a clear macromolecular phenotype. Reason why, despite the many global mutagenesis screens performed, no sRNA mutant with altered *Salmonella*

virulence has been discovered by this means. These screenings score the effect of individual gene disruption but do not take into account that a function of a sRNA can be backed up by an sRNA homologue or masked by the redundant activity of regulatory proteins. Recently, the application of the novel approach dual RNA-seq, lead to the identification of the sRNA PinT as a major regulator of *Salmonella* virulence (84).

1.3.5 CRP-cAMP and sRNA.

As previously introduced, CRP-cAMP is a transcriptional factor involved in many other cellular processes in addition to the carbon catabolite repression regulation. It regulates many genes that encode for metabolic enzymes invoved in metabolism. Interestingly, CRP-cAMP has been also described to modulate the expression of several sRNA. In *Yersinia pseudotuberculosis* it has been shown that CRP is a main regulator of sRNAs, by regulating several sRNAs involved in the genetic reprogramming of the cell upon temperature shifts (85).

In *Salmonella enterica* serovar Typhimurium, CRP-cAMP regulates the sRNA *cyaR*. This sRNA regulates the gene *ompX* by binding to its Shine-Dalgarno region and repressing the translational expression of the abundant porin OmpX, linking porin expression and nutrient availability (86). In *E. coli*, CRP-cAMP represses the expression of the sRNA Spot 42. It is remarkable that the sRNA Spot 42 forms a multioutput feedforward loop with CRP-cAMP to regulate catabolite repression in *Escherichia coli*. Spot 42 directly represses genes involved in central and secondary metabolism, redox balancing and the consumption of diverse non-preferred carbon sources (87). Many of the genes repressed by Spot 42 sRNA are transcriptionally activated by CRP-cAMP. This pathway seems to be required for the reduction of leaky expression from CRP-cAMP target genes. Under certain conditions (non-permissive), CRP-activated genes should be silenced. The cell responds by dropping the levels of CRP-cAMP and consequently activation is diminished. Moreover, the drop in CRP-cAMP will increase the levels of Spot 42 and the sRNA would then repress the basal expression of the CRP-cAMP target genes. The regulatory loop through the sRNA Spot 42 allows the bacteria to promptly

shut down expression of energetically less efficient metabolic pathways, allowing a fast use of more efficient metabolic pathways, such as metabolization of glucose (Fig. 10) (87–89).

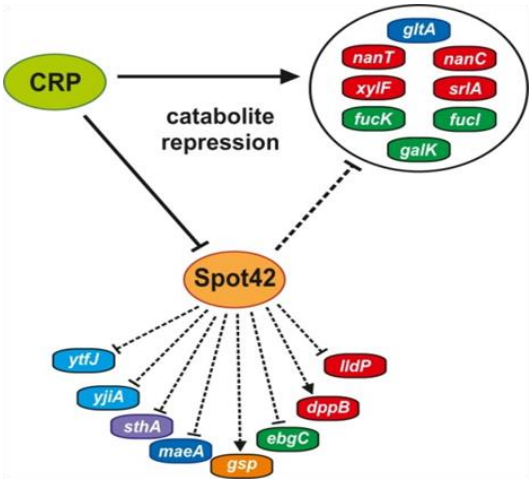

Figure 10. CRP-Spot42 regulation network in *Escherichia coli.* Genes positively regulated by CRP-cAMP are repressed by the CRP-cAMP-repressed sRNA Spot 42. Adapted from (89).

1.4 CsrA as a global regulator.

CsrA (carbon storage regulator) is an RNA binding protein involved in the regulation of carbon flux pathways. A *csrA* mutation has pleiotropic effects on gene expression. It has been reported to be involved in cell morphology and surface adhesion, motility, biofilm formation, virulence, etc (reviewed in (90)).

CsrA protein acts through the interaction with the target mRNA, It binds to GGA motifs, commonly located in apical loops. CsrA interaction can either activate or repress the target mRNA. To do so, CsrA modulates target mRNA translation by different molecular mechanisms as summarized in Figure 11. To repress targets, CsrA (RsmA in *Pseudomonas aeruginosa*) can i) bind to the 5'UTR and repress gene expression by avoiding the translation of the target mRNA (panel A and B), or ii) modulate Rho dependent transcriptional termination by affecting the terminator folding upon CsrA binding (panel C). On the other hand, CsrA can activate gene

expression by i) modulating the folding of the 5'UTR to enhance the mRNA translation, or ii) stabilizing the mRNA by protecting it from RNAse E cleavage.

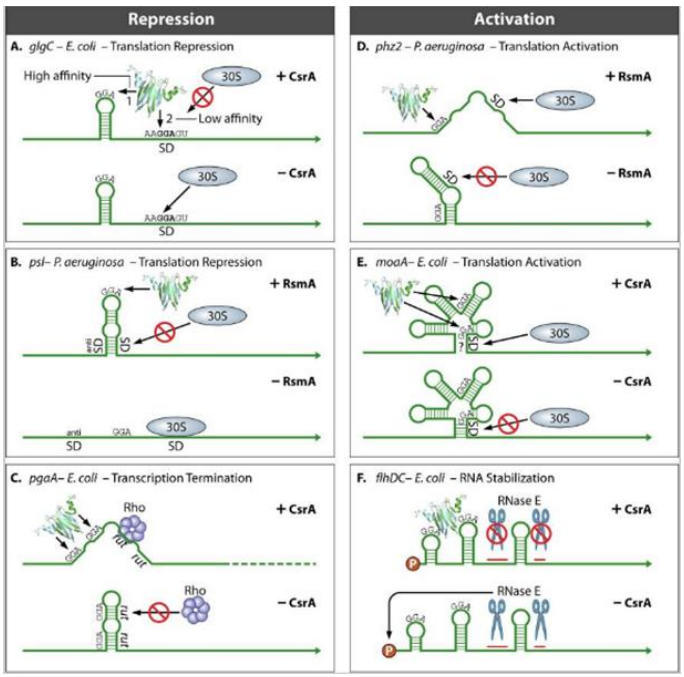

Figure 11. CsrA molecular mechanism of action. CsrA (RsmA in *P. aeruginosa*) examples of described molecular mechanism. **A** and **B)** Translation repression . **C)** Transcription termination. **D** and **E)** Translation activation. **F)** RNA stabilization. Adapted from (90).

CsrA regulates many bacterial processes in *Salmonella enterica* serovar Typhmurium. DNA microarray studies showed that the absence of CsrA deregulates up to 8 % of *Salmonella* genes (91). As examples, i) CsrA is involved in the expression of i) the flagellar synthesis operons *flg* and *fli*, ii) two metabolic pathways, the *pdu* operon involved in the utilization of 1, 2-propanediol and the *eut* operon involved in the ethanolamine catabolism. Accordingly, the *cob* operon involved in the synthesis of vitamin B12, required for the metabolism of both 1,2-propanediol and ethanolamine, is also affected by the RNA binding protein CsrA (91) and iii) CsrA is involved in the regulation of genes encoding for enzymes involved in the synthesis/turnover of the second messenger c-di-GMP turnover (92).

The role of CsrA in the virulence of *Salmonella enterica* serovar Typhimurium has been extensively studied. First studies revealed that CsrA was required for host cell invasion

(93). Later studies showed that the direct target of CsrA in *Salmonella* virulence is the transcriptional regulator HilD. It has been demonstrated that CsrA binds to the *hilD* mRNA and by blocking translation affects the steady state levels of *hilD* mRNA (30). Recently, the CsrA direct targets in *Salmonella* were elucidated by the use of CLIP-seq. In addition to *hilD,* CsrA seems to bind to many other SPI-1 encoded genes, further supporting the role of CsrA in virulence (94).

As CsrA affects many important regulatory pathways in the cell, the activity of CsrA it is strongly regulated. The regulation occurs at the post-translational level by two long non-coding RNA (lnc-RNA), CsrB and CsrC. These two lnc-RNA carry in their RNA sequence several GGA motifs, in a way that when *csrB* and *csrC* are highly expressed can sequester CsrA protein. In turn, that would cause a decrease in the level of free CsrA protein with ability to bind to its target RNA. Therefore, *csrB* and *csrC* repress the activity of CsrA. In addition to CsrB and CsrC, it has been described that the sRNA McaS can also titrate CsrA protein by direct binding in *E. coli* (95). In *Salmonella* it has been shown that CsrA can also be titrated by the 5'UTR region of the the type I fimbrial encoded mRNA (96).

The level of expression of these two long non-coding RNA is tightly regulated. In *Salmonella* it has been reported that the two-component system BarA-SirA is responsible for the transcriptional activation of *csrB* and *csrC*. According to Teplitski work, SirA binds to *csrB* leader but not to *csrC* (60). However, later studies performed in Bustamante lab showed that SirA can bind also to *csrC* leader region, although SirA seem to also activate *csrC* through an indirect mechanism not involving direct binding to its promoter region (30). There is crossregulation between CsrA, *csrB* and *csrC*. As shown by Fortune *et al.* (2006), CsrA activates the expression of *csrB* and *csrC*, and, it seems that *csrB* and *csrC* can crossactivate each other (97). A link between CsrA and CRP-cAMP mediated regulation has been suggested. As SirA seem to be under CCR control in some conditions. The absence of *crp* or *cya* reduces the expression of *sirA* and therefore of *csrB* and *csrC* (59). CRP-cAMP seems to also be regulating the expression of *csrB* and *csrC* in *E. coli* and *Y.pseudotuberculosis* (98,99). Further indicating the clear link between nutrient availability and virulence gene expression in bacteria.

2. OBJECTIVES

2. OBJECTIVES

The regulation of the expression of virulence genes in *Salmonella enterica* serovar Typhimurium is an intensively studied feature of *Salmonella* lifestyle. How *Salmonella* integrates environmental signals to activate virulence-related genes within the host has been explored. As mentioned in the introduction, the genes encoded in *Salmonella* pathogenicity island I (SPI-1) required for the invasion of epithelial cells are well characterized, and many regulators involved in the activation of SPI-1 genes have been described. However, little is known about mechanism involved in the repression of SPI-1 under conditions were *Salmonella* does not require the expression of virulence-related genes. The expression of SPI-1 encoded genes have been reported to be a burden for *Salmonella* physiology and therefore mechanism to control shut down of SPI-1 under non permissive conditions might play a crucial role in *Salmonella* physiology. Here we describe that at exponential phase, CRP-cAMP, which acts as an activator at stationary phase, represses the expression of SPI-1 genes. The overall objective of this thesis was to characterize how CRP-cAMP silences SPI-1 expression under non-permissive conditions and to describe the molecular mechanism behind this phenotypic observation. With this purpose, specific objectives were defined that will be addressed in the following results sections.

- Define the target gene for the CRP-mediated regulation of SPI-1.
- Elucidate at which level of regulation CRP-cAMP modulates the expression of the SPI-1 genes master regulator *hilD*.
- Characterize the involvement of CRP-cAMP dependent sRNA in *hilD* regulation.
- Characterize the interaction of the CRP-cAMP dependent sRNA Spot 42 with the *hilD* 3'UTR region to regulate SPI-1.
- Explore the role of CRP-cAMP in the modulation of the SPI-1 repressor CsrA through the regulation of the long non-coding RNA *csrB* and *csrC.*

3. MATERIALS AND METHODS

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3.1 Strains, plasmids and oligonucleotides.

Strains, plasmids and oligonucleotides are indicated in Annex I in Table S1, S2 and S3, respectively.

3.2 Media and growth conditions.

Salmonella enterica serovar Typhimurium SL1344 and derivative strains were cultivated either in Luria-Bertani (LB) broth or LB agar plates. LB contains 5 g/l of yeast extract, 10 g/l of tryptone and 10 g/l of NaCl in milliQ water, for LB agar plates, agar was added to 15 g/l.

After sterilization by autoclave, LB media was supplemented when required with ampicillin (Amp) 100 µg/ml, kanamycin (Km) 50 µg/ml, chloramphenicol (Cm) 15 µg/ml, tetracycline (Tc) 15 µg/ml. For gene expression induction from genetic constructs, up to 1 mM IPTG or 0.2 % L-arabinose was added to the liquid culture when indicated.

Liquid cultures were routinely inoculated up to an OD_{600nm} of 0.001 and incubated at 37^{o}C with vigorous shaking (200 rpm). An OD_{600nm} of 0.3-0.4 was considered logarithmic phase of growth, while an OD_{600nm} of 2.0 was considered early stationary phase of growth. For solid growth, single colonies were streaked on agar plates and incubated at 37^{o}C.

Composition of media and buffers are indicated in Annex 2.

3.3 Genetic manipulations

3.3.1 Polymerase chain reaction (PCR)

Routinely, the PCR was performed in a final volume of 25 µl. In detail, each reaction contains 12.5 µl of PCR master mix (containing nucleotide mix, polymerase buffer and Taq polymerase), 1 µl of a 10 µM solution of forward primer, 1 µl of a 10 µM solution of reverse primer, 1 µl of template suspension and 9.5 µl of nuclease-free water.

Standard PCR was performed by using the following cycling conditions. An initial denaturation at 95ºC for 5 min, followed by 25 cycles of i) denaturation 95ºC 30 s ii) annealing 50-60ºC 30 s and iii) extension 72ºC 1 min/Kb, and then a final extension at 72ºC for 10 min.

For some applications of the PCR, proofreading activity of the DNA polymerase was required. In those cases, the polymerase of use was Accuzyme, *pfu*turbo or phusion polymerase. Extension time and temperature was adjusted according to preferred conditions of the DNA polymerase in use.

3.3.1.1 Overlapping PCR

PCR principle has been applied to a broad range of applications. Two rounds of PCR can be used to *in vitro* fuse two DNA fragments, procedure known as overlapping PCR (100). For this purpose, the DNA polymerase of use was the *pfu* turbo DNA polymerase. Primers are designed that in a first PCR the two fragments generated carry complementary overhangs (Fig. 12).

In a first PCR, two fragments were obtained. The final reaction volume for the first PCR was set to 50 µl. Each reaction contains 5 µl of 10X *pfu* buffer, 2 µl of a 10 µM solution of forward primer (a or c), 2 µl of a 10 µM solution of reverse primer (b or d), 1 µl of *pfu* turbo polymerase, 1 µl of template suspension and 39 µl of nuclease-free water. The cycling conditions for the first PCR were the following. An initial denaturation at 95ºC for 5 min followed by 30 cycles of i) denaturation 95ºC 30 s ii) annealing 55-60ºC 30 s iii) extension 72ºC 1 min/Kb and then a final extension at 72ºC for 10 min.

Subsequently the specific PCR products were purified to remove residual primers and unspecific PCR products. A second round of PCR was performed using a mixture of the two PCR products obtained as a template. The reaction volume of the second PCR was also set to 50 µl. Each reaction contains 5 µl of 10X *pfu* buffer, 2 µl of a 10 µM solution of forward primer (a), 2 µl of a 10 µM solution of reverse primer (d), 1 µl of *pfu* turbo polymerase, 1 µl of template suspension (mix 1:1 of the two PCR products) and 39 µl of nuclease-free water.

The cycling conditions for the second PCR were the following. An initial denaturation at 95ºC for 5 min followed by 10 cycles of i) denaturation 95ºC 30 s ii) annealing 35ºC 30 s iii) extension 72ºC 1 min/Kb and then 25 cycles of i) denaturation 95ºC 30 s ii) annealing 50-60ºC 30 s iii) extension 72ºC 1 min/Kb and to end a final extension at 72ºC for 10 min.

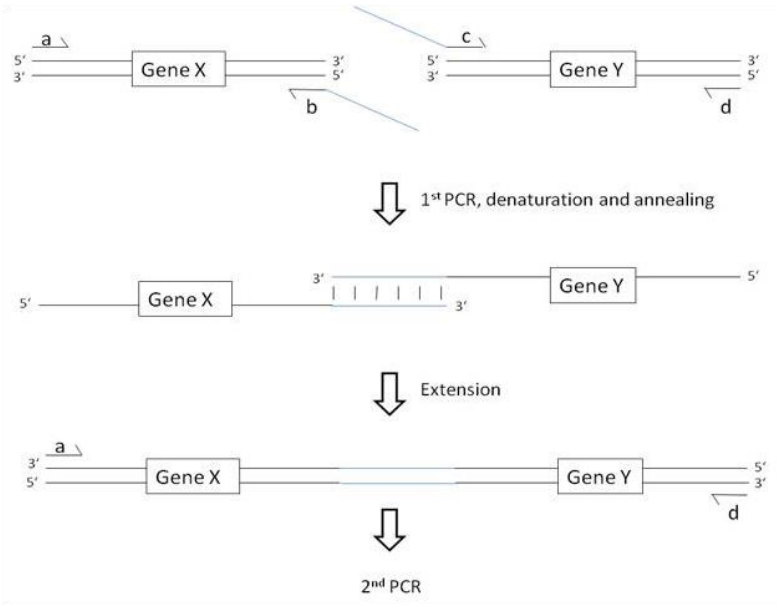

Figure 12. Overlapping PCR. In the first PCR round, Gene X and Gene Y are PCR amplified by using the pair of primers a/b and c/d respectively. The generated two PCR fragments carry complementary overhangs at the 3'end and 5'end, respectively. The second PCR used this two PCR fragments as templates to generate a fused PCR fragment by using the pair of primers a/d. Adapted from (100)

3.3.2 Transformation

3.3.2.1 Chemical transformation

Transformation of chemical competent cells was used for *E. coli* strain Top 10 and DH5α. Cells were grown on LB broth at 37ºC with vigorous shaking to an OD_{600nm} of 0.2-0.3 to subsequently be fast chilled on ice. Cells were pelleted by centrifugation and washed three times with an ice-cold solution of $CaCl_2$ 50 mM to finally resuspend the cells in 1:100 volume of the initial culture in $CaCl_2$ 50 mM.

For transformation, 50-70 µl of chemical competent cells were mixed in a pre-chilled microcentrifuge tube with 5-50 ng of desired DNA. Tubes were incubated 30 minutes

on ice. The samples were heat shocked at 42°C for 45 seconds. Next, 1 ml of LB broth was added to the tubes and incubated at 37°C for phenotypic expression for 90 minutes before to be spread on LB agar plates. Subsequently, single clones were streaked three times on LB agar plates in order to obtain a pure culture.

3.3.2.2 Electroporation based transformation

The standard procedure to transform DNA in *Salmonella* cells was electroporation. Cells were grown in LB broth at 37°C with vigorous shaking to an OD_{600nm} of 0.4-0.5 to subsequently be fast chilled on ice. Cells were pelleted by centrifugation and washed three times with an ice-cold solution of glycerol 10% to finally resuspend the cells in 1:100 volume of the initial culture in glycerol 10%. Electrocompetent cells were mixed with 20-100 ng of desired DNA in pre-chilled electroporation cuvettes. The cells were electroporated with a micropulse of 1.8 kv for plasmidic DNA and 2.5 kv for DNA fragments. Upon elctroporation, 1 ml of LB broth was added to the cuvette, transferred to a microcentrifuge tube and incubated 90 minutes at 37°C for phenotypic expression before to be spread on LB agar plates. Subsequently, single clones were streaked three times on LB agar plates in order to obtain a pure culture.

3.3.3 P22 transduction

3.3.3.1 P22 lysates

Strain that will act as a donor, carrying a genetic construct or a mutation of interest, will be grown in LB broth to an OD_{600nm} of 0.2-0.3, 10 µl of P22 lysate (TT1704) was added to the culture and incubated for 3-4 hours at 37°C. To lyse remaining cells, chloroform was added to the culture. The sample was centrifugated at 4°C for 10 minutes. Supernatant was transferred to a new tube and 500 µl of chloroform was added to obtain the final P22 lysate.

3.3.3.2 P22 transduction

A suspension of recipient cells in LB broth at a final OD_{600nm} of 5.0 was obtained from overnight streaks grown on LB agar plates. 100 µl of recipient cells were mixed with

100 µl of donor P22 lysate and incubated at 37^{o}C for 90 minutes. Transduction samples were subsequently spread on LB agar plates. Single colonies were streaked on EBU green agar plates twice in order to select lysogenic phage free bacteria. EBU plates allow differentiating between lysogenic phage carrying bacteria (green color) and lysogenic phage free bacteria (white).

3.3.4 Gene cloning

For gene cloning, the desired PCR fragment is amplified by using a proof reading polymerase (Accuzyme, *pfu* Turbo and Phusion polymerase). The resulting PCR fragment was purified using PCR purification kit (Qiagen) and subjected to restriction enzyme digestion (Thermo Fischer scientific). Digested fragments were purified again by using PCR purification kit and ligated with a vector digested with compatible enzymes by using T4 DNA ligase (Thermo Fisher scientific) at 22^{o}C overnight. The ligation was subsequently transformed into chemical competent cells.

The *cpdA* gene was cloned into the IPTG inducible vector pTRc99a (101). *cpdA* coding sequence was PCR amplified with the primers *cpdA_Xba*I_Fw and *cpdA_Sal*I6xHis_rev (Table S3), subsequently digested with *Xba*I and *Sal*I and ligated into *Xba*I/*Sal*I digested pTRc99a.

The *spf* gene encoding Spot 42 sRNA was cloned into pBRplac vector (102). *spf* was PCR amplified by using the primers *spf_Aat*II_Fw and *spf_Eco*RI_rev (Table S3), subsequently digested with *Aat*II and *Eco*RI and ligated into *Aat*II/*Eco*RI digested pBRplac.

3.3.5 Gene replacement

Deletion mutants were generated by gene replacement using the λ Red recombinase system described by Datsenko and Wanner, 2001 (103). For homologous recombination within a defined chromosomal locus, an antibiotic resistance cassette flanked by FRT sites and up to 40 nucleotides homologous extensions was introduced

into the bacterial cell. To replace the gene of interest, homologous extensions were designed so that recombination of the PCR fragment upstream and downstream of the respective gene will occur due to the activity of the λRed recombinase. As flanked by FRT sites, the antibiotic resistance cassette can be removed by Flp recombinase activity leaving behind one FRT scar at the site of the inactivated gene (Fig. 13).

In detail, antibiotic resistance cassettes carrying either Km^R or Cm^R resistance genes were PCR amplified from pKD4 and pKD3, respectively (Step 1). Primers used include up to 40 bp sequence complementary to the region where the insertion is desired. Purified fragments were electroporated into carrying pKD46 strains (Step 2).

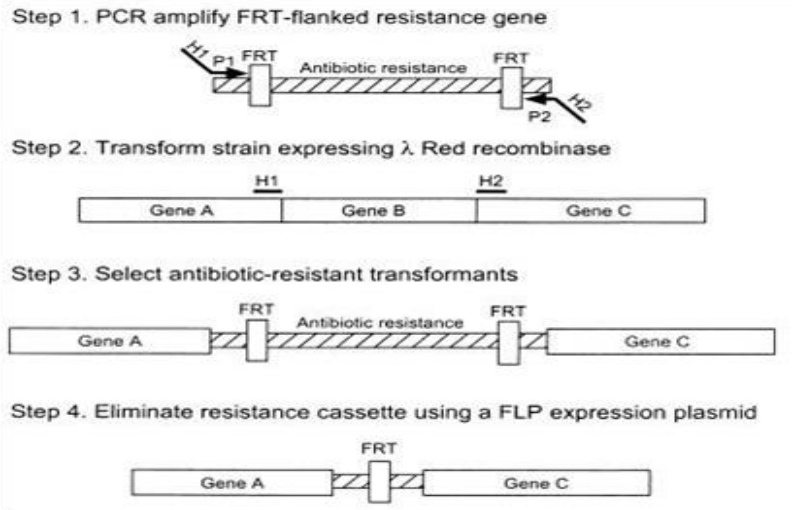

Figure 13. Gene replacement by λ Red recombinase. Step 1) The selected Frt- flanked antibiotic resistance was amplified from pKD3 or pKD4 (Cm^R or Km^R) with primers carrying overhangs complementary to the desired site of insertion. Step 2) Purified PCR fragment is transformed by electroporation into a strain carrying the plasmid pKD46, expressing λRed recombinase. Step 3) Selection of antibiotic-resistant transformants. Step 4) When desired, the FRT-flanked antibiotic resistance cassette can be removed by using the FLP-expressing plasmid pCP20. Schematic representation adapted from (103).

The vector pKD46 encodes for commonly referred λ Red genes, its expression interferes with the bacterial defense mechanisms and promote homologous recombination onto the chromosome. The acceptor strain carrying the pKD46 is grown in LB broth at 30°C to an OD_{600nm} of 0.2, at this point L-arabinose is added to the cell to induce the expression of λ Red genes. When reached OD_{600nm} 0.4-0.5 electrocompetent cells were obtained (2.3.2.2) and the purified DNA fragment was

electroporated. Positive clones were selected in presence of the required antibiotic (Step 3).

The FRT sites flanking the antibiotic cassette can recombine upon expression of Flp encoded into pCP20 to remove the antibiotic resistance cassette (Step 4) (104). pCP20 plasmid is thermosensitive (30^{o}C), it was mobilized into electrocompetent acceptor strains. Strains were grown on LB agar plates at 42^{o}C, at this temperature Flp will be highly expressed causing excision of the antibiotic resistance cassette, and it would promote the removal of pCP20 from the cell (Fig. 13).

3.3.6 Epitope FLAG-tagged proteins

Epitope tagged proteins were generated by a based λ Red recombinase system as described previously (105). The experimental procedure was identical to 2.3.5 but pSUB11 vector was used as a template (Fig. 14). pSUB11 carries a kanamycin resistance cassette downstream of a FLAG-tagged encoding sequence (106). When desired the Km^{R} cassette downstream of the 3XFLAG epitope was removed using the Flp recombinase as described above.

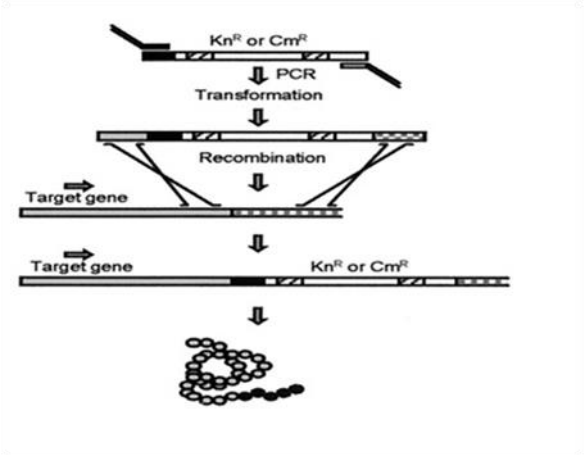

Figure 14. Epitope tagged proteins. λ Red recombinase based system was used to introduced Flag-tagged epitope at the C-term of a protein of interest. pSUB11 plasmid was used as template for PCR amplification of an antibiotic resistance cassette with FLAG-tag encoding sequence at the 5'end. PCR fragment flanked by 40 bp overlapping sequence with the desired site of insertion was transformed into pKD46 carrying strain. Generated construct will express a recombinant protein with a FLAG epitope tag at the C-term end of the protein that can be used for further applications as immunodetection by western blot, protein purification or immunoprecipitation. Adapted from (106).

Chromosomal constructs for FLAG-tagged proteins were generated for several genes. For the HilD-3 flag construct, when carrying the Km^R cassette, the 3'UTR of *hilD* mRNA locates far from the *hilD* mRNA and are not co-transcribed. When Km^R cassette is removed, the *hilD* 3'UTR locates downstream of the 3 flag epitope and the effect of the 3'UTR on *hilD* expression is clearly detected. Oligonucleotides used to generate the constructs are listed in Table S3. All strains were PCR confirmed and integrity of the sequence was checked by DNA sequencing.

3.3.7 Chromosomal *lacZ* transcriptional fusions

Deletion mutants obtained by gene replacement and carrying FRT scar were further used to generate reporter gene fusions. The remaining FRT-site was used to integrate plasmid pKG136 (107). pKG136 carries a promoterless reporter *lacZ* gene, a kanamycin resistance cassette and an FRT site. To do that, the vector pCP20 was transformed into the strain of interest carrying the gene with a single FRT site, and the resulting clones were transformed with pKG136. Upon expression of the Flp recombinase from pCP20 the FRT sites in the chromosome and in the pKG136 plasmid recombine leading to the integration of pKG136 thereby generating the chromosomal transcriptional fusion.

3.3.8 Plasmidic *gfp* translational fusions

A translational GFP reporter system was used for characterization of the base-pair interaction between a small RNA (sRNA) and its cognate target mRNA. The plasmid pXG1 generated by Urban and Vogel was used as a template (108) (Fig. 15). The *hilD* 3'UTR region was fused to *gfp* by overlapping PCR (2.3.1.1). Purified PCR fragment was subsequently digested with *Xba*I/*Nhe*I and ligated into a *Xba*I/*Nhe*I digested pXG1 vector.

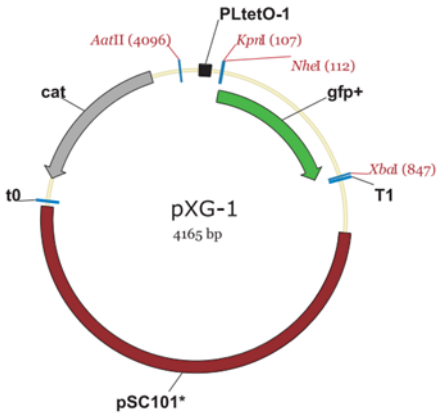

Figure 15. pXG1 vector. It is a low-copy translation GFP-based fusions plasmid. *Kpn*I and *Nhe*I can be used as cloning restriction sites for standard translational fusions. Note that *Nhe*I restriction site is located at the 3rd third codon of *gfp* ORF and therefore cloned regions must be in frame. Alternatively, regulatory regions can be also cloned at the 3'end of GFP. Adapted from (108).

3.3.9 Plasmidic *lacZ* transcriptional fusions

Plasmidic transcriptional fusions were generated for two long non-coding RNA, *csrB* and *csrC*. Transcriptional fusions were generated upon cloning of the regulatory regions of *csrB* and *csrC* in the vector pQF50. This cloning vector carries a promoterless *lacZ* reporter gene (Fig. 16) (109). Regulatory regions of *csrB* and *csrC* were PCR amplified using a proof reading polymerase, adding a *Bam*HI restriction site at the 5' end and a *Hin*dIII restriction site at the 3'end. DNA fragments were cloned in *Bam*HI/*Hin*dIII sites of pQF50 vector.

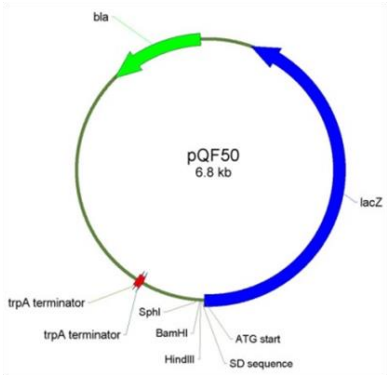

Figure 16. Map of pQF50 vector. Plasmidic transcriptional *lacZ* fusions can be generated in pQF50 by cloning the gene of interest regulatory regions using *Bam*HI and *Hin*dIII restriction enzyme sites. Gene expression levels were assessed by β-galactosidase assay as indicated below.

3.3.10 Nucleotide substitutions

Site directed mutagenesis procedures were performed to generate nucleotide substitutions in both plasmid cloned sequences and in chromosomal located sequences.

3.3.10.1 Quick-change site directed mutagenesis

To produce site-directed mutants of sequences cloned in a plasmid single to triple nucleotides substitutions were introduced (Fig. 17). Forward and reverse primers 30-40 nt nucleotides long with the desired mutation were used. The whole plasmid was PCR amplified using *pfu* turbo polymerase. The PCR product was *Dpn*I digested to remove the original plasmid template and the digestion product was transformed into *E. coli* Top 10 competent cells. Several transformants were isolated in pure cultures; and the sequence fidelity of the plasmid was assessed by DNA sequencing of the region of interest.

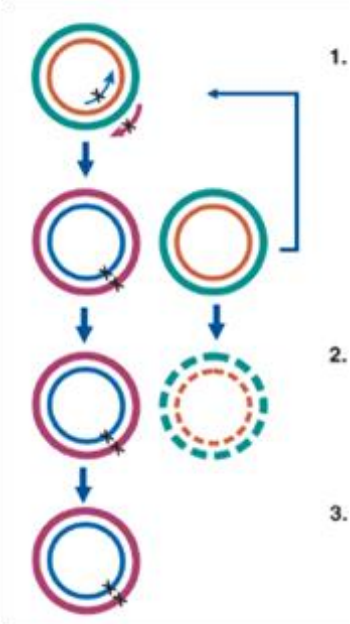

Figure 17. Quick-change site directed mutagenesis outline. By using this method, single to triple nucleotides substitutions can be generated in plasmid-encoded genes. **1)** First, primers containing the desired mutations were used to amplify the plasmid of interest by using proof reading polymerase. **2)** Next, the PCR product was digested with *DpnI* restriction enzyme to digest parental plasmids.**3)** Finally, *DpnI* digested PCR was transformed into competent *E. coli* Top 10 strain. Adapted from (110).

3.3.10.2 Chromosomal point mutations

Chromosomal mutations on *hilD* 3'UTR region were generated by a scarless method as described by I.Cota *et al.* (111). Briefly, 1 kb fragment containing the *hilD* 3'UTR sequence flanked by the *Sac*I and *Xba*I restriction sites was cloned into pGEM vector (112). Desired point mutations were generated by Quick change^{TM} method, using the *hilD* 3'UTR oligonucleotides from the Table S3. Subsequently, the vector was digested with *Sac*I/*Xba*I, and ligated into the suicide plasmid pDMS197 (113). The derivative pDMS197 was propagated in S17-1 lambda *pir*, and used as donor in mating experiments with SV5015 *sipC-lacZ* as a recipient strain. Transconjugants were selected for tetracycline resistance. Transconjugants have integrated the pDMS197 derivative within the *hilD* gene by homologous recombination. Selected clones were grown in salt-free nutrient broth supplemented with 5% sucrose and individual tetracycline-sensitive clones were selected. Those clones have lost the pDMS197 backbone by a recombination event. Theoretically, around 50% of clones will be *hilD* WT whereas the other 50% will have the substitutions generated by mutagenesis. Some clones were checked by PCR and subsequent DNA sequencing to select the clones carrying the desired chromosomal mutation.

3.4 Gene expression assays

3.4.1 B-galactosidase assay

β-galactosidase activity measurement has been widely used to assess transcriptional expression in this thesis. The *E. coli lacZ* gene encoding β-galactosidase is a standard reporter gene to monitor translational and/or transcriptional activity after being fused with the gene of interest. Thus, the expression of β-galactosidase is completely controlled at the transcriptional or translational level by the gene under study. The cellular activity of this enzyme can be easily quantified. To this end, the chromogenic substrate orthonitrophenyl-ß-D-galactopyranoside (ONPG) is hydrolysed by β-galactosidase to galactose and O-nitrophenol. The yellow color of the later can be recorded by measuring the absorbance at OD_{420nm}, representing proportionally the

transcriptional or translational activity of the fused gene. The absorbance at OD_{420nm} was normalized by the relative amount of cells (OD_{600nm}) and Miller units were calculated as indicated below. Strains carrying the *lacZ* transcriptional fusion of interest were grown in the desired conditions of study. Samples were taken and stored on ice up to 24 hours. To assess β-galactosidase activity, 100 µl of sample was mixed with 900 µl of buffer Z, in 15 ml glass tubes. To each tube, 10 µl of toluene was added and vortexed for 10 seconds in order to lyse the cells. Traces of toluene were eliminated by evaporation. Glass tubes were then incubated in a water bath at 28ºC. Next, 200 µl of ONPG (4 mg/ml) was added to each glass tube, when the sample color turned to yellow (OD_{420nm} 0.3-0.9), reaction was stopped by adding 500 µl of $NaCO_3$ (1 M). Activity determination was performed by duplicate for each of three biological replicates. Calculation of β-galactosidase activity was done by using Miller units calculation.

Miller units = $1000 \times [(OD_{420nm} - 1.75 \times OD_{550nm})] / (T \times V \times OD_{600nm})$

- OD_{420nm} and OD_{550nm} are read from the reaction mixture.
- OD_{600nm} reflects cell density of your original sample
- T = time of the reaction in minutes (from addition of ONPG to addition of $NaCO_3$).
- V = volume of culture used in the assay in ml.

3.4.2 GFP expression determination

GFP fluorescence was measured by using two different readout systems: direct visualization of single colonies on agar plates and/or in a quantitative manner by single cell analysis by flow citometry. Plates were visualized using a fluorescence imager allowing detection of proper fluorescence production for the different GFP-based genetic constructs.

For single cell analysis, cell cultures were grown to the desired analysis conditions. A volume corresponding to 1 ml of a culture with an OD_{600nm} 1.0 was collected (approximately $1x10^9$ bacterial cells). Bacterial suspensions were centrifuged (10000 rpm, 5 min, RT), the cells washed with 300 µl of phosphate-buffered saline (PBS, pH 7.4) and centrifuged again. The washed cells were resuspended in 300 µl of PBS

solution containing 4% formaldehyde. Bacterial cells were fixed by 15 minutes incubation at room temperature in the dark. Finally, the fixed cells were centrifuged (10000 rpm, 5 min, RT) and resuspended in 300 µl of PBS solution. For flow citometry analysis, the bacterial cell suspension was diluted 1/100 in PBS solution. The fluorescence was measured for 20,000 bacterial cells using preset parameters for GFP (excitation wavelength of 484 and emission wavelength of 512). Measurements were done by duplicate for three biological replicates; average of the mean was used to compare GFP expression.

3.5 RNA based methodology

3.5.1 RNA isolation

Three different methodologies were used to isolate RNA as they were the established methodologies in the three labs were RNA based experiments were performed during this thesis. Of note, for the study of small RNA, total RNA must be isolated by hot phenol method or Trizol method.

3.5.1.1 Hot phenol method

Bacterial cell cultures were grown to the desired conditions. For total RNA isolation bacterial cells from 4 units of OD_{600nm} were harvested (10 ml of OD_{600nm} 0.4). To the volume of sample, a 0.2 volume of STOP mix solution was added. The samples were mixed by inversion and stored at -80ºC. For RNA isolation, the bacterial cell samples were thawed on ice. Next, samples were centrifuged (10 min, 4000 rpm, 4ºC). The supernatant was carefully discarded. The cells were resuspended in 600 µl of TE (pH 8.0) containing 0.5 mg/ml lisozyme. The suspension was transferred to a 2 ml microcentrifuge tube. Then, 60 µl of 10 % SDS (w/v) was added; mixed by inversion and incubated at 64ºC for 1-2 minutes. After the incubation, 66 µl of sodium acetate pH 5.2 (1 M) was added and mixed by inversion.

For the hot phenol extraction, 750 µl of Roti-Aqua phenol was added to the samples, mixed by inversion and incubated at 64ºC for 6 minutes, during the incubation tubes were mixed by inversion 6-10 times. Afterwards, the tubes were placed on ice to cool

down and subsequently centrifuged (15 min, 13000 rpm, 4°C). After centrifugation, the top aqueous layer was transferred to a fresh 2 ml microcentrifuge tube, and 750 µl of chloroform was added. The samples were mixed by inversion and centrifuged (12 min, 13000 rpm, 15°C). Next, the upper aqueous layer was transferred into a new 2ml microcentrifuge tube and 1.4 ml of a 30:1 mix of ethanol and sodium acetate was added. The samples were incubated for 2-3 hours or overnight at -20°C. After ethanol precipitation, samples were centrifuged (30 min, 13000 rpm, 4°C). The supernatant was carefully removed. The pellet was washed with 450 µl of 75 % ethanol. The samples were then centrifuged (10 min, 13000 rpm, 4°C). Finally, the ethanol was carefully removed, the pellet air dried and the RNA resuspended in 50-100 µl of water. The resuspension was done by shaking for 5 minutes at 65°C; RNA samples were stored at -80°C.

3.5.1.2 Trizol method

Bacterial cell cultures were grown to the desired conditions. Harvested cells corresponding to an OD_{600nm} of 2.0 were centrifuged and supernatant removed. The pellet was resuspended in 100 µl of lisozyme (3 mg/ml in H_2O), and stored at -20°C. For total RNA isolation 1 ml of Trizol was added to the samples, solution was homogenized by pipetting and incubated 5 minutes at room temperature. Samples were then centrifuged (10 min, 13000 rpm, 4°C). Afterwards, supernatant was transferred to a new microcentrifuge tube. Subsequently, 200 µl of chloroform was added, samples were properly mixed by vortex. Next, samples were centrifuged (10 min, 13000 rpm, 4°C). Again, the supernatant was transferred to a new microcentrifuge tube and 500 µl of isopropanol was added. Tubes were mixed thoroughly and incubated for 10 minutes at room temperature. Samples were then centrifuged (10 min, 13000 rpm, 4°C). The supernatant was removed and the pellet washed with 400 µl of 70 % ice-cold ethanol. The tubes were mixed by inversion and centrifuged (5 min, 13000 rpm, 4°C). Supernatant was again discarded and the pellet air-dried. Finally, to resuspend RNA samples, 50-100 µl of water was added and incubated for 10 minutes with shaking at 65°C. RNA samples were stored at -80°C.

3.5.1.3 Total RNA isolation kit

For each strain, samples from three independent LB cultures, grown at 37ºC to exponential phase (OD_{600nm} of 0.4), were processed. Cells were pelleted and frozen at -80ºC. RNA extraction was performed using Total RNA isolation kit (Promega) following manufacturer instructions. RNA quality and concentration was assessed by an Agilent Technologies Bioanalyzer 2100.

3.5.2 Northern blot assay

Electrophoretic separation of total RNA samples was carried out in Tris-Borate-EDTA (TBE) 8% acrylamide gels containing 8.3 M urea. Samples were prepared by mixing 10 µl of RNA solution and 10 µl of urea dye (2x) loading buffer. Samples were incubated 10 minutes at 65ºC, immediately chilled on ice and loaded for electrophoretic separation at 300 V for 2 hours.

RNAs were transferred to Hybond N+ (GE Healthcare) filter by semy-dry TBE based transference for 1 hour at 50 V. RNAs were subsequently fixed to the filter by UV crosslinking or alternatively by incubating the membrane for 1 hour at 80ºC.

Filters were then hybridized with a 5' labeled DNA probe [ATP-[γP^{32}]]. DNA probes were produced by mixing 20 pmols oligonucleotide (20-25 bp long; 2 µl of 10 pmol/µl solution), 2 µl of 10X PNK buffer, 1 µl of PNK enzyme (T4 polynucleotide kinase), 2 µl of ATP-[γP^{32}] (10 µCi/µl) and 13 µl of nuclease free water.

Reaction was incubated for 1 hour at 37ºC. Unused nucleotides were removed by purifying the oligonucleotides using Sephadex G-25 (GE Healthcare) columns.

The membrane was then pre-hybridized for 1 hour at 42ºC with the hybridization buffer. Next, 5-10 µl of labeled probe was added to the hybridization buffer and the membrane was incubated overnight at 42ºC. Subsequently, hybridization buffer was removed and the membrane incubated with washing buffer for 30 minutes at 38ºC. The washing step was repeated twice with 15 minutes incubation each, respectively. Finally, membrane was exposed overnight in a Fuji screen cassette. Images of

radioactive filters were obtained with FLA-5100 imaging system (Fujifilm), and quantification was performed using Image J software.

3.5.3 RNA-RNA interaction

To assess RNA interaction it was applied a method based on the detection of sRNA-mRNA interactions by Electrophoretic Mobility Shift Assay (EMSA). First, RNA fragments of interest were obtained by *in vitro* RNA transcription. DNA templates for RNA transcription were obtained by PCR. As the RNA polymerase (RNApol) that will be used is T7 RNA polymerase, a putative T7 promoter was added at the 5' end of the PCR generated DNA templates. RNA was produced *in vitro* by using Megascript transcription procedure from Ambion. Briefly, DNA template containing T7 promoter at the 5' end was incubated at 37^{o}C for 4-6 hours in the presence of a T7 RNA polymerase and a nucleotide mix of: UTP, ATP, CTP and GTP. Resulting RNA samples were phenol:chloroform:Isopropanol precipitated in phase lock tubes (QuantaBio). The upper phase was purified in a G-25 column (Healthcare) to remove remaining nucleotides and the eluate was ethanol precipitated. RNA samples were subjected to electrophoresis, ethidium bromide stained and band of interest excised from the gel. RNA was eluted in RNA elution buffer and further ethanol-precipitated. RNA final concentration was measured by using a Nanodrop.

When required, either the sRNA (Spot 42) or the target RNA (*hilD* 3'UTR) was radiolabeled. First, 50 pmol of RNA were dephosphorylated by using 10 units of calf intestinal phosphatase (CIP, 10000 u/ml) for 1 hour at 37^{o}C. Next, 20 pmol of dephosphorylated RNA was labeled at the 5' end by using PNK as described in the northern protocol. Radiolabeled RNA was subjected to electrophoresis and band of interest was excised from the gel. RNA was eluted in RNA elution buffer and ethanol-precipitated. Final RNA concentration was measured by using Nanodrop.

To assess RNA-RNA interactions, the putative interacting RNA were incubated in structure buffer (Ambion). 4 nM of the radiolabeled RNA was incubated with increasing concentrations of the unlabelled RNA. Alternatively the radiolabeled RNA

was incubated with increasing concentrations of Hfq. Samples were incubated at 37ºC for 1 hour and subjected to electrophoresis in a native 4-6 % acrylamide gel. For specific RNA detection, acrylamide gels were dried and exposed as in the northern blot assay.

3.5.4 RNA structure probing

Same RNA samples used in the EMSA experiments were used for structure probing. 20nM of radiolabeled RNA was incubated with increasing concentration of unlabeled RNA in structure buffer (Ambion) for 1 hour at 37ºC. Samples were then treated differentially, either by RNAse T1 or RNAase III. I) RNAse T1 cuts single stranded RNA after G. Consequently, interaction with the non-labeled RNA will be read as protection of cleavage sites, in other words, disappearance of bands. II) RNAse III cuts double stranded RNA. Therefore, interaction with the non-labeled RNA will be read as generation of cleavage sites, in other words, appearance of bands.

For the ladders, 40 nM of denatured radiolabelled RNA was treated by i) alkaline hydrolysis (digestion after each nucleotide) and ii) RNAse T1 which cuts after G (all the molecule is single stranded after denaturation), and therefore cleavage will occur after all G of the sequence.

Samples and ladder were subjected to electrophoresis in 8 % acrylamide 8.3 M UREA containing gel at 40 W (approximately 1500 V) for 2 hours. The gel was dried and exposed as for the EMSA.

3.5.5 qRT-PCR

Quantitative reverse transcription-PCR (qRT-PCR) was performed as follows. Briefly, 0.1 µg of total RNA was reverse transcribed to generate cDNA using the High-capacity cDNA Reverse Transcription kit (Applied Biosystems) following manufacturer's guidelines. Reactions in which reverse transcriptase was not added to the reaction mixture were prepared as controls. Real-time PCR using SYBR green PCR master mix

(Applied Biosystems) was performed on the ABI Prism 7700 sequence detection system (Applied Biosystems). Analysis of amplification was carried out with ABI Prism software package. The relative amount of target cDNA was normalized using the *gapA (GAPDH)* gene as an internal control. Relative quantification of gene transcription was performed using the comparative threshold cycle (CT) method. Oligonucleotides used are listed in Table S3 (Supplementary material).

3.6 Protein based experiments

3.6.1 Total protein extracts

To obtain whole cell extracts, LB cultures were grown up to either exponential (OD_{600nm} of 0.4) or stationary (OD_{600nm} of 2.0) phase. Bacterial cells were recovered by centrifugation and resuspended in Laemmli sample buffer. Normalization of the loading samples was performed based on the culture biomass (OD_{600nm}). Coomassie staining to corroborate loading normalization was performed.

3.6.2 Secreted protein extracts

For extracts of secreted proteins, LB cultures were grown at 37^{o}C up to the desired OD_{600nm}. After cell removal by centrifugation, the culture supernatant was filtered through a 0.22 µm pore size filter. Subsequently, proteins were TCA precipitated (114). Precipitated proteins were resuspended in Laemmli sample buffer and subjected to SDS-PAGE separation. Normalization of the loading samples was performed based on the culture biomass (OD_{600nm}). Secreted proteins were stained with Coomassie blue solution or detected by western blot assay.

3.6.3 Western blot assay

Protein extracts were subjected to SDS-PAGE separation, transfer to PVDF filter and subsequent immunodetection using as primary antibodies either monoclonal Anti-Flag

(Sigma) or polyclonal Anti-SopE (115). As a secondary antibody, commercial polyclonal anti-mouse (Promega) and anti-rabbit (GE Healthcare) antisera conjugated to horseradish peroxidase were used. For detection, ECL™ Prime Western Blotting Detection Reagent (GE Healthcare) was used as a substrate. Chemiluminescence was detected using Chemidoc equipment (Bio-Rad). As a control prior to the immunodetection, all whole cell extract samples were analyzed by SDS-PAGE and Coomassie staining to ensure proper normalization of the amount loaded.

3.6.4 Protein identification

For protein identification, the protein bands from Coomassie stained SDS-PAGE were trypsin digested and analyzed by LC-MSMS by the Proteomic facility from the Scientific Park of Barcelona (PCB).

4. RESULTS

4. Results

4.1 CRP-cAMP dual role on SPI-1 expression.

This thesis is focused on the role that the transcriptional factor CRP-cAMP plays on the regulation of *Salmonella enterica* serovar Typhimurium virulence. As stated in the introduction section, epithelial cells invasion by *Salmonella* is mostly dependent on the expression of the genes present in the *Salmonella* pathogenicity island I (SPI-1). The role of CRP-cAMP as a regulator of the SPI-1 expression, and consequently, the ability of *Salmonella* to invade epithelial cells has been further explored.

4.1.1 CRP-cAMP represses HilA expression during the logarithmic growth phase.

To characterize the role of the metabolic sensor CRP-cAMP in the expression of SPI-1 genes, the transcriptional expression of the main SPI-1 regulator HilA was monitored in wild type and Δ*crp* derivative strains grown in LB at 37^{o}C. The expression pattern was determined in logarithmic cultures (OD_{600nm} 0.4, non-permissive conditions for SPI-1 expression) and after entering the stationary phase (OD_{600nm} 2.0, permissive conditions for SPI-1 expression) (Fig. 18A). Consistent with previous reports, a clear growth phase dependent profile was observed in the wild type strain (20). The *hilA* expression level was 8-fold higher after entry into the stationary phase when compared to log cultures.

Remarkably, a differential effect of the Δ*crp* mutation was detected depending on the growth phase of the culture. In agreement with previous work (59), the Δ*crp* mutation causes a near 2-fold drop in *hilA* transcriptional expression relative to the wild type strain in the stationary phase. Intriguingly, in logarithmic phase of growth, the Δ*crp* mutation causes a 4-fold upregulation of *hilA* expression relative to the wild type. The effect of the Δ*crp* mutation on *hilA* expression was also tested by immunodetection of a tagged HilA protein in cell extracts of wild type and Δ*crp* derivative strains grown in the same conditions as for the transcriptional studies (Fig. 18B). The data corroborate the transcriptional profile described. HilA expression was upregulated in a Δ*crp* mutant in log cultures and downregulated in stationary cultures. These results suggest that CRP is required for the silencing of *hilA* and presumably of the SPI-1 genes under non-

permissive conditions. While its role on the activation of *hilA* expression at permissive conditions its relevant, many regulators activating SPI-1 expression have been described. However, as earlier mentioned, very little is known about molecular mechanism that led to the silencing of SPI-1 genes under non permissive conditions. Therefore, in this thesis we decided to focus on the role of CRP-cAMP in the silencing of SPI-1 genes. Further experimental approaches will be carried out mostly at logarithmic growth phase (non-permissive conditions).

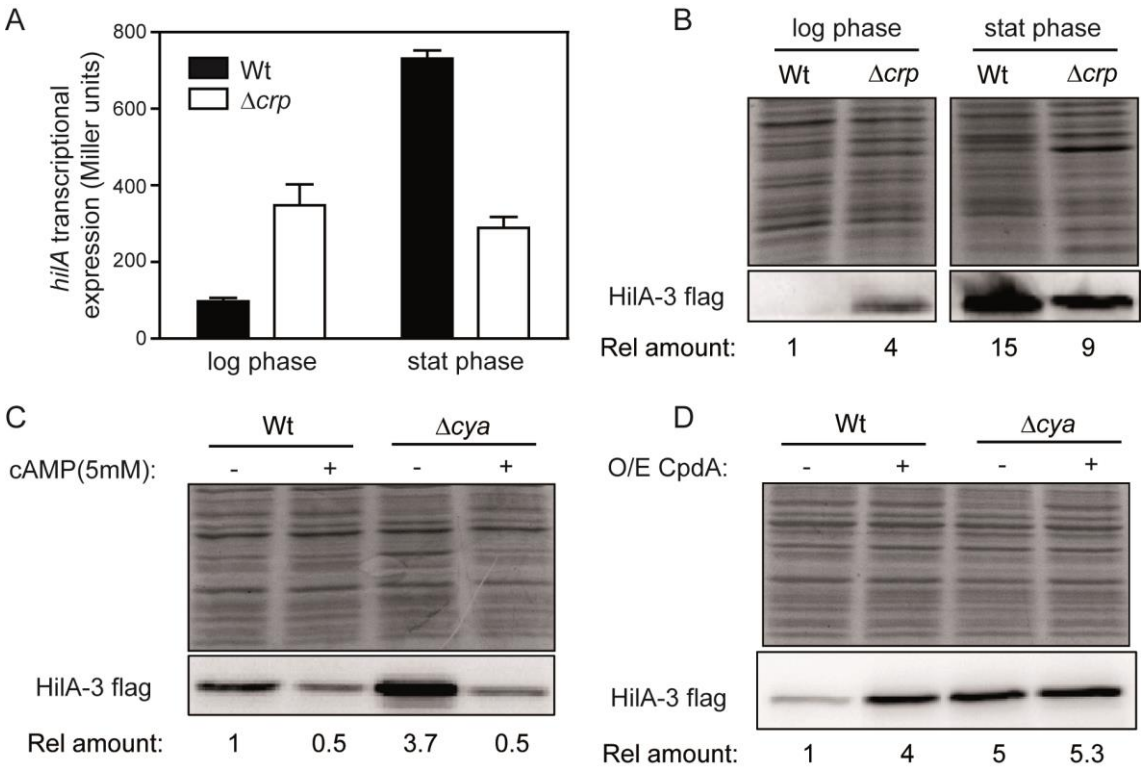

Figure 18. **CRP-cAMP represses *hilA* expression in logarithmic growing cells. A.** Transcriptional expression of *hilA* in a wild type (WT) and a Δ*crp* derivative strain. β-galactosidase activity from a *hilA-lacZ* fusion was assessed in LB cultures grown at 37ºC up to either logarithmic (OD_{600nm} 0.4) or early stationary (OD_{600nm} 2.0) phase of growth. Data from three independent experiments are averaged and the standard error of the mean is presented. **B.** Immunodetection of HilA-3 flag protein was performed in whole cell extracts of WT and Δ*crp* derivative strains, grown as in A. **C.** Immunodetection of HilA-3 flag in whole cell extracts from cultures of the WT and Δ*cya* derivative strains in the absence (-) or presence (+) of cAMP (5mM). Cultures were grown in LB at 37ºC up to an OD_{600nm} of 0.4. **D.** Effect of over expressing the cAMP-phosphodiesterase, CpdA. Immunodetection of HilA-3 flag was performed in cultures of the WT and Δ*cya* derivative strains carrying either pTrc99a (-, control vector) or pCpdA (+, pTrc99a+*cpdA* gene). Cultures were grown in LB supplemented with IPTG (0.1 mM) at 37ºC up to an OD_{600nm} of 0.4. In B, C and D the relative amount of HilA-3 flag is indicated. In each case the reference value was set as one.

As indicated in the introduction section, CRP is active upon binding of the cofactor cAMP (116). Therefore, lack of either CRP or cAMP should presumably have a similar effect on SPI-1 expression. To test that, HilA levels were monitored in a Δ*cya* mutant strain, which is deficient in the synthesis of cAMP (Fig. 18C). As expected, Δ*cya* mutation caused an almost 4-fold increase in the HilA levels in log phase cells. Chemical complementation was performed by monitoring HilA content after addition of cAMP (Fig. 18C). An 8-fold decrease in HilA level was observed when cAMP was added to cultures of the *cya*$^-$ strain. Interestingly, when cAMP was added to a culture of a *cya*$^+$ strain, a 2-fold drop in HilA levels was also observed. These results may indicate that the intracellular cAMP was not saturating all CRP molecules. Consequently, external addition of the cofactor would lead to an increase in the amount of CRP-cAMP complex, causing further repression of HilA expression.

To further corroborate the involvement of cAMP in the control of HilA expression, the intracellular levels of cAMP were lowered by ectopically overexpressing CpdA from *Salmonella*, a putative cAMP phosphodiesterase that degrades cAMP (117). CpdA expression in a *cya*$^+$ strain caused a 4-fold increase in HilA expression. To demonstrate that overexpression of CpdA alters HilA expression through its role in cAMP turnover, control experiments were performed. CpdA was also overexpressed in a *cya*$^-$ strain and no effect was detected on HilA expression (Fig. 18D).

4.1.2 The CRP-cAMP-mediated regulation of HilA causes concomitant attenuation in the expression level of SPI-1-encoded effector proteins

HilA regulates the transcriptional expression of most SPI-1 genes, including those required for the synthesis of a type III secretion system (T3SS) and several effector proteins that are translocated to the host cell during *Salmonella* infection (1,15). Consequently, Δ*hilA* mutant strain is impaired in secretion of SPI-1 effector proteins (24). Comparative studies of the secreted protein profile in extracts of cell free supernatants from cultures of wild type and Δ*hilA* mutant strains grown up to early stationary phase (permissive SPI-1 conditions) were performed to identify protein bands corresponding to SPI-1 effector proteins (Fig. 19).

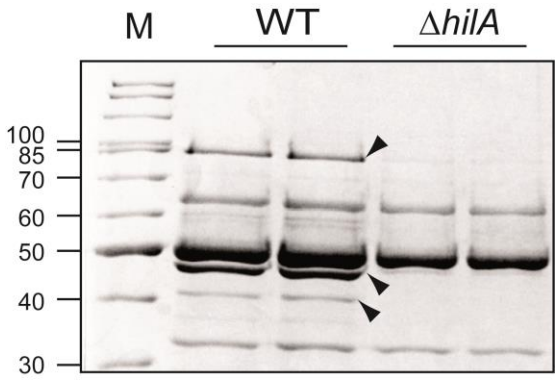

Figure 19. Effect of *hilA* mutation in the expression of SPI-1 encoded secreted effector proteins. Cell-free supernatants from two independent LB cultures of the wild type (WT) and *hilA* strain grown up to early stationary phase (OD_{600nm} 2.0) were TCA precipitated. The resulting extracts were analyzed by SDS-PAGE and Coomassie staining. Arrowheads indicate from the top the bottom the protein bands corresponding to SipA, FliD, a flagellar protein and SipC as identified by LC-MSMS. Size in kDa of some molecular mass marker (M) is indicated.

The major protein bands only detected in extracts of the wild type were identified by liquid chromatography mass spectrometry (LC-MS/MS). The bands indicated in Figure 19, were identified as the SPI-1-encoded proteins SipA and SipC and the flagellar protein FliD. The secretome of wild type and CRP-cAMP-deficient derivative strains was characterized in LB cultures grown to the logarithmic and early stationary phase (Fig. 20A). When comparing extracts of CRP-cAMP-deficient and -proficient cells grown up to the stationary phase, a lower amount of those secreted proteins was observed in CRP-cAMP-deficient cells relative to the wild type, as expected from the *hilA* transcriptional data (Fig. 18A) and from previous reports (118). In extracts from log phase cultures, there was a clear increase in the intensity of protein bands identified as SipA and SipC in the extracts from CRP-cAMP-deficient strains. To further assess the CRP effect on expression of SPI-1 effector proteins, the amount of SipA was monitored using a Flag-tagged construct as indicated in the methods section. Immunodetection of the SipA-3 flag variant confirmed that SipA expression was induced in the log phase of growth in CRP-cAMP-deficient strains relative to the wild type (Fig. 20B). Consistent with previous data, in the stationary phase, lower SipA expression was detected in Δ*crp* mutant strain (Fig. 20B).

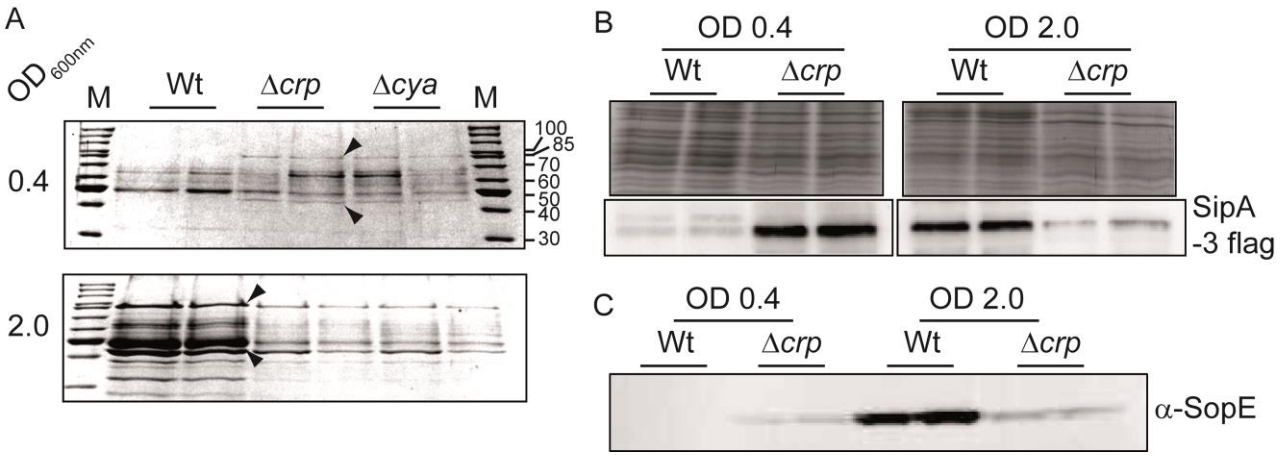

Figure 20. Effect of *crp* and *cya* mutations in the expression of SPI-1 effector proteins. A. Cell-free supernatants from cultures of the wild type (WT), Δ*crp* and Δ*cya* derivative strains grown up to logarithmic (OD_{600nm} 0.4) and stationary (OD_{600nm} 2.0) phase were TCA precipitated. The resulting extracts were analyzed by SDS-PAGE and Coomassie Blue staining. Size in kDa of the molecular mass markers (M) is indicated. Arrowheads indicate the protein bands corresponding to SipA and SipC, secreted effector proteins from *Salmonella*. **B.** Immunodetection of SipA-3 flag was performed in whole cell extracts from cultures of the WT and Δ*crp* derivative strains grown up to logarithmic (OD_{600nm} 0.4) and stationary (OD_{600nm} 2.0) growth phase. Additional panels with Coomassie blue stained SDS-PAGE are presented as loading control of the whole cell extracts. **C.** Immunodetection of SopE protein was performed in same extracts as in A. In all cases extracts from two independent cultures were analyzed.

It has been reported that HilA also regulates the expression of SopE, an effector protein encoded outside the SPI-1 locus, but that is indeed secreted by the SPI-1 encoded T3SS (119). SopE levels were monitored in wild type and Δ*crp* mutant strains (Fig. 20C). As for SipA, in the Δ*crp* mutant strain, SopE protein level increases in the log phase of growth and decreases in the stationary phase as compared to wild type. As expected, the overall expression of the secreted effector proteins is higher in permissive conditions (stationary phase) than in non-permissive conditions (exponential phase).

The fact that CRP-cAMP-mediated repression of *hilA* expression has a concomitant effect on the expression and secretion of SPI-1 effector proteins highlights the biological relevance of CRP-cAMP and its apparent role as a repressor of SPI-1 genes in the control of *Salmonella* virulence under non-permissive conditions.

4.1.3 CRP-cAMP regulation of SPI-1 during logarithmic growth phase occurs upstream of HilA by repressing *hilD*, *hilC* and *rtsA* expression

Three AraC-like transcriptional activators, HilD, HilC and RtsA, are directly involved in *hilA* transcriptional regulation. As mentioned in the introduction section, HilD, HilC and RtsA is a regulatory triad located at the top of SPI-1 regulation cascade. These activators form a positive feed forward loop, as each of them can activate *hilA* expression and there is crossactivation among them (120). To determine whether CRP-cAMP-mediated silencing of SPI-1 has as a main target HilA or by contrast acts upstream of *hilA,* the transcriptional expression of *hilD*, *hilC* and *rtsA* was monitored in a Δ*crp* mutant strain. RNA was extracted from exponentially growing cultures (OD_{600nm} 0.4) of both wild type and Δ*crp* derivative strains and the relative amounts of mRNA of all three AraC-like regulators were determined by qPCR. As shown in Fig. 21A, *hilD*, *hilC* and *rtsA* transcripts levels were upregulated in the Δ*crp* derivative strain compared to wild type, indicating that CRP-cAMP affects the regulatory cascade that control SPI-1 expression upstream from HilA.

HilD, HilC and RtsA can stimulate its own expression as well as the expression of the other two. In order to elucidate the target of the CRP-mediated regulation of SPI-1, mutants for the three regulators were generated in both *crp*+ and Δ*crp* genetic backgrounds and the expression of *hilA* monitored. According to previous studies, RtsA, HilC and HilD are required to different extents for the induction of *hilA* transcription (120). As seen in Fig. 21B, induction of *hilA* expression in the Δ*crp* derivative strains was in the *rtsA*$^-$ similar to wild type, whereas a relevant drop in the derepression caused by Δ*crp* mutation was observed when either HilC or HilD were absent in a Δ*crp* genetic background.

These results indicate that these two regulators are required for the reported CRP-mediated regulation of *hilA.* To further assess the involvement of *hilD* and *hilC* in the regulation of *hilA*.

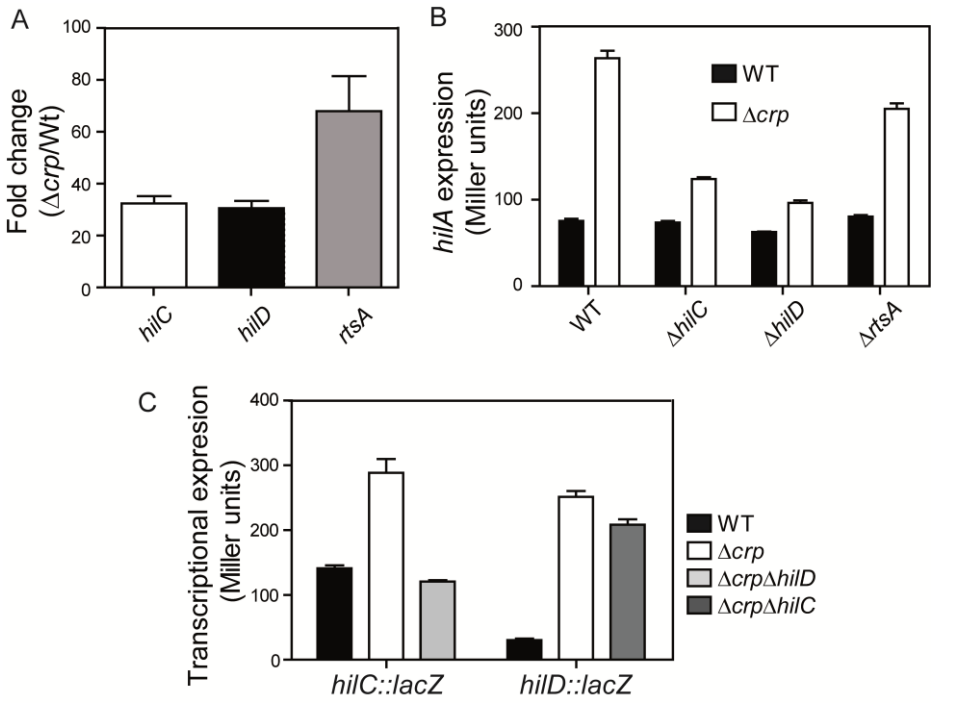

Figure 21. Effect of *crp* mutation in the expression of *hilA* activators. **A.** Relative mRNA quantification by qPCR for *hilD*, *hilC* and *rtsA* in a Δ*crp* derivative strain compared to the wild type (WT). Results are expressed as fold change between WT and Δ*crp* derivative strains. Detection of *gapA* (GAPDH) was used as an internal control (see Material and Methods). RNA samples were extracted from cultures of the WT and the Δ*crp* derivative strains grown in LB at 37ºC up to an OD_{600nm} of 0.4. qPCR quantification was performed for 3 independent cultures. **B.** *hilA* transcriptional expression was monitored in *crp*$^+$ and *crp*$^-$ strains in different genetic backgrounds: WT, Δ*hilC*, Δ*hilD* and Δ*rtsA*. **C.** *hilC* and *hilD* transcriptional expression was monitored. For *hilC*, WT, Δ*crp* and Δ*crp*Δ*hilD* derivative strains carrying a *hilC-lacZ*, chromosomal fusion were used. For *hilD*, WT, Δ*crp* and Δ*crp*Δ*hilC* carrying the *hilD*1235-*lacZ* were used. For both B and C, strains were grown in LB at 37ºC up to an OD_{600nm} of 0.4. β-galactosidase activity was determined for three independent cultures, average and standard error of the mean is presented.

The expression of *hilC* and *hilD* was further studied using transcriptional *lacZ* fusions. As expected, both *hilC* and *hilD* were upregulated in a Δ*crp* mutant background (Fig. 21C). Remarkably, HilD seems to be required for the upregulation of *hilC* observed in the Δ*crp* derivative strain whereas *hilD* induction does not require HilC. Taken together, these results suggest that HilD is the pivotal target for the CRP-cAMP-mediated regulation of SPI-1 expression. Although it cannot be ruled out that CRP-cAMP does also act at the level of *hilA* transcription, the abolishment of the CRP-mediated regulation on *hilA* transcription in absence of *hilD* suggest that the regulation of CRP-cAMP on *hilD* plays a major role in the reported regulation of SPI-1 genes. The role of HilD as a hub for the regulatory pathways that modulate virulence in *Salmonella* has previously been suggested (121). Regulatory mechanisms acting at all levels of *hilD*

expression have been described, including transcriptional, post-transcriptional, translational and post-translational mechanisms (27,122–125) (see introduction).

4.1.4 CRP-cAMP-mediated transcriptional regulation of HilD requires the 3'UTR of the *hilD* transcript

As mentioned in the introduction it has been reported that *hilD* carries a 310 bp long 3'UTR that appears to be important for the regulation of *hilD* mRNA turnover (123). The 3'UTR confers to the *hilD* mRNA a greater susceptibility for degradation. Accordingly, *hilD* mRNA accumulates in the absence of the 3'UTR, which in turn causes the concomitant induction of the SPI-1 genes.

The effect of CRP-cAMP on *hilD* transcription described in the previous section (Fig. 21C) was elucidated using a *hilD-lacZ* fusion at position +1235, containing the *hilD* coding sequence and the 3'UTR. To determine whether the *hilD* 3'UTR is important in CRP-mediated regulation, a proximal fusion at position +76 was constructed (Fig. 22A). Remarkably, when using this proximal fusion no differential activity was detected between the wild type and the Δ*crp* derivative strains. This indicates either that CRP-cAMP does not regulate *hilD* expression at the transcription initiation level or that the HilD protein is required for the induction of transcription initiation in a *crp*-deficient strain. To discriminate between these two possibilities, a *hilD-lacZ* fusion at position +965 was constructed, carrying the whole *hilD* coding sequence but lacking the *hilD* 3'UTR. As shown in Fig. 22A, no remarkable derepression in a Δ*crp* mutant was observed in the absence of the 3'UTR regardless of the absence (*hilD*76-*lacZ*, *hilD*$^+$, 3'UTR$^+$) or presence (*hilD*965-*lacZ*, *hilD*$^+$, 3'UTR$^-$) of HilD protein, whereas a 6-fold induction in the Δ*crp* mutant was detected when the 3'UTR was present (*hilD*1235-*lacZ*, *hilD*$^+$, 3'UTR$^+$). Although it cannot be ruled out that CRP might contribute to the regulation of *hilD* expression at the level of transcription initiation, the different behavior of *hilD*965-*lacZ* and *hilD*1235-*lacZ* clearly indicates that the 3'UTR is crucial for the CRP regulation of *hilD* and suggests that CRP-cAMP effect on *hilD* regulation takes place mainly at the post-transcriptional level. Remarkably, when overall transcriptional activity of the *lacZ* fusions is compared, it can be observed that the

fusions carrying the HilD gene (*hilD*$^{+}$, *hilD*965-*lacZ*, 5193.01 +/- 334.63 Miller units) show higher transcriptional expression than the fusion lacking the complete *hilD* ORF (*hilD*$^{-}$, *hilD*76-*lacZ*, 63.61 +/- 2.03 Miller units). These results are in agreement with the described role of *hilD* as a positive regulator of its own expression (24). Moreover, when comparison is based on the presence of the *hilD* 3'UTR, it is observed that in absence of the *hilD* 3'UTR (3'UTR^{-}, *hilD*965-*lacZ*, 5193.01 +/- 334.63 Miller units) the transcriptional expression is higher than in the presence of the *hilD* 3'UTR is present (3'UTR^{+}, *hilD*1235-*lacZ*, 35.12 +/- 2.88 Miller units). These results support the role attributed to *hilD* 3'UTR as a negative motif for *hilD* expression (31).

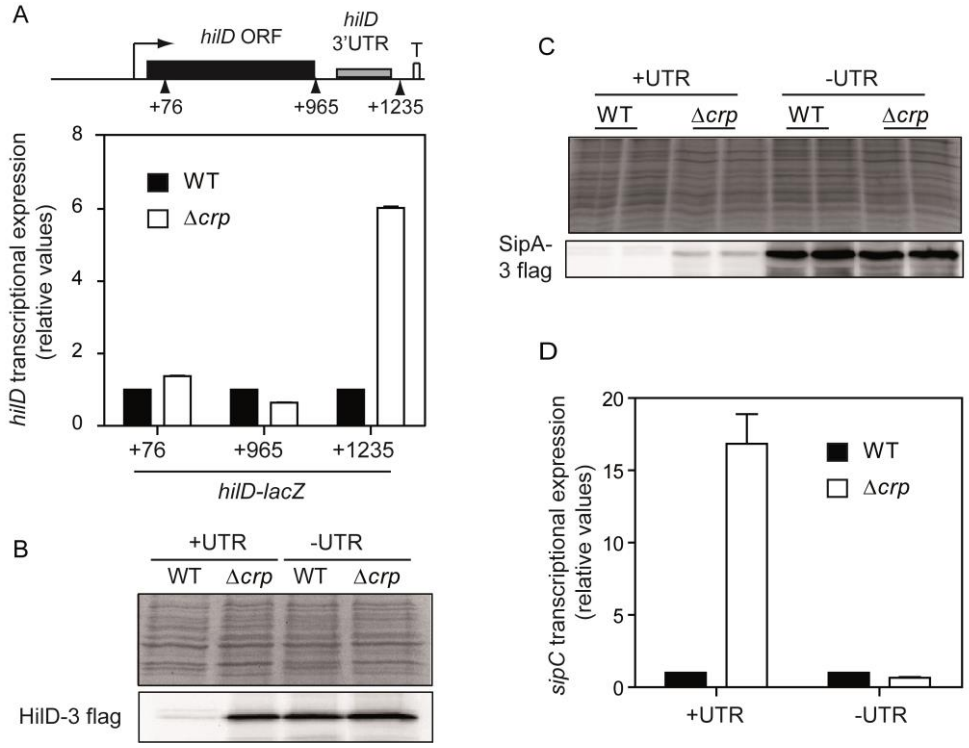

Figure 22. CRP-cAMP-mediated repression of HilD at logarithmic phase of growth requires the 3'UTR of the *hilD* gene. A. *hilD* transcriptional expression was monitored using 3 different *hilD*-lacZ reporter fusions: at position +76 (within the *hilD* ORF), at position +965 (including the whole *hilD* ORF) and at position +1235 (including the *hilD* ORF and the *hilD* 3'UTR). Transcriptional studies were performed in a wild type (WT) and a Δ*crp* derivative strain. The transcriptional expression is shown in relative values. In each case the reference (WT) value was set as one. Miller units in WT strains, *hilD*76-*lacZ* 63.61 +/- 2.03; *hilD*965-*lacZ* 5193.01 +/- 334.63; *hilD*1235-*lacZ* 35.12+/- 2.88. **B.** Immunodetection of HilD-3 flag. Two different genetic constructs were used: one containing the *hilD* 3'UTR (+UTR) and the other lacking that region (-UTR). Immunodetection was assessed in whole cell extracts from cultures of wild type (WT) and Δ*crp* derivative strains. **C.** Immunodetection of the SPI-1 encoded SipA-3 flag protein. Immunodetection was performed in whole cell extracts from cultures of WT and Δ*crp* derivative strains, either in the presence (+UTR) or the absence (-UTR) of the *hilD* 3'UTR. In B and C, additional panels with Coomassie blue stained SDS-PAGE of the whole cell extracts are presented as loading control. **D.** *sipC* transcriptional expression was performed in cultures of a WT and Δ*crp* derivatives carrying a *hilD* native gene or a derivative *hilD* lacking the 3'UTR. Miller units in WT strains, UTR^{+} 82.36 +/- 12.66; UTR^{-} 11967.07 +/- 507.01. In A and D β-galactosidase activity was determined for three independent cultures, average and

standard error of the mean is presented. In all cases, cultures were grown in LB at 37ºC up to an OD_{600nm} of 0.4.

The relevance of the 3'UTR in the CRP-mediated regulation of HilD expression was clearly corroborated when HilD levels were determined in both the wild type and Δ*crp* derivative strains in presence and absence of the 3'UTR (Fig. 22B). Genetic constructs allowing immunodetection of a HilD-3 flag variant expressed from *hilD* mRNA with or without the 3'UTR were obtained (see Methods for details). Consistent with the described role of the 3'UTR in *hilD* mRNA turnover, in the wild type strain, the amount of HilD produced from the *hilD* variant lacking the 3'UTR was higher than in the variant carrying the 3'UTR. Derepression by the Δ*crp* mutation was only observed when the 3'UTR is present; further supporting the hypothesis that the *hilD* 3'UTR is the target sequence of the CRP-mediated regulation. To estimate levels of functional HilD in the cell, and to confirm the pivotal role of the *hilD* 3'UTR in the CRP-mediated control of SPI-1 related genes, the expression of the SPI-1 effectors SipA and SipC was monitored. SipA expression was determined by immunodetection and *sipC* transcriptional expression was assessed using a *lacZ* fusion. Consistent with previous results, in cells carrying a native *hilD* gene, SipA and *sipC* were upregulated in a Δ*crp* mutant background, whereas, in strains lacking the *hilD* 3'UTR, CRP-mediated regulation was abolished (Fig. 22C-D).

Our data also show that, according to the role attributed to the 3'UTR in the expression of *hilD* mRNA (123), there was an increase in the levels of HilD-3 flag, SipA-3 flag and *sipC-lacZ* when the 3'UTR was removed as compared to the parental strains carrying the native *hilD* mRNA containing the 3'UTR. The *sipC-lacZ* activity in a strain carrying a *hilD* variant lacking the *hilD* 3'UTR shows an overall expression of 11,967.07 +/- 507.01 Miller units, while in strains carrying the *hilD* native gene it shows an overall activity of 82.36 +/- 12.66.

4.2 A role for small RNA in the CRP-mediated regulation of *hilD*

The *hilD* 3'UTR is 310 nt long, carries a *rho* independent terminator, as mentioned it plays a major role on the overall expression of HilD. Therefore, the *hilD* 3'UTR can be defined as a negative regulatory motif for *hilD* expression. Although Casadesus lab showed that the effect of *hilD* 3'UTR on *hilD* expression depends on the presence of a functional RNAase E and the presence of the sRNA chaperone Hfq, the molecular mechanism by which the 3'UTR modulates *hilD* expression remains elusive, Furthermore, partial deletions of the *hilD* 3'UTR performed by Casadesus lab indicated that the presence of the first 100 nt of the *hilD* 3'UTR are sufficient to have a negative effect on *hilD* expression (31).

Additionally, in our lab we have shown that the transcription elongation factors GreA and GreB affect *hilD* transcription at stationary phase by a mechanism derived from the anti-backtracking activity of the Gre factors (27). Shortly, during transcription the RNA polymerase might get paused and backtracking of the RNA polymerase (RNApol) occurs (126). The Gre factors through interaction with the secondary channel of the RNApol allow transcription to continue by rescuing transcriptional pause events (127). Remarkably, our genetic analysis suggest that the putative transcriptional pause liberated by the Gre factors during *hilD* transcription occurs at the *hilD* 3'UTR. In fact, it seems to occur within the first 100 nt of the *hilD* 3'UTR (27).

How CRP-cAMP modulates *hilD* expression through its 3'UTR will be explored in this section. Having in consideration that CRP-cAMP is defined as a transcriptional factor, it is intriguing that the CRP-mediated regulation of HilD does not occur at the transcriptional initiation level, but instead occurs at the post-transcriptional level in a 3'UTR-dependent manner. These data suggest that CRP-cAMP modulates *hilD* expression by an indirect mechanism. Since the small RNA binding protein Hfq is required for *hilD* expression and that requirement vanishes in the absence of the 3'UTR (31,10), it was suggested that small RNAs may participate in the *hilD* regulation mediated by the 3'UTR. Accordingly, whether any small RNA might be involved in the CRP-mediated repression of *hilD* was explored.

First, the possible requirement of Hfq in the CRP-mediated regulation of *hilD* was studied by monitoring i) *hilD* transcriptional expression (*hilD*1235-*lacZ*, 3'UTR$^+$, HilD$^+$) and ii) HilD functional levels in the cell by measuring *sipC* transcriptional expression. Expression from both the *hilD*1235-*lacZ* fusion containing the 3'UTR (Fig. 23A) and the *sipC-lacZ* fusion (Fig. 23B) was determined in wild type and Δ*crp* mutant in either Hfq proficient or deficient strains. The results indicate that Hfq deficiency causes a downregulation in *hilD* transcriptional expression and that the upregulation observed in the Δ*crp* mutant drops in the absence of Hfq. Consistently, the results on *sipC* transcriptional expression indicate that the increase in the HilD amount in the cell in the Δ*crp* mutant strictly requires the presence of Hfq. Altogether, these results suggest a possible involvement of sRNA in the CRP-mediated silencing of HilD expression during logarithmic growth phase.

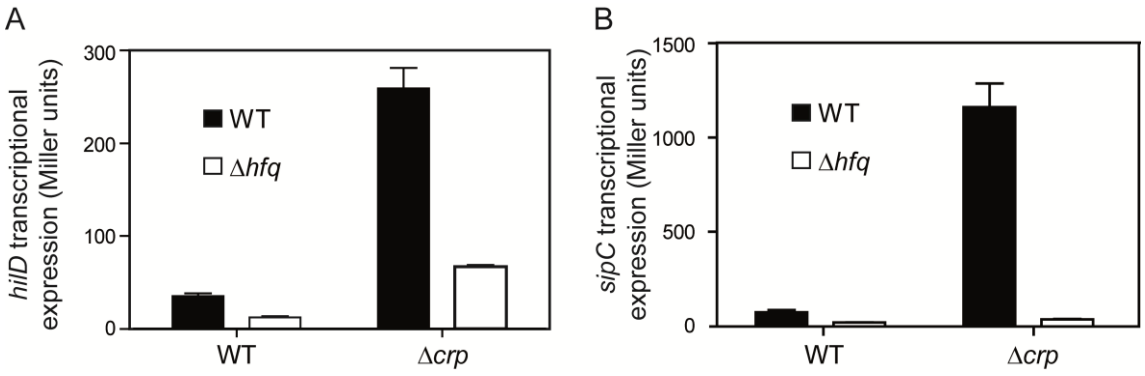

Figure 23. CRP-cAMP-mediated repression of *hilD* requires the presence of Hfq. *hilD* (*hilD*1235-*lacZ*) (**A**) and *sipC* (**B**) transcriptional expression was monitored in wild type (WT) and Δ*crp* derivative strains in either an *hfq*$^+$ or *hfq*$^-$ genetic background. Cultures were grown in LB at 37ºC up to an OD_{600nm} of 0.4. β-galactosidase activity was determined for three independent cultures, average and standard error of the mean is presented.

Several small RNAs have been identified in *Salmonella enterica* serovar Typhimurium, among them Spot 42 sRNA, encoded by the *spf* locus. In *E. coli*, Spot 42 is controlled by catabolite repression in a CRP-cAMP dependent manner (129). Spot 42 plays a role in the multi-output feed-forward loop in CRP-mediated catabolite repression by modulating the expression of several genes from the CRP regulon at the post-transcriptional level (see introduction) (130). To determine whether Spot 42 is under CRP-cAMP control in *Salmonella,* total RNA was isolated from logarithmic cultures and the amount of Spot 42 in the cell was determined by northern blot assays. Detection of

5S RNA was used as loading control. The data shown in Fig. 7 clearly indicate that CRP-cAMP strongly represses the expression of Spot 42 in *Salmonella*. A 7-fold upregulation of Spot 42 sRNA was detected in the CRP-deficient strain (Fig. 24A). As a control, RNA sample from Δ*spf* strain was analyzed. As expected no Spot 42 RNA was detected. Transcriptional studies were also performed using a chromosomal *spf-lacZ* fusion. Accordingly with the northern data, upregulation of *spf-lacZ* fusion was detected in the Δ*crp* mutant strain in logarithmic growth phase (Fig. 24B).

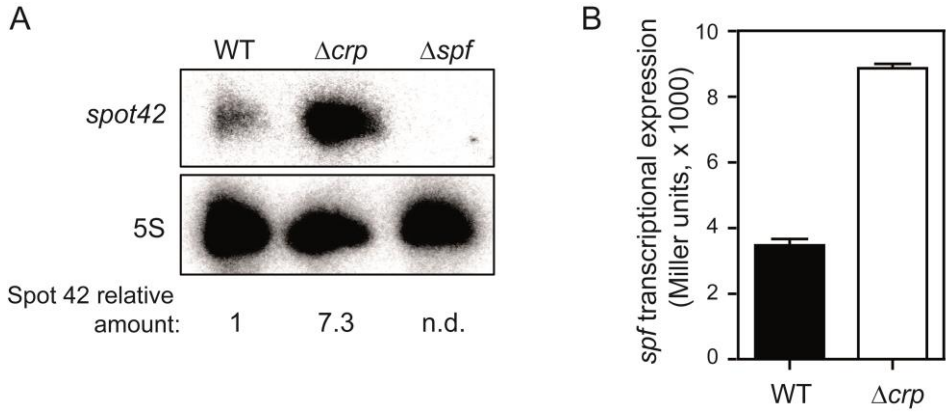

Figure 24. Spot 42 is under the control of CRP-cAMP in *Salmonella.* A. Northern blot analysis for Spot 42 sRNA using 5S RNA as loading control. Total RNA samples were extracted from cultures of the strains wild type (WT), Δ*crp* and Δ*spf* grown in LB at 37ºC up to an OD_{600nm} of 0.4. For quantification, ratio Spot 42/5S was normalized to 1 in the WT strain. In the Δ*crp* derivative strain a 7.3-fold induction of Spot 42 was quantified as compared to WT. n.d (no detected) **B.** *spf* transcriptional expression was assessed in WT and Δ*crp* derivative strain carrying a *spf-lacZ* chromosomal fusion, cultures were grown as in A. β-galactosidase activity was determined for three independent cultures, average and standard error of the mean is presented.

To establish whether Spot 42 is involved in the CRP-cAMP-mediated regulation of *hilD*, expression studies were performed in strains either deficient in Spot 42 or overexpressing the sRNA. In a Δ*crp* derivative strain, there was a partial but relevant drop in the upregulation of *hilD* in the Spot 42-deficient strain (Δ*spf*), suggesting that Spot 42 is involved in the CRP-mediated repression of *hilD* expression (Fig. 25A). Moreover, ectopical overexpresion of Spot 42 in a Δ*spf* background causes a stimulatory effect on *hilD* expression (3-fold increase). Remarkably, the induction of *hilD* expression upon overexpression of Spot 42 was only detected in the presence of the *hilD* 3'UTR. Overexpression of Spot 42 induced transcriptional expression from *hilD*1235-*lacZ* ($HilD^+$, $3'UTR^+$) but had no positive effect on *hilD*965-*lacZ* ($HilD^+$, $3'UTR^-$)

expression, strongly indicating that 3'UTR is the target of Spot 42-mediated regulation of *hilD* (Fig. 25B).

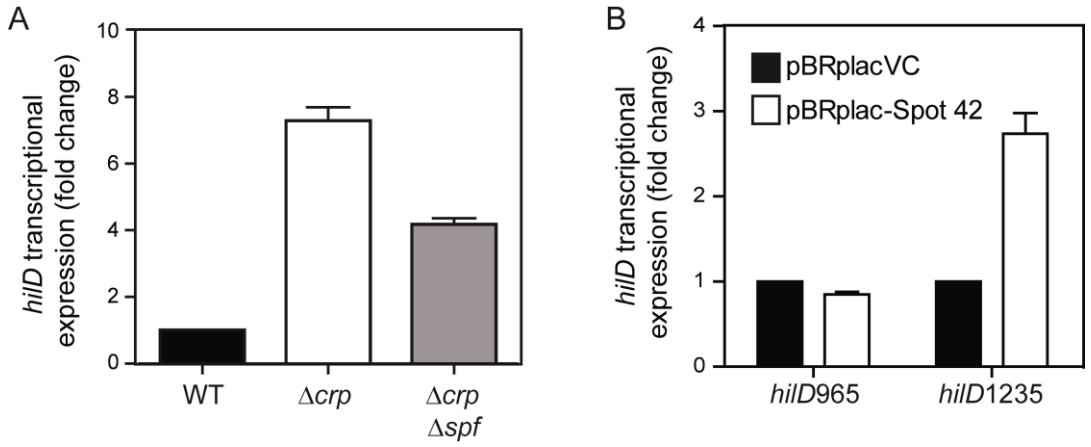

Figure 25. Spot 42 is involved in *hilD* regulation. A. *hilD* transcriptional expression was assessed in wild type (WT), Δ*crp* and Δ*crp*Δ*spf* derivative strains carrying a *hilD*1235-*lacZ* chromosomal fusion (+UTR). The transcriptional expression is presented in relative values, the reference value (WT) was set as one. Miller units WT *hilD*1235-*lacZ*, 32.45+/-1.03. **B.** *hilD* transcriptional expression was assessed in WT strain upon overexpression of Spot 42 in WT strains carrying either the *hilD*965-*lacZ* (lacking the 3'UTR) or the *hilD*1235-*lacZ* (containing the 3'UTR). The transcriptional expression is shown in relative values; the reference value (strain containing the pBRplacVC) was set as one. Miller units for *hilD*965-*lacZ* 4381.58 +/- 490.32, for *hilD*1235-*lacZ* 33.61 +/- 1.65. In all cases, cultures were grown in LB at 37°C up to an OD_{600nm} of 0.4. β-galactosidase activity was determined for three independent cultures, average and standard error of the mean is presented.

The stimulatory effect of Spot 42 on *hilD* expression was corroborated by studies at the protein level. Immunodetection of HilD 3-Flag shows a 2.5 fold increase when Spot 42 was ectopically overexpressed (Fig. 26A).

In order to show whether the described regulation plays a physiological relevant role by affecting the levels of functional HilD in the cell and the subsequent control of SPI-1 gene expression, the transcriptional activity of *sipC* was monitored. Transcription of *sipC-lacZ* was monitored in strains carrying the native *hilD* (3'UTR$^+$) and in strains with a *hilD* variant where the 3'UTR was deleted. In agreement with previous results, a 5-fold induction of *sipC-lacZ* was detected upon overexpression of Spot 42 in the +UTR background whereas no induction was observed when Spot 42 was overexpressed in a background lacking the *hilD* 3'UTR (3'UTR$^-$). These results clearly indicate that the effect of Spot 42 on *hilD* expression requires the *hilD* 3'UTR and the derepression of

hilD expression is sufficient to affect downstream SPI-1 effector gene expression (Fig. 26B).

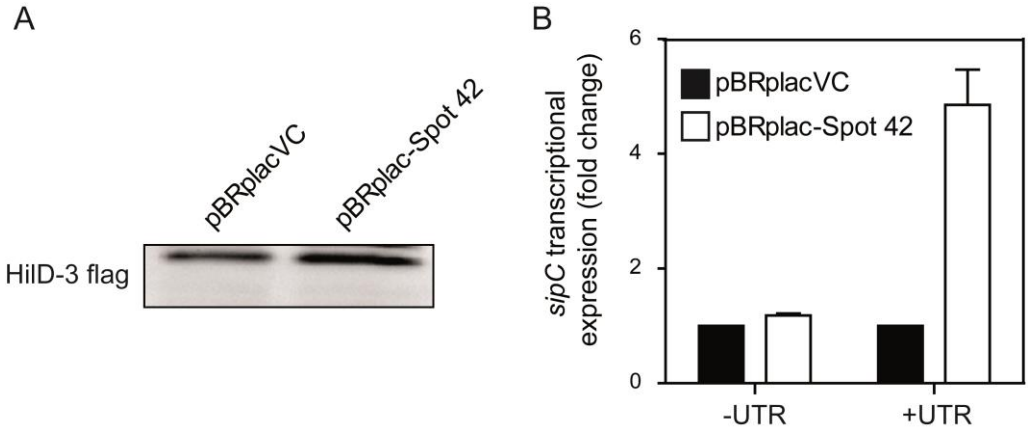

Figure 26. HilD functional levels upon Spot 42 overexpression. A. Immunodetection of HilD-3 flag. Immunodetection was assessed in whole cell extracts from cultures of wild type (WT) strain carrying the pBRplacVC (control vector) or upon overexpression of the sRNA Spot 42 (pBRplac-Spot 42). **B.** *sipC* transcriptional expression was assessed upon overexpression of Spot 42 in strains carrying a *hilD* gene either in presence (+UTR) or absence (-UTR) of the *hilD* 3'UTR. The transcriptional expression is presented in relative values. In each case the reference value (wild type, pBRplac VC) was set as one. Miller units in presence of (+UTR) 82.36 +/- 12.66, and in absence of (-UTR) 11967.07 +/- 507.01. Cultures were grown in LB at 37°C up to an OD_{600nm} of 0.4. β-galactosidase activity was determined for three independent cultures, average and standard error of the mean is presented.

4.2.1 Use of *in vivo* reporters to characterize the effect of Spot 42 on *hilD* 3'UTR.

The *hilD* 3'UTR is a regulatory motif that plays a negative role regardless of its genomic location since its fusion downstream of a plasmidic encoded *gfp* leads to a downregulation on *gfp* expression (31). As Spot 42 seems to act directly on the *hilD* 3'UTR, experiments were performed to show whether Spot 42 can act on an heterologous gene carrying the *hilD* 3'UTR. So that, the *hilD* 3'UTR was fused downstream of a *gfp* gene by overlapping PCR and further cloned into pXG1 vector as described in the Methods section. Cells carrying pXG1 *gfp-hilD* 3'UTR in the presence of either pBRplac-Spot 42 or pBRplacVC, were grown up to an OD_{600nm} 0.4 and GFP expression was numbered by fluorescence quantification by flow citometry. Upon overexpression of Spot 42, the fluorescence derived from the construct of *gfp*-3'UTR was induced near 1.8 fold compared to the strain carrying the control vector (pBRplac VC) indicating increased stability of *hilD* 3'UTR in the presence of Spot 42 (Fig. 27A).

The effect of Spot 42 on *hilD* 3'UTR was further assessed by monitoring the accumulation of *hilD* 3'UTR *solo* fragments in the cell upon overexpression of Spot 42.

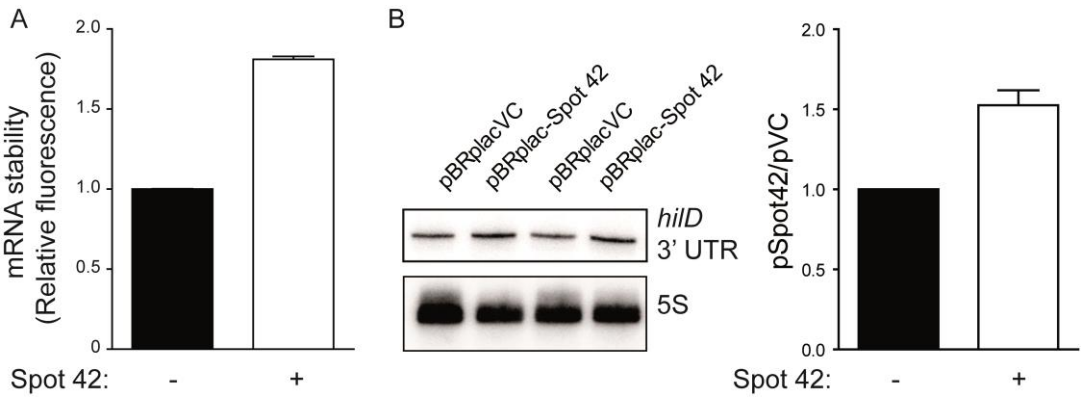

Figure 27. Spot 42-mediated derepression of *hilD* 3'UTR motif. A. Gfp fluorescence assessment by flow citometry of GFP-*hilD* 3'UTR upon overexpression of the sRNA Spot 42. **B.** Northern blot detection of *hilD* 3'UTR solo fragments in the cell expressed constitutively from pXG1 vector upon overexpression of the sRNA Spot 42. In all cases cultures of Δ*spf* strains carrying the VC (Spot 42 -) or upon overexpression of the sRNA Spot 42 (Spot 42 +) grown in LB at 37°C up to an OD_{600nm} of 0.4.

Briefly, the *hilD* 3'UTR region was cloned into a low copy plasmid (pXG1 backbone) and expressed constitutively in the cell in the presence of either pBRplac-Spot 42 (overexpression of Spot 42) or pBRplacVC (not overexpression). Cultures were grown up to exponential phase (OD_{600nm} 0.4) and subsequently total RNA was extracted. The amount of *hilD* 3'UTR was directly detected by northern blot as read out of the stability of *hilD* 3'UTR RNA in the cell. As shown in Figure 27, the level of *hilD* 3'UTR *solo* fragments in the cell is slightly higher in the strain overexpressing Spot 42 than in the strain carrying the control vector. We conclude that the turnover of the *hilD* 3'UTR seems to be lower upon overexpression of Spot 42, causing accumulation of *hilD* 3'UTR *solo* fragments (Fig. 27B).

It might be argued that this fold activation is modest. However, it has to be underlined, that small differences in the stabilization of *hilD* 3'UTR by Spot 42 might led to larger effects downstream. HilD has an important positive autoregulatory feedforward loop. Small increase in the HilD protein production is amplified by this loop. As HilD is at the

top of SPI-1 regulation hierarchy, discrete differences in *hilD* expression led to larger differences at the level of SPI-1 effector proteins.

4.2.2 Spot 42 mediated derepression of *hilD* requires Hfq and RNAse E

As mentioned previously, the *hilD* 3'UTR is a motif that seem to play an overall negative effect on *hilD* expression. In addition to Hfq, RNAse E seems to play an important role in the mechanism by which the *hilD* 3'UTR regulates *hilD* expression. In absence of a functional RNAse E, the levels of *hilD* mRNA are induced in a strain carrying the *hilD* native gene whereas no further accumulation of *hilD* mRNA is observed when the *hilD* 3'UTR has been deleted in an RNase E deficient background (31,131). All these data suggest that the negative effect of *hilD* 3'UTR on the overall *hilD* expression is mediated by the RNAse E activity (31).

The involvement of RNAse E and Hfq in the Spot 42 mediated derepression of *hilD* expression was assessed. To do so, the transcriptional expression of *hilD*1235-*lacZ* (HilD$^+$, 3'UTR$^+$) was monitored upon overexpression of Spot 42 in a genetic background lacking the sRNA chaperone Hfq (Δ*spf Δhfq*) or a functional RNAse E (Δ*spf rne537*). As *rne* is essential, a C-terminus RNAse E mutant was used (132).

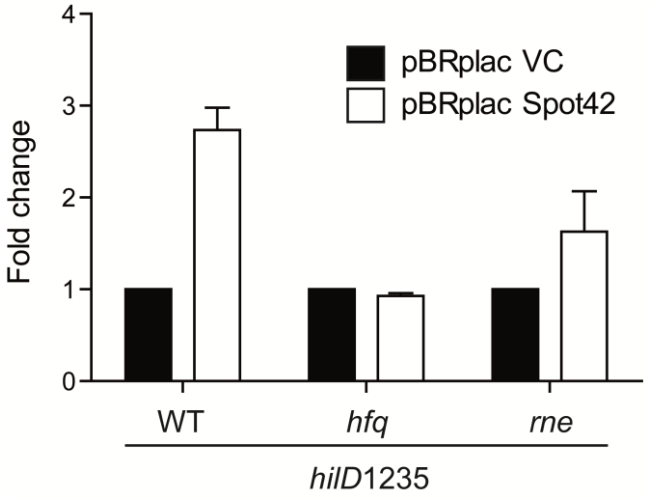

Figure 28. Spot 42-mediated derepression of *hilD* requires Hfq and RNAse E. *hilD* transcriptional expression was assessed in wild type (WT), *hfq* and *rne*537 derivative strains carrying the *hilD*1235-*lacZ* (HilD$^+$, 3'UTR$^+$) chromosomal fusion. The transcriptional expression is presented in relative values, in each of the genetic backgrounds (WT, *hfq* ,*rne*537) the expression of the pBRplacVC carrying strain was set as one. β-galactosidase activity was determined for three independent cultures, average and standard error of the mean is presented.

Ectopic expression of Spot 42, as shown previously, induced 3-fold the *hilD*1235-*lacZ* (HilD$^+$, 3'UTR$^+$) transcriptional expression in a wild type background. Of note, Spot 42 ectopic expression had no positive effect on *hilD*1235-*lacZ* (HilD$^+$, 3'UTR$^+$) transcriptional expression when overexpressed in a background lacking a functional RNAse E or the sRNA chaperone Hfq (Fig. 28).

These results led us to conclude that Spot 42 sRNA can directly or indirectly activates *hilD* expression in a manner that requires i) the sRNA chaperone Hfq, ii) the endonuclease RNAse E and iii) the presence of the *hilD* 3'UTR.

Recently, the global RNA recognition pattern of Hfq in *Salmonella* has been revealed by UV crosslinking *in vivo* (94). Shortly, cells from a strain expressing Flag-tagged Hfq were *in vivo* crosslinked, Hfq 3-flag was then immunoprecipitated and Hfq bound RNA was analyzed by RNA deep sequencing (CLIP-seq). Remarkably, Holmqvist et al. 2016 detect two interactions sites of Hfq in both the *hilD* 3'UTR and the sRNA Spot 42. Supporting our data on the requirement of Hfq in the Spot 42 mediated regulation of *hilD* through the *hilD* 3'UTR.

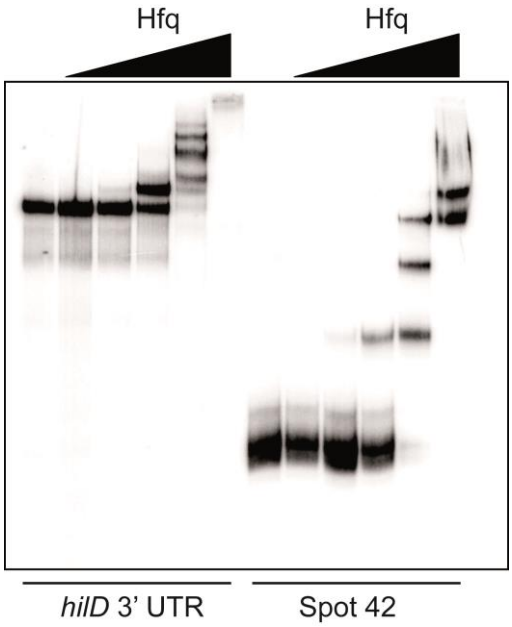

Figure 29. Hfq binding assessment to *hilD* 3'UTR and Spot 42 by EMSA. *In vitro* transcribed RNA was radiolabelled. 4 nM of the radiolabelled RNA was incubated with increasing concentration of purified Hfq (0, 1.3 nM, 4 nM, 13 nM, 40 nM, 130 nM) and subjected to electrophoresis in a native gel. Bandshift was observed upon drying and exposure of the gel.

Electrophoretic mobility shift assays were performed in order to corroborate the Hfq binding detected by CLIP-seq. *In vitro* transcribed RNA for Spot 42 and *hilD* 3'UTR was generated and radiolabelled at the 5'end. The generated RNA was incubated with increasing concentrations of Hfq in a binding buffer and subjected to electrophoresis in a native gel. As shown in Figure 29, Hfq binds to the Spot 42 sRNA and to the *hilD* 3'UTR. In fact, the multiple band shift supports the reported data on multiple binding sites for Hfq in both Spot 42 and *hilD* 3'UTR.

4.3 Spot 42- *hilD* 3'UTR interaction

4.3.1 The unstructured region III of Spot 42 is required for the HilD-mediated regulation of SPI-1 expression.

Three unstructured regions of Spot 42 have been identified to participate in gene regulation through base-pairing in *E. coli* (88) (Fig. 30A, upper panel). To shed light in the mechanism of action of Spot 42 on the regulation of SPI-1 expression, it was studied if specific base pairing of Spot 42 within the *hilD* 3'UTR region was required for the Spot 42-mediated derepression of *hilD* expression. The software IntaRNA (133), designed to search for putative interactions between two given RNA molecules was initially used to predict a putative interaction site between Spot 42 and the *hilD* 3'UTR. IntaRNA predicts a putative interaction site between the *hilD* 3'UTR base pairing with the unstructured region III of Spot 42 (Fig. 30A, lower panel).

To test whether base pairing mediated by Spot 42 through the region III was relevant for the regulation of SPI-1 genes, two independent Spot 42 mutant alleles were generated, the *spf*mut 1 and *spf*mut 2 both altering Spot 42 unstructured region III. The effect of overexpressing the mutant *spf* alleles on *hilD* expression was monitored in exponentially growing cultures (OD_{600nm} 0.4) by determination of i) *hilD*1235-*lacZ* expression, ii) detection of *hilD* mRNA by qRT-PCR and iii) *sipC*-*lacZ* expression monitoring HilD functional levels. Overexpression of Spot 42 mutant derivatives was performed in strains carrying a deletion of the chromosomal *spf* gene. As shown previously, overexpression of wild type Spot 42 upregulates *hilD*1235-*lacZ* and *sipC*-*lacZ* expression. Accordingly, relative *hilD* mRNA levels are also induced upon Spot 42 overexpression (Fig. 30B). Overexpression of either Spot42 mut1 (*spf*mut1, mutation in region III) or Spot42 mut2 (*spf*mut2, mutation in region III) did not induce *hilD*1235-*lacZ* expression. Similarly, no induction of *hilD* mRNA levels was detected by qRT-PCR and accordingly, *sipC* expression was not induced upon Spot 42 mut1 or Spot42 mut2 overexpression (Fig. 30B).

Same substitutions as introduced in the allele *spf*mut1 (GUA-CAU) and *spf*mut2 (GGA-CAC) have been previously described in *E. coli* to abolish base pairing mediated regulation and to no compromise Spot 42 expression (87,88).

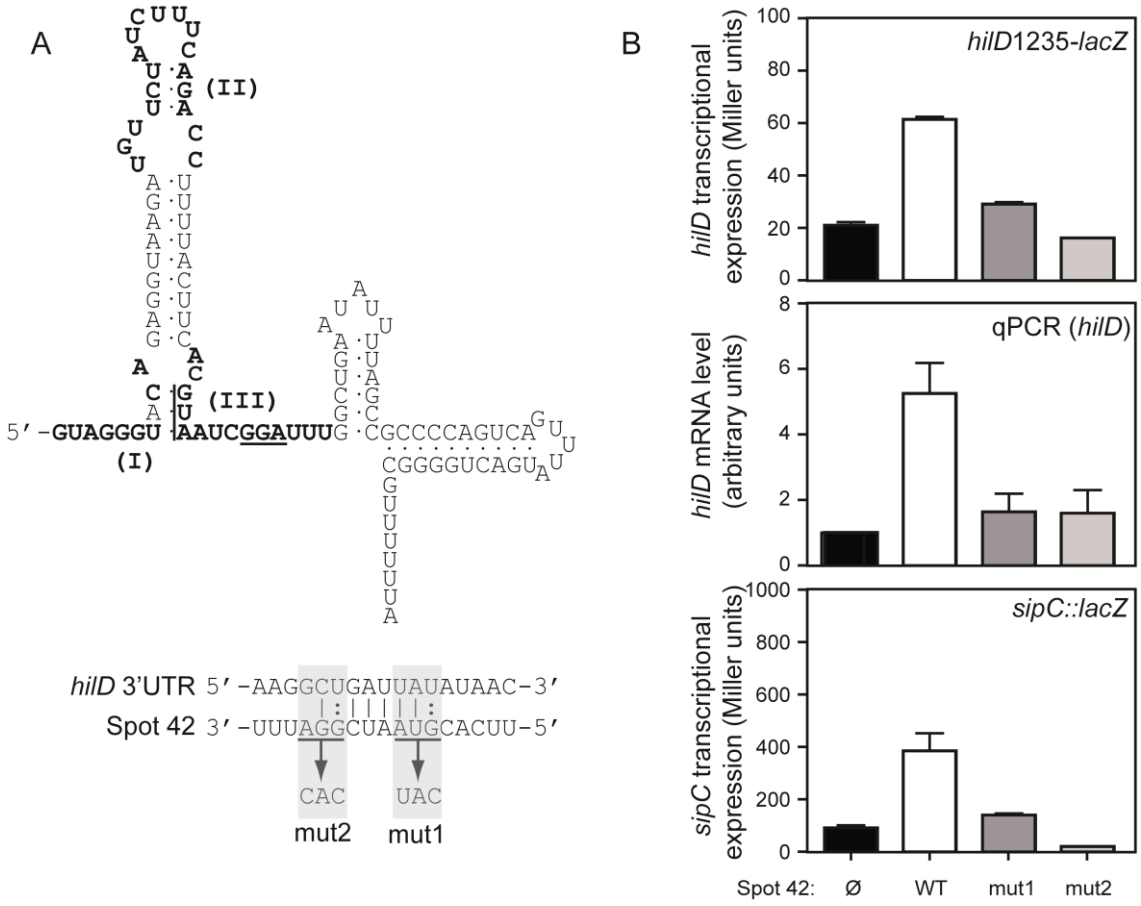

Figure 30. Unstructured region III of Spot 42 is required for *hilD* derepression. A. Structure of the sRNA Spot 42, the three described seed regions are indicated in bold (upper panel). IntaRNA prediction of the RNA-RNA interaction site between the sRNA Spot 42 and the *hilD* 3'UTR (lower panel). **B.** *hilD* expression was assessed by i) transcription of *hilD*1235-*lacZ*, ii) qRT-PCR of *hilD* transcript and iii) transcription of *sipC-lacZ.* In all cases cultures of Δ*spf* strains carrying the pBRplac control vector or upon overexpression of the different Spot 42 variants were grown in LB at 37°C up to an OD_{600nm} of 0.4.

The absence of effect on *hilD* expression by overexpressing Spot 42 mut1 and Spot 42 mut 2 could be explained by: i) the nucleotides substitutions cause the disruption of the base pairing with the *hilD* 3'UTR or ii) the nucleotides substitutions make the Spot 42 mutant derivatives highly unstable and consequently under overexpressing conditions only low levels of the Spot 42 variants in the cell are achieved. To discriminate between the two possibilities, the level of expression of Spot 42 mut1 and Spot 42 mut2 was monitored by northern blot. As shown in Figure 31, in the pBRplac VC carrying strain there is no expression of Spot 42 as the experiments were performed in a Δ*spf* background. The allele *spf*mut1 and *spf*mut2 were detected and the expression level was similar to the expression of Spot 42 WT (Fig. 31).

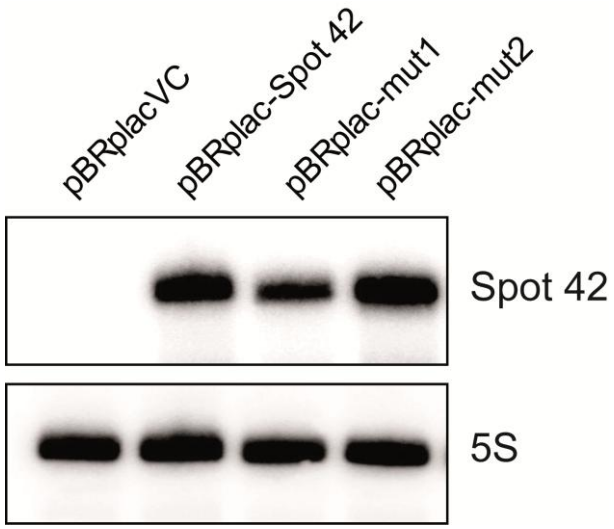

Figure 31. Spot 42 mutants expression level. Northern blot detection of Spot 42 and derivative mutants expression, mut1 and mut 2**.** In all cases cultures of the Δ*spf* strain carrying the VC or upon overexpression of the sRNA Spot 42 (or mutants) were grown in LB at 37^{o}C up to an OD_{600nm} of 0.4.

We can conclude that the substitutions introduced in Spot 42 mut1 and Spot 42 mut 2 cause a loss of the ability to induce *hilD* expression.

As previously described, Spot 42 mediated activation of *hilD* through the *hilD* 3'UTR requires the presence of Hfq. As mentioned previously, Holmqvist *et al*. (2016) reported by CLIP-seq two binding sites of Hfq in both the *hilD* 3'UTR and Spot 42, indicated in green in Figure 32 (94). In yellow, the putative interaction site between Spot 42 and the *hilD* 3'UTR detected by IntaRNA is indicated. Remarkably, the putative interaction site in the *hilD* 3'UTR is located next to one of the described Hfq binding sites. In the case of Spot 42, this predicted sequence interacting with *hilD* 3'UTR overlaps with one of the Hfq interaction sites (Fig. 32). Those findings raise the question of whether the mutants Spot42 mut1 and Spot42 mut2 do not induce *hilD* by causing the nucleotide substitutions impaired ability of Hfq to bind Spot 42 rather than by the interference of the interaction Spot 42-*hilD* 3'UTR.

To rule out that possibility, Hfq binding to both mutants, Spot42 mut1 and Spot mut2, was assessed by EMSA as previously described. The modification of the residues in GUA-CAU and GGA-CAC for Spot 42 mut1 and Spot 42 mut2 respectively did not alter the ability of Hfq to bind these Spot 42 mutants (Fig. 33), suggesting that the

abolishment of regulation of *hilD* expression by these mutants is due to interference of Spot 42 base pairing mechanism of action.

***hilD* 3'UTR**

CATTTTTTGTATCTGTCACTTAAGTAAAGATTTTTATTAAAATTGTAATA
ATTTAAAATTCAGACTGCGCATTAACACGCTCTATCAGGATGGGAGG
CTATTCAATATCATTGTTCTGTCCGGAAGACAGCTTATACTGATATCTAT
GGTAATTTAAAGTAAGGCTGATTATATAACACGATTTTTGTGAACTTG
TCATCGCTATGATGACTGGTAAAACGATATTGCCTTATTCACATCGTAA
GAATTCGTCCAGATGACACTATCTCCTTCCGGCTTTAACCCTGTGGAT
TAAGGCCGGCATTTTATT

Spot42

GTAGGGTACAGAGGTAAGATGTTCTATCTTTCAGACCTTTTACTTCAC
GTAATCGGATTTGGCTGAATATTTTAGCCGCCCCAGTCAGTTTATGAC
TGGGGCGTTTTTTA

Figure 32. Hfq binding sites in the *hilD* 3'UTR and in the sRNA Spot 42. CLIP-seq reported Hfq binding site in the *hilD* 3'UTR and in the sRNA Spot 42 (green). IntaRNA RNA-RNA interaction prediction between *hilD* 3'UTR and Spot 42 (yellow).

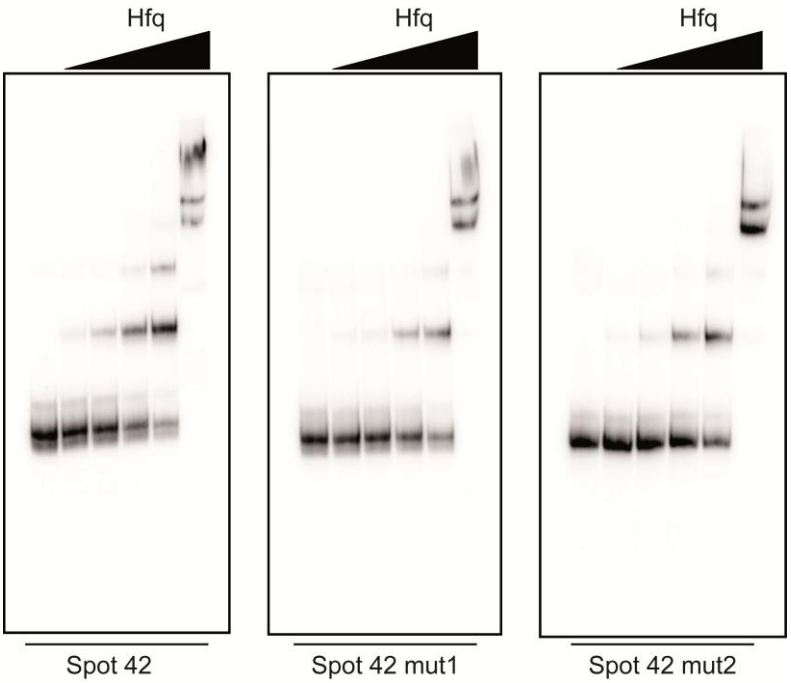

Figure 33. Hfq binding to Spot 42 mutants by EMSA. Hfq binding to Spot 42 and derivative mutants by EMSA. *In vitro* transcribed RNA was radiolabelled. 4 nM of the radiolabelled RNA was incubated with increasing concentration of purified Hfq (0, 1.3 nM, 4 nM, 13 nM, 40 nM, 130 nM) and subjected to electrophoresis in a native gel. Bandshift was observed upon dryng and exposure of the gel.

Taken together, our data show that the specific effect of Spot 42 on *hilD* expression is base pairing dependent and that the region III of Spot 42 sRNA is involved in the Spot 42-dependent regulation of *hilD*.

In silico prediction proposes that region III of Spot 42 interacts within the *hilD* 3'UTR. To assess the existence of direct base pairing between Spot 42 and *hilD* 3'UTR in the predicted sequence, two independent chromosomal compensatory mutations were generated in the *hilD* 3'UTR that restore the putative base pairing with Spot 42 mut1 and Spot 42 mut2 respectively. Those *hilD* alleles were named, *hilD* 3'UTR mut1 and *hilD* 3'UTR mut2 (Fig. 34A). *sipC* expression was used as a reporter in the strains carrying the chromosomal compensatory mutations as *sipC-lacZ* expression finely correlate with the levels of *hilD* expression. Overexpression of Spot 42 WT in both *hilD* 3'UTR mut1 and *hilD* 3'UTR mut2 genetic backgrounds induced expression of *sipC-lacZ*, indicating that substitution of those residues did not impair the ability of Spot 42 to affect SPI-1 expression.

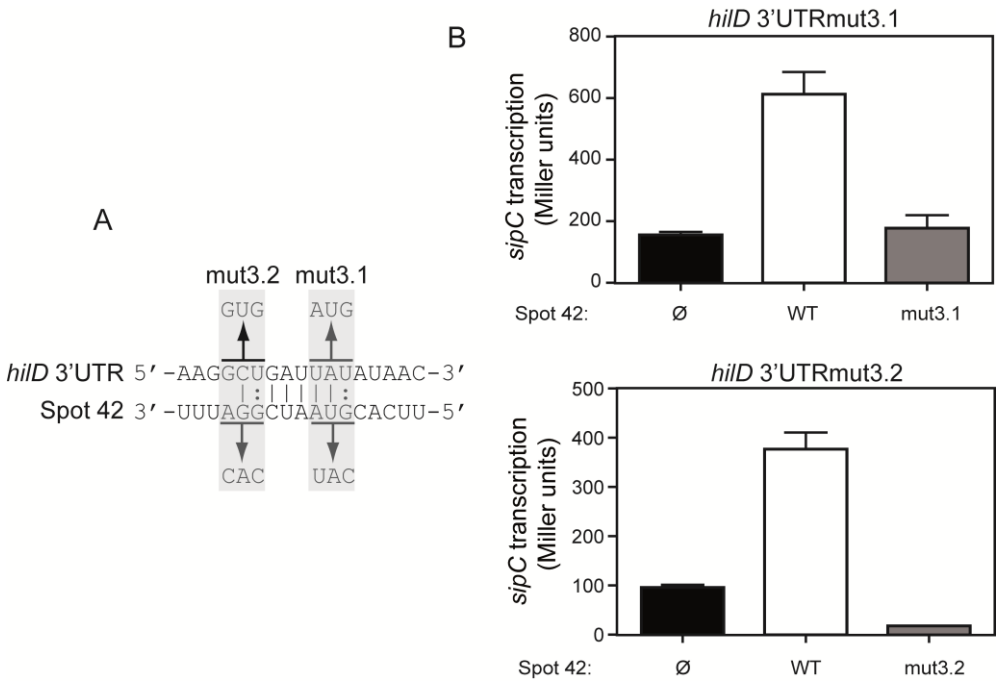

Figure 34. *hilD* 3'UTR compensatory mutants. *sipC* transcriptional expression was assessed in wild type (WT) upon overexpression of Spot 42 or derivative mutants (Spot 42 mut 1 and Spot 42 mut 2) in the *hilD* 3'UTR chromosomal mutant backgrounds *hilD* 3'UTR mut1 (upper panel) or *hilD* 3'UTR mut2 (lower panel). In all cases cultures were grown in LB at 37°C up to an OD_{600nm} of 0.4. β-galactosidase activity was determined for three independent cultures, average and standard error of the mean is presented.

Accordingly, overexpression of either Spot 42 mut1 or Spot 42 mut2 in both *hilD* 3'UTR mut1 and *hilD* 3'UTR mut2 backgrounds did not reestablish the ability of Spot 42 to induce *sipC* expression through *hilD* (Fig. 34B). These results suggest that, despite of region III of Spot 42 being responsible for the reported *hilD* derepression, the putative interaction site predicted with IntaRNA within the *hilD* 3'UTR does not seem to be the target sequence. Whether Spot 42 interacts somewhere else in the *hilD* 3'UTR will be further explored in the next section.

4.3.1.1 Bioinformatic predictions of Spot42-*hilD* 3'UTR interaction site

While the region in Spot 42 required for the base pairing has been properly identified. Complementary mutations in the identified putative interaction site in *hilD* 3'UTR did not reconstitute a functional interaction, indicating that Spot 42 might be interacting somewhere else in the *hilD* 3'UTR region.

To further assess the interaction site between the Spot 42 and the *hilD* 3'UTR, RNA-RNA interaction predictions were done by using as input target RNA, different fragments of the *hilD* 3'UTR rather than the whole 310 nt UTR sequence. NUPACK and IntaRNA prediction softwares were used. The Casadesus lab has shown that the first 100 nt of the 3'UTR seem to play a major role on *hilD* expression (31). So that, predictions were done by using the first 50 nt or 100 nt of the *hilD* 3'UTR sequence as input target regions. The output from the first 50 nt overlap between the NUPACK and IntaRNA predictions, while the output when using the first 100 nt of the *hilD* 3'UTR as input target region differ between the softwares. Therefore, three new putative interaction regions were detected between Spot 42 and the *hilD* 3'UTR. The three new putative interaction sites in addition to the previously reported in the above section are schematically represented in Figure 35.

In order to decipher the location of the Spot 42 interaction site within the *hilD* 3'UTR, the experimental strategy aimed to firstly generate mutations in the *hilD* 3'UTR that abolish Spot 42-mediated derepression and subsequently compensate those mutations by modifying the complementary nucleotides in the sRNA Spot 42. In each of the new putative interaction sites (purple, red and grey in Fig. 35) two independent

triplet nucleotide substitutions were generated in the chromosome. Therefore, in addition to the previously generated *hilD* 3'UTR mut 1 and *hilD* 3'UTR mut 2, six new triplet mutations were generated in the *hilD* 3'UTR in the chromosome (Fig 36A). Nucleotides substituted are indicated in Figure 36.

Figure 35. Bioinformatic RNA-RNA interaction predictions. RNA-RNA interaction predictions between *hilD* 3'UTR and the sRNA Spot 42 by using either IntaRNA or NUPACK softwares. In purple, interaction of Spot 42 within the first 50 nt of the *hilD* 3'UTR. In grey, interaction within the first 100 nt of *hilD* 3'UTR (Nupack). In red, interaction within the first 100 nt of *hilD* 3'UTR (IntaRNA prediction). In yellow, interaction prediction between Spot 42 and the whole *hilD* 3'UTR. In white, residues mutates to generate *hilD* 3'UTR chromosomal mutations are indicated.

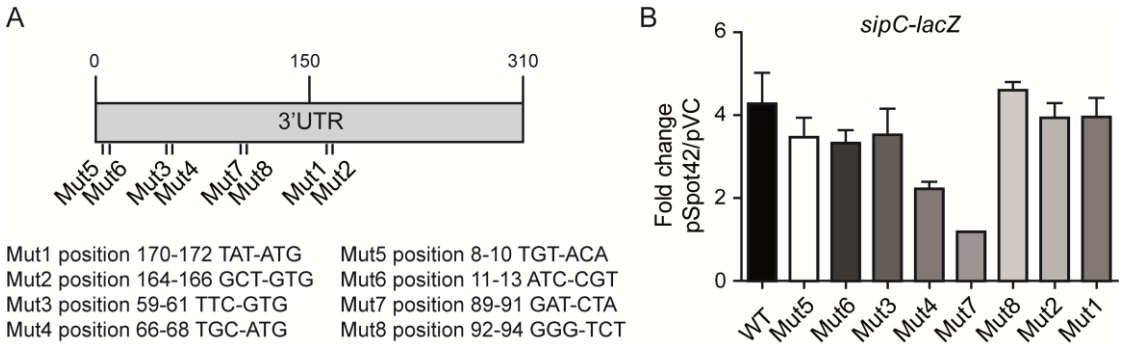

Figure 36. *hilD* 3'UTR chromosomal mutations. A. Map of the *hilD* 3'UTR chromosomal mutations. **B.** *sipC* transcriptional expression was assessed in wild type (WT) upon overexpression of Spot 42 in the different *hilD* 3'UTR chromosomal mutant backgrounds. In each case the reference value (wild type, pBRplac VC) was set as one, and results are expressed as fold change upon overexpression of Spot 42 respective to VC carrying strain. Cultures were grown in LB at 37°C up to an OD_{600nm} of 0.4. β-galactosidase activity was determined for three independent cultures, average and standard error of the mean is presented.

When those chromosomal genetic backgrounds were challenged by Spot 42 overexpression, the *sipC-lacZ* activity was induced in a similar fold as compared to the

induction observed for the wild type *hilD* variant, with exception of *hilD* 3'UTR mut7. Overexpression of Spot 42 did not induce the expression of the downstream gene *sipC* in the *hilD* 3'UTR mut7 background (Fig. 36B), suggesting that mut7 is generated in a region required for Spot 42-mediated regulation of *hilD*. Compensatory mutation of *hilD* 3'UTR mut7 was generated in Spot 42 to generate the Spot 42 mut7. As predicted by IntaRNA, this mutation would reconstitute the perfect base pairing of Spot 42 and *hilD* 3'UTR mut 7. Spot 42 mut7 was overexpressed in the *hilD* 3'UTR mut7 background in order to reconstitute the Spot 42-mediated induction of *hilD* and subsequently *sipC* (Fig. 37B). Regrettably, the compensatory mutation did not lead to *sipC* induction in the *hilD* 3'UTR mut7 chromosomal background. Suggesting that the predicted sequence within Spot 42 by IntaRNA did not base pair with the region where *hilD* 3'UTR mut7 is generated.

When comparing transcriptional expression of *sipC-lacZ* in the different chromosomal backgrounds carrying the point mutations generated in the *hilD* 3'UTR (Mut 3-Mut8), it can be observed that the mutants show differential basal expression of *sipC-lacZ* (Fig. 36A). It is induced in *hilD* 3'UTR mut 3, 4, 5 and 6 while diminished in *hilD* 3'UTR mut 7 and 8. For *hilD* 3'UTR mut 1 and 2, as shown before, the expression of *sipC-lacZ* reported is similar to the wild type background (Fig. 37A).

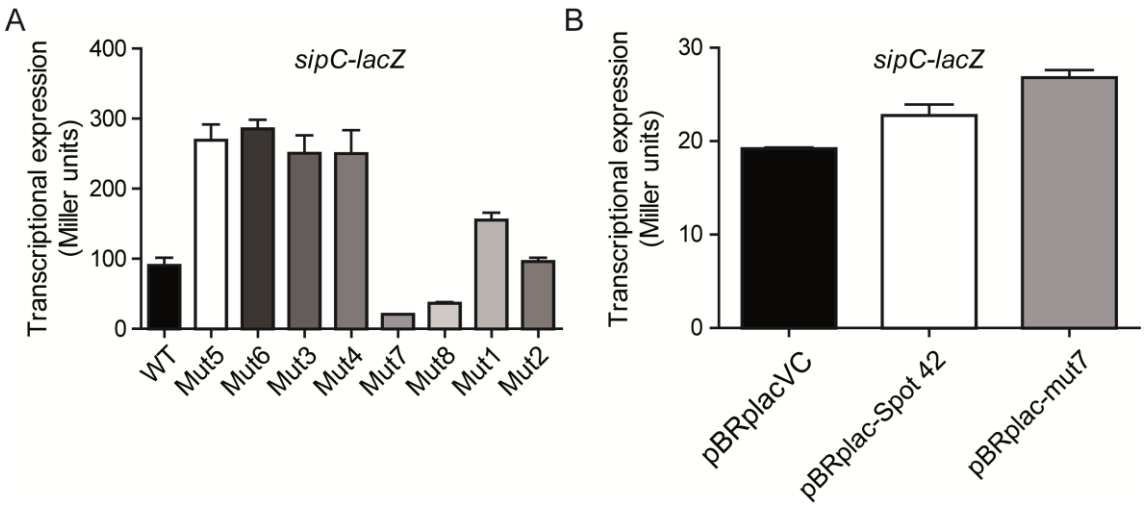

Figure 37. *hilD* 3'UTR chromosomal mutations basal expression. A, *sipC* transcriptional expression was assessed in the different *hilD* 3'UTR chromosomal background mutations. **B.** *sipC* transcriptional expression in the *hilD* 3'UTR mut 7 background upon overexpression of Spot 42 and Spot 42 mut 7. In all cases cultures were grown in LB at 37^{o}C up to an OD_{600nm} of 0.4. β-galactosidase activity was determined for three independent cultures, average and standard error of the mean is presented.

It is remarkable the drop on *sipC* expression between Mut4 (position 66-68) and Mut7 (position 89-91) *hilD* 3'UTR genetic backgrounds. Suggesting, that the mutants *hilD* 3'UTR mut7 and *hilD* 3'UTR mut8 (position 92-94) might be affecting a regulatory motif within the *hilD* 3'UTR. It has been highlighted previously, that the first 100 nt of the *hilD* 3'UTR seem to be crucial for the *hilD* 3'UTR mediated regulation of *hilD* expression. To elucidate whether the region where *hilD* 3'UTR mut7 and *hilD* 3'UTR mut8 were generated is a regulatory region, transcriptional expression of *hilD-lacZ* fusions generated within the *hilD* 3'UTR was compared. The following fusions were used: i) *hilD*965-*lacZ* (HilD$^+$, UTR$^-$), ii) *hilD*1044-*lacZ* (HilD$^+$, UTR position 79), iii) *hilD*1065-*lacZ* (HilD$^+$, UTR position 100) and iv) *hilD*1235-*lacZ* (HilD$^+$, UTR$^+$). As it can be observed in Figure 37, the overall activity of *hilD-lacZ* fusions is different. As shown previously, *hilD*965-*lacZ* (HilD$^+$, UTR$^-$) show a much higher activity compared to *hilD*1235-*lacZ* (HilD$^+$, UTR$^+$) in agreement with the negative role attributed to the presence of the *hilD* 3'UTR on the expression of *hilD.* Remarkably, the *hilD*1044-*lacZ* (HilD$^+$, UTR position 79) monitored transcriptional expression was much higher than the *hilD*1065-*lacZ* (HilD$^+$, UTR position 100) expression (Fig. 37). Suggesting, that within the sequence between position 79 and 100 of the *hilD* 3'UTR, where *hilD* 3'UTR mut7 and *hilD* 3'UTR mut8 were generated, an important regulatory motif for *hilD* 3'UTR mediated regulation of *hilD* might exist.

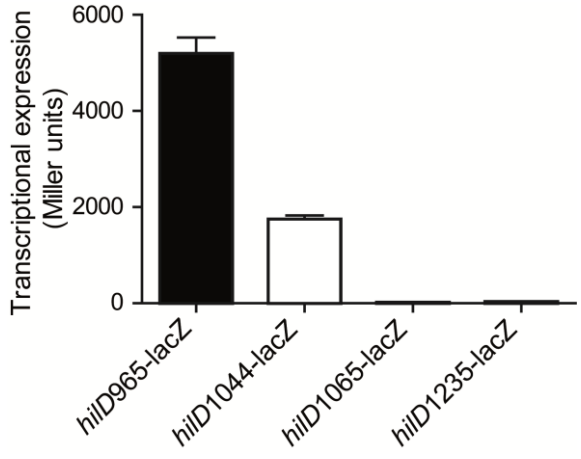

Figure 37. *hilD* transcriptional fusions. *hilD* transcriptional expression was assessed by using different transcriptional constructs, *hilD*965-*lacZ* (HilD$^+$, UTR$^-$), *hilD*1044-*lacZ* (HilD$^+$, UTR position 79), *hilD*1065-*lacZ* (HilD$^+$, UTR position 100) and *hilD*1235-*lacZ* (HilD$^+$, UTR$^+$). Cultures were grown in LB at 37^{o}C up to an OD_{600nm} of 0.4. β-galactosidase activity was determined for three independent cultures, average and standard error of the mean is presented.

4.3.2 *In vitro* analysis of Spot 42-*hilD* 3'UTR interaction

As it can be observed in Figure 36, the *hilD* UTR mut7 was generated in the first 100 nt of the *hilD* 3'UTR while the unstructured region III of Spot 42 putatively interacts with a region at position 170 nt within the *hilD* 3'UTR. Moreover, the Hfq binding sites locate far downstream of position 170. Of note, the *hilD* 3'UTR mut7 could not be complemented by restoring mutations in Spot 42. Similarly, the mutations in the unstructured region III of Spot 42 (Spot 42 mut1, Spot 42 mut2) could not be complemented by the predicted restoring mutations in the *hilD* 3'UTR. On one hand, it is clear that both *hilD* 3'UTR mut 7 and Spot 42 mut1 and mut 2 abolish the Spot 42 mediated derepression of *hilD*. Although the existence of alternative molecular mechanisms cannot be ruled out, the results suggest direct interaction between Spot 42-*hilD* 3'UTR but the exact interaction site remains elusive.

Alternatively to the genetic analysis to demonstrate RNA-RNA interaction *in vivo*, biochemical approaches were used for further characterization of Spot 42-*hilD* 3'UTR interaction. Electrophoretic mobility shift assay (EMSA) experiments were performed to display evidences on the physical interaction between Spot 42 and *hilD* 3'UTR.

The ability of Spot 42 to bind to *hilD* 3'UTR fragment was assessed. *hilD* 3'UTR RNA fragment and different variants of Spot 42 were generated *in vitro* as described in the Methods section. EMSA experiments were performed with *hilD* 3'UTR radiolabeled RNA upon addition of increasing concentrations of either Spot 42 or Spot 42 mut2. The EMSA showed (Fig. 38A) a faint shift of the *hilD* 3'UTR upon addition of Spot 42. Remarkably, the observed shift with Spot 42 did not appear upon addition of Spot 42 mut 2, indicating that although faint the shift is rather specific (Fig. 38A).

EMSA experiments were also performed by adding *hilD* 3'UTR RNA fragment in excess to a radiolabeled Spot 42 RNA. In agreement with the previous results a shift was observed upon addition of *hilD* 3'UTR (Fig. 38B).

As a positive control, it was used 310 nt fragment from the mRNA of *mglB*. *mglB* is a gene that has been described to be regulated by Spot 42 in *Salmonella* (94), being identified the base pairing site Spot 42-*mglB*. The band shift observed although more

prominent for *mglB* than for the *hilD* 3'UTR, was still faint and only causes the bandshift of a small amount of the labeled *hilD* 3'UTR (Fig. 38C).

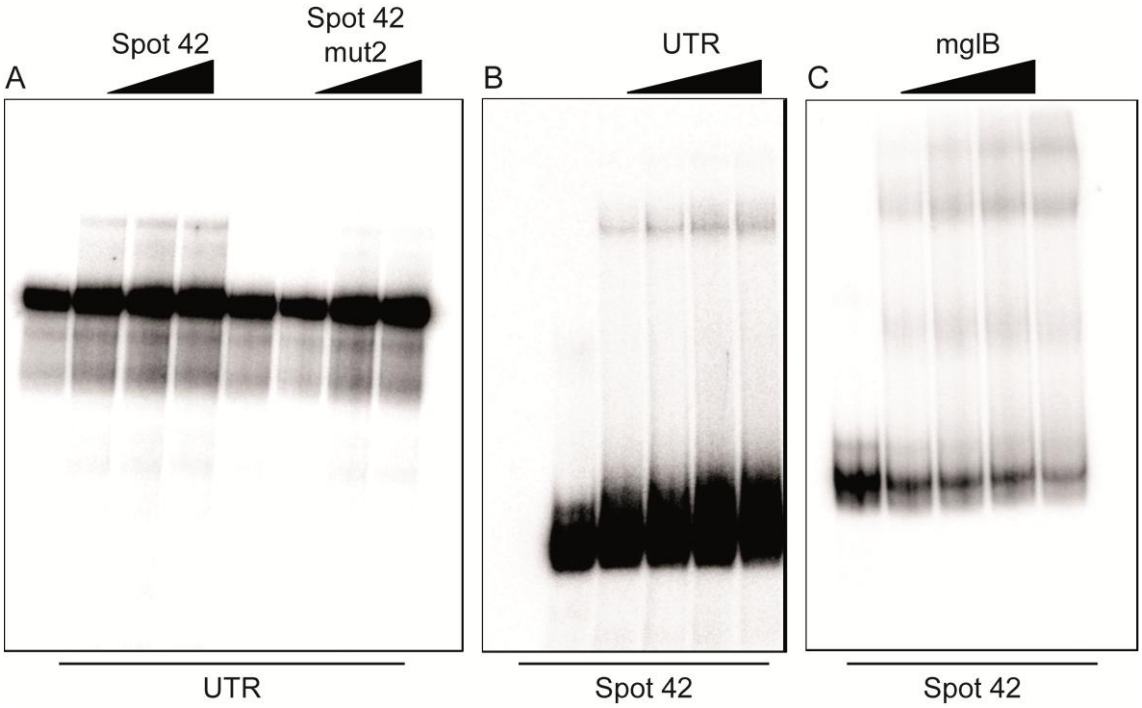

Figure 38. Spot 42 interacts with the *hilD* 3'UTR. A. *In vitro* transcribed *hilD* 3'UTR RNA was radiolabelled. 4 nM of the radiolabelled RNA was incubated with increasing concentration of either Spot 42 or Spot 42 mut2 *in vitro* transcribed RNA (0, 56, 280, 560, 1700 nM). **B.** and **C.** *In vitro* transcribed Spot 42 RNA was radiolabeled. 4 nM of the radiolabeled RNA was incubated with increasing concentration of either *hilD* 3'UTR or *mglB in vitro* transcribed RNA (0, 56, 280, 560, 1700 nM). In all cases, samples were subjected to electrophoresis in a native gel. Bandshift was observed upon drying and exposure of the gel.

In order to improve the EMSA efficiency, the *hilD* 3'UTR was divided in two fragments (UTR^L and UTR^R) that were generated by T7 *in vitro* transcription as described in the Methods section. The UTR^L includes the first half of the *hilD* 3'UTR, from position -38 to +149 of the *hilD* 3'UTR sequence, being +1 the first nucleotide after the stop codon. This fragment includes the region where the *hilD* 3'UTR mut7 and mut8 was generated. The UTR^R includes the second half of the *hilD* 3'UTR, from position +149 to position +310. This fragment includes the Hfq binding sites and the putative interaction site with the unstructured III region of Spot 42 (mut1 and mut2).

Additionally, the sRNA Spot 42 was also generated by T7 *in vitro* transcription. Spot 42 sRNA was 5' radiolabeled and EMSA experiments were performed. 4 nM of radiolabeled Spot 42 was incubated with increasing concentrations of either UTR^L or UTR^R in structure buffer at 37°C for 1 hour, and then subjected to electrophoresis in a native gel. Remarkably, no shift of the Spot 42 sRNA can be observed in the presence of the UTR^L whereas there was a concentration dependent shift upon increasing amounts of UTR^R (Fig. 39A, left panel). These results suggest that Spot 42 interacts with the second half region of the *hilD* 3'UTR. To further assess the interaction of Spot 42 with the *hilD* 3'UTR fragments, an additional experiment was performed. Both UTR^L and UTR^R RNA fragments were radiolabeled, incubated with increasing concentrations of Spot 42 and subjected to electrophoresis as mentioned above. Accordingly, increasing concentrations of Spot 42 did not lead to band shift for UTR^L radiolabeled RNA but led to a UTR^R bandshift in a concentration dependent manner (Fig. 39B). The overall results indicate that in fact, Spot 42 does physically interact with the *hilD* 3'UTR, this interactions takes place within the second half of the *hilD* 3'UTR (UTR^R).

It has to be highlighted, that UTR^R includes the Hfq binding sites in addition to the putative interaction site for the unstructured region III of Spot 42. In order to demonstrate specificity of interaction Spot 42-*hilD* 3'UTR a Spot 42 mutant was used as a negative control. As shown previously, the Spot 42 mut1 and mut2 abolish the Spot 42 mediated derepression of *hilD in vivo*, being the strongest phenotype shown for Spot 42 mut2 (Fig. 30B). EMSA experiments were performed by assessing the interaction between radiolabeled Spot 42 mut2 incubated with increasing concentrations of UTR^R. Remarkably, the interaction affinity was significantly lower. No shift was observed when UTR^R is added in excess to Spot 42 mut2 labeled RNA (Fig. 39A right panel).

Our results indicate, that the abolishment of regulation shown by Spot 42 mut2 *in vivo*, it is due to interference with direct base pairing interaction of Spot 42 with the *hilD* 3'UTR, concretely with the second half of *hilD* 3'UTR (UTR^R).

As shown previously, the sRNA chaperone Hfq interacts with the *hilD* 3'UTR and with the sRNA Spot 42. The Hfq binding sites locate in the second half *hilD* 3'UTR, and therefore are present in the *in vitro* transcribed fragment UTR^R.

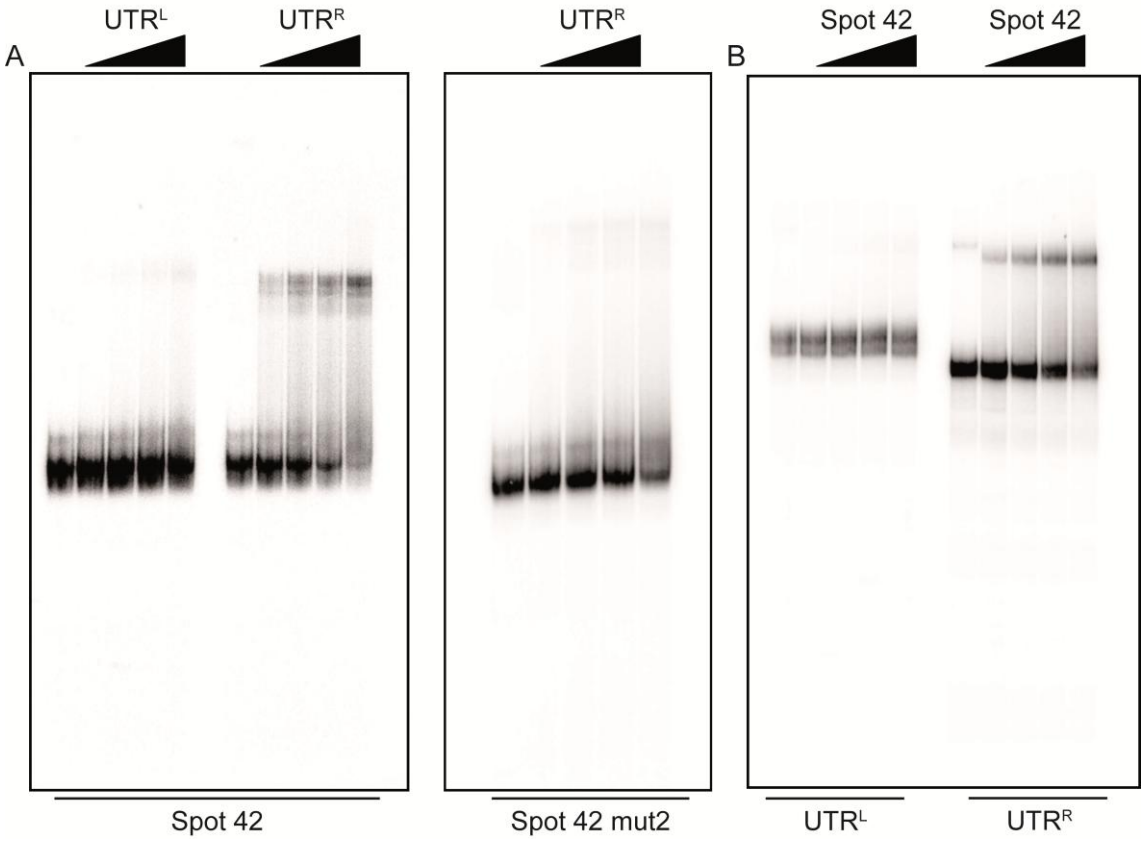

Figure 39. Spot 42-*hilD* 3'UTR fragments interaction by EMSA. A. Either Spot 42 or Spot 42 mut2 *in vitro* transcribed RNA was radiolabeled. 4 nM of radiolabeled Spot 42 or Spot 42 mut2 RNA was incubated with increasing concentration (0, 56, 280, 560, 1700 nM) of either UTR^R or UTR^L *in vitro* transcribed RNA. **B.** Either UTR^R or UTR^L *in vitro* transcribed RNA was radiolabeled. 4 nM of radiolabeled UTR^R or UTR^L was incubated with increasing concentration (0, 56, 280, 560, 1700 nM) of Spot 42 *in vitro* transcribed RNA. In all cases, samples were subjected to electrophoresis in a native gel. Bandshift was observed upon drying and exposure of the gel.

The ability of Hfq to form a complex with the sRNA Spot 42 and the UTR^R was assessed by EMSA (Fig. 40). Hfq was added to a concentration of 20 nM, as it can be observed in lane 2, Hfq bind to the UTR^R. Spot 42 was added in excess and as it can be observed a shift resembling UTR^R-Spot42 appears, identified by comparison of the migration of

the complex UTR^R-Spot42 in other EMSA shifts. Remarkably, an upper shift also occurs, indicating that the complex Hfq-Spot42-UTR^R is formed. The complexes that are formed upon addition of Hfq and Spot 42 to a radiolabeled UTR^R are indicated in the right side of the Figure 40. It might be argued that the UTR^R shift in Figure 40 it is not as prominent as the shown in Figure 39B, but it has to be underlined that Hfq does also bind Spot 42, and therefore some Spot 42 might be binding only Hfq. As a consequence, less Spot 42 is free to bind UTR^R or the complex UTR^R-Hfq.

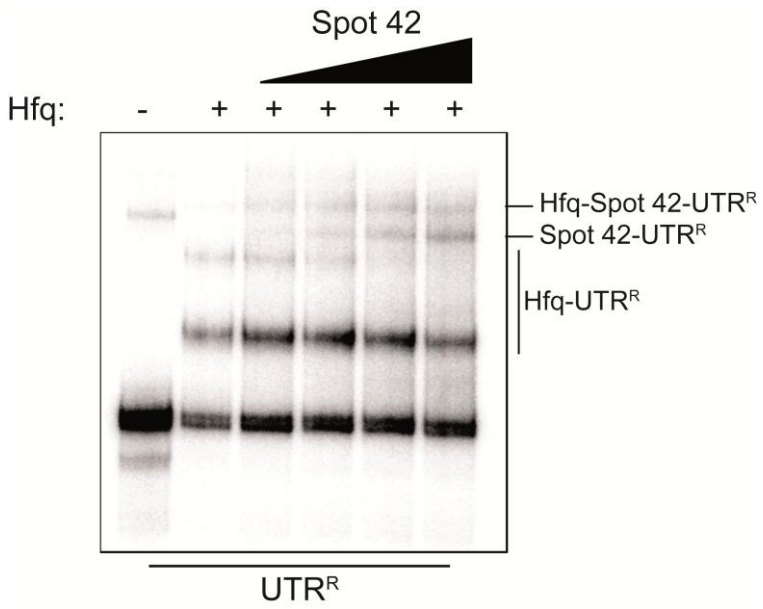

Figure 40. Spot 42 forms a complex with UTR^R and Hfq. *In vitro* transcribed UTR^R RNA was radiolabeled. 4 nM of the radiolabeled RNA was incubated with increasing concentration (0, 56, 280, 560, 1700 nM) of Spot 42 *in vitro* transcribed RNA in the presence of 20 nM of purified Hfq. Samples were subjected to electrophoresis in a native gel. Bandshift was observed upon drying and exposure of the gel.

Taken together, all the results obtained by EMSA indicate that Spot 42 physically interact with the *hilD* 3'UTR. This interaction occur within the second half of the *hilD* 3'UTR as it interacts with UTR^R fragment but not UTR^L. Additionally, it seems that unstructured region III of Spot 42 is involved in the interaction as Spot mut 2 is impaired in the ability to interact with the *hilD* 3'UTR.

In order to elucidate the residues within the *hilD* 3'UTR required to interact with Spot 42, structure probing experiments were performed as described in the methods

section. Shortly, a radiolabeled RNA, in our case UTR^R, is incubated with increasing concentration of the interacting RNA, in our case the sRNA Spot 42. After incubation, samples are treated differentially depending on the band profile desired: i) RNAse T1 which cuts single stranded RNA after G, interaction with the non labeled RNA will be read as protection of cleavage sites, in other words, disappearance of bands and ii) RNAse III which cuts double stranded RNA, interaction with the non labeled RNA will be read as generation of cleavage sites, in other words, appearance of bands.

For each of the samples, 20 nM of radiolabeled UTR^R was incubated as in the EMSA experiments with only Hfq (20 nM), only Spot 42 (1000-fold increase) or in presence of both Hfq (20 nM) and Spot 42 (1000-fold increase). After incubation, samples were treated by RNAse T1 or RNAse III.

For the ladders, 40 nM of denatured radiolabeled UTR^R was treated by i) alkaline hydrolysis, digestion after each nucleotide and ii) RNAse T1 causing cleavage after all G of the sequence.

The samples were subjected to electrophoresis in a denaturing gel. As it can be observed in Figure 41, from guanosine 102 (G102) to guanosine 134 (G134), a putative interaction site for Spot 42 on *hilD* 3'UTR can be observed. Some cleavage bands of RNAse T1 are fainter in the samples containing Spot 42. Additionally, for the same region, in the RNAse III treated samples some new cleavage sites are generated for Spot 42 mediated interaction and therefore new bands can be observed in the Spot 42 containing samples. The presence of Hfq does not seem to affect the cleavage pattern as no evident differences can be observed between presence or absence of Hfq containing samples band profile.

Genetic approaches for the demonstration of Spot 42-*hilD* 3'UTR interaction led us show that the unstructured region III of Spot 42 is required for the Spot 42-mediated regulation of *hilD*. *In vitro* experiments let us to finely show that Spot 42 physically interacts with *hilD* 3'UTR and that interaction occurs within the second half of the *hilD* 3'UTR. Structure probing experiments led us propose a more defined region of Spot 42 binding within the *hilD* 3'UTR. It has to be underlined, that UTR^R, does also include the predicted interaction region with Spot 42 unstructured region III studied in the genetic

approach of the Spot 42-*hilD* 3'UTR interaction, where complementary mutations of Spot 42 mut 1 and Spot mut2 did not lead to reconstitution of regulation. The exact residues required in the *hilD* 3'UTR will need further experiments to be deciphered. Mutations to lose regulation and complementary mutations to reconstitute regulation are the gold standard to show RNA-RNA interactions. Nonetheless, the direct interaction of Spot 42 on the *hilD* 3'UTR has been demonstrated and it represents the first example of a trans-encoded RNA regulating expression through interaction with a 3'UTR region.

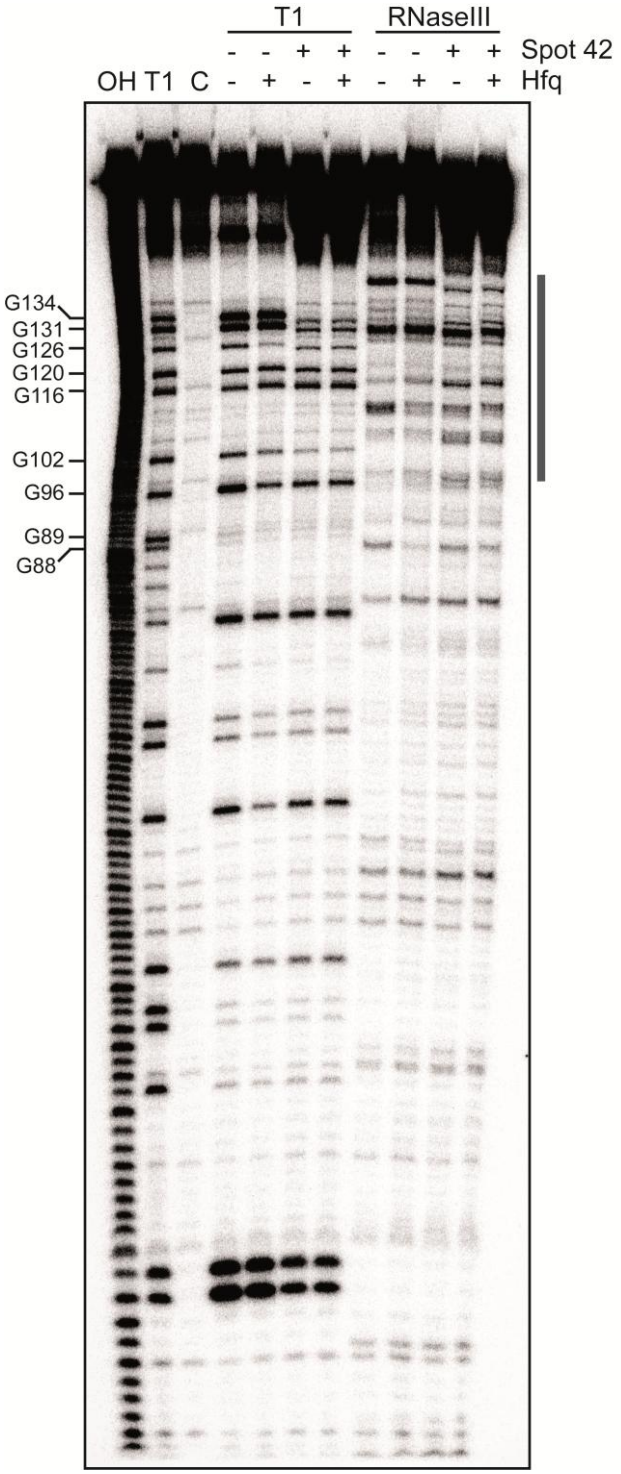

Figure 41. *hilD* 3'UTR-Spot 42 Structure probing. *In vitro* transcribed RNA was radiolablled. 20 nM of the radiolabeled UTR^{R} RNA was incubated with i) 20 nM Hfq, ii) 20 µM Spot 42 and iii) 20 nM Hfq + 20 µM Spot 42. Samples were treated by RNAse T1 or RNAse III and subjected to electrophoresis in a denaturing gel. Structure probing was visualized upon drying and exposure of the gel. In addition, as ladders were used i) alkaline hydrolysis treated denatured sample (OH) and ii) RNAse T1 treated denatured sample (T1) and untreated samples as a control (C).

4. 4 CRP-cAMP mediated regulation of the Csr network.

4.4.1 CRP-cAMP does not induce *csrB* and *csrC* in stationary growing cells

The Csr network is formed by the RNA binding protein CsrA and the two long non-coding RNA CsrB and CsrC. CsrA binds to GGA motifs in apical loops in the target mRNA leading to either activation or repression of the target mRNA expression. The long non-coding RNAs CsrB and CsrC carry on their sequence several GGA motifs, which lead to the sequestration of free CsrA. As mentioned in the Introduction section, the level of expression of CsrB and CsrC, as they can sequester CsrA, is the main regulatory feature on the activity of CsrA.

The role of CRP-cAMP in the regulation of Csr has been studied previously. It has been reported the involvement of CRP-cAMP in the regulation of the Csr network, being CRP-cAMP an activator of both CsrB and CsrC expression in some conditions. Ahmer lab reported that on agar plates grown cells, CRP-cAMP seem to activate both CsrB and CsrC expression (59). In this thesis, the transcriptional expression of *csrB* and *csrC* was assessed. To do so, transcriptional fusions were generated in vector pQF50 by cloning a region including all regulatory elements controlling either *csrB* (-345, +45) or *csrC* (-345, +32).

The transcriptional expression of the generated *csrB-lacZ* and *csrC-lacZ* gene fusion was monitored in a wild type and Δ*crp* mutant background. When *lacZ* activity was assessed on X-Gal supplemented agar plates, both *csrB* and *csrC* expression it is strongly diminished in a Δ*crp* derivative strain when compared to WT, as it can be extrapolated by a white phenotype on X-Gal agar plates in the *Δcrp* mutant background (Fig. 42A). These results are in agreement with previous report (59).

As CsrB and CsrC downregulates the activity of CsrA, CsrB and CsrC downregulation would in turn, increase the levels of CsrA, a known regulator of SPI-1 expression. To test the involvement of this CRP-mediated regulation of *csrB* and *csrC* in the SPI-1 expression, *csrB* and *csrC* transcriptional activity was assessed in SPI-1 permissive conditions, defined as stationary phase (DO_{600nm} 2.0). Intriguingly, on SPI- 1 permissive conditions, *csrB* and *csrC* expression were not diminished in a Δ*crp* mutant background

when compared to WT (Fig. 42B). The reason why CRP-cAMP affects the expression of CsrB and CsrC on solid culture but not on liquid culture at stationary phase remains elusive. Bacterial growth as single colonies in solid media lead to physiological heterogenicity within the colony which might explain the differences in the expression pattern observed.

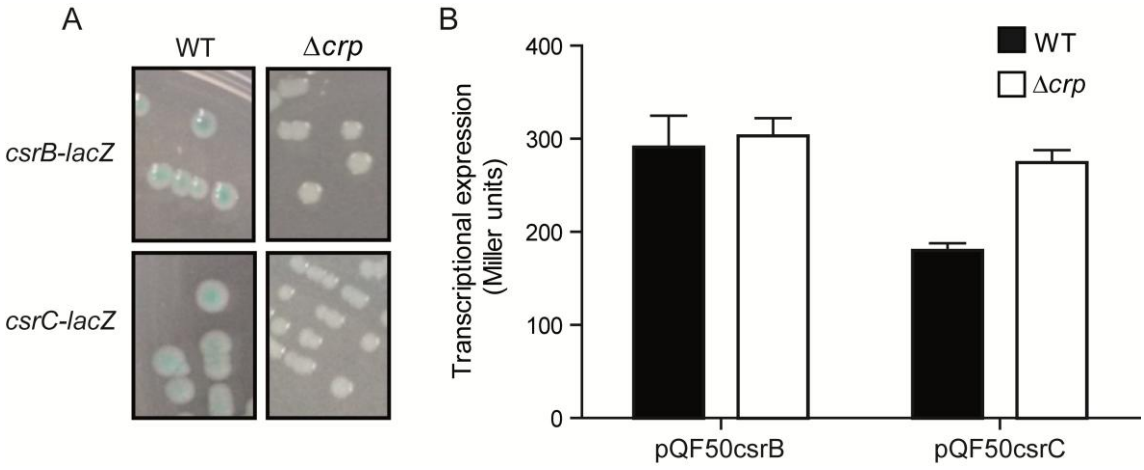

Figure 42. CRP-cAMP does not regulate *csrB* and *csrC* transcription at stationary phase. A. Strains carrying *csrB-lacZ* or *csrC-lacZ* fusions in the vector pQF50, in either in wild type (WT) or Δ*crp* mutant genetic backgrounds were streaked on LB agar plates supplemented with X-gal (20 mg/ml). Colony phenotype was monitored after 12 hours incubation at 37°C. **B.** Transcriptional expression of *csrB-lacZ* and *csrC-lacZ* fusions was monitored in WT and Δ*crp* mutant genetic backgrounds. Cultures were grown in LB at 37°C up to an OD_{600nm} of 2.0. β-galactosidase activity was determined for three independent cultures, average and standard error of the mean is presented.

4.4.2 CRP-cAMP represses CsrC but not CsrB at logarithmic growth phase

In this thesis, CRP-cAMP has been defined to play a dual role in SPI-1 expression, being an activator at stationary phase (OD_{600nm} 2.0) but a repressor at exponential phase (OD_{600nm} 0.4). To further assess the role of CRP-cAMP on Csr, the transcriptional expression of *csrB* and *csrC* was also assessed at exponential growth phase. Remarkably, *csrB* expression was not affected when compared WT and Δ*crp* mutant background, whereas *csrC* expression was induced by 3-fold in the Δ*crp* mutant background when compared to WT (Fig. 43A).

Direct RNA detection of *csrB* and *csrC* at exponential growth phase was performed to confirm the differential regulation of these long non-coding RNA by CRP-cAMP. In the

wild type strain, it can be observed that the overall levels of CsrB and CsrC are different, being CsrB much more abundant than CsrC.

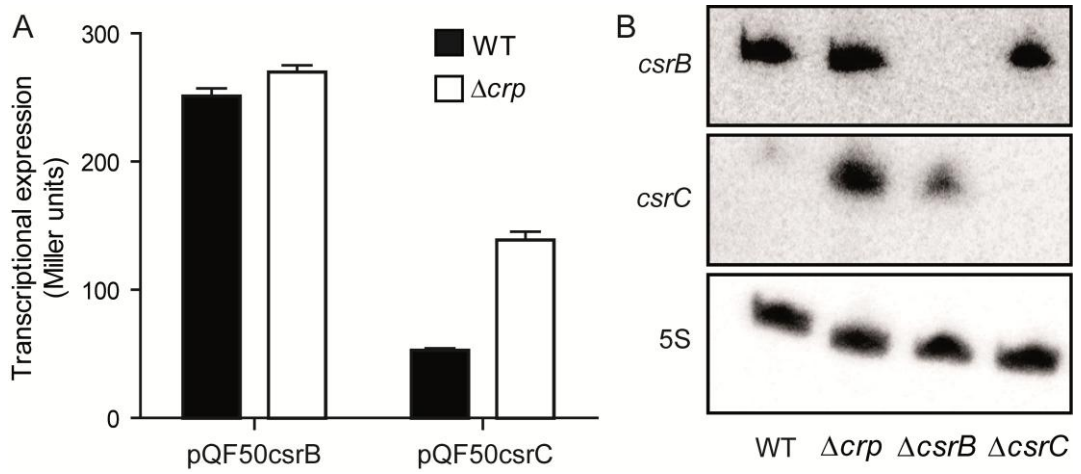

Figure 43. CRP-cAMP represses CsrC but not CsrB at exponential growth phase. A. Transcriptional expression of *csrB-lacZ* and *csrC-lacZ* fusions was monitored in wild type (WT) and Δ*crp* mutant genetic backgrounds. β-galactosidase activity was determined for three independent cultures, average and standard error of the mean is presented. **B.** Northen blot detection of CsrB and CsrC was carried out in WT, Δ*crp*, Δ*csrB* and Δ*csrC* mutant genetic backgrounds. 5S RNA was detected as a loading control. In all cases cultures were grown in LB at 37°C up to an OD_{600nm} of 0.4

As mentioned in the introduction, CsrB, CsrC and CsrA are crossregulated. At exponential phase it can be observed that the amount of CsrB is slightly lower in the Δ*csrC* mutant compared to the WT. On the other hand, the amount of CsrC in the Δ*csrB* mutants is higher than in the WT. Taken together, that would indicated that at exponential phase, CsrB represses the expression of *csrC* while CsrC positively regulates the expression of *csrB*, however, further experiments will be required to confirm that observation.

When monitored the amounts of CsrB and CsrC in the Δ*crp* mutant background, it can be observed that *csrC* it is clearly induced in the Δ*crp* mutant background when compared to WT, while no differences are observed in the levels of CsrB. Again our results indicate the differential regulation of CsrB and CsrC by CRP-cAMP at exponential phase (Fig. 43B).

4.4.3 CRP-cAMP-mediated repression of CsrC is partially independent from SirA.

To further assess the putative repression of *csrC* by CRP-cAMP at exponential growth, a chromosomal *lacZ* fusion was generated for *csrC*. As expected, *csrC* chromosomal *lacZ* fusion activity was induced (7-fold) in a Δ*crp* mutant background when compared to WT (Fig. 44A). Furthermore, when the transcriptional expression was assessed at stationary phase, no differences were observed in Δ*crp* mutant background compared to WT (Fig. 44A). According to Ahmer Lab, the two component system Bar-SirA is required for the expression of CsrC (59). In agreement, when SirA was deleted, the overall transcriptional expression of the *csrC-lacZ* fusion was significantly lower (28.18 +/- 0.83 Miller units) than the WT background (308.75 +/- 19.29 Miller units). However, although the overall expression level was lower, deletion of *crp* in a Δ*sirA* deletion background led to similar fold increase in the transcriptional expression of *csrC-lacZ*. This results indicate that i) SirA is required for full expression of CsrC and ii) the CRP-mediated regulation of CsrC is partially independent of the role of SirA on CsrC expression (Fig. 44B).

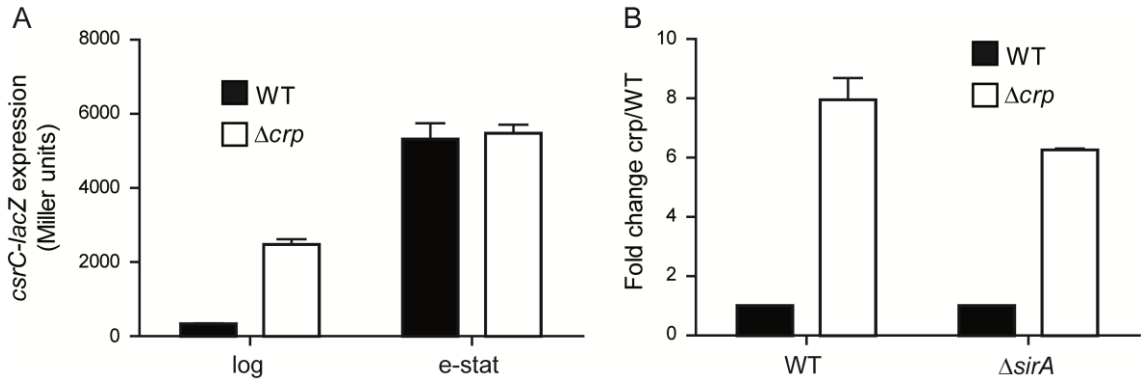

Figure 44. CRP-cAMP mediated repression of *csrC* is partially independent of SirA. A. Transcriptional expression of the chromosomal *csrC-lacZ* fusion was monitored in wild type (WT) and Δ*crp* mutant genetic backgrounds. Cultures were grown in LB at 37°C up to an OD_{600nm} of either 0.4 or 2.0. **B.** Transcriptional expression of the chromosomal *csrC-lacZ* fusion was monitored in WT, Δ*crp,* Δ*sirA* and Δ*crp*Δ*sirA* genetic backgrounds. The transcriptional expression is shown in relative values. In each case the reference (WT) value was set as one. Miller units of the WT background, WT (308.75 +/- 19.29) Δ*sirA* (28.18 +/- 0.83). Cultures were grown in LB at 37°C up to an OD_{600nm} of 0.4. In all cases β-galactosidase activity was determined for three independent cultures, average and standard error of the mean is presented.

4.4.4 The sRNA Spot 42 positively regulates CsrC expression.

As shown in previous sections, CRP-cAMP represses the expression of the sRNA Spot 42 at exponential growth phase. Remarkably, the *spf* gene encoding for Spot 42 is located 700 bp upstream of the gene encoding for CsrC, with only the locus *engB* encoding for a hypothetical protein in between (Fig. 45A). CRP-cAMP seems to be repressing both Spot 42 and CsrC at exponential phase, due to its genomic location, we assessed whether Spot 42 and CsrC are co-transcribed. Total RNA was extracted from cultures grown up to exponential phase (OD_{600nm} 0.4), the pool of RNA was subjected to reverse transcription by using primers within *engB* or *csrC* that let to retrotranscribe from a theoretical spf-engB-csrC transcript (RT primers). As negative control, same samples were processed but by previously inactivating the retrotranscriptase enzyme. This cDNA was subjected to PCR amplification by using the RT primers as reverse primers and primers within *spf*, *engB* or *csrC* as forward primers (Fig. 45A).

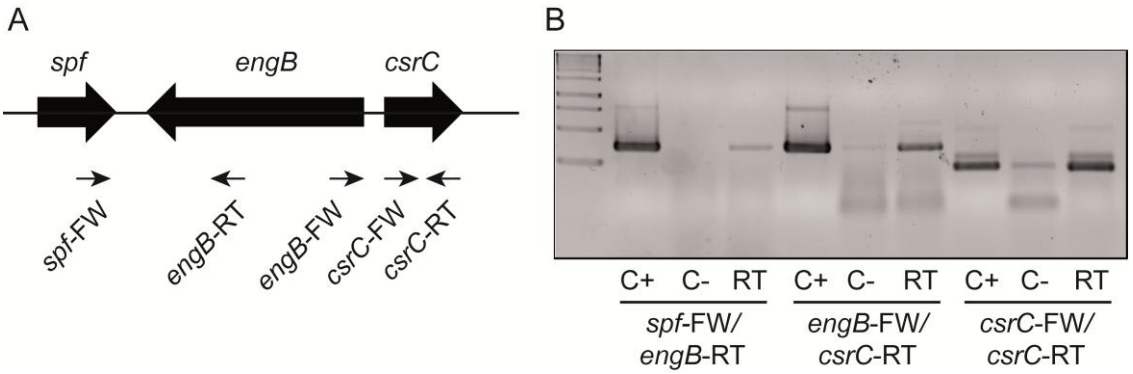

Figure 45. Spot 42 seems to be co-transcribed with CsrC. A. Genomic location of Spot 42 (*spf*) and CsrC, adapted from Salcom genomic browser. **B.** Retrotranscription (RT) of RNA extracted from culture grown up to exponential phase (OD_{600nm} 0.4). The existence of *spf-engB*, *engB-csrC* and *csrC-csrC* transcripts was characterized. As a positive control a PCR from genomic DNA was performed (C+), for the negative control (C-), the retrotranscriptase activity of the polymerase was inactivated.

Remarkably, by using a primer within *spf* as forward primer and a primer within *engB* as reverse primer, a PCR fragment was obtained, indicating that the transcript *spf-engB* exists in the cell. Furthermore, by using a primer within *engB* as forward primer and a primer within *csrC* as reverse primer, a PCR fragment was obtained indicating that the transcript *engB-csrC* exist in the cell. As an internal control, PCR amplification

with two primers within *csrC* was used. The size of the fragment is the expected as indicated by PCR amplification using genomic *Salmonella* DNA as template (lanes labeled as C+) (Fig. 45B). As a negative control, same pair of primers were used to PCR-amplify by using as template the negative control of the RT reaction (lanes indicated as C-). While there is evidence that *spf-engB-csrC* loci might be co-transcribed, further experiments need to be carried out (Fig. 45B).

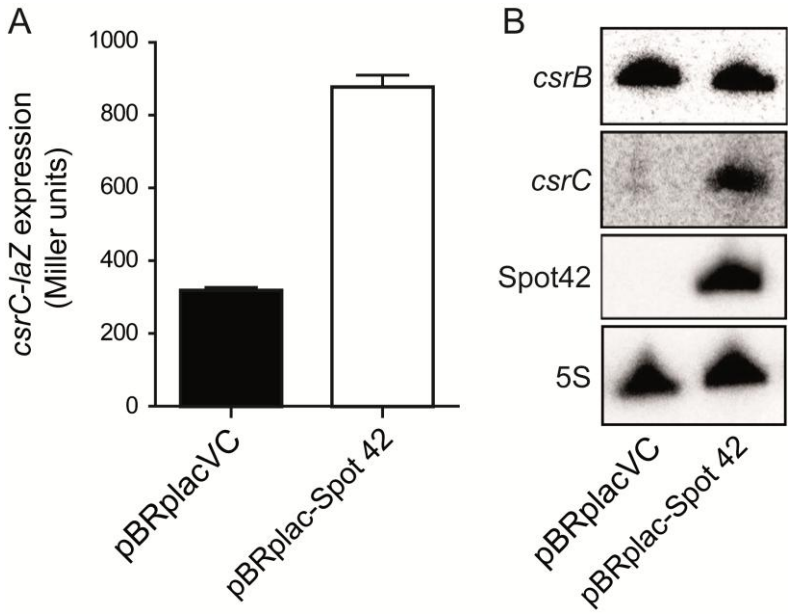

Figure 46. Spot 42 positively regulates the expression of CsrC but not CsrB. A. Transcriptional expression of the chromosomal *csrC-lacZ* fusion upon ectopic expression of the sRNA Spot 42 compared to the strain carrying the control vector (pBRplacVC). β-galactosidase activity was determined for three independent cultures, average and standard error of the mean is presented. **B.** Northen blot detection of CsrB, CsrC and Spot 42 was carried out in strains overexpressing the sRNA Spot 42 (pBRplac Spot 42). 5S RNA was monitored as loading control. In all cases cultures were grown in LB at 37^{o}C up to an OD_{600nm} of 0.4.

Our data indicate that CRP-cAMP represses CsrC at exponential phase, but no CRP-cAMP binding site has been detected in the promoter region of CsrC. Whether CRP-cAMP regulates CsrC through the modulation of the expression of the sRNA Spot 42 was assessed. First, the transcriptional expression of the chromosomal *csrC-lacZ* fusion was monitored upon ectopic expression of the sRNA Spot 42. Interestingly, the expression of *csrC-lacZ* is induced up to 3-fold upon overexpression of Spot 42 when compared to the strain carrying the control vector (Fig. 46A). To confirm the Spot 42-

mediated induction of CsrC, total RNA was extracted from cultures grown up to exponential phase (OD_{600nm} 0.4) and direct CsrC RNA detection was carried out by northern blot. Remarkably, the amount of CsrC is higher in the strain overexpressing Spot 42 compared to the strain carrying the control vector. Furthermore, when CsrB levels were assessed, no accumulation of CsrB was detected upon Spot 42 over expression (Fig. 46B). These results indicated that Spot 42 positively regulates CsrC but not CsrB, indicating that Spot 42 might be involved in the molecular mechanism by behind the CRP-cAMP differential regulation of CsrB and CsrC.

4.4.5 CsrA represses *hilD* expression at exponential phase of growth

As mentioned in previous sections, CRP-cAMP represses the expression of the SPI-1 encoded genes through the modulation at the post-transcriptional level of the SPI-1 master regulator HilD. The induction of *hilD* reported in a Δ*crp* mutant was partially lost by additional deletion of the Spot 42 encoding locus *spf* (Fig. 25A).

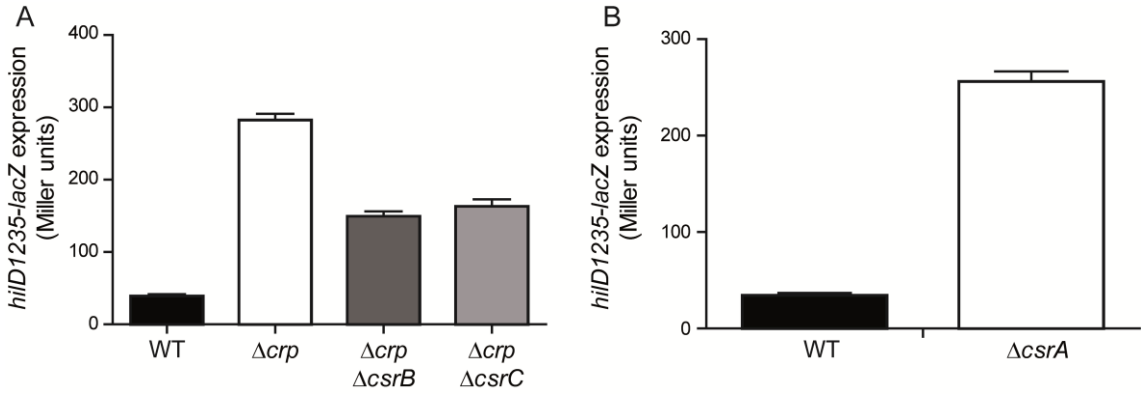

Figure 47. CsrA represses *hilD* expression in exponential growth phase. A. Transcriptional expression of the chromosomal *hilD*1235-*lacZ* fusion was monitored in wild type (WT), Δ*crp*, Δ*crp* Δ*csrB*, Δ*crp* Δ*csrC* mutant genetic backgrounds. **B.** Transcriptional expression of the chromosomal *hilD*1235-*lacZ* fusion was monitored in WT and Δ*csrA* mutant genetic backgrounds. In all cases cultures were grown in LB at 37°C up to an OD_{600nm} of 0.4 and β-galactosidase activity was determined for three independent cultures, average and standard error of the mean is presented.

The factors responsible of the remaining derepression of *hilD* in a Δ*crp* remain elusive. In this section we introduced the CRP-cAMP-mediated repression of *csrC*, which in turns promotes the activity of the known SPI-1 repressor CsrA. So that, the role of the

long non-coding RNA CsrB and CsrC in the CRP-mediated regulation of *hilD* was assessed. Deletion of either *csrB* or *csrC* in the Δ*crp* mutant background led to a partial lost of the derepression of *hilD* detected in the Δ*crp* mutant (Fig. 47A). These results indicate that in addition to Spot 42, CsrC and CsrB might play a role in the CRP-cAMP mediated control of SPI-1 expression through HilD.CsrB and CsrC modulate the activity of the RNA binding protein CsrA. CsrA binds to *hilD* mRNA and promotes its degradation. The transcriptional expression of *hilD*1235-*lacZ* ($HilD^+$, $3'UTR^+$) was assessed upon deletion of Δ*csrA.* Remarkably, the deletion of CsrA at exponential phase led to a derepression of *hilD* (Fig. 47B). Of note, it has to be underlined that the CRP-mediated repression of *hilD* strictly requires of the presence of the *hilD* 3'UTR (Fig. 25), while CsrA seem to act by binding to the 5'UTR of *hilD* (30). Our data indicate that CsrA may somehow affect *hilD* expression by a mechanism involving the 3'UTR. To test that possibility, *hilD* expression studies in a Δ*csrA* strain carrying a *hilD* variant lacking the 3'UTR would be very useful. Regrettably, Δ*csrA* mutant present a growth defect, as it does the strain lacking the *hilD* 3'UTR (31), combination of the two genetic backgrounds lead to a strain with a severe growth defect that could not be used for genetic experiments. However, CRP-cAMP seem to be repressing the expression of SPI-1 at several levels, by modulating Spot 42 regulates *hilD* at post transcriptional level and by modulating CsrC regulates *hilD* at the translational level through CsrA. The role of CRP-cAMP as a major repressor of virulence in *Salmonella* will be further discussed below.

5. DISCUSSION

5. Discussion

The role of CRP-cAMP on the regulation of *Salmonella* virulence has been addressed in this thesis. During the infection process, *Salmonella* requires the expression of a huge number of genes, many of them located in discrete regions of *Salmonella* chromosome, the so-called *Salmonella* Pathogenicy Islands (SPIs). During the first steps of the infection process it is required the ability to invade epithelial cells, which involves expression of a T3SS and many effector proteins. Most of the genes required for cell invasion are encoded within the *Salmonella* pathogenicity island I (SPI-1). The fitness cost associated with SPI-1 gene expression has been studied previously (19). Although SPI-1 expression is crucial for *Salmonella* during infection, it is a burden for its physiology as it has a deleterious effect on the growth rate, as a consequence of presumably being a remarkably energetically demanding process. Accordingly, the expression of SPI-1 genes is expected to be tightly regulated. Most relevant studies on SPI-1 expression control have focused on describing the regulatory pathways devoted to efficiently induce SPI-1 under permissive conditions. As SPI-1 expression affects cell fitness, mechanisms involved in its silencing in non-permissive conditions, for instance in actively growing cells (logarithmic growth phase), are also required to avoid potentially deleterious expression.

We found CRP-cAMP to be a key player in the repression of SPI-1 gene expression under exponentially growing cells. CRP-cAMP is a metabolic sensor that responds to, among other environmental conditions, carbon availability, and promotes changes in the gene expression profile of the cell (116). Coordination of metabolism and stress-related functions is crucial for the evolutionary success of bacterial populations. In pathogenic bacteria, the cross talk between virulence factors, which can be considered within-host stress-related factors, and physiology is crucial for efficient colonization. A sudden shift between the expression of genes involved in active growth and genes involved in adaptation to stress might be required for quick adaptation to changing conditions during the infection process. Secondary messengers such as cAMP, the intracellular levels of which can be quickly altered by the action of both synthetases (adenylate cyclases) and degrading enzymes (phosphodiesterases), provide a rapid response system that can promote sudden changes in the gene expression profile.

Although cAMP has traditionally been described as a metabolism regulator, its role in the modulation of virulence-related functions has been extensively studied in several pathogens, as reviewed by McDonough & Rodriguez (134). In a former report in our research group it was described in *E. coli* that CRP-cAMP represses type 1 fimbriae expression during logarithmic growth phase (135). Other secondary messengers, such as ppGpp, have also been reported to participate in the interplay between cell metabolism and virulence control, in this case by mostly promoting the expression of virulence related functions when cell conounter environmental stress (136). C-di-GMP, a widely distributed second messenger among bacteria, it regulates virulence in some pathogenic bacteria in addition to sessility-motility lifestyle (137).

We describe that CRP-cAMP represses *hilA* transcription under logarithmic growth phase, and consequently maintain low level of expression of SPI-1 T3SS-1 and SPI-encoded effector proteins. CRP-cAMP-mediated *hilA* regulation occurs through the repression of *hilD*, *hilC* and *rtsA*, encoding for the three components of the feed-forward regulatory loop that activates *hilA* expression. Within this triad, a major role has been attributed to the HilD protein (120). Consistently, our results indicate that the CRP-mediated regulation of SPI-1 is also centered on the *hilD* gene. Although it cannot be ruled out that CRP-mediated regulation of SPI-1 genes, as exemplified by *sipC*, might be also mediated by mechanisms involving additional CRP-related regulators, the fact that in absence of *hilD* the regulation vanishes suggests that CRP-mediated regulation of SPI-1 occurs through the modulation of *hilD* levels.

Studies on the transcriptional expression profile of *Salmonella* during infection show that SPI-1 expression is required for initial steps of infection but also is crucial for the establishment of long-term systemic infections (138). It has been shown that HilD mediated a complex crosstalk among virulence associated genetic elements. The role of HilD in the control of *Salmonella* pathogenicity overcomes its major function in SPI-1 expression control since is able to also activate SPI-2 expression - required for survival within macrophages -, and other genes present in different genome locations (20,139). Hauterfort et al. (2008) reported that within different cell types *Salmonella* expresses a differential intracellular transcriptomic signature. It was reported that SPI-1 and SPI-2 genes are simultaneously expressed at late stages of epithelial cells infection, while

within macrophage-like cells SPI-1 genes are downregulated and SPI-2 genes are induced (140). Interestingly, the replication state of intracellular *Salmonella* cells and the expression of SPI-2 genes in macrophages seem to promote the polarization of macrophages to anti-inflammatory M2 state. So, low replication rate lead to pro-inflammatory M1 state in macrophages, while high replication within macrophages, which depends on the SPI-2 expression, promotes polarization to anti-inflamatory M2 state (141).

CRP-cAMP is a regulator prompted to mediate rapid response to environmental changes and our data indicate that is relevant for the HilD-mediated regulation of virulence in response to environmental conditions that *Salmonella* encounters through the infection process.

Interestingly, the CRP-mediated regulation of *hilD* does not occur at the transcriptional initiation level. Our data clearly indicate that the target of the CRP-mediated regulation of SPI-1 is the 3'UTR of the *hilD* gene, a motif that is essential for defining the steady-state level of *hilD* mRNA (31). In the absence of the *hilD* 3'UTR the upregulation of SPI-1 expression detected in the Δ*crp* mutation vanished, highlighting the biological relevance of this newly described regulatory motif for *hilD* expression.

These results suggest that CRP-cAMP modulates *hilD* expression at the post-transcriptional level through the long 3'UTR (310 bp) of the *hilD* gene. Post-transcriptional regulation seems to be an extensively used mechanism to regulate virulence in bacteria (142,143). A feature of gene that is regulated at the post-transcriptional level is to carry long untranslated regions. The role of the 5'UTR in gene expression has been extensively studied. It should be noted that SPI-1 regulators, such as *invF*, *hilA* and *hilE*, carry long 5'UTR suggesting that those genes may be targets of post-transcriptional regulation of SPI-1 expression (37,144,145). Recently, the involvement of the 3'UTRs in post-transcriptional regulation has raised attention, although it is yet poorly understood. It has been reported that in *Staphylococcus aureus*, one-third of the bacterial RNA carry 3'UTRs longer than 100 nt, being the long 3'UTR of *icaR* mRNA a regulatory motif by interacting with its own 5'UTR (146). In *Salmonella*, it has been suggested that 3'UTR regions might function as a reservoir of

sRNA (71). Of note, a sRNA is in fact annotated in the *hilD* 3'UTR as stnc600 although it could not be detected by northern blot (147). Whether the *hilD* 3'UTR is processed in some conditions to generate a sRNA or whether the *hilD* mRNA exist in more than one specie (+UTR, -UTR) remains elusive.

The *hilD* 3'UTR constitutes a silencing module, since its deletion causes significant *hilD* upregulation. Although the molecular mechanism has not yet been elucidated, its observed Hfq dependency suggests that sRNA may be crucial for *hilD* 3'UTR function (31). The CRP-mediated repression of *hilD* requires both the presence of the *hilD* 3'UTR and Hfq, indicating that CRP regulates *hilD* expression through the *hilD* 3'UTR presumably in a sRNA-mediated manner.

The sRNA Spot 42 has been reported to participate in CRP-mediated gene regulation in *E. coli*. Moreover, its own expression is repressed by CRP-cAMP (148). Our data demonstrate that expression of Spot 42 in *Salmonella* is also repressed by CRP-cAMP. It also suggests the involvement of Spot 42 in the CRP-mediated regulation of *hilD* expression, since upregulation of *hilD* expression was observed when overexpressing Spot 42. Remarkably, Spot 42-mediated regulation of *hilD* requires the presence of the *hilD* 3'UTR. The increase in HilD levels after increasing the intracellular levels of Spot 42 was further corroborated by the induction of *sipC* expression. Moreover, the induction of *hilD* in a *crp*$^{-}$ strain was diminished in the absence of Spot 42. The fact that the absence of Spot 42 did not completely abolish the upregulation caused by Δ*crp* mutation indicates that other factors may also be involved. It should be noted that these other factors, if they exist, will also act through the *hilD* 3'UTR.

The sRNA chaperone Hfq and the major endonuclease RNAse E seem to play a major role in the *hilD* 3'UTR mediated regulation of *hilD*. Consistently, the derepression of *hilD* by Spot 42 requires of the presence of Hfq, RNAse E and the *hilD* 3'UTR (Fig. 28). While it has been clearly shown that Hfq binds both to *hilD* 3'UTR and Spot 42 (Fig. 29), no direct evidence of direct involvement of RNAse E has been obtained. Nonetheless, our in vivo data let us conclude that Spot 42 does not induce *hilD* expression in a background lacking a functional RNAse E. Although Casadesus lab claims that within the *hilD* 3'UTR there are several putative RNAse E cleavage sites, it has to be

highlighted that no RNAse E cleavage site has been reported in the *hilD* 3'UTR (31,131). Recently an *in vivo* library of RNAse E cleavage sites in *Salmonella* has been reported (131). Shortly, *Salmonella* was grown up to stationary phase and temperature shifted to 44^{o}C in order to inactivate the temperature sensitive mutant of RNAse E TS (*rne*-3071) (149), RNA was extracted and subjected to deep sequencing. The approach was coined TIER-seq for transiently inactivating endonucleases followed by RNA seq. When RNA-seq is performed, based on the phosphorylation state at the 5' end one can discriminate between processed transcripts and true transcripts. For TIER-seq, transcripts of interest are the ones that are processed in the WT strain but not in the RNAse E TS mutant, indicating that for its processing RNAse E is required. The overall expression data, in agreement with our data and Casadesus lab data, showed that *hilD* accumulates in absence of a functional RNAse E. However, when looking for the RNAse E cleavage sites, no reads are detected in the *hilD* 3'UTR in the WT strain compared to the RNAse E TS strain. Intriguingly, when looking at the raw data, cleavage sites are detected in the *hilD* 3'UTR in the RNAse E mutant but not in the wild type strain. The biological significance of this feature remains elusive. It has to be highlighted that the TIER-seq experiments were performed at stationary phase, which are permissive conditions for SPI-1 expression. It cannot be ruled out whether RNAse E cleaves *hilD* 3'UTR under non-permissive conditions, when levels of *hilD* mRNA should be kept low in the cell.

The gold standard for demonstration of RNA-RNA interactions is the generation of point mutations that abolish regulation, and subsequent reconstitution of the regulation by generating complementary point mutations that again restore the RNA-RNA interaction. Our data indicate that the effect of Spot 42 on SPI-1 expression is mediated by a base pairing mechanism, involving the unstructured region III of Spot 42. However, complementary mutations did not restore the interaction (Fig. 34). Alternatively, based on further bioinformatic predictions, triplet nucleotide substitutions were generated in the chromosomal *hilD* 3'UTR. Of note, the triplet mutants generated show differential basal activity (Fig. 37). Remarkably, a triplet mutation within the 3'UTR (*hilD* 3'UTR mut7) abolishes the Spot 42 mediated regulation of *hilD* (Fig. 36). However, this mutation could not be complemented by

modification of the putative interacting nucleotides within Spot 42 sequence (Fig. 37). In fact, according to our *in vitro* data that we will discuss below, Spot 42 does not interact with this region. That raised the question, why the mutation *hilD* 3'UTR mut 7 abolishes Spot 42 mediated regulation of *hilD*. It has been previously reported that the first 100 nt of the *hilD* 3'UTR plays the major role on the *hilD* 3'UTR mediated regulation of *hilD* (31). We have shown that in fact, the nucleotidic sequence between position 71 and 100 plays a major role in the mechanism by which the *hilD* 3'UTR causes a downregulation of *hilD* expression (Fig. 38). Interestingly, hilD 3'UTR mut7 introduce changes within that sequence.

In another project out of the scope of this thesis (annex 3), we assessed the role of the transcriptional elongation factors GreA and GreB in the regulation of SPI-1 (27). At stationary phase, SPI-1 permissive conditions, the Gre factors regulate SPI-1 expression by modulating the *hilD* expression levels. To do so, Gre factors interact with the secondary channel of the RNA polymerase to rescue the polymerase from backtracking due to a transcriptional pause occurring in the first 100 nt of the *hilD* 3'UTR. In fact, our data suggest that the transcriptional pause occurs within the 20 nucleotides mentioned before between position 71 and 100 of the *hilD* 3'UTR. As the expression of SPI-1 at exponential phase is already low, there is no difference in SPI-1 expression when comparing a wild type strain and a Δ*greA*Δ*greB* strain. Interestingly, when Spot 42 is overexpressed at exponential phase in a background lacking the Gre factors, it is unable to induce the expression of *hilD*. The molecular mechanism by which transcriptional pauses rescue regulate gene expression it is not well understood. We have shown that Gre factors mediated regulation of *hilD* partially requires the presence of the sRNA chaperone Hfq and the endonuclease RNAse E (27). Transcriptional pauses might be a source of structure variation that will lead to allow or not interaction with non-coding RNA. At exponential phase, when *hilD* expression is silenced, Gre factors do not play an evident role on *hilD* expression, but its absence interferes with the ability of Spot 42 to derepress *hilD* expression. One may propose a mechanism by which the transcriptional pause rescued by Gre factors led to a specific folding of *hilD* 3'UTR that allow Spot 42 direct interaction, while in absence of Gre factors, the *hilD* 3'UTR fold differently and does not allow Spot 42 interaction. Taking

the results together, one can hypothesize that *hilD* 3'UTR mut7, located in the same region where the transcriptional pause occurs, abolished Spot 42 mediated *hilD* derepression by affecting the transcriptional pause. In fact, one can observe that *hilD* 3'UTR mut7 has a lower SPI-1 expression, as monitored by determination of *sipC-lacZ* expression, compared to the wild type background (Fig. 37). The role of this modification on Spot 42 mediated derepression of *hilD* might be due to interference with the role of Gre factors in the *hilD* 3'UTR rather than direct interference with Spot 42 base pairing within the *hilD* 3'UTR.

In vitro approaches led us to demonstrate direct base pairing between Spot 42 and the *hilD* 3'UTR, being the first example of a trans-encoded sRNA positively regulating an mRNA through direct base pairing with its 3'UTR region. To our knowledge, there is only one example of sRNA that might be regulating through interaction with the 3'UTR. GadY, a sRNA cis-encoded in the 3'UTR of GadX seem to positively regulate *gadX* through interaction with its 3'UTR (70).

Spot 42 physically interacts within the second half of the *hilD* 3'UTR (UTR^R). This fragment includes the two Hfq binding sites reported by CLIP-seq (94). It also includes the putative binding site involved in base pairing with Spot 42 first predicted by IntaRNA by using as input target RNA the whole *hilD* 3'UTR sequence (yellow in Fig. 33). As stated in the results section, complementation of the mutations in the unstructured region III of Spot 42 in the *hilD* 3'UTR did not lead to reconstitution of regulation. As the *in vitro* data confirm that Spot 42 interact with a fragment of the *hilD* 3'UTR including that region, we believe that two major possibilities may explain the lack of complementation. First, it can be that triplet modification might lead to structural changes that cannot be reconstituted by complementary mutations. Or second, the Spot 42 sRNA interacts within the second half (UTR^R) of the *hilD* 3'UTR but in another region. Structural probing experiments support the second possibility. As shown in the results section, Spot 42 seem to interact within position G102-G134 of the second half of the *hilD* 3'UTR, far downstream of the putative interaction site predicted by IntaRNA. Further experiments generating point mutations within that region and look for loss of regulation by Spot 42 are required, as well as reconstitution of regulation by modification of residues within Spot 42 sequence.

The residues required within the *hilD* 3'UTR to interact with Spot 42 could be also identified by genetic approaches. We have shown that Spot 42 can regulate *hilD* 3'UTR regardless of its genetic location. A *gfp* gene fused to *hilD* 3'UTR it is positively regulated upon ectopic over expression of Spot 42. So, technically, random mutagenesis performed on this construct would allow us to look for lost-of-function *hilD* 3'UTR mutants which would lead us to infer the interaction site of Spot 42. Regrettably, as the molecular mechanism under study occurs at logarithmic growth phase, random mutagenesis with selection on plates cannot be used as a read out. Wagner lab has developed a methodology based in FACS to use saturation mutagenesis to look for base pairing residues of sRNA interaction within target 5'UTR which technically could be applied for further characterization of *hilD* 3'UTR-Spot42 interaction in logarithmic phase grown cells (150). However, the slight induction of *gfp-hilD* 3'UTR upon overexpression of Spot 42 it is not sufficient to look for lost-of-function mutants sorted by flow citometry. The fact that Spot 42 has only a slight positive effect on *hilD* 3'UTR when fused to *gfp* might be argued by the importance of the positive autoregulation of *hilD* expression. In fact, when Casadesus lab assess the effect of the *hilD* 3'UTR on *hilD* expression by using a strain carrying a *hilD* gene with a constitutive promoter, they can observe that the overall effect of *hilD* 3'UTR on *hilD* expression is smaller than when the *hilD* promoter is present (31).

Our findings allow us to propose a model on the Spot 42-mediated derepression of *hilD*. Under non-permissive conditions, there is basal low level expression of *hilD*. However, the presence of the long 3'UTR in the *hilD* transcript would confer greater susceptibility to degradation, in a polynucleotide phosphorylase (PNP) and RNAseE dependent manner (10). Consequently, even being transcribed no significant levels of HilD protein will be found (Fig. 48 panel I). This silencing mechanism is further supported by CRP-cAMP, which maintains low levels of Spot 42 sRNA in the cell. Enviromental and/or physiological conditions may change, requiring triggering expression of the SPI-1 gens. Expression of Spot 42 occurs and upon binding directly to the 3'UTR, Spot 42 would protect the RNA from degradation in an Hfq-dependent manner, thereby inducing HilD protein expression. As HilD is under a positive feed-forward loop, expression of some copies of *hilD* amplifies the final output by increasing

its own transcription (Fig. 48 panel II). In this scenario, a drop in the level of CRP-cAMP in response to physiological/environmental alterations would trigger *hilD* expression by causing derepression of Spot 42, promoting *hilD* mRNA stability, allowing HilD protein expression and positive feed forward loop on *hilD* expression.

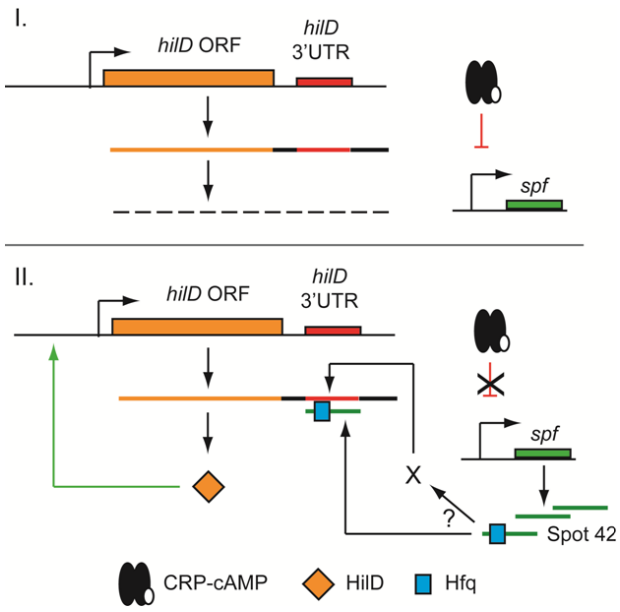

Figure 48. Spot 42-mediated derepression of *hilD* model.

During infection, *Salmonella* encounters a complex environment, with a wide range of environmental signals that affect virulence expression. How *Salmonella* integrates the different pathways reported to regulate *hilD* expression remains elusive.

sRNA seem to play a relevant role in the regulation of virulence in *Salmonella*. The sRNA SgrS regulates the expression of the SPI-1 effectors *sopD*, interestingly it is also involved in metabolic regulation as it plays a role in phosphosugar stress. SgrS downregulates *ptsG*, a major glucose transporter, and positively regulates *yigL*, a sugar phosphatase (151,152). In general, the role of sRNA is to fine-tune gene expression which in occasion difficult the assessment of its role on the cell by using standard phenotypic assessment. Dual-RNA seq of *Salmonella* infecting cells allowed the identification of the sRNA PinT as a major regulator of virulence in *Salmonella*. The sRNA stnc404 renamed as PinT for PhoP-activated sRNA, it is induced up to 100-fold upon invasion of *Salmonella* cells (84). The authors characterize the RNA pool from both host and bacterial cells at different time points during an invasion assay of

epithelial cells by *Salmonella*. Upon invasion, PinT it down regulates the expression of the two SPI-1 effector proteins SopE and SopD. The expression of PinT is regulated by the two component system PhoP-PhoQ, and it seems to be induced when SPI-2 genes are induced. Interestingly, the role of PinT during invasion seems to be through the transcriptional factor CRP-cAMP. PinT represses the expression of SPI-2 genes in intracellular bacteria, but this regulation is abolished upon deletion of Δ*crp*. How CRP-cAMP regulates SPI-2 genes in *Salmonella* remains elusive and whether the described regulatory pathway in this thesis plays a role in the PinT mediated regulation of virulence remains to be studied. An interesting feature of PinT, is its genomic location. It is encoded approximately 2 kb upstream the SPI-1 transcriptional regulator RtsA. As mentioned previously, SPI-1 is regulated by three major transcriptional factors, HilD, HilC and RtsA. HilD and HilC are encoded within the SPI-1 while RtsA is encoded in another genomic location, resembling and horizontally transferred island. While the authors do not discuss on that (84), it is particularly interesting how *Salmonella* evolved to generate molecular mechanism to cross regulate the horizontally transferred genes of its genome. Altogether, the described role of the CRP-regulated Spot 42 derepressing *hilD* and PinT regulating SPI-2 through CRP-cAMP puts CRP-cAMP as a major regulator of virulence in *Salmonella* by mechanisms involving non-coding RNA.

Moreover, the role of CRP-cAMP in the regulation of the non-coding RNA *csrB* and *csrC* highlights its relevance as a component in post-transcriptional mediated regulation molecular mechanism. In this thesis, we showed that CRP-cAMP represses the expression of *csrC* but not *csrB* in exponentially growing cells. While the transcriptional activator SirA seems to be required for full activation of csrC, the CRP –cAMP mediated repression seem to be independent of SirA, as a similar fold induction in the Δ*crp* mutant can be observed in both $sirA^{+}$ and $sirA^{-}$ backgrounds. It is not known whether CRP-cAMP directly acts on *csrC* leader or it rathers regulate *csrC* through an indirect mechanism, although there is no consensus sequence of CRP-cAMP binding in the promoter region of *csrC.*

We have shown that *csrC* is encoded in a locus 700 bp downstream of *spf* encoding for Spot 42. Again, genomic location seems to play a role. We have shown that Spot 42

induces expression of *csrC* upon ectopic overexpression. However, we have also some indications that Spot 42 and *csrC* might be co-transcribed. As mentioned before, the cleavage map of RNAse E on *Salmonella* transcriptome has been elucidated (131), and Spot 42 has one RNAse E cleavage site on its sequence while csrC has up to six RNAseE cleavage sites on its sequence (131). One may propose a model where at low levels of CRP-cAMP, Spot 42 is co-transcribed as a single RNA molecule with *csrC*. Upon processing of the RNA, Spot 42 can bind to CsrC and protect the long non coding RNA for further processing by RNAse E. Independently of the molecular mechanism by which CRP-cAMP affects *csrC*, it is notable that a crosstalk regulation between CRP-cAMP and CsrA exist. It has to be underlined, that the only role described for *csrB* and *csrC* is the modulation of CsrA activity, CsrB and CsrC sequester free CsrA and consequently downregulates its activity. CsrA has been described to repress SPI-1 expression through interaction with the *hilD* leader. Bustamante lab has shown that CsrA in fact bind to *hilD* mRNA (30). The observation that CsrA represses SPI-1 is supported mainly for the repression of *hilD* observed upon overexpression of CsrA. Upon deletion of *csrA* only a slight and inconsistent induction of *hilD* can be observed. It should be mentioned that all those experiments were performed using stationary phase bacterial cultures. Having in consideration that CsrA is acting presumably as a repressor, genetic analysis performed under permissive conditions are perhaps not suitable for proper characterization. Accordingly, when the authors explored the molecular mechanism using logarithmic cultures they report that CsrA affects negatively *hilD* mRNA stability (30). It seems that CsrA acts as a repressor of SPI-1, but consistent with its role as a repressor, it acts at non-permissive conditions. When assessed the expression of *hilD* in a Δ*csrA* mutant at exponential phase, a clear induction could be observed.

The induction of *hilD* observed in the Δ*crp* mutant was only partially lost upon deletion of the sRNA Spot 42 (Δ*spf*). The involvement of *csrC* and therefore CsrA in the CRP-cAMP-mediated regulation might be the answer to the remaining induction of *hilD* in the Δ*crp* mutant in absence of Spot 42. In absence of CRP-cAMP, the increased level of CsrC will sequester CsrA promoting *hilD* expression. However, it needs to be highlighted that the CRP-cAMP-mediated regulation of *hilD* requires the presence of

the *hilD* 3'UTR. As it has been shown in a Δ*crp* mutant background the deletion of Δ*csrB* or Δ*csrC* leads to partial lose of the observed *hilD* induction. It would be interesting to monitor the upregulation of *hilD* in a Δ*crp* background in absence of both Spot 42 and CsrB/CsrC. Regrettably, the strain Δ*crp* Δ*csrB* Δ*csrC* Δ*spf* could not be technically obtained for reasons that escape to our understanding. Anyhow, if part of the CRP-cAMP mediated regulation is due to modulation of the level of free CsrA, that would indicate that the downregulation of *hilD* by CsrA requires of the presence of the *hilD* 3'UTR. However, the use of *csrA* deletion mutants in combination with strains lacking the *hilD* 3'UTR could not be used for genetic experiments. The growth defect of a Δ*csrA* mutant in combination with the growth defect of a strain lacking the *hilD* 3'UTR leads to the generation of a strain with severe growth defect that easily generates suppressors. The use of strains overexpressing either *csrB* or *csrC* in substitution of the Δ*csrA* mutant might allow the study of the involvement of *hilD* 3'UTR on the CsrA mediated repression of *hilD.*

Overall data in this thesis let us to underline the importance of post-transcriptional regulation of *hilD* by sRNA through the *hilD* 3'UTR. We have introduced a new key player in SPI-1 regulation, being important in the silencing of SPI-1 expression under non-permissive conditions in a CRP-dependent manner. CRP-cAMP as a regulator of non-coding RNA expression that post transcriptionally regulates *Salmonella* virulence.

6. CONCLUSIONS

6. Conclusions

- CRP-cAMP represses *hilA* expression at exponential phase (non-permissive conditions for SPI-1 expression) and acts as an activator at stationary phase (permissive conditions for SPI-1 expression).
- CRP-cAMP mediated repression of *hilA* causes a concomitant attenuation in the expression level of SPI-1 encoded effector proteins.
- CRP-cAMP regulation of SPI-1 during logarithmic growth phase occurs upstream of HilA by repressing *hilD*, *hilC* and *rtsA* expression.
- CRP-cAMP repression of SPI-1 is mediated by regulating *hilD* expression at the post transcriptional level through the *hilD* 3'UTR.
- CRP-cAMP mediated regulation of *hilD* requires, in addition to the *hilD* 3'UTR, the sRNA chaperone Hfq and the major endonuclease RNAse E.
- CRP-cAMP represses the expression of the sRNA Spot 42 at exponential phase.
- Spot 42 positively regulates *hilD* expression at exponential growth phase.
- Spot 42 mediated derepression of *hilD* at exponential phase requires of the presence of the *hilD* 3'UTR, the sRNA chaperone Hfq and the major endonuclease RNAse E.
- Spot 42 positively regulate *hilD* 3'UTR levels regardless of its genomic location.
- Spot 42 and the *hilD* 3'UTR region physically bind to Hfq.
- Spot 42 unstructured region III is required for the regulation of *hilD.*
- Spot 42 physically interacts with the last 150 nt of the *hilD* 3'UTR.
- CRP-cAMP represses *csrC* but not *csrB* expression at exponential phase.
- Spot 42 positively regulates the expression of *csrC***.**

7.SUMMARY IN SPANISH

7. Summary in Spanish

Salmonella enterica serovar Typhimurium es un bacteria Gram negativa, patógena, causante de gastroenteritis en humanos. La infección empieza normalmente mediante la ingestión de alimentos contaminados. Al llegar al intestino, *Salmonella* es capaz de infectar las células del epitelio intestinal. Para ello, *Salmonella* expresa una batería de genes relacionados con la virulencia. Mediante la secreción de proteínas efectoras, *Salmonella* es capaz de modificar el citoesqueleto de las células del epitelio y permite la internalización de las células de *Salmonella* en el epitelio del hospedador. *Salmonella* es capaz de sobrevivir y replicarse dentro de la célula, en unos compartimentos membranosos citoplasmaticos del huésped llamadas SCVs del inglés *Salmonella containing vacuoles*.

Los genes necesarios tanto para la invasión de las células del epitelio intestinal como para la supervivencia en el huésped están codificados en regiones genómicas concretas llamadas islas de patogenicidad. Estas islas de patogenicidad se han adquirido mediante mecanismos de transferencia génica horizontal (HGT) entre bacterias.

Salmonella codifica para almenos cinco islas de patogenicidad, llamas SPIs del inglés *Salmonella* Pathogenicity Island. Los genes necesarios para la invasión de las células del epitelio intestinal están codificados en la isla de patogenicidad I (SPI-1) mientras que los genes necesarios para la supervivencia dentro de las células del hospedador están codificados en la isla de patogenicidad II (SPI-2). La función de los genes codificados en las islas 3, 4 y 5 está menos estudiado y juegan un papel secundario en el proceso de infección.

La isla de patogenicidad I está regulada jerárquicamente. En la cima de la regulación se encuentran tres reguladores transcripcionales HilD, HilC y RtsA, estos se autoregulan positivamente y se regulan positivamente entre si. Los tres regladores activan la expression del regulador transcripcional HilA. HilA es el principal activador de la expression de los genes codificantes para las proteínas efectoras. HilA activa los operones *prg*/*org* y *inv*/*spa* directamente mediante la union a sus promotores y activa *sic*/*sip* mediante la activación transcripcional de otro regulador, InvF. Las proteínas

efectoras son las encargadas de interactuar con las células huésped y promover la invasión por parte de *Salmonella*. Es por eso, que la delección de *hilA*, como regulador de los genes codificantes para células efectoras, en relación al fenotipo de invasión es equivalente a la delección de toda la SPI-1.

Los genes codificados en la SPI-1 están fuertemente regulados. SPI-1 codifica para 40 genes que son secuencialmente expresados en diferentes estadios de la invasión. Se ha demostrado, que al ser un proceso que conlleva un elevado gasto energético, la expresión de la SPI-1 conlleva un efecto negativo sobre el *fitness* de la célula bacteriana. En condiciones de laboratorio, la expresión de la SPI-1 sigue un claro patrón dependiente de la curva de crecimiento bacteriano. En la fase logarítmica de crecimiento la expresión de SPI-1 es baja, en esta tesis consideradas condiciones no permisivas de expresión. Por otro lado, al entrar en la fase estacionaria de crecimiento, los genes de SPI-1 están fuertemente inducidos, en esta tesis consideras condiciones permisivas de expresión.

Se ha descrito la implicación de muchos reguladores en el control de expresión de la SPI-1. Principalmente se han estudiado mecanismos moleculares que llevan a la activación de los genes de SPI-1 en *Salmonella*. Pero como hemos mencionado previamente, al suponer tan elevado coste energético, mecanismos de represión de la expresión de SPI-1 en condiciones no permisivas deben tener una importancia capital para la supervivencia de *Salmonella*.

Entre los muchos reguladores descritos que afectan a la invasión, en esta tesis nos hemos centrado en el regulador metabolico CRP-cAMP.

CRP-cAMP es un factor de transcripción ampliamente conocido por su función global en la regulación del metabolismo. Como es sabido, CRP-cAMP está implicado en la regulación por catabolito. Regula la expresión de vías alternativas del metabolismo en ausencia del azúcar más fácilmente metabolizable, la glucosa. Siendo un regulador principalmente descrito en metabolismo, parece estar implicado en la regulación de otros procesos celulares tales como la virulencia. En lo que se refiere a *Salmonella*, parece que la delección de este regulador genera una cepa de *Salmonella* que es incapaz de infectar. El mecanismo molecular por el cual esto ocurre parece implicar los

genes codificados en la SPI-1, ya que se ha mostrado que en ausencia de CRP-cAMP, en condiciones permisivas de infección, los genes de SPI-1 se encuentran reprimidos.

Para nuestra sorpresa, observamos que en ausencia de CRP-cAMP la expresión de SPI-1 se encuentra inducida en la fase logarítmica de crecimiento, es decir, en condiciones no permisivas de expresión de SPI-1. Parece que CRP-cAMP juega un papel diferencial a lo largo de la curva de crecimiento en lo que a expresión de SPI-1 se refiere.

La expresión del factor de transcripción HilA, al igual que ocurre con el resto de genes de la SPI-1, se encuentra poco expresado en la fase logarítmica de crecimiento y se induce en fase estacionaria. Observamos que tanto a nivel de transcripción como a nivel de proteína, la expresión de HilA se encuentra inducida en fase exponencial en ausencia de CRP-cAMP, mientras que en fase estacionaria se encuentra reprimida en ausencia de CRP-cAMP. Es decir, CRP-cAMP actúa como represor de la SPI-1 en fase exponencial (condiciones no permisivas), y como activador en fase estacionaria (condiciones permisivas). Como hemos mencionado, se conocen muchos de los mecanismos moleculares para la activación/represión de SPI-1 en condiciones permisivas pero poco se sabe de mecanismos de represión en condiciones no permisivas. Es por ello que esta tesis se ha centrado en la caracterización del mecanismo molecular por el cual CRP-cAMP reprime HilA y por extensión la SPI-1 en fase logarítmica de crecimiento.

Como ya hemos comentado, la expresión de HilA regula los niveles de expresión de las proteínas efectoras de la SPI-1. Para monitorizar si la represión mediada por CRP-cAMP de HilA tiene un efecto fisiológico relevante, la expresión de las proteínas efectoras en ausencia de CRP-cAMP fue estudiada. Observamos que como ocurre con HilA, en ausencia de CRP-cAMP, la producción y secreción de proteínas efectoras se encuentra inducida, indicando que CRP-cAMP juega un papel funcional sobre la represión de SPI-1.

CRP-cAMP reprime HilA en fase exponencial, para discernir si el efecto de CRP-cAMP sobre la expresión de SPI-1 es directo sobre HilA o por el contrario ocurre aguas arriba de la red de regulación de la SPI-1, se estudió la expresión de los tres reguladores que controlan la expresión de *hilA*, HilD, HilC y RtsA. Se observó que en ausencia de CRP-

cAMP la expresión transcripcional de los tres reguladores *hilD*, *hilC* y *rtsA* determinada mediante qRT-PCR se encuentra inducida. Mediante experimentos de epistasia se observó que para la inducción de *hilA* en ausencia de CRP-cAMP se requiere de la presencia de HilC y HilD pero no de RtsA. El análisis de la expresión de *hilC* y *hilD* en ausencia del otro regulador en un fondo genético mutante para CRP llevó a determinar que para la inducción de *hilC* que se observa en ausencia de CRP-cAMP se requiere de la presencia de HilD. Por el contrario, para la inducción de *hilD* que se observa en ausencia de CRP-cAMP no se requiere de la presencia de HilC. Indicando que CRP-cAMP reprime la SPI-1 en fase logarítmica de crecimiento a través del principal regulador de la SPI-1, HilD.

Al analizar el efecto de CRP-cAMP sobre la expresión de *hilD* mediante el uso de fusiones génicas se observa una característica interesante. Usando una fusión transcripcional al inicio de la pauta de lectura del mRNA de *hilD*, observamos que la expresión no se ve afectada por la presencia/ausencia de CRP-cAMP. Siendo CRP-cAMP un factor de transcripción, resulta interesante que la regulación de *hilD* no ocurra a nivel de inicio de transcripción. Al generar la fusión al final de la pauta de lectura para la proteína HilD, por lo tanto recuperando la auto-regulación positiva que tiene HilD sobre su propio promotor, tampoco observamos inducción de la expresión en ausencia de CRP-cAMP.

Una característica interesante del gen *hilD* es que posee una región inusualmente larga en el extremo 3' del mRNA (3'UTR, del inglés 3' untranslated region), esta región parece tener un efecto negativo sobre la expresión global de *hilD*. En estirpes donde la 3'UTR es deleccionada se observa una mayor expresión de *hilD* y por lo tanto de la SPI-1. Es por esto, que se genero otra fusión transcripcional en *hilD* que incluyera la presencia de la 3'UTR en el mRNA que se generaba. Sorprendentemente, en este caso si se observa inducción de la expresión de *hilD* en ausencia de CRP-cAMP, lo que finalmente corroboraba los datos previamente obtenidos mediante qRT-PCR. Es remarcable que solo en la fusión transcripcional que presenta la región 3'UTR del mRNA de *hilD* se observe la inducción en ausencia de CRP-cAMP. Esto llevaba a pensar que la regulación de CRP-cAMP sobre *hilD* no ocurría a nivel de inicio de transcripción sino que por otro lado ocurría a nivel post-transcripcional, mediante un mecanismo

que requería de la presencia de la región *hilD* 3'UTR. Esta observación quedó confirmada a nivel de proteína mediante la generación de construcciones que permitían la detección de HilD unida a un epítopo tanto en presencia como en ausencia de la *hilD* 3'UTR. Cabe añadir, que cuando se analizó la expresión de genes codificantes para proteínas efectoras de la SPI-1 se observó que estos genes solo se encontraban reprimidos por CRP-cAMP en la fase exponencial de crecimiento cuando la región *hilD* 3'UTR estaba presente. Indicando de nuevo que la regulación de la SPI-1 a través de HilD y mediada por CRP-cAMP ocurría a nivel post transcripcional y requería de la región *hilD* 3'UTR.

Previamente, se había descrito por parte del laboratorio del Prof. Josep Casadesús que la regulación de *hilD* a través de su región 3'UTR estaba mediada por la chaperona de RNAs pequeños no codificantes Hfq y por la endonucleasa RNAsa E. El requerimiento de Hfq para la regulación a través a de la *hilD* 3'UTR indicaba que posiblemente RNAs no codificantes estaban implicados en el mecanismo molecular por el que la secuencia 3'UTR ejercía su efecto en la expresión de *hilD*. Para la regulación aquí descrita de CRP-cAMP sobre *hilD*, observamos que en ausencia de Hfq no se detectan diferencias de expresión en *hilD* en presencia/ausencia de CRP-cAMP. Indicando, que el mecanismo molecular por el cual CRP-cAMP regula *hilD* a nivel post transcripcional podría estar mediado por RNAs pequeños no codificantes (sRNAs, del inglés small RNAs)

Se han descrito sRNAs que están bajo el control transcripcional de CRP-cAMP, entre ellos el sRNA Spot 42. En *Escherichia coli* este sRNA se encuentra reprimido por CRP-cAMP. En esta tesis se observó que de manera similar, este sRNA se encuentra reprimido por CRP-cAMP en la fase exponencial de crecimiento en *Salmonella*. La delección de Spot 42 en combinación con la ausencia de CRP-cAMP permitió observar que la inducción de *hilD* se veía parcialmente disminuida, indicando que Spot 42 juega un papel en el mecanismo por el cual CRP-cAMP afecta la expresión de *hilD*.

La sobreexpresión de Spot 42 induce la expresión de *hilD*. Es interesante observar que esta inducción de *hilD* solo se produce cuando la región *hilD* 3'UTR está presente. La inducción por parte de Spot 42 se puede observar también cuando se estudian los niveles de proteína HilD, o cuando se determina el nivel de expresión de los genes bajo

control de HilD. Todos los resultados obtenidos sugerían que Spot 42 actuaba a través de la *hilD* 3'UTR.

El efecto de Spot 42 sobre *hilD* 3'UTR fue desconectado de su localización genómica como parte del mRNA de *hilD*. Spot 42 es capaz de regular positivamente *hilD* 3'UTR independientemente de su localización cromosómica. Spot 42 regula positivamente una construcción donde la secuencia *hilD* 3'UTR fue fusionada al extremo 3' de un gen codificante para la proteína fluorescente GFP, *gfp*.

Como se ha mencionado previamente, la regulación mediada por *hilD* 3'UTR parece requerir de la presencia de Hfq y de la endonucleasa RNAsa E. Es interesante observar que en estirpes con fondos donde o bien Hfq o bien la RNAsa E funcional están ausentes, Spot 42 no es capaz de inducir la expresión de *hilD*. Sugiriendo que efectivamente el mecanismo molecular por el cual Spot 42 regula positivamente *hilD* involucra tanto a Hfq como a RNAsa E.

Se ha descrito recientemente que Hfq parece tener sitios de unión tanto en la *hilD* 3'UTR como en Spot 42, lo que explicaría el requerimiento de Hfq para el mecanismo de regulación aquí explicado. Se determinó la capacidad de unión de Hfq tanto a *hilD* 3'UTR como Spot 42 mediante EMSA, del inglés electrophoretic mobility shift assay. Observamos que Hfq se une tanto al RNA *hilD* 3'UTR como al sRNA Spot 42.

El mecanismo por el cual Spot 42 regula *hilD* requiere de la presencia de Hfq y de la región *hilD* 3'UTR, lo que sugería que el sRNA Spot 42 podría estar regulando *hilD* mediante unión a su región 3'UTR en complejo con Hfq y en consecuencia protegiendo el mRNA de la degradación por parte de RNAsa E.

Spot 42 contiene en su secuencia tres regiones conocidas de interacción con los genes a los que regula (región I, II y III). Predicciones bioinformáticas de interacción RNA-RNA nos permitieron obtener una posible región de interacción entre el sRNA Spot 42 y *hilD* 3'UTR. Basándonos en los residuos nucleotídicos que formaban parte de esta predicción (región III en Spot 42) se generaron dos mutantes puntuales en la secuencia de sRNA, Spot 42 mut1 y Spot 42 mut2. Es interesante observar que estas mutaciones conllevaron la pérdida de regulación de *hilD* por parte de Spot 42, sugiriendo que los

residuos modificados era importantes para el mecanismo molecular por el cual Spot 42 activa *hilD*. Posteriormente se procedió a la complementación de estas mutaciones en la secuencia de *hilD* 3'UTR, de manera que atendiendo a la predicción de interacción, estas nuevas mutaciones en *hilD* 3'UTR conllevarían la restitución de la interacción con los mutantes Spot 42 mut1 y Spot 42 mut2 y por lo tanto se esperaría la restitución de la regulación ya descrita. Desgraciadamente, estas mutaciones en *hilD* 3'UTR no restituyen la regulación por parte de los mutantes de Spot 42 lo que sugiere que la interacción de Spot 42 con *hilD* 3'UTR está localizada en otra secuencia nucleotídica diferente a la modificada.

De manera alternativa a la estrategia genética (*in vivo*) para la demostración de la interacción Spot 42-*hilD* 3'UTR, se utilizaron ensayos bioquímicos para la demostración de la interacción de las dos secuencias *in vitro.* Se generaron los RNA de Spot 42 y *hilD* 3'UTR mediante transcripción *in vitro*. La interacción de las dos moléculas de RNA se estudio mediante EMSA. Los resultados obtenidos indican que la interacción entre Spot 42 y *hilD* 3'UTR ocurre *in vitro*. Si bien la interacción detecteda es leve, es específica ya que no se produce cuando en el ensayo de interacción se utiliza el Spot 42 mut2, que como ya se ha mencionado, pierde la capacidad de regular *hilD in vivo*.

Para determinar a qué región de la *hilD* 3'UTR se une Spot 42, se generaron dos fragmentos de RNA a partir de la secuencia de *hilD* 3'UTR, llamados UTR^{L} y UTR^{R}. Fragmentos que contenían aproximadamente la primera y segunda mitad de la región *hilD* 3'UTR, respectivamente. Se procedió a realizar ensayos de EMSA con estos fragmentos. Se pudo observar que Spot 42 interacciona con la región UTR^{R} pero no con la región UTR^{L}. Es decir, la interacción de Spot 42 con *hilD* 3'UTR se produce con la segunda mitad del fragmento. Al igual que sucedía con el fragmento de *hilD* 3'UTR, esta interacción es específica ya que no se produce cuando en el ensayo de interacción se utiliza Spot 42 mut2.

Como ya hemos demostrado, Hfq se une tanto a *hilD* 3'UTR como a Spot 42. Es interesante que la región de unión de Hfq a *hilD* 3'UTR se encuentra en la segunda mitad de *hilD* 3'UTR, y por lo tanto esta región se encuentra en el fragmento UTR^{R}, fragmento con el que Spot 42 interacciona. Se procedió a demostrar la capacidad de

Hfq de formar complejo con Spot 42 y UTR^R, como muestra de lo que debe suceder en la célula cuando Spot 42 regula positivamente *hilD* a través de su *hilD* 3'UTR y con el requerimiento de la chaperona de sRNA Hfq. Se llevo a cabo un EMSA como el antes descrito, esta vez en presencia de Hfq. Se pudo observar que el complejo Hfq-UTR^R-Spot 42 se forma *in vitro*. Respaldando los resultados previamente descritos.

Los residuos requeridos en Spot 42 para regular *hilD* 3'UTR ya fueron identificados (Spot 42 mut1, Spot 42 mut2). Pero desconocíamos los residuos necesarios en la *hilD* 3'UTR para la interacción con Spot 42. Por otra parte, sabemos que la interacción tiene lugar en la segunda mitad de la *hilD* 3'UTR, en el fragmento UTR^R. Se procedió a la identificación de los residuos necesarios en la *hilD* 3'UTR mediante structure probing. Una técnica que permite mapear residuos de interacción entre dos moléculas de RNA. Mediante esta técnica observamos que los residuos necesarios para la interacción con Spot 42 en el fragmento UTR^R, se encuentran entre las G102 a la G134, cerca ya del terminador de la *hilD* 3'UTR. Nuevos experimentos generando mutaciones puntuales en esa región serán necesarios para la demostración del sitio exacto de unión de Spot 42 a *hilD* 3'UTR. Cabe resaltar que Spot 42 regula positivamente la expresión de *hilD* mediante la unión directa de Spot 42 a la región *hilD* 3'UTR. Esta es la primera vez que se describe la interacción física de un sRNA con una región 3'UTR en bacterias.

En esta tesis, también se estudió el papel que juega CRP-cAMP en la regulación del sistema Csr. El sistema Csr está formado por una proteína de unión al RNA llamada CsrA y por dos RNA no codificantes llamados CsrB y CsrC. CsrA regula muchos genes en *Salmonella* a través de la unión a sus mRNA, CsrA se une a motivos GGA que se encuentran en regiones no estructuradas del mRNA. La regulación de la actividad de CsrA es principalmente a nivel post-traduccional a través de la acción de CsrB y CsrC. Estos RNA no codificantes contienen varias regiones de unión a CsrA en su secuencia lo que les lleva a unir CsrA y en consecuencia modular los niveles de CsrA libre en la célula. Por tanto, los niveles de expresión de CsrB y CsrC son los que dictaminan los niveles de actividad de CsrA.

En esta tesis hemos demostrado que CRP-cAMP regula la expresión de estos RNA no codificantes de manera diferencial. Se ha observado que la transcripción de estos

genes en crecimiento en placa se ve positivamente regulada por CRP-cAMP. En cambio, en cultivo liquido en fase estacionaria, CRP-cAMP parece que no juega un papel en la regulación de estos RNA no codificantes.

Cuando los experimentos se realizaron en fase exponencial de crecimiento se observaron unos resultados interesantes. CRP-cAMP parece reprimir en estas condiciones la expresión de CsrC pero no de CsrB. Siendo el factor de transcripción SirA el principal activador de la expresión de tanto CsrB como CsrC, la inducción de CsrC que se observa en fase exponencial de crecimiento en ausencia de CRP-cAMP parece ser independiente de SirA.

Interesantemente, el sRNA Spot 42 se encuentra codificado cerca de de CsrC en el cromosoma, aproximadamente a 1 Kb aguas arriba en el mismo sentido de expresión transcripcional y separados por un único gen, *engB*. Ensayos de RT-PCR en regiones intergénicas indican que posiblemente Spot 42 se este co-transcribiendo con CsrC, lo que explicaría que ambos genes estuvieran reprimidos por CRP-cAMP siendo Spot 42 el único de los dos que contiene un sitio de unión a CRP-cAMP en su promotor. Es interesante observar también, que la sobreexpresión de Spot 42 induce la expresión de CsrC, sugiriendo que al igual que sucede con *hilD* 3'UTR, Spot 42 podría unirse al RNA CsrC y protegerlo de la degradación.

Por último, cabe indicar que el mecanismo molecular aquí descrito parece formar parte también de la represión de la SPI-1 en fase logarítmica de crecimiento por parte de CRP-cAMP. La delección de o bien *csrB* o *csrC* en ausencia de CRP-cAMP promueve una pérdida parcial de la inducción de *hilD*, indicando que CsrB y CsrC podrían jugar un papel en el mecanismo por el cual CRP-cAMP reprime la expresión de *hilD*. Consecuentemente, la delección de la proteína CsrA en fase exponencial induce la expresión de *hilD*.

A modo de conclusión general, en esta tesis hemos introducido el regulador metabólico CRP-cAMP como principal represor de la SPI-1 en condiciones no permisivas. Al reprimir CRP-cAMP la expresión del sRNA Spot 42, promueve la represión de *hilD* a nivel post transcripcional. Además mediante la represión de CsrC podría promover la represión de *hilD* a nivel traduccional. Hemos descrito por primera

vez un mecanismo molecular por el cual un sRNA, en este caso Spot 42 regula positivamente un gen mediante a la unión directa a su región 3'UTR. Añadiendo más relevancia al papel que la *hilD* 3'UTR parece jugar en la expresión global de los genes de invasión en *Salmonella*.

Conclusiones

• CRP-cAMP reprime la expresión de hilA en la fase exponencial (condiciones no permisivas para la expresión de SPI-1) y actúa como un activador en fase estacionaria (condiciones permisivas para la expresión de SPI-1).

• La represión mediada por CRP-cAMP de *hilA* causa una atenuación en el nivel de expresión de las proteínas efectoras codificadas por SPI-1.

• La regulación de CRP-cAMP de SPI-1 durante la fase de crecimiento logarítmico se produce aguas arriba de HilA reprimiendo la expresión *hilD*, *hilC* y *rtsA*.

• La represión de CRP-cAMP de SPI-1 está mediada por la regulación de la expresión *hilD* a nivel post-transcripcional a través de la secuencia *hilD* 3'UTR.

• La regulación mediada por CRP-cAMP de *hilD* requiere la presencia de la *hilD* 3'UTR, la chaperona de sRNA Hfq y la endonucleasa principal RNAse E.

• CRP-cAMP reprime la expresión del sRNA Spot 42 en la fase exponencial.

• Spot 42 regula positivamente la expresión de *hilD* en la fase de crecimiento exponencial.

• La derepresión mediada por Spot 42 de *hilD* en fase exponencial requiere de la presencia de la secuencia *hilD* 3'UTR, la chaperona de sRNA Hfq y la endonucleasa principal RNAse E.

• Spot 42 regula positivamente los niveles de *hilD* 3'UTR independientemente de su localización genómica.

• Spot 42 y la región *hilD* 3'UTR se unen físicamente a Hfq.

• La región III no estructurada de Spot 42 es necesaria para la regulación de *hilD*.

• Spot 42 interactúa físicamente con los últimos 150 nt de la secuencia *hilD* 3'UTR.

• CRP-cAMP reprime *csrC* pero no *csrB* expresión en la fase exponencial.

• Spot 42 regula positivamente la expresión de CsrC.

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9. ANNEXES

- ANNEX I Strains, Plasmids and Oligonucleotides.
- ANNEX II Media and buffers.
- ANNEX III Related publications

9.1 Annex I

Table S1. Bacterial strains.

Strains	Genotype	Source
SV5015	*Salmonella enterica* serovar Typhimurium SL1344 His+	(153)
TGC200	SV5015 Δ*crp*:frt	This study
TGC201	SV5015 Δ*cya*:frt	This study
SV5015UB2	SV5015 *hilA-lacZ*	(154)
TGC202	SV5015 Δ*crp*:frt *hilA-lacZ*	This study
JPTM7	SV5015 HilA-3 flag::*km*	(20)
TGC203	SV5015 Δ*crp*:frt HilA-3 flag::*km*	This study
TGC244	SV5015 Δ*cya*:frt HilA-3 flag::*km*	This study
TGC204	SV5015 HilA-3 flag*::km* pTRC99a VC	This study
TGC205	SV5015 Δ*cya*:Frt HilA-3 flag*::km* pTRc99aVC	This study
TGC206	SV5015 HilA-3 flag*::km* pTRc99a CpdA	This study
TGC207	SV5015 Δ*cya*:*frt* HilA-3 flag*::km* pTRc99a CpdA	This study
MHS2	SV5015 SipA-3 flag	(154)
TGC208	SV5015 Δ*crp*:*frt* SipA-3 flag	This study
TGC14	SV5015 Δ*rtsa*::*cm hilA-lacZ*	This study
TGC209	SV5015 Δ*crp*:frt Δ*rtsA*::*cm hilA-lacZ*	This study
TGC10	SV5015 Δ*hilD*::*cm hilA-lacZ*	This study
TGC210	SV5015 Δ*crp*:*frt* Δ*hilD*::*cm hilA-lacZ*	This study
TGC8	SV5015 Δ*hilC*:*frt hilA-lacZ*	This study
TGC211	SV5015 Δ*crp*:frt Δ*hilC*:frt *hilA-lacZ*	This study
TGC212	SV5015 *hilC-lacZ*	This study
TGC213	SV5015 Δ*crp*:*frt hilC-lacZ*	This study
TGC214	SV5015 Δ*crp*:*frt* Δ*hilD*::*cm hilC-lacZ*	This study
TGC35	SV5015 *hilD*1235-*lacZ*	This study
TGC215	SV5015 Δ*crp*:*frt hilD*1235-*lacZ*	This study
TGC216	SV5015 Δ*crp*:*frt* Δ*hilC*:*frt hilD*1235-*lacZ*	This study
TH20428	*Salmonella enterica* serovar Typhimurium LT2 HilD-3 flag	(155)
TGC217	SV5015 HilD-3 flag::*km*	This study
TGC218	SV5015 Δ*crp*:*frt* HilD-3 flag*::km*	This study
TGC219	SV5015 HilD-3 flag:*frt*	This study

TGC220	SV5015 Δ*crp*:*frt* HilD-3 flag:*frt*	This study
SV6190	SV5015 *hilD*ΔUTR:*km*	(31)
TGC221	SV5015 *hilD*ΔUTR:*frt* SipA-3 flag	This study
TGC222	SV5015 Δ*crp*:*frt hilD*ΔUTR:*frt* SipA-3 flag	This study
TGC25	SV5015 *hilD*76-*lacZ*	This study
TGC223	SV5015 Δ*crp*:*frt hilD*76-*lacZ*	This study
TGC38	SV5015 *hilD*965-*lacZ*	This study
TGC224	SV5015 Δ*crp*:*frt hilD*965-*lacZ*	This study
SV5293	14028 Φ(*sipC'-lacZ'*)	(156)
TGC40	SV5015 *Φ(sipC'-lacZ')*	This study
TGC225	SV5015 Δ*crp*:*frt* Φ(sipC'-lacZ')	This study
TGC226	SV5015 *hilD*ΔUTR:*frt Φ(sipC'-lacZ')*	This study
TGC227	SV5015 Δ*crp*:*frt hilD*ΔUTR:*frt Φ(sipC'-lacZ')*	This study
TGC228	SV5015 Δ*hfq*::*cm hilD*1235-*lacZ*	This study
TGC229	SV5015 Δ*crp*:*frt* Δ*hfq*::*cm hilD*1235-*lacZ*	This study
TGC230	SV5015 Δ*hfq*::*cm Φ(sipC'-lacZ')*	This study
TGC231	SV5015 Δ*crp*:*frt* Δ*hfq*::*cm* Φ(sipC'-lacZ')	This study
TGC232	SV5015 *hilD*965-*lacZ* pBRplacVC	This study
TGC233	SV5015 *hilD*965-*lacZ* pBRplac Spot 42	This study
TGC234	SV5015 *hilD*1235-*lacZ* pBRplacVC	This study
TGC235	SV5015 *hilD*1235-*lacZ* pBRplac Spot 42	This study
TGC236	SV5015 Δ*hfq*::*cm hilD*1235-*lacZ* pBRplacVC	This study
TGC237	SV5015 Δ*hfq*::*cm hilD*1235-*lacZ* pBRplac Spot 42	This study
TGC238	SV5015 *hilD*ΔUTR:*frt Φ(sipC'-lacZ')* pBRplacVC	This study
TGC239	SV5015 Δ*crp*:*frt hilD*ΔUTR:*frt Φ(sipC'-lacZ')* pBRplacVC	This study
TGC240	SV5015 *hilD*ΔUTR:*frt Φ(sipC'-lacZ')* pBRplac Spot 42	This study
TGC241	SV5015 Δ*crp*:*frt hilD*ΔUTR:*frt Φ(sipC'-lacZ')* pBRplac Spot 42	This study
TGC242	SV5015 Δ*spf*::*cm hilD*1235-*lacZ*	This study
TGC243	SV5015 Δ*crp*:*frt* Δ*spf*::*cm hilD*1235-*lacZ*	This study
TGC244	SV5015 *spf-lacZ*	This study
TGC245	SV5015 Δ*crp*:*frt spf-lacZ*	This study
TGC246	SV5015 HilD-3 flag:*frt* pBRplacVC	This study
TGC247	SV5015 HilD-3 flag:*frt* pBRplac Spot 42	This study
TGC248	SV5015 Δ*rne*537::*cm hilD*1235-*lacZ* pBRplacVC	This study

TGC249	SV5015 Δ*rne*537::*cm hilD*1235-*lacZ* pBRplac Spot 42	This study
TGC250	SV5015 Δ*spf*::*frt* pXG1 *gfp-hilD* 3'UTR pBRplacVC	This study
TGC251	SV5015 Δ*spf*::*frt* pXG1 *gfp-hilD* 3'UTR pBRplac Spot 42	This study
TGC252	SV5015 Δ*spf*::*frt* pXG1 *hilD* 3'UTR pBRplacVC	This study
TGC253	SV5015 Δ*spf*::*frt* pXG1 *hilD* 3'UTR pBRplac Spot 42	This study
TGC254	SV5015 *hilD*1235-*lacZ* pBRplac Spot 42 mut 1	This study
TGC255	SV5015 *hilD*1235-*lacZ* pBRplac Spot 42 mut 2	This study
TGC256	SV5015 *Φ(sipC'-lacZ')* pBRplacVC	This study
TGC257	SV5015 *Φ(sipC'-lacZ')* pBRplac Spot 42	This study
TGC258	SV5015 *Φ(sipC'-lacZ')* pBRplac Spot 42 mut 1	This study
TGC259	SV5015 *Φ(sipC'-lacZ')* pBRplac Spot 42 mut 2	This study
TGC260	SV5015 *hilD* 3'UTR mut1 *Φ(sipC'-lacZ')*	This study
TGC261	SV5015 *hilD* 3'UTR mut2 *Φ(sipC'-lacZ')*	This study
TGC262	SV5015 *hilD* 3'UTR mut1 *Φ(sipC'-lacZ')* pBRplacVC	This study
TGC263	SV5015 *hilD* 3'UTR mut1 *Φ(sipC'-lacZ')* pBRplac Spot 42	This study
TGC264	SV5015 *hilD* 3'UTR mut1 *Φ(sipC'-lacZ')* pBRplac Spot 42 mut 1	This study
TGC265	SV5015 *hilD* 3'UTR mut2 *Φ(sipC'-lacZ')* pBRplacVC	This study
TGC266	SV5015 *hilD* 3'UTR mut2 *Φ(sipC'-lacZ')* pBRplac Spot 42	This study
TGC267	SV5015 *hilD* 3'UTR mut2 *Φ(sipC'-lacZ')* pBRplac Spot 42 mut 2	This study
TGC268	SV5015 *hilD* 3'UTR mut3 *Φ(sipC'-lacZ')*	This study
TGC269	SV5015 *hilD* 3'UTR mut4 *Φ(sipC'-lacZ')*	This study
TGC270	SV5015 *hilD* 3'UTR mut5 *Φ(sipC'-lacZ')*	This study
TGC271	SV5015 *hilD* 3'UTR mut6 *Φ(sipC'-lacZ')*	This study
TGC272	SV5015 *hilD* 3'UTR mut7 *Φ(sipC'-lacZ')*	This study
TGC273	SV5015 *hilD* 3'UTR mut8 *Φ(sipC'-lacZ')*	This study
TGC274	SV5015 *hilD* 3'UTR mut3 Δ*spf*::*frt Φ(sipC'-lacZ')* pBRplac VC	This study
TGC275	SV5015 *hilD* 3'UTR mut4 Δ*spf*::*frt Φ(sipC'-lacZ')* pBRplac VC	This study
TGC276	SV5015 *hilD* 3'UTR mut5 Δ*spf*::*frt Φ(sipC'-lacZ')* pBRplac VC	This study
TGC277	SV5015 *hilD* 3'UTR mut6 Δ*spf*::*frt Φ(sipC'-lacZ')* pBRplac VC	This study
TGC278	SV5015 *hilD* 3'UTR mut7 Δ*spf*::*frt Φ(sipC'-lacZ')* pBRplac VC	This study
TGC279	SV5015 *hilD* 3'UTR mut8 Δ*spf*::*frt Φ(sipC'-lacZ')* pBRplac VC	This study
TGC280	SV5015 *hilD* 3'UTR mut3 Δ*spf*::*frt Φ(sipC'-lacZ')* pBRplac Spot 42	This study
TGC281	SV5015 *hilD* 3'UTR mut4 Δ*spf*::*frt Φ(sipC'-lacZ')* pBRplac Spot 42	This study
TGC282	SV5015 *hilD* 3'UTR mut5 Δ*spf*::*frt Φ(sipC'-lacZ')* pBRplac Spot 42	This study

TGC283	SV5015 *hilD* 3'UTR mut6 Δ*spf*::*frt Φ(sipC'-lacZ')* pBRplac Spot 42	This study
TGC284	SV5015 *hilD* 3'UTR mut7 Δ*spf*::*frt Φ(sipC'-lacZ')* pBRplac Spot 42	This study
TGC285	SV5015 *hilD* 3'UTR mut8 Δ*spf*::*frt Φ(sipC'-lacZ')* pBRplac Spot 42	This study
TGC286	SV5015 *hilD*3'UTR mut7 Δ*spf*::*frt Φ(sipC'-lacZ')* pBRplac Spot 42 mut 7	This study
TGC287	SV5015 *hilD*1044-*lacZ*	This study
TGC288	SV5015 *hilD*1065-*lacZ*	This study
TGC289	SV5015 pQF50 *csrB-lacZ*	This study
TGC290	SV5015 Δ*crp*:*frt* pQF50 *csrB-lacZ*	This study
TGC291	SV5015 pQF50 *csrC-lacZ*	This study
TGC292	SV5015 Δ*crp*:*frt* pQF50 *csrC-lacZ*	This study
TGC293	SV5015 Δ*csrB*::*cm*	This study
TGC294	SV5015 Δ*csrC*::*cm*	This study
TGC295	SV5015 *csrC-lacZ*	This study
TGC296	SV5015 Δ*crp*:*frt csrC-lacZ*	This study
TGC297	SV5015 Δ*sirA*:*cm*	This study
TGC298	SV5015 Δ*sirA*:*cm csrC-lacZ*	This study
TGC299	SV5015 Δ*crp*:*frt* Δ*sirA*:*cm csrC-lacZ*	This study
TGC300	SV5015 *csrC-lacZ* pBRplac VC	This study
TGC301	SV5015 *csrC-lacZ* pBRplac Spot 42	This study
TGC302	SV5015 Δ*crp*:*frt* Δ*csrB*::*cm hilD*1235-*lacZ* pBRplacVC	This study
TGC303	SV5015 Δ*crp*:*frt* Δ*csrC*::*cm hilD*1235-*lacZ* pBRplacVC	This study
TGC304	SV5015 Δ*csrA*:*frt hilD*1235-*lacZ*	This study

Table S2 Plasmids.

Plasmids	Antibiotic resistance	Source
pTRc99a	Amp^R	(101)
pTRc99a CpdA	Amp^R	This study
pKD3	Amp^R	(103)
pKD4	Amp^R	(103)
pKD46	Amp^R	(103)
pCP20	Amp^R	(103)
pBR plac	Amp^R	(102)

pBRplac Spot 42	Amp^R	This study
pBRplac Spot 42 mut1	Amp^R	This study
pBRplac Spot 42 mut2	Amp^R	This study
pBRplac Spot 42 mut7	Amp^R	This study
pXG1	Cm^R	(108)
pXG1 *gfp-hilD* 3'UTR	Cm^R	This study
pQF50	Amp^R	(109)
pQF50 *csrB*	Amp^R	This study
pQF50 *csrC*	Amp^R	This study
pSUB11	Km^R	(106)
pKG136	Km^R	(107)
pGEM-T easy	Amp^R	(112)

Table S3 Oligonucleotides

Primers	Sequence
	Gene deletions
*crp*_P1	ATGGTGCTTGGCAAACCGCAAACAGACCCGACTCTTGAATGGGTGTAGGCTGGAGCTGCTTC
*crp*_P2	TTAGCGTGTCTTAATAACCAGACGATTACTCTGTTTTACCTCCATATGAATATCCTCCTTAGT
*cya*_P1	TTGTACCTCTATATTGAGACTCTGAAACAGAGACTGGATGCCGTGTAGGCTGGAGCTGCTTC
*cya*_P2	TTACGAAAAATACTGCTGCAATAGCGGCGCGTCATGATCCTGCATATGAATATCCTCCTTAGT
*hilC*_P1	ATGGTATTGCCTTCAATGAATAAATCAGTTGAGGCCATTAGCGTGTAGGCTGGAGCTGCTTC
*hilC*_P2	TCAATGGTTCATTGTACGCATAAAGCTAAGCGGTGTAATCTTCATATGAATATCCTCCTTAGT
*rtsA*_P1	GCACATTTAATAAAAGGAAATTATCATGCTAAAAGTATTTAATCCCTCACCGTGTAGGCTGGAGCTGCTTC
*rtsA*_P2	TCTTATACTGCATTGTCAGATATCTCAATTAACATATTGATGACGAGAGGCATATGAATATCCTCCTTAGT
*hilD*_P1+76	ATGGAAAATGTAACCTTTGTAAGTAATAGTCATCAGCGTCCTGTGTAGGCTGGAGCTGCTTC
*hilD*_P2+76	TTAATGGTTCGCCATTTTTATGAATGTCGATGGCGTAGTTTTCATATGAATATCCTCCTTAGT
*hilD*_P1+1235	GATATTGCCTTATTCACATCGTAAGAATTCGTCCAGATGACACTATCTCCGTGTAGGCTGGAGCTGCTTC
*hilD*_P2+1235	TATAAATATGAATAAAATGCCGGCCTTAATCCACAGGGTTAAAGCCGGAACATATGAATATCCTCCTTAGT
*hilD*_P2+1044	TTTTTATTAAAATTGTAATAATTTAAAATTCAGACTGCGCATTAACACGCGTGTAGGCTGGAGCTGCTTC
*hfq*_P1	GTACAATTGAGACGTATCGTGCGCAATTTTTCAGAATCGAGTGTAGGCTGGAGCTGCTTC
*hfq*_P2	CCCGACATGGATAAACAGCGCGTGAACTTATTCAGTCTCTTGATATGAATATCCTCCTTA
*spf*_P1	GTAATTAAGCAACATAAGCACGGGGGTTTTGTGATGGGTATTGTGTAGG
*spf*_P1_lacZ	GAGGTAAGATGTTCTATCTTTCAGACCTTTTACTTCACGTGTGTAGGCTGGAGCTGCTTC

*spf*_P2	GTCTTATCCGGCCTACGGTGTGAGCGAAACTTTTCTTGCGCACATATGAATATCCTCCTTA
*csrB*_P1	GTACAACGAAGCGAACGTCAGGATGATGACGCTTCAGCAGGTGTAGGCTGGAGCTGCTTC
*csrB*_P2	CATCCGTGACAACTTTTCCTGTGACCTTACGGCCTGTTCACATATGAATATCCTCCTTA
*csrC*_P1	GAGGACGCTAACAGGATCAACGACTCAGGATGAGGGTGTAGGCTGGAGCTGCTTC
*sirA*_P1	CAGGGATACGACGCATTCTTGAAGATATAAAGGGCATTAAAGGTGTAGGCTGGAGCTGCTTC
*sirA*_P2	CCAGGTGAGTCAGCTCAACATCACCATGAATGTTTAATTTACCATATGAATATCCTCCTTA
	Northern blot probes
*csrC*_probe	CATCCTGAGTCGTTGATCCTG
*csrB*_probe	GGAGGTGTCCTTTAACGCATC
*spf*_probe	CAAATCCGATTACGTGAAGT
*hilD*_probe	CTTCCGGACAGAACAATGATATTG
5S_probe	CTACGGCGTTTCACTTCTGAGTTC
	RT-PCR
*spf_engB*Fw	CAGTCAGTTTATGACTGGG
*spf_engB*rev	CAGATGATCCAGTGGGCTGTA
*engB_csrC*rev	GAATATCAGGCGCACTCATC
*engB_csrC*rev	GTTGATCCTGTTAGCGTCCTC
*csrC*fwRT	GAGGACGCTAACAGGATCAAC
*csrC*revRT	GACAGAGTAATCTGCCGCCTT
	Gene cloning
*csrB*fw_pQF50	CGCGGATCCTAGGCCACCTGGTCACGC
*csrB*rv_pQF50	CGCAAGCTTCGACTCCCTGTCGACGAAG
*csrC*fw_pQF50	CGCGGATCCGCAATCGGAAGGTAAATGG
*csrCrv*_pQF50	CGCAAGCTTCGCCTCCTGGCGCTCC
*hilD*UTR_Fw	CGCTCTAGAGATACACGCAAAGGTTGCAGTAAC
*hilD*UTR_rev	CGCGAGCTCCTCGAAGATTTCCACTAAATGACC
*cpdA_XbaI*_Fw	CGCTCTAGAGTTTATTAGCGTCGTGAAACC
*cpdA_SalI*_Rev	GCGGTCGACATCAGTGATGGTGATGGTGATGGTATCCTTCCGAAGCGGTATC
	Point mutations
UTR_mut1fw	GGTAATTTAAAGTAAGGCTGATATGATAACACGATTTTTGTG
UTR_mut1fw	CACAAAAATCGTGTTATCATATCAGCCTTACTTTAAATTACC
UTR_mut2fw	GGTAATTTAAAGTAAGGTGGATTATATAACACGATTTTTGTG
UTR_mut2rev	CACAAAAATCGTGTTATATAATCCACCTTACTTTAAATTACC
UTR_mut3fw	TTGTAATAATTTAAAAGTGAGACTGCGCATTAACACGCTCTATC
UTR_mut3rev	GATAGAGCGTGTTAATGCGCAGTCTCACTTTTAAATTATTACAA
UTR_mut4fw	TTGTAATAATTTAAAATTCAGACATGGCATTAACACGCTCTATC
UTR_mut4rev	GATAGAGCGTGTTAATGCCATGTCTGAATTTTAAATTATTACAA
UTR_mut5fw	GGCGAACCATTAACATTTTTACAATCTGTCACTTAAGTAAAG

UTR_mut5rev	CTTTACTTAAGTGACAGATTGTAAAAATGTTAATGGTTCGCC
UTR_mut6fw	GGCGAACCATTAACATTTTTTGTCGTTGTCACTTAAGTAAAG
UTR_mut6rev	CTTTACTTAAGTGACAACGACAAAAAATGTTAATGGTTCGCC
UTR_mut7fw	CATTAACACGCTCTATCAGCTAGGGAGGCTATTCAATATC
UTR_mut7rev	GATATTGAATAGCCTCCCTAGCTGATAGAGCGTGTTAATG
UTR_mut8fw	CATTAACACGCTCTATCAGGATTCTAGGCTATTCAATATC
UTR_mut8rev	GATATTGAATAGCCTAGAATCCTGATAGAGCGTGTTAATG
*spf*_mut7.fw	GTACAGAGGTAAGATGTTCTTAGTTTCAGACCTTTTACTTCACG
*spf*_mut7.rev	CGTGAAGTAAAAGGTCTGAAACTAAGAACATCTTACCTCTGTAC
*spf.*mut1fw	GACCTTTTACTTCACCATATCGGATTTGGCTGAATATTTTAG
*spf.*mut1rev	CTAAAATATTCAGCCAAATCCGATATGGTGAAGTAAAAGGTC
*spf.*mut2fw	GACCTTTTACTTCACGTAATCCACTTTGGCTGAATATTTTAG
*spf.*mut2rev	CTAAAATATTCAGCCAAAGTGGATTACGTGAAGTAAAAGGTC
	T7 RNA transcription
*hilD*UTRT7.fw	TAATACGACTCACTATAGGGCTACGCCATCGACATTCATAA
*hilD*UTRT7.rev	GAATAAAATGCCGGCCTTAATC
*hilD*UTRR.fw	TAATACGACTCACTATAGGGACAGCTTATACTGATATCTATGG
*hilD*UTRLrev	CCATAGATATCAGTATAAGCTGTC
Spot42T7.fw	TAATACGACTCACTATAGGGTAGGGTACAGAGGTAAGATG
Spot42T7.rev	GTCTTATCCGGCCTACGGTG
*mglB*T7.fw	TAATACGACTCACTATAGGGCAAAACGCGTTAGCGTTTTGAAC
*mglB*T7.rev	GAAAGGGTCAGTACCTTCTTATTC

Annex 2 Media and buffers

Media

LB

Luria-Bertrani growth media was prepared dissolving yeast extract at concentration of 5 g/l, tryptone at 10 g/l and NaCl at 10 g/l in milliQ water. For sterilization, media was autoclaved

LB agar

Luria-Bertrani agar plates were prepared dissolving yeast extract at concentration 5g/l, tryptone at 10 g/l, NaCl at 10 g/l and agar at 15 g/l in milliQ water. For sterilization, media was autoclaved. When required, upon cooling down to 50^{o}C, media can be supplemented with antibiotics and other components. Approximately, 25 to 30 ml of media was poured on agar plates for polymerization.

EBU plates

For EBU plates preparation, LB agar media was supplemented with blue evans to a concentration of (), fluorescein to a concentration of (), glucose to a concentration of () and K_2HPO_4 to a concentration of (). When required, antibiotics were additionally added to the media.

Long-term storage of microbial cells

For long-term storage of microbial cells, an OD_{600nm} of 5-6 was supplemented with 20% glycerol. Approximately 1 ml of the mixture was stored in cryotubes at -80^{o}C.

Solutions

IPTG

IPTG was dissolved in ddH_2O at a concentration of 1 M. The solution was sterilized by filtration using 0.22 µM pore size filters.

Arabinose

Arabinose was dissolved in ddH_2O at a concentration of 20 %. The solution was sterilized by filtration using 0.22 µM pore size filters.

Antibiotics

Antibiotics were dissolved in the appropriate solvent and sterilized by filtration using 0.22 µM pore size filters.

Antibiotic	Solvent	Use concentration
Kanamycin	ddH_2O	50 ng/ml
Chloramphenicol	Ethanol	15 ng/ml
Ampicillin	ddH_2O	50 ng/ml
Tetracyclin	Ethanol 50 %	12,5 ng/ml

ONPG

ONPG was dissolved in buffer Z at a concentration of 4 mg/ml. ONPG solution was freshly prepared before use.

Buffers

Hybridization buffer

Hybridization buffer for northen blot was prepared in ddH2O. Sodium phosphate buffer pH 7.2 was added to a concentration of 0.5 M, EDTA pH 8.0 was added to a concentration of 10 mM and SDS was added to a concentration of 7 %. This buffer shoul be stored at 40-50^{o}C as the SDS precipitates.

Washing Buffer

The washing buffer for northern blot assay was prepared in ddH2O. Sodium phosphate buffer pH 7.2 was added to a concentration of 0.4 M and SDS was added to a concentration of 1 %. This buffer should be stored at 40-50^{o}C as SDS precipitates.

Transfer buffer

The transfer buffer for western blot was prepared in ddH2O by dissolving Tris to a concentration of 48 mM, glycine to a concentration of 39 mM and SDS to a concentration of 1.3mM. Finally, methanol was added to a concentration 20 % v/v. This buffer can be stored at room temperature.

Running buffer 10X

The running buffer for SDS acrylamide gels was prepared in ddH2O by dissolving Tris to a concentration of 0.25 M, SDS to a concentration of 1 % and Glycine to a concentration of 1.92 M.

TBE 10X

TBE buffer for nucleic acids electrophoresis was prepared in ddH2O by dissolving Tris to a concentration of 450 mM, Boric acid to a concentration of 450 mM and EDTA to a concentration of 10mM. The buffer was autoclaved before use.

Buffer Z

To prepare buffer Z for β-galactosidase, Na_2HPO_4 was dissolved in ddH_2O to a concentration of 60 mM, NaH_2PO_4 to a concentration of 40 mM, KCl to a concentration of 10 mM and $MgSO_4$ to a concentration of 1 mM. Buffer Z solution was stored at 4^oC. Before use, β-mercaptoetanol was added to the solution at a concentration of 50mM.

PLOS | PATHOGENS

RESEARCH ARTICLE

Gre factors-mediated control of *hilD* transcription is essential for the invasion of epithelial cells by *Salmonella enterica* serovar Typhimurium

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OPEN ACCESS

Citation: Gaviria-Cantin T, El Mouali Y, Le Guyon S, Römling U, Balsalobre C (2017) Gre factors-mediated control of *hilD* transcription is essential for the invasion of epithelial cells by *Salmonella enterica* serovar Typhimurium. PLoS Pathog 13(4): e1006312. https://doi.org/10.1371/journal.ppat.1006312

Editor: Joan Mecsas, Tufts University, UNITED STATES

Received: November 1, 2016

Accepted: March 23, 2017

Published: April 20, 2017

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by the Spanish Ministry of Economy and Competitiveness (grants BIO2010-15417 and AGL2013-45339-R, CB), the Catalonian government (grant 2014SGR1260, CB), the RecerCaixa program (grant 2012/ACUP/00048, CB), the Swedish Research Council Natural Sciences and Engineering (grant D0480901, UR)

Abstract

The invasion of epithelial cells by *Salmonella enterica* serovar Typhimurium is a very tightly regulated process. Signaling cascades triggered by different environmental and physiological signals converge to control HilD, an AraC regulator that coordinates the expression of several virulence factors. The expression of *hilD* is modulated at several steps of the expression process. Here, we report that the invasion of epithelial cells by *S*. Typhimurium strains lacking the Gre factors, GreA and GreB, is impaired. By interacting with the RNA polymerase secondary channel, the Gre factors prevent backtracking of paused complexes to avoid arrest during transcriptional elongation. Our results indicate that the Gre factors are required for the expression of the bacterial factors needed for epithelial cell invasion by modulating expression of HilD. This regulation does not occur at transcription initiation and depends on the capacity of the Gre factors to prevent backtracking of the RNA polymerase. Remarkably, genetic analyses indicate that the 3'-untranslated region (UTR) of *hilD* is required for Gre-mediated regulation of *hilD* expression. Our data provide new insight into the complex regulation of *S*. Typhimurium virulence and highlight the role of the *hilD* 3'-UTR as a regulatory motif.

Author summary

Salmonella enterica serovar Typhimurium is a foodborne pathogen that causes gastroenteritis in humans. To successfully trigger infection, *S*. Typhimurium invades epithelial cells, a process that requires the coordinated expression of a set of genes. HilD is a pivotal regulator of *S*. Typhimurium pathogenicity, as it activates the expression of the genes required for invasion of intestinal epithelium. Expression and activity of HilD are tightly regulated and respond to several environmental and physiological conditions. In this report, we introduce the transcription elongation as a novel level of regulation of *hilD*. We

and the Karolinska Institutet (KID-funding, UR). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interest exist.

describe that the Gre factors, proteins that prevent backtracking of paused RNA polymerase complexes during transcription elongation, are required for the expression of HilD and the subsequent expression of genes involved in the invasion of epithelial cells.

Introduction

Salmonella enterica serovar Typhimurium *(S.* Typhimurium*)*, an enteric bacterial pathogen that infects both humans and animals, is extensively used as a model organism in pathogenicity studies [1,2]. *S.* Typhimurium infection is asymptomatic in poultry, but causes gastroenteritis in humans [3]. Its infectious cycle is complex and requires the expression of a large number of virulence factors that are mostly encoded by chromosomal genes clustered in discrete regions known as *Salmonella* pathogenicity islands (SPIs). SPIs have been acquired through different evolutionary processes via horizontal gene transfer, with the successive acquisition of different genetic elements playing a determinative role in host adaptation [4]. Comparative genomic studies identify up to 21 SPIs in the *S.* Typhimurium genome, SPI-1 and SPI-2 being the best characterized [5]. SPI-1 contains genes required during the first steps of epithelial cell infection, while SPI-2 encodes genes needed for *S.* Typhimurium survival and replication inside host cells [6]. SPI-1 and SPI-2 genes encode their respective type three secretion systems (TTSS), TTSS-1 and TTSS-2, secreted effector proteins and regulators that coordinate the optimal expression of virulence genes [7–9]. In SPI-1, the HilA protein directly regulates the expression of secretion machinery components and various TTSS-1 effectors (S1 Fig) [10]. Moreover, HilA induces the expression of the regulator InvF, a transcriptional activator of *sic/sip* operons, encoding effector proteins [11,12]. HilA transcriptional expression is autoregulated and tightly modulated by the combined action of three AraC-like transcriptional activators: HilC, HilD and RtsA [13,14]. Each of these three regulators are positively autoregulated and can induce the expression of the other two, producing a positive feed-forward loop that controls SPI-1 gene expression [15]. HilD plays a major role in regulating *hilA* expression. Its expression and activity is targeted by many signaling pathways, with HilD acting as a hub that integrates diverse environmental and physiological cues to trigger *S.* Typhimurium invasion of epithelial cells [16]. Of note, HilD-mediated regulation is not restricted to the SPI-1 genes, as HilD also modulates the expression of genes located outside this genetic locus such as *sopE*, which encodes an effector protein secreted via TTSS-1, and *ssrAB*, which encodes the two-component system that acts as the central positive regulator of the SPI-2 genes [10,17,18]. Therefore, HilD plays a key role in *S.* Typhimurium pathogenicity.

Transcription, the first step in gene expression, is tightly regulated. Regulation of transcription initiation is crucial in determining the genomic response to physiological and environmental signals. Likewise, regulation during transcription elongation and termination has a pronounced effect on the steady state expression levels of particular genes and regulons, but is less well understood [19]. Brief transcriptional pauses in RNA polymerase (RNAP) activity that occur during elongation can quickly resolve spontaneously; however, sustained pauses may cause backtracking of the transcription elongation complex. In *E. coli*, the Gre factors, GreA and GreB, resolve backtracked complexes by interacting with the secondary channel of the RNAP and inducing endoribonuclease activity. These actions restore the proper positioning of the 3'-end of the nascent transcript within the RNAP active center [20,21]. Transcriptional pauses may act as regulatory events that affect the expression levels of specific genes [22]. In addition to their role in suppressing transcriptional pauses, Gre factors stimulate RNAP promoter escape and enhance transcriptional fidelity [23]. Gre factors occur widely in

prokaryotes. The presence of two distinct clades among the Gre family members sharing high structural and functional homology, GreA and GreB, has been detected among proteobacteria. Members of the family outside proteobacteria resemble GreA more closely than GreB [24]. In *S*. Typhimurium, GreA and GreB share 34% identity and 57% similarity, numbers which are nearly identical to those observed in *E. coli* (35% and 56%, respectively).

In this report, we explored whether Gre factors are relevant in regulating pathogenicity in *S*. Typhimurium. We found that the Gre factors are required for the proper expression of the SPI-1 effector proteins and subsequent cell invasion as well as organ colonization in a mouse model of systemic infection. The dissection of the regulatory pathway let us conclude that Gre factors are essential for the expression of HilD, a major regulator of SPI-1 genes. Regulation of *hilD* does not occur at transcription initiation, but the 3'-untranslated region (UTR) is required for Gre-mediated regulation of *hilD* expression. This suggests that regulation depends on the ability of Gre factors to prevent backtracking of paused RNA polymerase complexes possibly coupled with downstream events. Our data provide new insights into the complex regulation of *S*. Typhimurium virulence and the role of the 3'-UTR of *hilD* as a regulatory motif.

Results

S. Typhimurium invasion of epithelial cells is impaired in bacterial cells lacking *greA* and *greB*

To elucidate whether Gre factors regulate *S*. Typhimurium pathogenicity, invasion assays were performed using strains deleted for *greA* and/or *greB*. The HT-29 epithelial cell line was infected with the wild-type (WT) SV5015 strain, the Δ*greA* strain lacking GreA, the Δ*greB* mutant deficient in GreB, and the double mutant Δ*greA*Δ*greB* strain lacking both Gre factors. The Δ*motA* and Δ*hilA* derivatives, deficient in invasion, were used as controls [25,26]. Determination of the percentage of intracellular bacterial cells after one hour of infection showed that, as expected, the WT strain did invade epithelial cells, whereas the Δ*motA* and Δ*hilA* derivatives showed impaired invasion (Fig 1A). Although the Δ*greA* and Δ*greB* mutants were invasive, they displayed only 23% and 58% of WT invasiveness, respectively. Remarkably, invasion was abolished in the strains lacking both GreA and GreB. To confirm that the inability to invade was caused by the absence of the Gre proteins, *trans*-complementation of the Δ*greA*Δ*greB* double mutant with a pBR322-based plasmid containing the *greA* and *greB* genes (pBR*greAB*) restored invasiveness. To validate the invasion defect mediated by Gre deficiency, a different strain and cell line background was used. Similar results were obtained when the effect of the Gre factors on invasion was also tested in HT-29 cells using virulent *S*. Typhimurium strain ATCC14028 and when performed with Caco-2 cells (S2 Fig and Fig 1B).

To corroborate the *in vitro* defect in epithelial cell invasion and to address the biological significance of the Gre factors in *Salmonella* pathogenesis, the potential of the Gre-deficient strain to cause systemic infection in *Salmonella* susceptible mice was investigated (Table 1). The bacterial load in livers and spleens of BALB/c mice (n = 5) inoculated orally with a 1:1 mixture of the WT and the Δ*greA*Δ*greB* strain was estimated four days after infection. While the WT strain was able to colonize the internal organs, the Δ*greA*Δ*greB* mutant was unable to do so, only being recovered from liver and spleen of one mouse (1000-fold lower than the wild type). Thus, our *in vivo* data highlight the biological significance of the Gre proteins in *Salmonella* pathogenesis.

Invasion of epithelial cells requires a battery of effector proteins encoded mainly by genes in SPI-1 that are secreted through the TTSS encoded by the same genetic locus. Therefore, our

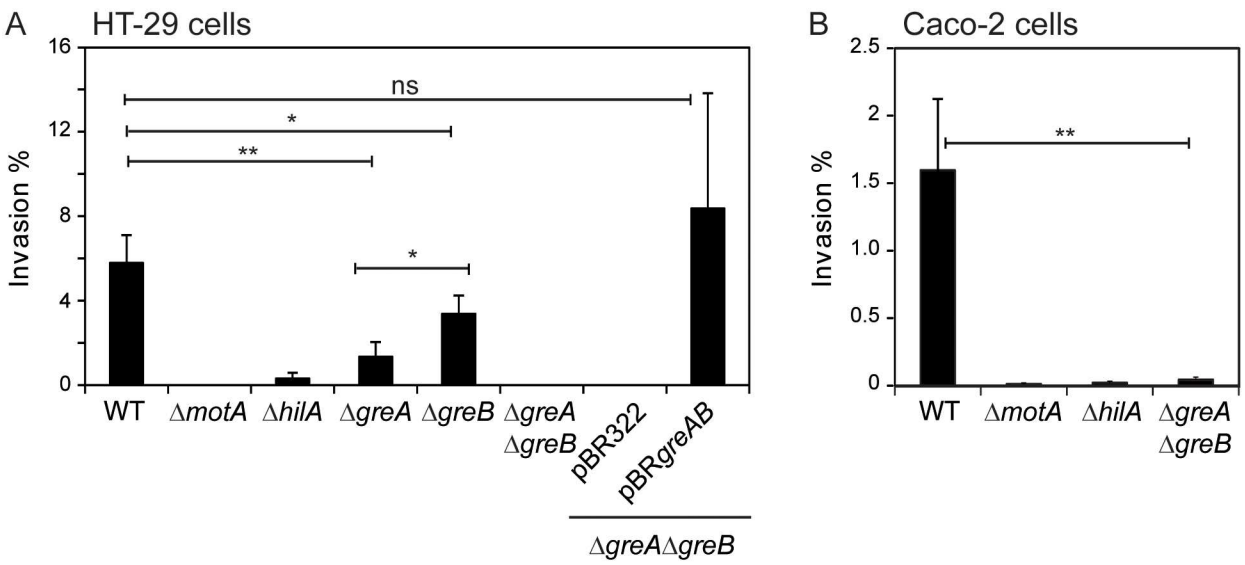

Fig 1. Invasion of epithelial cells by *S.* Typhimurium is impaired in strains deficient for the Gre factors. Cultures of the WT (SV5015) and the Δ*greA*, Δ*greB* and Δ*greA*Δ*greB* derivatives were assessed for invasion of HT-29 (A) and Caco-2 (B) cell lines. As a control, cultures of the invasion impaired mutants Δ*hilA* and Δ*motA* were used. A bar shows the arithmetic mean of experimental results and the error bar indicates the standard deviation. Significance was tested by an unpaired two–sided Student's t-test. Statistical significance is indicated by *p<0.05, **p<0.01, ns: non-significant.

https://doi.org/10.1371/journal.ppat.1006312.g001

results suggest that Gre factors are involved in the efficient expression of SPI-1 genes and that their absence elicits the avirulent phenotype of the Δ*greA*Δ*greB* strain.

Gre factors are required for the expression of SPI-1 effector proteins

Translocation of specific SPI-1-encoded effector proteins, such as the Sip proteins, has been associated with hemoglobin release during *S.* Typhimurium infection of erythrocytes *in vitro* [27]. Contact-dependent hemolysis was monitored with cultures of WT and Gre-deficient strains. The Δ*greA*Δ*greB* mutant could not lyse erythrocytes when compared to the parental strain (Fig 2A). Secreted proteins from WT, Δ*greA*, Δ*greB* and Δ*greA*Δ*greB* strains grown under conditions that induce invasiveness (cultivated in LB broth at 37˚C with aeration to the early stationary phase (OD_{600nm} of 2.0), [18]) were analyzed by SDS-PAGE and Coomassie staining. Comparison of the secreted protein profiles (Fig 2B) indicated that: (i) extracts from the Δ*greA* mutant strain showed a significant decrease in the intensity of three protein bands (labeled with an asterisk in the Figure); (ii) extracts from the Δ*greB* mutant strain were

Table 1. Competition assay of the Δ*greA*Δ*greB* mutant strain versus WT. Amount of bacteria in liver and spleen was determined at 4 days post infection. A total of ~2E+7 colony forming units (cfu) of WT and the Δ*greA*Δ*greB* strain at a 1:1 ratio was administered orally to 5 mice.

Mice	Liver (CFU/g)			Spleen (CFU/g)		
	WT	Δ*greA*Δ*greB*	CI	WT	Δ*greA*Δ*greB*	CI
#1	1.8E+5	4.7E+1	4.0E-4	2.4E+6	1.6E+3	1.0E-3
#2	3.0E+4	n.d.	< E-4	3.5E+5	n.d.	< E-3
#3	1.9E+4	n.d.	< E-4	2.2E+5	n.d.	< E-3
#4	1.8E+4	n.d.	< E-4	2.2E+5	n.d.	< E-3
#5	1.7E+2	n.d.	-	3.3E+2	n.d.	-

n.d.: non-detected

https://doi.org/10.1371/journal.ppat.1006312.t001

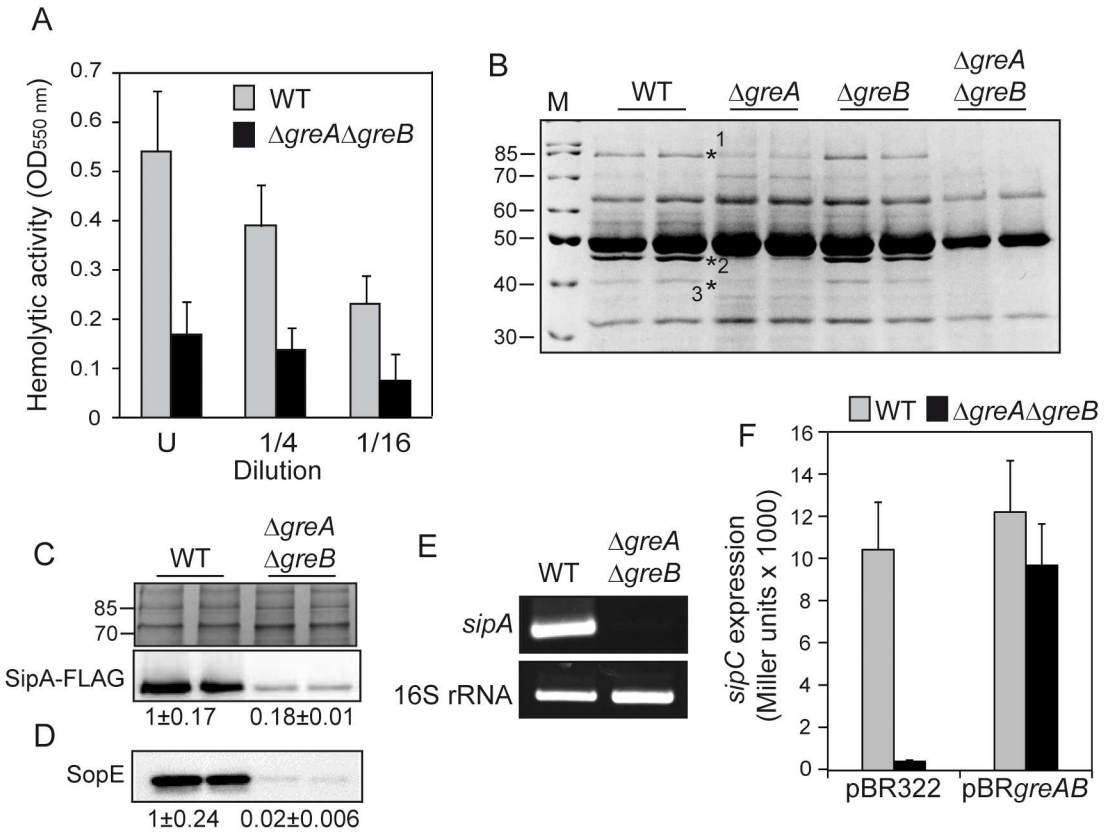

Fig 2. The Gre factors affect the expression of SPI-1 effector proteins. (A) Contact haemolysis assays with cell-free supernatants from cultures of the WT and Δ*greA*Δ*greB* derivative strains. The haemolytic activity of LB bacterial cultures of WT and Δ*greA*Δ*greB* strains was monitored as an increase in the OD_{550nm} of the supernatant of a blood suspension with serial dilutions of cell-free supernatants. U: undiluted supernatant. Three independent bacterial cultures were tested. (B) Cell-free supernatants of two independent LB cultures of WT (SV5015) and its Δ*greA*, Δ*greB* and Δ*greA*Δ*greB* derivatives. Extracts were analyzed by Coomassie blue stained 12.5% SDS-PAGE. Lane M: molecular mass markers (size in kDa indicated). The bands labelled were identified as SipA (1), FliD (2) and SipC (3) by LC-MS/MS. (C) Immunodetection (lower panel) of the SPI-1 encoded SipA-FLAG protein in whole culture extracts from two independent cultures of WT and Δ*greA*Δ*greB* derivative strains. The upper panel is a section of a Coomassie stained gel as a loading control. (D) Immunodetection of SopE protein was performed in extracts from WT and Δ*greA*Δ*greB* strains obtained from cell-free supernatants of two independent LB cultures. (E) Semiquantitative RT-PCR of *sipA* in total RNA samples from LB cultures of the WT (SV5015) and Δ*greA*Δ*greB* strains. 16S RNA was used as endogenous control to confirm that equivalent quantities of templates were used. (F) *sipC* transcriptional expression was tested in cultures of WT and Δ*greA*Δ*greB* derivative strains carrying a chromosomal *sipC*::*lacZ* fusion and either pBR322 or pBR*greAB*. A bar shows the arithmetic mean of experimental results and the error bar indicates the standard deviation from three biological replicates. All cultures were grown in LB at 37˚C with vigorous shaking (200 rpm) up to an OD_{600nm} of 2.0.

https://doi.org/10.1371/journal.ppat.1006312.g002

apparently identical to those from the WT strain; and (iii) extracts from the Δ*greA*Δ*greB* mutant showed an overall decrease in protein secretion including the three above mentioned protein bands. To determine if these protein bands corresponded to SPI-1 effector proteins, extracts from WT and a Δ*hilA* strain were compared (S3 Fig). Since HilA is a transcriptional regulator that controls the expression of SPI-1 genes, the Δ*hilA* mutant is phenotypically equivalent to a deletion of the entire SPI-1 locus [15]. Interestingly, the three major protein bands that were missing in the extracts from the Δ*greA*Δ*greB* mutant were also absent in the Δ*hilA* extracts. LC-MS/MS (Liquid chromatography-mass spectrometry) revealed that the protein bands corresponded to the SPI-1 effector protein SipA, the flagellar cap protein FliD and the SPI-1 effector protein SipC.

These results are in agreement with the different levels of invasiveness presented by the mutant strains and indicate that Gre factors are required for either the expression or secretion of SPI-1 effector proteins. The amount of a SipA-FLAG tagged protein from whole culture extracts (cellular + extracellular proteins) was measured by immunodetection (Fig 2C). There was a significant drop (5.5-fold) in the total production of SipA in the Δ*greA*Δ*greB* strain compared to WT, demonstrating that Gre factors are required for the optimal expression of SPI-1 effector proteins. Expression of the SopE protein, encoded outside SPI-1 but secreted through TTSS-1, is co-regulated with the SPI-1-encoded effector proteins [10]. The amount of SopE secreted by the Δ*greA*Δ*greB* strain was diminished >25-fold when compared to WT (Fig 2D), demonstrating a crucial role of Gre factors in modulating SopE expression.

To determine the level at which Gre factors affect SPI-1 gene expression, transcriptional studies were performed. Semi-quantitative RT-PCR indicated a significant decrease in *sipA* mRNA steady state levels in the Δ*greA*Δ*greB* strain compared to WT (Fig 2E). This was corroborated using a chromosomal *sipC*::*lacZ* fusion, which demonstrated that there was a 10-fold reduction in *lacZ* expression in the Δ*greA*Δ*greB* strain compared to WT. This transcriptional phenotype can be complemented in the presence of pBR*greAB* (Fig 2F).

Transcriptional expression of the major SPI-1 regulator HilA is impaired in strains lacking Gre factors

As the expression of several SPI-1 effector proteins depends on the presence of Gre factors, we hypothesized that Gre factors may be acting upstream in the regulatory pathway controlling SPI-1 expression. The expression of most SPI-1 genes requires the transcriptional activator HilA, encoded in the SPI-1. HilA binds to target promoters to induce the expression of many genes involved in TTSS biogenesis, including *invF*, which promotes the subsequent expression of the *sic*/*sip* genes (reviewed by [26]). The effect of Gre factors on *hilA* transcriptional expression was studied using a chromosomal *hilA*::*lacZ* fusion (Fig 3A). Consistent with previous reports, *hilA* expression was induced in the WT strain in cells entering the stationary phase [18]. By contrast, *hilA* transcriptional expression was impaired in the Δ*greA*Δ*greB* mutant, suggesting that Gre factors are required for HilA transcriptional expression. This was further corroborated by qPCR analysis of *hilA* transcript levels, which showed a 10-fold drop in the Δ*greA*Δ*greB* strain compared to WT (Fig 3B). The effect of Gre factors on *hilA* transcriptional expression was also observed when looking at the levels of HilA-FLAG (Fig 3C). Accordingly, the expression of InvF, the AraC activator that is directly activated at the transcriptional level by HilA, was significantly reduced in the Δ*greA*Δ*greB* strain compared to WT (Fig 3C).

Our data show that Gre factors are involved in the transcriptional expression of both *hilA* and HilA-regulated genes. We aimed to determine whether Gre factors are required directly for the efficient transcription of genes encoding effector proteins or whether their effects are mediated only through regulating *hilA* expression. HilA expression was ectopically induced and its effect on effector protein production monitored in WT and the Δ*greA*Δ*greB* double mutant. As seen in Fig 3D, *hilA* ectopic expression elicited effector protein expression independently of Gre factors, suggesting that Gre factors are not essential for the transcription of the effector protein genes, but are required for regulating HilA expression or other regulator (s) that modulate *hilA* expression.

Gre factors affect SPI-1 gene expression by modulating *hilD* expression

The transcriptional expression of *hilA* is tightly modulated by three AraC activators, HilD, HilC and RtsA, which form an auto-inducing regulatory loop where each protein can activate its own expression, as well as expression of the other two regulators [15]. Genetic

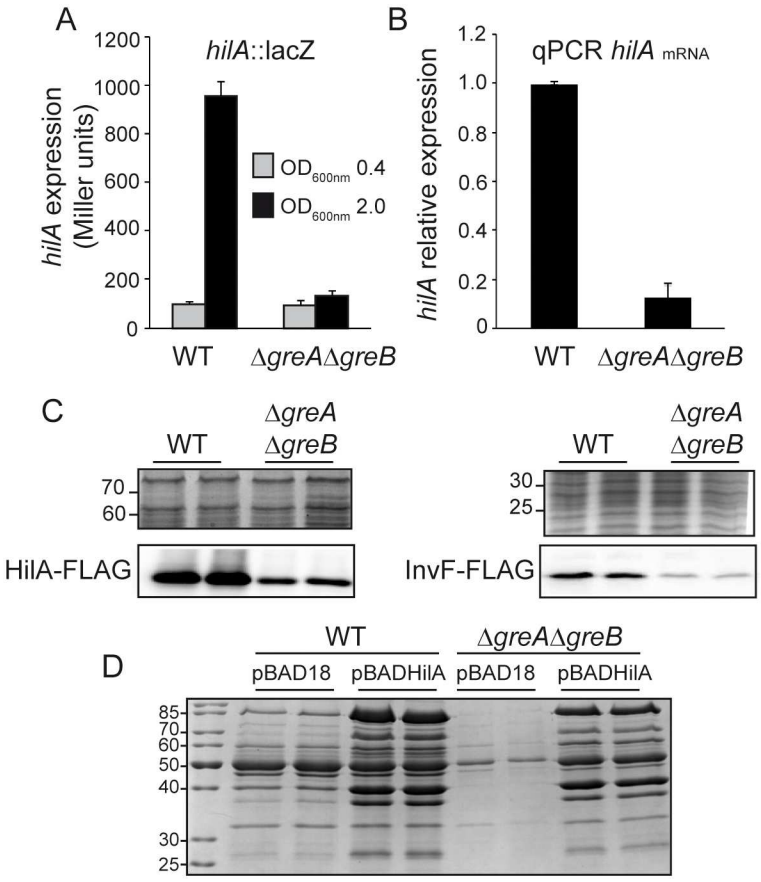

Fig 3. Gre factors are essential for HilA expression. (A) Transcriptional expression of *hilA* in WT and Δ*greA*Δ*greB* derivative strains. β-galactosidase activity from a *hilA*::*lacZ* fusion was assessed in LB cultures grown at 37˚C up to logarithmic (OD_{600nm} 0.4) and stationary growth phase (OD_{600nm} 2.0). (B) Relative *hilA* mRNA quantification by qPCR in WT and Δ*greA*Δ*greB* derivative strains. Results are normalized with *gapA* (GAPDH) as an endogenous control. RNA samples were extracted from cultures of WT and Δ*greA*Δ*greB* derivative strains grown in LB at 37˚C up to an OD_{600nm} 2.0. In A and B, a bar shows the arithmetic mean of experimental results and the error bar indicates the standard deviation from three biological replicates. (C) Immunodetection of HilA-FLAG (lower left panel) and InvF-FLAG (lower right panel) proteins in whole cell extracts from cultures of WT and Δ*greA*Δ*greB* derivative strains grown as in B. The upper panels are sections of Coomassie stained gels as loading controls. (D) Cell-free supernatants of LB cultures, grown at 37˚C up to an OD_{600nm} of 2.0, of WT and Δ*greA*Δ*greB* derivative strains carrying either pBAD18 or pBADHilA. Arabinose (0.02%) was added in all cultures. Extracts were analyzed by Coomassie blue stained 12.5% SDS-PAGE.

https://doi.org/10.1371/journal.ppat.1006312.g003

analyses indicated that Gre factors are required for the transcriptional expression of these three activators (Fig 4A). Expression of *hilA* was determined in the WT and the Δ*greA*Δ*greB* mutant as well as in combination with deletion of each of the three different activators (Fig 4B). Under the experimental conditions used, HilC and RtsA were not needed to achieve high *hilA* expression; however, HilD was essential since *hilA* expression was abolished in Δ*hilD* mutants regardless of the presence or absence of Gre factors. *Trans*-complementation of the Δ*hilD* mutant with a pBR322-based plasmid containing the *hilD* gene restored *hilA* expression (S4 Fig), confirming the lack of *hilA* expression to be caused by the Δ*hilD* mutation. The requirement of HilD for *hilA* expression was further studied by monitoring HilA-FLAG expression in the presence or absence of HilD (Fig 4C). A drastic drop in intracellular HilA-FLAG levels in the Δ*greA*Δ*greB* mutant can only be detected in the $HilD^+$ strains. Altogether, these results suggest that HilD plays a pivotal role in activating *hilA* expression under

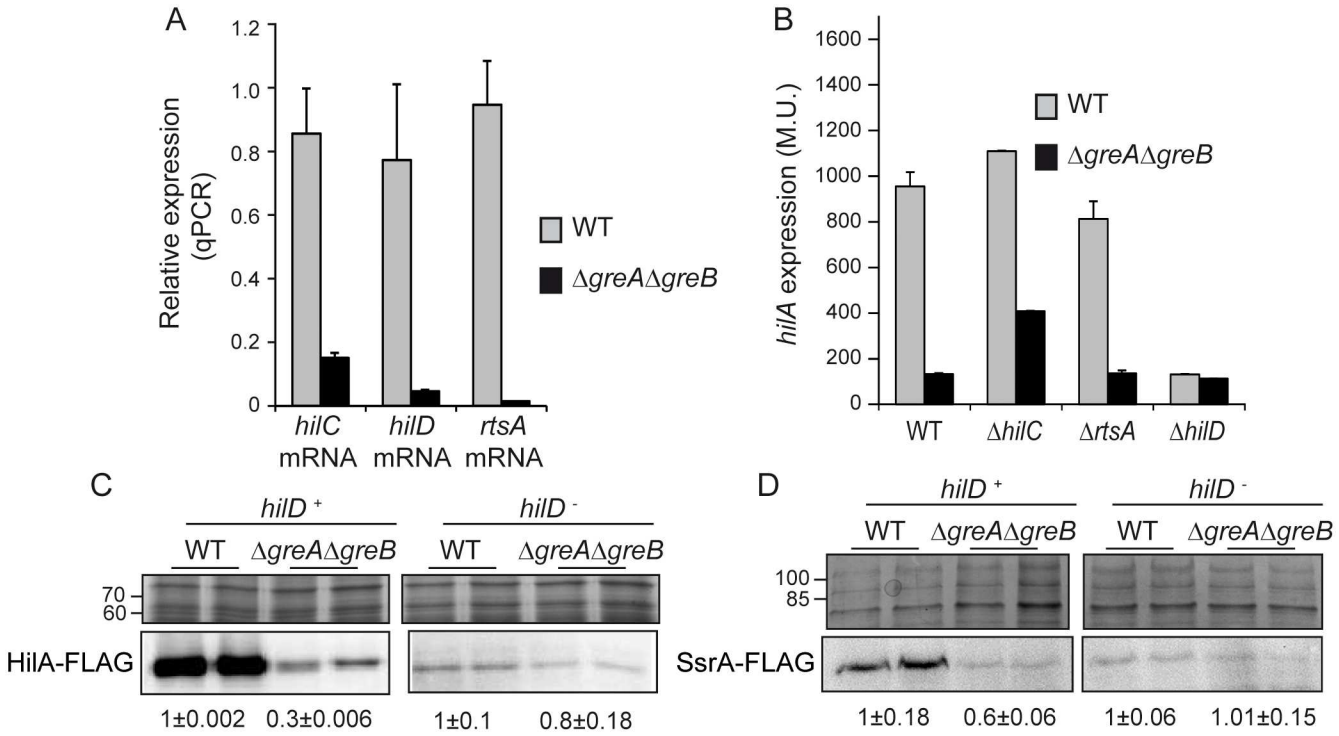

Fig 4. Gre factors-mediated regulation of *S.* Typhimurium virulence is focused in the regulation of the master regulator HilD. (A) Relative *hilC*, *hilD and rtsA* mRNA quantification by qPCR in WT and Δ*greA*Δ*greB* derivative strains. Results are normalized after detection of *gapA* (GAPDH) that was used as an endogenous control. Same RNA samples as in Fig 3B. (B) Transcriptional expression of *hilA* in WT, *hilC*, *rtsA* and *hilD* derivative strains either proficient (grey bars) or deficient (black bars) in the Gre factors was monitored by β-galactosidase activity determination from a *hilA*::*lacZ* fusion. In A and B, a bar shows the arithmetic mean of experimental results and the error bar indicates the standard deviation from three biological replicates. Immunodetection (lower panels) of HilA-FLAG protein (C) and the SPI-2 encoded SsrA-FLAG protein (D) in whole cell extracts from cultures of WT and Δ*greA*Δ*greB* derivative strains in a *hilD*$^+$ and *hilD*$^-$ genetic backgrounds. The upper panels are sections of Coomassie stained gels as loading controls. In all cases bacterial cultures were grown in LB at 37˚C up to an OD_{600nm} of 2.0.

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the experimental conditions used. A central role of HilD in regulating SPI-1 gene expression has been previously described since many signaling pathways that influence SPI-1 expression converge in controlling HilD [28]. Additionally, HilD not only regulates bacterial entry into host cells, but also coordinates the bacterial response to the intracellular milieu by modulating the expression of *ssrAB*, which encodes the central positive regulator of the SPI-2 genes [17]. SsrA expression was evaluated in WT and the Δ*greA*Δ*greB* mutant (Fig 4D), with the latter showing lower SsrA levels. Under the conditions used, *ssrAB* expression depended strongly on HilD, since there was no expression in a *hilD*-deficient strain regardless of the presence or absence of Gre factors. Our results suggest that Gre-mediated regulation of *hilD* is not restricted to SPI-1, but also affects other genetic loci, having a pronounced effect on the expression of virulence traits in *S.* Typhimurium.

We aimed to determine whether *hilA* downregulation and all the downstream effects are a consequence of Gre-mediated regulation of *hilD*. As shown in Fig 5A, ectopic induction of *hilD* expression elicited *hilA* expression even in the absence of Gre factors. Consistent with these results, the Δ*greA*Δ*greB* strain efficiently produced and secreted SPI-1 effector proteins and recovered the ability to invade epithelial cells when HilD expression was ectopically induced (S5 Fig and Fig 5B). Motility is required for effective invasion by *Salmonella* [25]. Motility, though, moderately 2-fold downregulated in the absence of Gre factors, was not

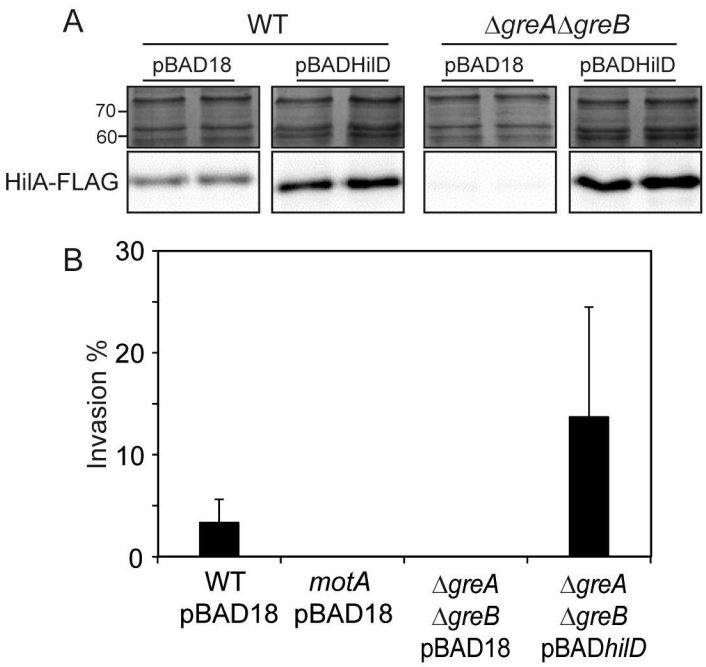

Fig 5. Overexpression of HilD restores HilA expression and epithelial cell invasiveness in Δ*greA*Δ*greB* strains. (A) In lower panels, immunodetection of the HilA-FLAG protein in whole cell extracts from cultures of WT and Δ*greA*Δ*greB* derivative strains carrying either pBAD18 or pBADHilD grown in LB at 37˚C up to an OD_{600nm} of 2.0, arabinose (0.02%) was added in all cultures. The upper panels are sections of Coomassie stained gels as loading controls. (B) Invasion assays using WT, Δ*motA* and Δ*greA*Δ*greB* strains carrying the indicated plasmids. Bacterial cultures were grown as in Fig 1. A bar shows the arithmetic mean of experimental results and the error bar indicates the standard deviation.

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restored upon overexpression of HilD (S6 Fig). Thus, Gre factors specifically regulate SPI-1 expression and epithelial invasiveness via *hilD* expression.

The 3'-UTR of *hilD* is required for Gre-mediated regulation of *hilD* expression

Several regulatory mechanisms acting at the transcriptional, post-transcriptional, translational and post-translational levels of HilD expression have been described. It should be noted that in contrast to the qPCR data (Fig 4A), there was no differences in *hilD* transcriptional expression between WT and the Δ*greA*Δ*greB* mutant when a chromosomal transcriptional *hilD*::*lacZ* fusion at position +76 (41 bp within the *hilD* open reading frame (ORF)) was assessed (Fig 6A). This discrepancy indicates that Gre factors modulate *hilD* transcriptional expression after initiation of transcription. Other features that need to be taken in account when assessing *hilD* transcription are the presence of an intact *hilD* ORF, as the HilD protein positively regulates its own expression [15] and the presence of the newly described regulatory motif *hilD* 3'-UTR, that modulates *hilD* mRNA stability by promoting its rapid degradation [29]. Both regulatory units, the entire ORF and the 3'-UTR are absent in the $hilD_{76}$::*lacZ* construct.

To assess the involvement of the *hilD* ORF and the 3'-UTR in Gre factor-mediated regulation of *hilD* two more constructs were investigated: i) the $hilD_{965}$::*lacZ* fusion (at position +965, after the TAA codon of the *hilD* ORF, $HilD^+$ $3'UTR^-$) and ii) the $hilD_{1235}$::*lacZ* fusion (at position +1235, just upstream of the transcriptional terminator, $HilD^+$ $3'UTR^+$) (Fig 6A). When comparing β-galactosidase activity levels among the different fusions in the WT strain,

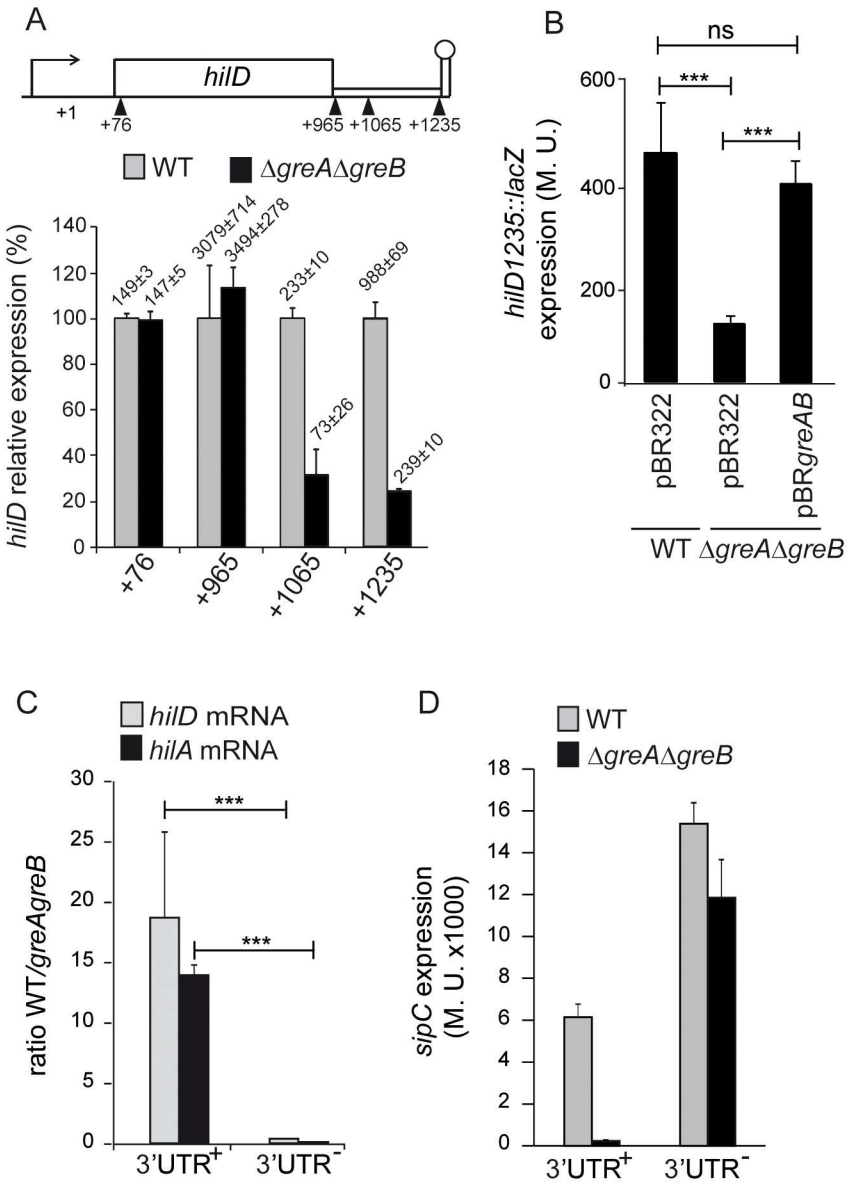

Fig 6. The 3'UTR of *hilD* is required for the Gre-mediated regulation. (A) Transcriptional expression from *hilD*::*lacZ* chromosomal fusions at positions +76, +965, +1065 and +1235 (relative location in the *hilD* gene is indicated in the upper panel). β-galactosidase activity was monitored in LB cultures grown at 37˚C up to an OD_{600nm} of 2.0.of both WT and Δ*greA*Δ*greB* strains carrying the indicated fusions. (B) $hilD_{1235}$::*lacZ* transcriptional expression was tested in cultures of WT and Δ*greA*Δ*greB* derivative strains carrying the indicated plasmids. β-galactosidase activity was determined as in A. (C) Ratio in the levels of *hilD* and *hilA* transcripts between WT and Δ*greA*Δ*greB* strains in *hilD* 3'UTR+ and *hilD* 3'UTR- genetic backgrounds. mRNA levels were monitored by qPCR using detection of *gapA* (GAPDH) as an endogenous control. Total RNA was isolated from cultures grown as in A. (D) Transcriptional expression from a *sipC*::*lacZ* chromosomal fusion. β-galactosidase activity was monitored as in A in cultures of both WT and Δ*greA*Δ*greB* strains in both *hilD*3'UTR^+ and *hilD*3'UTR^- genetic backgrounds. In all panels, a bar shows the arithmetic mean of experimental results and the error bar indicates the standard deviation from three biological replicates. In B and C, to assess differences in the values, an unpaired, two-sided Student's test was performed. Statistical significance is indicated by ***p<0.001, ns: non-significant.

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the expression of $hilD_{965}$::*lacZ* was higher than that of $hilD_{76}$::*lacZ*. The production of HilD from the $hilD_{965}$::*lacZ* fusion and the subsequent positive *hilD* autoregulation may explain this result. Moreover, $hilD_{1235}$::*lacZ* showed lower transcriptional activity than $hilD_{965}$::*lacZ*. This result is consistent with the assigned role of the 3'-UTR in promoting mRNA degradation.

Next, the transcriptional expression of *hilD* in the WT and its Δ*greA*Δ*greB* double mutant was compared. As for the $hilD_{76}$::*lacZ*, no difference was detected with the fusion $hilD_{965}$::*lacZ*. Remarkably, a clear drop in *hilD* expression in the absence of the Gre factors can only be detected with the $hilD_{1235}$::*lacZ* fusion (Fig 6A). Confirming that downregulation was due to the absence of Gre factors, $hilD_{1235}$::*lacZ* expression recovered to WT levels in the presence of the pBR*greAB* plasmid (Fig 6B). Since the absence of Gre factors had no effect on *hilD* expression when the 3'-UTR was not present ($hilD_{76}$::*lacZ* and $hilD_{965}$::*lacZ*), the 3'-UTR of *hilD* is therefore required for Gre-factor modulation of *hilD* expression. To narrow down the region, the effect of the Gre factors was localized within the first 100 nucleotides of the 3' UTR with fusion $hilD_{1065}$::*lacZ* (Fig 6A). Consistently, the first 100 nucleotides of the 3' UTR of *hilD* are sufficient to modulate *hilD* expression [29].

To further assess the requirement of the 3'-UTR for Gre-mediated regulation of *hilD*, the mRNA levels of both *hilD* and *hilA* were monitored by qPCR in a strain carrying a deletion of the *hilD* 3'-UTR, UTR^-, and compared to the WT, UTR^+ background. The results are given as a transcript ratio of WT versus Δ*greA*Δ*greB* (Fig 6C). Interestingly, only in the presence of the 3'-UTR a relative drop in *hilD* and *hilA* expression in the Δ*greA*Δ*greB* strain can be detected and consequently the specific transcripts were 18- and 14-fold more abundant in WT as compared to the Gre-deficient strains. These results are in agreement with the data derived from the *hilD*::*lacZ* fusions (Fig 6A).

Furthermore, the transcriptional expression of *sipC* was monitored (Fig 6D). Consistent with the assigned role of the *hilD* 3'-UTR [29], the results revealed that the lack of the *hilD* 3'-UTR upregulates *sipC* expression in the presence and absence of Gre factors, with higher expression than in the WT strain containing $3'UTR^+$. These results were generalized by the assessment of secreted SPI-1 effector proteins (S7 Fig).

GreA-mediated rescue of backtracked paused complexes during transcription is crucial in promoting *hilD* expression

In *E. coli*, the acidic residues D41 and E44 of the GreA protein are required to prevent backtracking of paused complexes thereby suppressing transcriptional pauses. A *greA** (D41A, E44Y) mutant, devoid of the rescue function for transcriptional arrest, has been described previously [30]. The GreA protein of *E. coli* and *S.* Typhimurium are highly conserved, sharing 96.83% identity including the above mentioned acidic residues. We used the *greA* (WT) and *greA** (D41A, E44Y) from *E. coli* to determine if the anti-backtracking activity of GreA is associated with the regulation of SPI-1 genes. As shown in Fig 7A, transcriptional expression of *hilD*, *hilA* and *sipC* was fully restored when the *greA* gene of *E. coli* was introduced into the *S.* Typhimurium Δ*greA*Δ*greB* double mutant. However, this was not observed with the *greA** variant, indicating that the anti-backtracking activity of GreA is needed for the transcriptional expression of *hilD* and, consequently, SPI-1 genes. Immunodetection using a monoclonal anti-GreA antibody confirmed equal expression of *greA* and *greA** in *S. typhimurium* (Fig 7B).

Our results suggest that Gre factors affect SPI-1 expression by a mechanism that includes prevention of a transcriptional arrest located in the *hilD* 3'-UTR. To explore the possible presence of a transcriptional arrest site within the 3'-UTR, we used an *in vivo* approach involving a pQF-50 based vector carrying a P_{BAD} promoter upstream of the promoter-less *lacZ* gene. Three different fragments of *hilD* were cloned between the P_{BAD} promoter and the *lacZ* gene

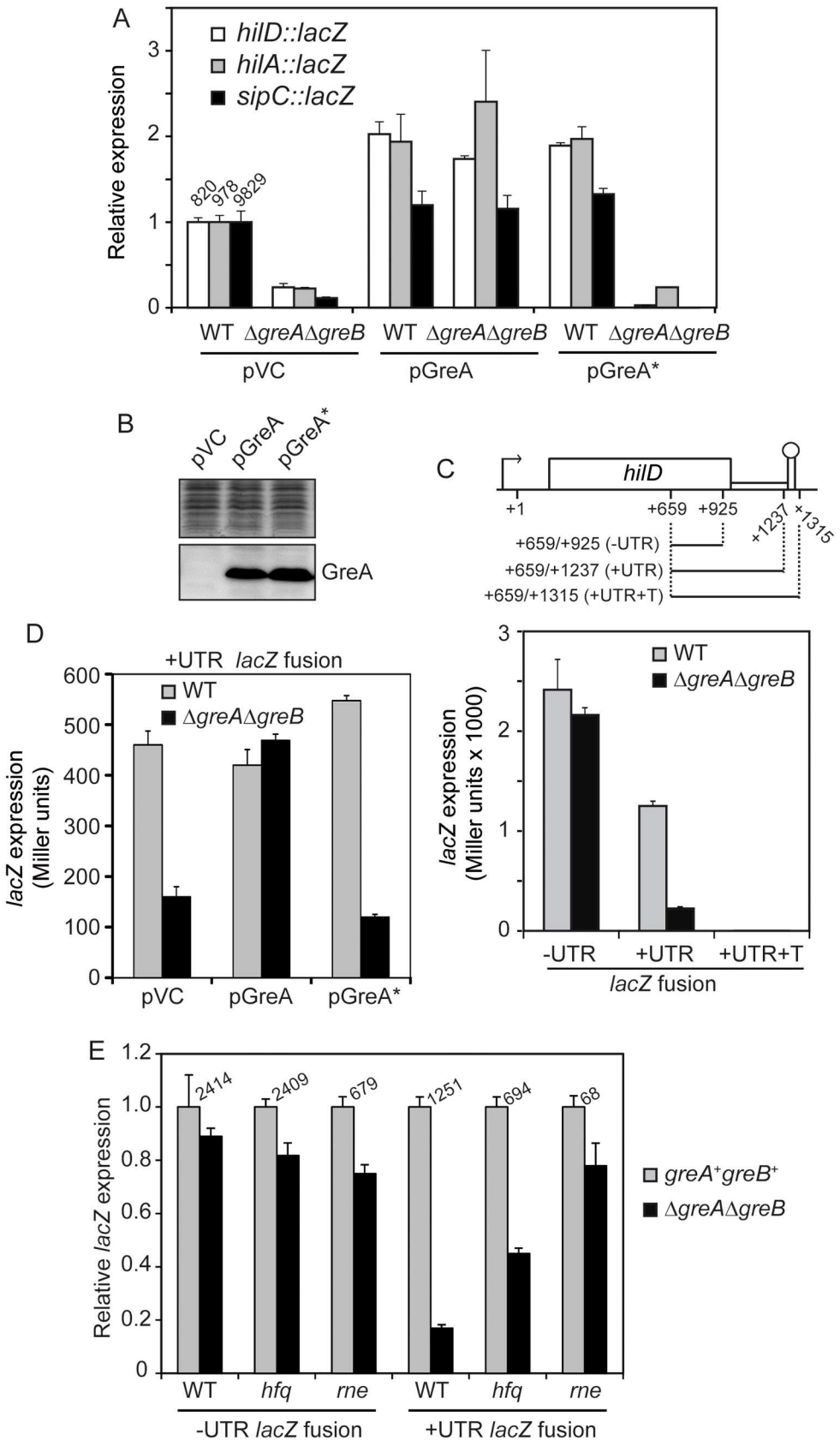

Fig 7. The anti-backtracking activity of the Gre factors is required for the expression of SPI-1 genes by alleviating a transcriptional pause located within the 3'-UTR of *hilD*. (A) Transcriptional expression

from *hilD*:: *hilA*:: and *sipC*::*lacZ* chromosomal fusions. β-galactosidase activity was monitored in cultures of both WT and Δ*greA*Δ*greB* strains carrying the following plasmids pHM1883 (pVC), pHM1873 (pGreA) and pHM1854 (pGreA*). (B) Immunodetection of GreA protein was performed in extracts from the Δ*greA*Δ*greB* strain with plasmids as in A. (C) *lacZ* expression from plasmids constructs carrying the indicated *hilD* fragments (-UTR, +UTR and +UTR+T) cloned in pTT68 vector downstream of a P_{BAD} promoter and upstream of a promoter less *lacZ* gene in both WT and Δ*greA*Δ*greB* strains. (D) Expression from the +UTR *lacZ* fusion, as denoted in panel C, in WT and Δ*greA*Δ*greB* strains carrying the plasmids pHM1883, pHM1873 and pHM1854. (E) Effect of the Gre factors on expression of the -UTR and +UTR *lacZ* fusions, as denoted in panel C, in WT and its *hfq* and *rne*537 mutants. In all cases, cultures were grown in LB at 37˚C up to an OD_{600nm} of 2.0. In B, D and E, 0.02% arabinose was added to the LB medium. In A and C-E panels, a bar shows the arithmetic mean of experimental results and the error bar indicates the standard deviation from three biological replicates.

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(Fig 7C, upper panel): a 267-bp fragment from *hilD* encoding the sequence upstream of the 3'-UTR (+659/+925); a 579-bp fragment (+659/+1237) carrying the entire *hilD* 3'-UTR; and a 657-bp fragment (+659/+1315) carrying both the 3'-UTR and the native *hilD* transcriptional terminator downstream of the 3'-UTR. These constructs were introduced into *araBAD araC* SV5015 derivatives to increase sensitivity to arabinose induction and *lacZ* expression was monitored under permissive conditions (0.02% arabinose) in both WT and the Δ*greA*Δ*greB* mutant (Fig 7C). With the construct carrying the 267-bp fragment lacking the 3'-UTR (-UTR), the expression level was not affected by the presence or absence of Gre factors. For the construct carrying the 3'-UTR and the native terminator (+UTR+T), there was no β-galactosidase activity, demonstrating the effectiveness of the *hilD* Rho-independent transcriptional terminator. Remarkably, the absence of Gre factors had a clear effect when using the construct carrying the 3'-UTR, but lacking the terminator (+UTR). Expression decreased more than 5-fold in the absence of Gre factors, suggesting that during transcription of the 3'-UTR sequence an arrested transcription complex is formed which is released by the Gre factors. Consistently, expression of *lacZ* preceded by the +UTR raises to WT levels upon *greA* overexpression, whereas no recovery was detected with the *greA** (D41A, E44Y) variant (Fig 7D).

The 3'-UTR of *hilD* plays a role in *hilD* mRNA turnover and in Hfq-mediated modulation of *hilD* expression [29]. To explore whether mRNA turnover and Hfq are involved in the Gre-mediated control of *hilD* expression, Δ*hfq* and *rne*537 mutations were generated in *greA*$^+$*greB*$^+$ WT and Δ*greA*Δ*greB* double mutant backgrounds. Expression of *lacZ* preceded by -UTR fragment was only moderately altered in the absence of Gre factors in WT, *hfq* and *rne*537 (Fig 7E), consistent with the fusion $hilD_{965}$::*lacZ*, lacking the 3'-UTR, to be Gre factor independent (Fig 6A). In contrast, while expression of *lacZ* preceded by +UTR is 5.5-fold downregulated in the absence of the Gre factors, absence of an intact RNAseE reduced this difference to 1.3-fold suggesting that mRNA turnover plays a relevant role in Gre-mediated control of transcriptional regulation involving the +UTR. In the *hfq* genetic background a 2.2-fold drop was observed indicating a partial role for Hfq. This is consistent with mRNA turnover and Hfq-mediated regulation playing a relevant role in the control of genetic constructs containing the *hilD* 3'-UTR [29]. The Gre factors regulate expression when the 3' UTR of *hilD* is present, presumably through its anti-backtracking activity. The molecular mechanism by which *hilD* expression is effectively downregulated in the absence of the Gre factors remains elusive, but RNA processing seems to play a crucial role.

Discussion

Gre factors were initially described to prevent backtracking of paused complexes during transcription elongation thereby preventing transcriptional arrest, with further studies assigning

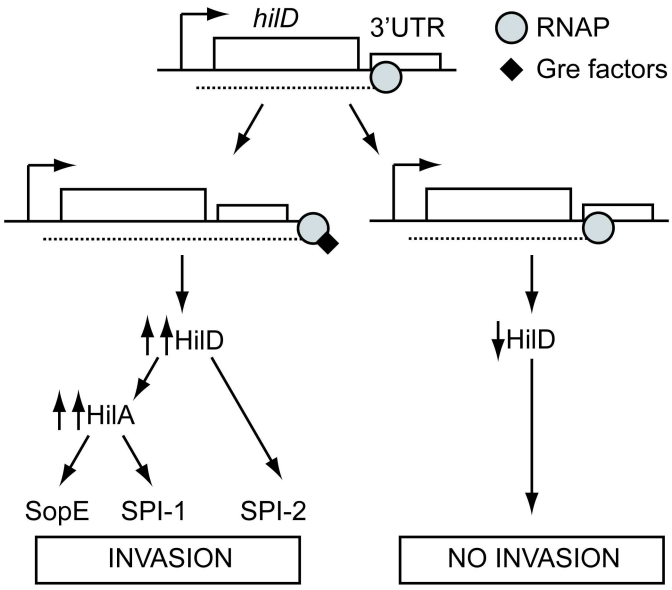

Fig 8. Summary of the effects of Gre factor deficiency in the expression of virulence in *S.* Typhimurium.

https://doi.org/10.1371/journal.ppat.1006312.g008

additional roles in transcription initiation and fidelity [31]. Genes encoding Gre proteins are found in most bacteria. It has been reported that mutations in genes encoding Gre factors elicit hypersensitivity to ionic detergents, high temperatures and osmotic shock, suggesting that Gre factors are involved in adapting to harsh environments [32–34]. Interestingly, *greA* is a member of the sigma E regulon in both *E. coli* and *S.* Typhimurium, further indicating a potential role of GreA in cell stress response [35–37]. Moreover, *greA* expression is upregulated during the stress response to hypoxia and acid in *Mycobacterium avium* and *Streptococcus mutans*, respectively [38,39]. However, a direct relationship between the molecular mode of action of Gre factors during transcription and their physiological role in the cell is not clear. Few studies on the *in vivo* function of the Gre factors exist. In *E. coli* K12 strains only a discrete effect on the global gene expression pattern was detected when the level of GreA production was altered [23]. In this report, we demonstrate that Gre factors are required for the optimal expression of virulence factors in *S.* Typhimurium, since the ability to invade epithelial cells and produce SPI-1-encoded effector proteins was fully impaired in strains lacking both GreA and GreB. The absence of either GreA or GreB had partial effects compared to those elicited by the absence of both Gre factors. Moreover, the absence of only GreA had a greater impact on *S.* Typhimurium virulence than the absence of only GreB. This suggests that Gre factors might be functionally exchangeable up to a certain point, whereby GreA seems to play a more relevant role in *S.* Typhimurium pathogenesis than GreB. Similar observations on the functional redundancy of the Gre factors have also been described in *E. coli* [23,30].

We observed that the major regulator of SPI-1, HilD, was indeed the target of Gre-mediated regulation. As summarized in Fig 8, Gre factors affect *hilD* expression during transcription elongation, targeting the recently described 3'-UTR regulatory motif of the *hilD* gene. Although there is currently no physical evidence for an arrested or backtracked complex during *hilD* transcription on which the Gre factors act, the requirement of the anti-backtracking activity of Gre factors suggest a model where Gre factors contribute to the regulation of *hilD* expression by rescuing an arrested or backtracked complex that occurs in the *hilD* 3'-UTR

during transcription elongation (Fig 8). Further studies will be needed to address how backtracked RNAP complex potentially in combination with other mechanisms affects *hilD* expression.

HilD expression and activity are very tightly regulated, highlighting its pivotal role in the biology of *S*. Typhimurium. HilD acts as a regulatory hub for virulence coordinating the expression of several virulence factors encoded inside and outside SPI-1 [18,28,40–42]. Indeed, in this report we provide further corroboration of the role of HilD in regulating SPI-2 gene expression. Given the significant impact of HilD on gene expression in *S*. Typhimurium, HilD-mediated activation of gene expression only occurs when several environmental and physiological cues provide permissive conditions. Multiple signaling pathways control *hilD* transcription initiation [28]. CsrA post-transcriptionally regulates *hilD* [41]. At the post-translational level, factors such as HilE and the Lon protease affect HilD activity and stability, respectively, while different metabolites, including L-arabinose and fatty acids, have also been shown to modulate HilD activity [43–46]. In this report, we provide evidence that HilD regulation also occurs during transcription elongation in the 3'-UTR.

Pauses during transcription elongation can be targeted in gene expression regulation. In eukaryotes, paused complexes with RNAPII are affected by environmental cues. In bacteria, transcriptional pauses are involved in transcription attenuation, transcription termination and coupling transcription and translation [22]. Studies in *Streptococcus pneumoniae* suggest that transcription elongation is a highly regulated step of gene expression, whereby GreA plays a relevant role by preventing long-living pauses during transcription [47]. Regulatory pausing events are mainly localized in proximal promoter sequences, often in the 5'-UTR, but in *hilD*, it is the 3'-UTR that putatively contains a transcriptional pause. In bacteria, investigation of the features of 3'-UTRs of genes is a growing field of study, with the 3'-UTR possibly acting as a reservoir for sRNAs [48]. 3'-UTRs are rather abundant as in the *Staphylococcus aureus* genome, where up to a third of the genes carry long 3'-UTRs [49].

The *hilD* 3'-UTR is a regulatory sequence that overall affects transcript stability, since its absence increases *hilD* transcript levels. Furthermore, positive regulation of *hilD* expression by Hfq has been reported to require the 3'-UTR sequence [29]. As Hfq is a major sRNA chaperone, it probably regulates *hilD* expression through its 3'-UTR by a mechanism involving sRNA-based post-transcriptional regulation. In the present study, we propose that *hilD* expression is also regulated during transcription elongation of the 3'-UTR. Further studies are needed to elucidate whether changes in the kinetics of transcription elongation in the 3'-UTR affect *hilD* expression. Taking into consideration the described role of the *hilD* 3'-UTR, our results suggest that the anti-backtracking activity of Gre factors beyond *hilD* ORF transcription elongation may decrease susceptibility to mRNA degradation, possibly by stimulating the generation of certain RNA structures or promoting optimal interactions with sRNAs to protect the *hilD* transcripts from degradation. Thus, our report provides evidence for an additional mechanism of regulation of *hilD* expression at the post-transcriptional level.

Materials and methods

Bacterial strains, plasmids and growth conditions

Bacteria strains and plasmids used in this study are listed in S1 Table. Bacteria were grown in LB (5 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract), and LB agar plates with 15 g/L agar. Lac phenotype from strains with *lacZ* fusions was monitored on LB agar plates supplemented with X-gal (40 μg/mL). When needed, antibiotics were added at the following concentrations: chloramphenicol (Cm) 15 μg/mL, kanamycin (Km) 50 μg/mL, ampicillin (Amp) 50 μg/mL,

streptomycin (Sm) 20 μg/mL and spectinomycin (Sp) 25 or 50 μg/mL. Arabinose 0.02% (w/v) was added to the cultures for induction of genetic constructs with the P_{BAD} promoter.

Construction of mutants

Chromosomal mutants were generated by one step gene replacement by homologous recombination [50]. In general, entire open reading frames except 40 nucleotides at the beginning and at the end of the gene were replaced by either a kanamycin (Km) or chloramphenicol (Cm) resistance marker. The Km or Cm resistance gene along with target gene homologous overhangs was PCR-amplified from pKD4 or pKD3, respectively, and electroporated into *S*. Typhimurium carrying pKD46. Primers used in this work are listed in S2 Table. Recovered colonies were purified on LB medium containing the corresponding antibiotics. To obtain tagged proteins for immunodetection purposes, a 3×FLAG tag linked to a kanamycin cassette was amplified from pSUB11 plasmid [51] and inserted in frame upstream of the stop codon of the protein of interest. For transcriptional studies, *lacZ* fusions were constructed as described by Ellemeier et al [52]. Briefly, first the resistance cassette was removed expressing the FLP protein encoded in plasmid pCP20 that promotes site-specific recombination between the FRT sites. Next, either plasmid pKG136 or plasmid pKG137 were transformed into the resulting strains. Again, FLP-mediated recombination achieved from plasmid pCP20 results in an integrated *lac* fusion into the gene of interest.

Phage transduction of mutant alleles into a novel strain background was carried out with phage P22 HT/int4. Transductants were colony purified twice on EBU LB agar plates containing 0.25% (p/v) glucose, 0.25% (p/v) K_2HPO_4, 0.0125 g/L evans blue and 0.0250 g/L fluorescein [53]; and appropriate antibiotics. All constructed mutants were verified by PCR with control primers located in the genes flanking the deleted open reading frame.

Plasmid construction

The *greA*, *greB* and *hilD* genes were cloned in pBR322 vector in order to perform complementation experiments. Primers greASalmUP/greASalmDOWN, greBSalmUP/greBSalmDOWN and hilDFw322/hilDRv322A were used to PCR amplify the different genes. After cloning the fragments in pGEM-T, either the *Eco*RI-*Bam*HI fragments (*greA* and *greB)* or the *Bam*HI-*Sal*I fragment (*hilD*) were cloned subsequently in pBR322, resulting plasmids pBR*greA*, pBR*greB* and pBR*hilD*. For genotyping purposes, the primers pBR-FW/pBR-RV were used. To generate the pBR*greAB* plasmid, primers greASalIUP and greABamHIDOWN, were used to amplify the *greA* gene. The PCR fragment was cloned into *Sal*I-*Bam*HI pBR*greB*. For genotyping the primers pBRFw/greASalIUP or greBSalmUP/greASalIUP were used.

The ORF of *hilD* was PCR amplified using the primers hilDBADFw and hilDBADRev and cloned in the *Eco*RI and *Xba*I sites of pBAD18. Different fragments of the *hilD* gene were cloned in the pTT68 vector, downstream of the arabinose inducible promoter P_{BAD} and upstream of a promoter less *lacZ* gene. The *hilD* fragments were PCR amplified with the primer pairs hilDNcoI1/UTRSalI3, hilDNcoI1/UTRSalI8 and hilDNcoI1/UTRSalI6. The *Nco*I-*Sal*I PCR fragments were cloned in the same restriction sites of pTT68. The generated plasmids were named pTTORF, pTT3'UTR and pTT3'UTR+T, respectively.

Invasion assay

Invasion assays were performed as previously described [25]. The human colon adenocarcinoma cell lines HT-29 (ATCC HTB 38) and Caco-2 (ATCC HTB 37) were grown to confluence in 24-well plates in RPMI-1640 medium (Life Technologies) supplemented with 25 mM HEPES, 2 mM L-glutamine and 10% fetal calf serum (Hyclone) at 37˚C in 5% CO_2. *S*.

Typhimurium was grown in LB containing 0.3 M NaCl in standing culture for 16h, diluted 1:100 in fresh medium and grown until OD_{600nm} of 0.6 at 37˚C statically. Bacteria were washed with cold PBS and resuspended in RPMI-1640 medium. A 100 μL aliquot of the bacterial suspension were added to each well of confluent epithelial cells (MOI of 1.7, approximately). One hour post infection, supernatant was removed and RPMI-1640 medium containing gentamicin at a final concentration of 100 μg/mL was added to the cells for 1 h to kill remaining extracellular bacteria. Cells were gently washed twice with PBS and disrupted with 1% Triton X-100 (Sigma). The number of intracellular bacteria was determined by counting colony-forming units (CFU). The Δ*motA* and Δ*hilA* mutants were used as a negative control in all assays. The invasion percentage was calculated as CFU recovered inside cells after 1 h incubation with reference to the inoculum CFU at time of inoculation as 100%. Results are based on at least two biological replicates consisting of two technical replicates each.

Animal studies in BALB/c mice

WT SV5015 and its Km-resistant Δ*greA*Δ*greB* derivative (TGC65) were grown in LB medium at 37˚C up to an OD_{600nm} of 2.0. The bacteria were pelleted and diluted 1/10 in sterile PBS. The input ratio of a 1:1 mixture of WT and Δ*greA*Δ*greB* suspensions was determined by plating serial dilutions onto LB-Sm for total CFU and on LB-SmKm plates for estimation of CFU for the Δ*greA*Δ*greB strain*. A group of 5 8-week-old female BALB/c mice was infected orally with ~ 2E+7 CFU in 200 μL. Mice were sacrificed by CO_2 asphyxiation four days post infection and liver and spleen were removed and homogenized. The homogenates were treated with sodium deoxycholate (0.01%), serially diluted and plated onto LB-Sm and LB-SmKm in triplicates. CFU/g were determined and CI values were calculated as the mean ratio of mutant versus WT CFU, normalized to the input ratio [54]. All animal care and handling were performed according to Federation of European Laboratory Animal Science Associations (FELASA) guidelines and were under the approval of the University of Barcelona (UB) Ethical committee.

Secreted protein extracts

Protein extracts of the cell free supernatants, corresponding to the secreted protein fraction, was analyzed. Bacterial strains were cultured in LB medium at 37˚C up to an OD_{600nm} of 2.0 (early-stationary phase). A 5 mL culture aliquot was centrifuged for 10 min at 4000 g at room temperature. The supernatant was filtered through a 0.22 μm filter and the proteins precipitated using trichloroacetic acid (TCA) [55]. When whole culture extracts were obtained, bacterial cultures (cells and medium) were TCA precipitated.

SDS-PAGE and western blot analysis

Secreted protein extracts and whole cell extracts were separated on a 12.5% SDS-PAGE gel. To detect total protein, Coomassie staining was performed. When specific proteins were detected, proteins were electrotransferred onto a PVDF membrane (Bio Rad). For immunodetection the following antisera were used; monoclonal anti-FLAG (Sigma) polyclonal anti-SopE [56] and monoclonal anti-GreA (Neoclone). Detection was with a HRP-conjugated secondary antibodies and ECL Prime Western Blotting detection reagent (GE Healthcare). Visualization of the detected bands was performed using Molecular Imager ChemiDoc XRS System and Quantity One software (Bio Rad). Prior to western blot analysis from whole cell extracts, the protein content of samples normalized by OD_{600nm} (biomass of original cultures) was corroborated by Coomassie staining. Equally, loading of secreted protein extract was normalized to the culture biomass (OD_{600nm}). Routinely, normalization by biomass of cultures grown to obtain secreted protein extracts was corroborated by SDS-PAGE and Coomassie staining of the cell extract

from the cultures. Moreover, immunodetection of the cytoplasmic cyclic AMP receptor protein CRP was performed in secreted protein extracts to exclude contamination with cellular proteins. A representative control experiment performed on a secreted protein extract is shown in S8 Fig.

Haemolytic activity

The haemolytic activity was determined as described [57]. Briefly, the strains were grown in LB medium at 37˚C up to an OD_{600nm} of 2.0. An aliquot of 5 mL was centrifuged 15 min at 4000 g at room temperature, and the supernatant was filtered by 0.22 μm filter and kept on ice. The defribrinated sheep blood was centrifuged at 1500 g during 5 min at 4˚C and the blood cells resuspended with cold PBS in order to eliminate debris from broken cells. This process was repeated as many times as required, until supernatant was transparent. In a 96-well plate, aliquots (50 μL) of different serial dilutions of the cell-free supernatant were mixed with 50 μL of defribrinated sheep blood stock solution. Next, mixtures were incubated statically at 37˚C during 2.5 hours. After incubation, 150 μL of PBS were added to each well and the plates were centrifuged (10 min, 400 g, 4˚C). A 100 μL aliquot of the supernatant was removed to another plate and the hemoglobin was monitored measuring the optical density at 550nm.

β-Galactosidase assay

β-Galactosidase assays were performed as described [58]. Data are mean values of duplicate determinations in at least three independent experiments plotted with standard deviations.

Expression analysis by qPCR

RNA was purified from three independent cultures grown in LB medium at 37˚C up to an OD_{600nm} of 2.0 by using Total RNA Isolation kit (Promega) according to the manufacturer's protocol. Samples were analyzed by Bioanalyzer 2100 from Agilent in order to verify RNA quality. After determination of the RNA concentrations using the NanoDropND-1000 V3.3.0 Spectrophotometer, 1 μg RNA was reverse transcribed in a 20 μL reaction using High-Capacity cDNA Reverse Transcription kit (Life Technologies). Primers used for quantification of the *hilA*, *hilD*, *hilC* and *rtsA* transcripts are listed in S2 Table. cDNA was diluted 1:100 and used as template in the real-time PCR reaction using SYBR Green PCR Master Mix X2 kit (Life Technologies). The cycling reaction was performed with a Step One Real-Time PCR system (Life Technologies). Individual gene expression profiles were normalized against the *gapA* gene (GAPDH) as endogenous control. In all experiments, the change in expression was measured relative to a WT strain, which was set to 1.0. The data values presented in all figures represent the mean values calculated from at least three independent experiments performed with three technical replicates. The error bars represent the standard deviations.

Expression analysis by RT-PCR

The mRNA levels of *sipA* were monitored by RT-PCR using the Transcriptor One-Step RT-PCR Kit (Roche) and the primer pairs SipAFor/SipARev (S2 Table). The RT-PCR was carried out in a Bio-Rad T100 thermal cycler. First, the RNA was reversely transcribed for 15 min at 50˚C, following by reverse transcriptase inactivation by incubation for 7 min at 94˚C. The cDNA was amplified by 35 cycles of denaturation for 10 s at 94˚C, annealing for 30 s at 54˚C, and extension for 30 s at 68˚C, with a final extension step of 7 min at 68˚C. 16S rRNA was used as the internal control, using primers SalI16S/SalII16S (S2 Table). In all cases, the amount of total RNA used was defined by performing saturation curves with increasing amounts of

total RNA to determine the interval of lineal increase in the relative amount of RT-PCR product and total RNA.

Supporting information

S1 Fig. Schematic representation of the regulatory pathway that control expression of the TTSS and effector proteins of the SPI-1 of *S*. Typhimurium.
(PDF)

S2 Fig. Invasion of epithelial cells by *S*. Typhimurium ATCC14028 is impaired in strains deficient for the Gre factors. Invasion assays used HT-29 epithelial cells. Cultures of the WT (UMR1) and the Δ*greA*, Δ*greB* and Δ*greA*Δ*greB* derivatives were assessed. As a control, cultures of the invasion impaired mutant Δ*motA* were used. A bar shows the arithmetic mean of experimental results and the error bar indicates the standard deviation. Significance was tested by an unpaired two–sided Student's t-test. Statistical significance is indicated by $^{**}p<0.01$, ns: non-significant.
(PDF)

S3 Fig. Profile of secreted proteins in a Δ*hilA* derivative strain. Protein extracts from cell-free supernatants of two independent LB cultures of WT (SV5015) and its Δ*hilA* derivative. Extracts were analyzed by Coomassie blue stained 12.5% SDS-PAGE. Lane M: molecular mass markers (size in kDa indicated). The bands labelled were identified as SipA (1), FliD (2) and SipC (3) by LC-MS/MS.
(PDF)

S4 Fig. The Δ*hilD*-cmR mutant strain is trans-complemented by a pBR322-based plasmid carrying the *hilD* gene. Cultures of the strains SV5015UB2 and TGC-10 were grown in LB at 37˚C up to an OD_{600nm} of 2.0. Data are the average and error bars represent standard deviations from three biological replicates.
(PDF)

S5 Fig. Ectopic induction of *hilD* expression elicited SPI-1 effector proteins even in the absence of the Gre factors. Cell-free supernatants of LB cultures of WT and Δ*greA*Δ*greB* strains carrying either pBAD18 or pBADHilD grown in LB at 37˚C up to an OD_{600nm} of 2.0, arabinose (0.02%) was added in all cultures. Extracts were analyzed by Coomassie blue stained 12.5% SDS-PAGE.
(PDF)

S6 Fig. Effect of the Gre factors on *Salmonella* swimming motility. Single colonies of the indicated strains were inoculated on either 0.3% LB agar plates (A) or 0.3% LB agar plates supplemented with 0.2% L-arabinose and 50 μg/ml of ampicillin (B). Plates were incubated at 37˚C for 5 hours and swimming motility diameter was measured. A bar shows the arithmetic mean of experimental results and the error bar indicates the standard deviation from 5 replicates.
(PDF)

S7 Fig. The absence of 3'-UTR of *hilD* causes a severe upregulation of the secreted SPI-1 effector protein levels even in the absence of Gre factors. Cell-free supernatants of LB cultures of WT and Δ*greA*Δ*greB* strains in both *hilD* 3'UTR+ and *hilD* 3'UTR- genetic backgrounds. Cultures were grown at 37˚C up to an OD_{600nm} of 2.0. Extracts were analyzed by Coomassie blue stained 12.5% SDS-PAGE.
(PDF)

S8 Fig. Representative control experiment for loading normalization of secreted extracts. A. Coomassie stained SDS-PAGE of either cell extracts (upper panel) or secreted protein extracts (lower panel) from two cultures of the strains SV5015 (WT) and TGC3 (Δ*greA*Δ*greB*) grown in LB at 37˚C up to an OD_{600nm} of 2.0. Lane M: molecular mass markers (size in kDa indicated). B. Immunodetection of CRP, a cytoplasmic protein, in the indicated extracts from the same cultures as in A.
(PDF)

S1 Table. Strains and plasmids used in this study.
(PDF)

S2 Table. Primers used in this study.
(PDF)

Acknowledgments

We are indebted to Dr Casadesus (Universidad de Sevilla) by providing strains SV5293, SV6190, SV5961, SV6212 and SV6197; to Dr. Huttener (IBEC) by providing MHS-1, MHS-2, MHQ-2 and plasmid pBADhilD; to Dr Bustamante (Universidad Nacional Autónoma de México) for kindly providing the strains JPTM7 and JPTM8; to Dr. Cashel (Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH) by providing the plasmids pHM1883, pHM1873 and pHM1854. We thank Dr. Wolf-Dietrich Hardt (Institute of Microbiology, ETH Zurich) for providing the SopE antisera used in this study.

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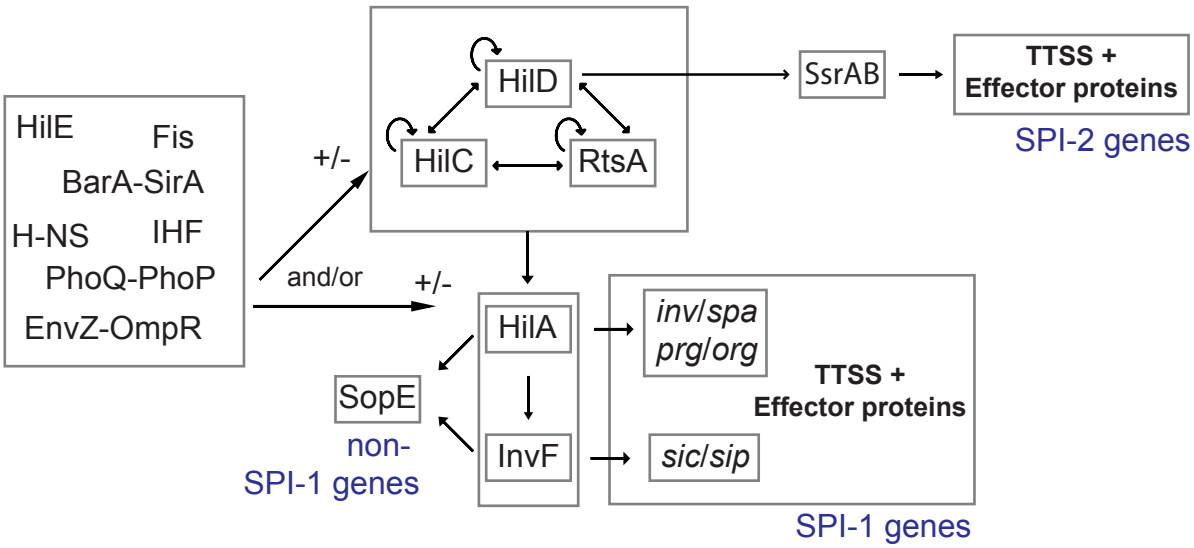

Figure S1. **Schematic representation of the regulatory pathway that control expression of the TTSS and effector proteins of the SPI-1 of *S.* Typhimurium.**

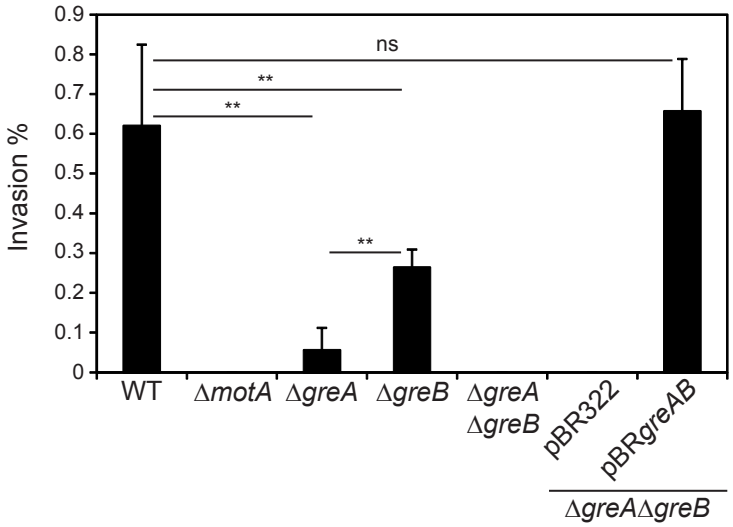

Figure S2. Invasion of epithelial cells by *S*. Typhimurium ATCC14028 is impaired in strains deficient for the Gre factors. Invasion assays used HT-29 epithelial cells. Cultures of the WT (UMR1) and the Δ*greA*, Δ*greB* and Δ*greA*Δ*greB* derivatives were assessed. As a control, cultures of the invasion impaired mutant Δ*motA* were used. A bar shows the arithmetic mean of experimental results and the error bar indicates the standard deviation. Significance was tested by an unpaired two–sided Student's t-test. Statistical significance is indicated by **p<0.01, ns: non-significant.

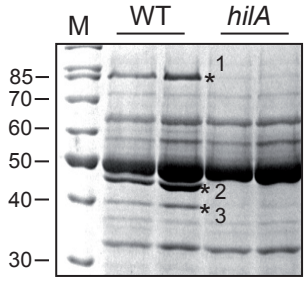

Figure S3. **Profile of secreted proteins in a Δ*hilA* derivative strain.**
Protein extracts from cell-free supernatants of two independent LB cultures of WT (SV5015) and its Δ*hilA* derivative. Extracts were analyzed by Coomassie blue stained 12.5 % SDS-PAGE. Lane M: molecular mass markers (size in kDa indicated). The bands labelled were identified as SipA (1), FliD (2) and SipC (3) by LC-MS/MS.

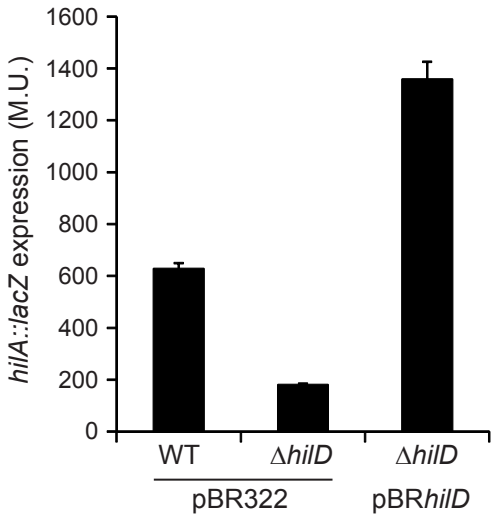

Figure S4. The Δ*hilD*-cm^R mutant strain is trans-complemented by a pBR322-based plasmid carrying the *hilD* gene. Cultures of the strains SV5015UB2 and TGC-10 were grown in LB at 37ºC up to an OD_{600nm} of 2.0. Data are the average and error bars represent standard deviations from three biological replicates.

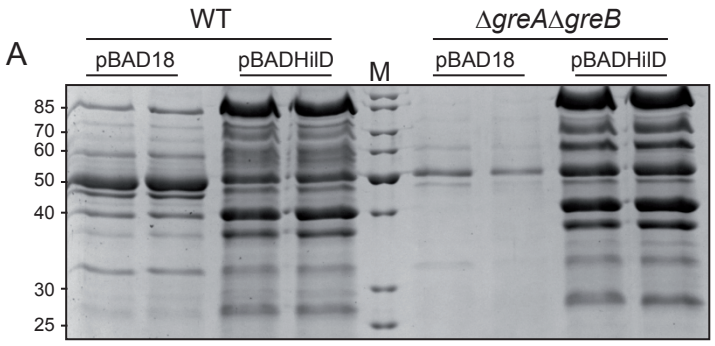

Figure S5: Ectopic induction of *hilD* expression elicited SPI-1 effector proteins even in the absence of the Gre factors. Cell-free supernatants of LB cultures of WT and Δ*greA*Δ*greB* strains carrying either pBAD18 or pBADHilD grown in LB at 37ºC up to an OD_{600nm} of 2.0, arabinose (0.02 %) was added in all cultures. Extracts were analyzed by Coomassie blue stained 12.5 % SDS-PAGE.

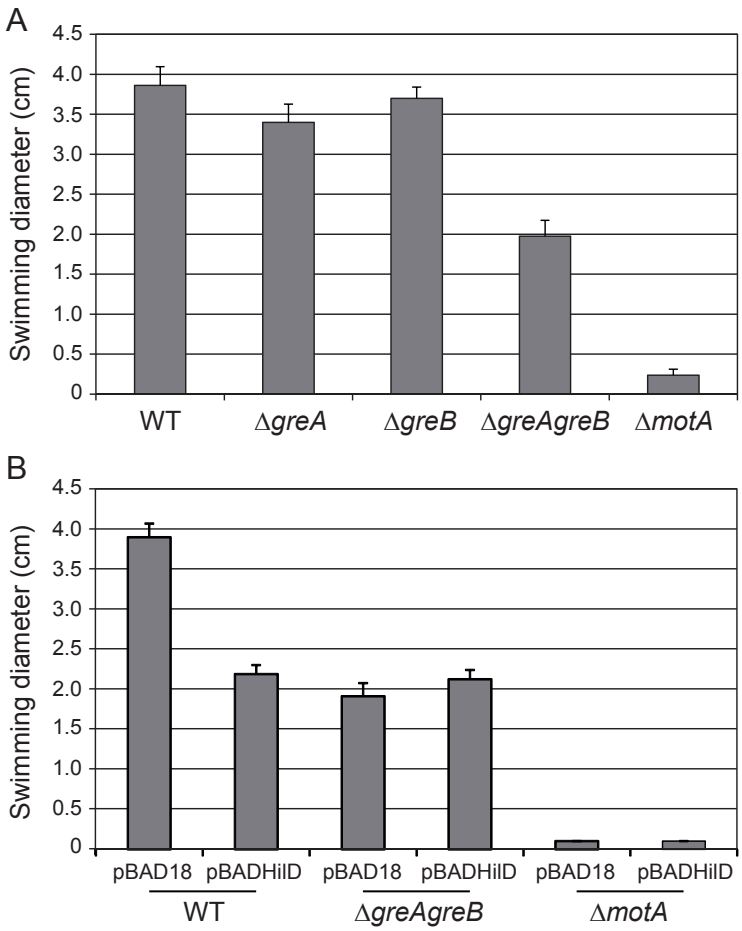

Figure S6. Effect of the Gre factors on *Salmonella* swiming motility. Single colonies of the indicated strains were inoculated on either 0.3% LB agar plates (A) or 0.3% LB agar plates supplemented with 0.2% L-arabinose and 50 μg/ml of ampicillin (B). Plates were incubated at 37ºC for 5 hours and swimming motility diameter was measured.
A bar shows the arithmetic mean of experimental results and the error bar indicates the standard deviation from 5 replicates.

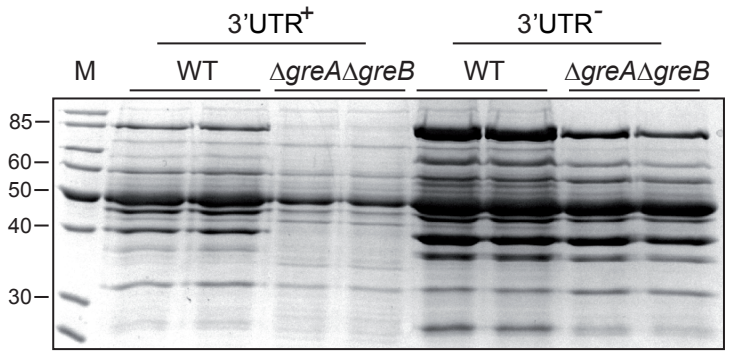

Figure S7: The absence of 3'-UTR of *hilD* causes a severe upregulation of the secreted SPI-1 effector protein levels even in the absence of Gre factors. Cell-free supernatants of LB cultures of WT and Δ*greA*Δ*greB* strains in both *hilD*3'UTR+ and *hilD*3'UTR- genetic backgrounds. Cultures were grown at 37ºC up to an OD_{600nm} of 2.0. Extracts were analyzed by Coomassie blue stained 12.5 % SDS-PAGE.

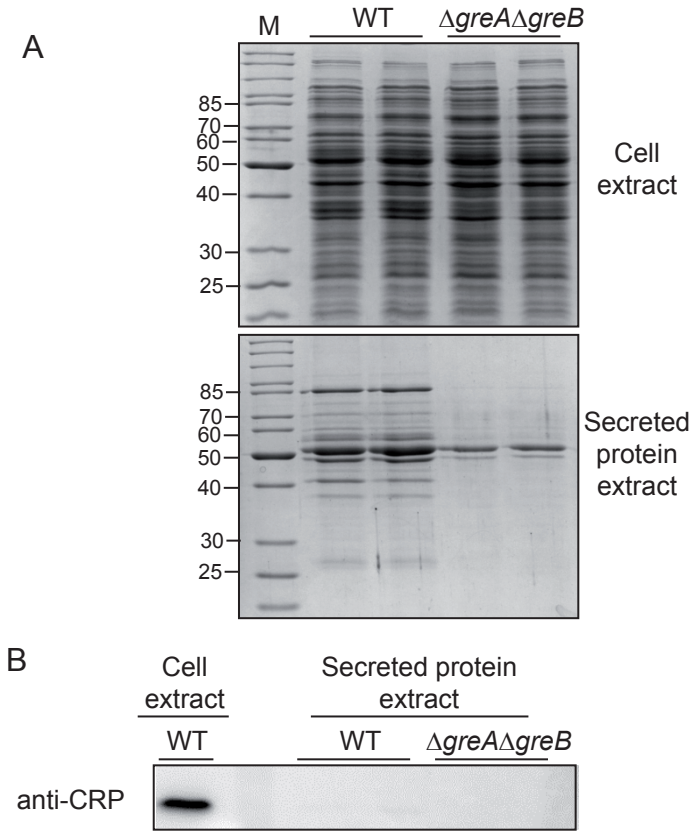

Figure S8: Representative control experiment for loading normalization of secreted extracts.
A. Coomassie stained SDS-PAGE of either cell extracts (upper panel) or secreted protein extracts (lower panel) from two cultures of the strains SV5015 (WT) and TGC3 (Δ*greA*Δ*greB*) grown in LB at 37ºC up to an OD_{600nm} of 2.0. Lane M:molecular mass markers (size in kDa indicated).
B. Immunodetection of CRP, a cytoplasmic protein, in the indiacted extracts from the same cultures as in A.

Table S1. Strains and plasmids used in this study

Strain	Relevant characteristics or description	Reference
SV5015	*Salmonella enterica* serovar Typhimurium SL1344 *his*$^{+}$	(1)
TGC-1	SV5015 *greA*	This study
TGC-2	SV5015 *greB*	This study
TGC-3	SV5015 *greAgreB*	This study
TGC-65	SV5015 *greA*-km^{R} *greB*	This study
SV5293	14028 *sipC::lacZ*-km^{R} (Δ 76 to 1112)	(2)
TGC-40	SV5015 *sipC::lacZ*-km^{R}	This study.
TGC-41	SV5015 *greAgreB sipC::lacZ*-km^{R}	This study
MHS-1	SV5015 *hilA*	(3)
MHQ-2	SV5015 *hilC*	(3)
TGC-4	SV5015 *hilD*-cm^{R}	This study
TGC-6	SV5015 *rtsA*-cm^{R}	This study
SV5015UB2	SV5015 *hilA::lacZ*-km^{R} (Δ 44 to 1621)	(4)
TGC-7	SV5015 *greAgreB hilA::lacZ*-km^{R}	This study
TGC-8	SV5015 *hilC hilA::lacZ*-km^{R}	This study
TGC-9	SV5015 *hilC greAgreB hilA::lacZ*-km^{R}	This study
TGC-10	SV5015 *hilD*-cm^{R} *hilA::lacZ*-km^{R}	This study
TGC-11	SV5015 *greAgreB hilD*-cm^{R} *hilA::lacZ*-km^{R}	This study
TGC-14	SV5015 *rtsA*-cm^{R} *hilA::lacZ*-km^{R}	This study
TGC-15	SV5015 *greAgreB rtsA*-cm^{R} *hilA::lacZ*-km^{R}	This study
MHS-2	SV5015 *sipA::*3Flag-km^{R}	(5)
TGC-16	SV5015 *greAgreB sipA::*3Flag-km^{R}	This study
JPTM7	SV5015 *hilA::*3Flag-km^{R}	(6)
TGC-17	SV5015 *greAgreB hilA::*3Flag-km^{R}	This study
TGC-18	SV5015 *hilD*-cm^{R} *hilA::*3Flag-km^{R}	This study
TGC-19	SV5015 *greAgreB hilD*-cm^{R} *hilA::*3Flag-km^{R}	This study

TGC-20	SV5015 *invF::*3Flag-km^R	This study
TGC-21	SV5015 *greAgreB invF::*3Flag-km^R	This study
JPTM8	SV5015 *ssrA::*3Flag-km^R	(6)
TGC-22	SV5015 *hilD*-cm^R *ssrA::*3Flag-km^R	This study
TGC-23	SV5015 *greAgreB ssrA::*3Flag-km^R	This study
TGC-24	SV5015 *greAgreB hilD*-cm^R *ssrA::*3Flag-km^R	This study
TGC-25	SV5015 *hilD*$_{+76}$*::lacZ*-km^R	This study
TGC-26	SV5015 *greAgreB hilD*$_{+76}$*::lacZ*-km^R	This study
TGC-35	SV5015 *hilD*$_{+1235}$*::lacZ*-km^R	This study
TGC-36	SV5015 *greAgreB hilD*$_{+1235}$*::lacZ*-km^R	This study
TGC-38	SV5015 *hilD*$_{+965}$*::lacZ*-km^R	This study
TGC-39	SV5015 *greAgreB hilD*$_{+965}$*::lacZ*-km^R	This study
SV6190	14028 *hilD*Δ3'UTR-km^R	(7)
TGC-53	SV5015 *hilD*Δ3'UTR-km^R	This study
TGC-37	SV5015 *greAgreB hilD*Δ3'UTR	This study
TGC-54	SV5015 *hilD*Δ3'UTR *sipC::lacZ*-km^R	This study
TGC-55	SV5015 *greAgreB hilD*Δ3'UTR *sipC::lacZ*-km^R	This study
SV6212	14028 *araBAD*-cm^R *hilA::lacZ*-km^R	(8)
SV6197	14028 *araC*-km^R	(8)
TGC-61	SV5015 *araBAD*-cm^R *araC*-km^R	This study
TGC-62	SV5015 *greAgreB araBAD*-cm^R *araC*-km^R	This study
TGC-66	SV5015 *hfq*-cm^R	This study
TGC-67	SV5015 *greAgreB hfq*-cm^R	This study
SV5961	14028 *rne*-cm^R (*rne537*)	(2)
TGC-68	SV5015 *rne*-cm^R	This study
TGC-69	SV5015 *greAgreB rne*-cm^R	This study
TGC-42	SV5015 *motA*	This study
TT1704	Δ*his*-9533	(9)

UMR1	ATCC 14028 nal^R	(10)
LB5000	*rLT*$^-$ *rSA*$^-$ *rSB*$^-$	(11)
Plasmids	**Relevant characteristics or description**	**Reference**
pKD3	*bla* FRT cm^R PS1 PS2 oriR6K	(12)
pKD4	*bla* FRT km^R PS1 PS2 oriR6K	(12)
pKD46	*bla* P_{BAD} *gam bet exo* pSC101 oriTS	(12)
pCP20	*bla* cm^R *cI*857 λP_R *flp* pSC101 oriTS	(12)
pKG137	*ahp* FRT *lacZY*+ t_{his}, oriR6K	(13)
pKG136	*ahp* FRT *lacZY*+ t_{his}, oriR6K	(13)
pSUB11	3xFLAG- and Km^R-coding template vector	(14)
pGEM-T easy	oripMB1 Cb^R	(15)
pBAD18	p*ara*BAD oripMB1Amp^R	(16)
pBADHilA	pBAD18 + *hilA* ORF $_{SV5015}$	(5)
pBADHilD	pBAD18 + *hilD* ORF $_{SV5015}$	This study
pBR322	oripMB1 Tc^R, Amp^R	(17)
pBR*greA*	pBR322+$greA_{SV5015}$	This study
pBR*greB*	pBR322+$greB_{SV5015}$	This study
pBR*greAB*	pBR322+$greAgreB_{SV5015}$	This study
pBR*hilD*	pBR322+$hilD_{SV5015}$	This study
pHM1883	Ptrc expression vector, oripGB2 $Spec^R$	(18)
pHM1873	pHM1883+$greA_{MG1655}$	(18)
pHM1854	pHM1883+*greA* $_{MG1655}$ (D41A E44Y)	(18)
pTT68	P_{BAD}-MCS-*lacZ*, oriRO1600/MB1 Amp^R	This study
pTTORF	pTT+ *hilD* ORF	This study
pTT3'UTR	pTT+ *hilD* 3'UTR	This study
pTT3'UTR+T	pTT+ *hilD* 3'UTR+Terminator	This study

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Table S2. Primers used in this study

Name	Sequence (5' to 3')
	One-step inactivation of chromosomal genes
greAP1	CCCTACAGGAATGTTCAAGAGGTATAACAAATGCAAGCTGTGTAGGCTGG AGCTGCTTC
greAP2	TACACCAACAATTTGCGTATTGAGTACTGCTTAAAGGTACATATGAATATC CTCCTTAGT
greAP1UP	TGACCCTGGTATGGCATAT
greAP2DOWN	TCTGTGTAAAACGAGGGGTT
greBP1	CAATATCGACAGCAAAGGTAAATCAACGAGATGAAAACGGTGTAGGCTGG AGCTGCTTC
greBP2	CATCAGCGGGGGCTTAGGATTCTTCTTGTCTTATTTGACCATATGAATATC CTCCTTAGT
greBP1UP	CTTAAATATACAATTAATCGGC
greBP2DOWN	AAAATCAGGGGATAGTTATAC
hilDP1+76	ATGGAAAATGTAACCTTTGTAAGTAATAGTCATCAGCGTCCTGTGTAGGCT GGAGCTGCTTC
hilDP2.1	TTAATGGTTCGCCATTTTTATGAATGTCGATGGCGTAGTTTTCATATGAATA TCCTCCTTAGT
hilDP1UP	GTAGGATACCAGTAAGGAAC
hilDP2DOWN	GCGTGTTAATGCGCAGTCTG
hilDP1+1235	GATATTGCCTTATTCACATCGTAAGAATTCGTCCAGATGACACTATCTCCG TGTAGGCTGGAGCTGCTTC
hilDP2.2	TATAAATATGAATAAAATGCCGGCCTTAATCCACAGGGTTAAAGCCGGAA CATATGAATATCCTCCTTAGT
rtsAP1	GCACATTTAATAAAAGGAAATTATCATGCTAAAAGTATTTAATCCCTCACC GTGTAGGCTGGAGCTGCTTC
rtsAP2	TCTTATACTGCATTGTCAGATATCTCAATTAACATATTGATGACGAGAGGC ATATGAATATCCTCCTTAGT
Hfq-P1	GTACAATTGAGACGTATCGTGCGCAATTTTTCAGAATCGAGTGTAGGCTG GAGCTGCTTC
Hfq-P2	CCCGACATGGATAAACAGCGCGTGAACTTATTCAGTCTCTTGATATGAATA TCCTCCTTA
rtsAP1UP	GATCGCCACCTGATACCTTA
rtsAP2DOWN	GCGAGCAACAGAATCCCATC
KT	CGGCCACAGTCGATGAATCC
K2	CGGTGCCCTGAATGAACTGC
C1	CCTTGTCGCCTTGCGTATAA
C2	CCTACCTGTGACGGAAGATC
LACZR	GATGACCTGCAAGGCGATTA
	Epitope tagging of chromosomal genes in *Salmonella*
InvFP13Flag	GCCGCGGAAATTATCAAATATTATTCAATTGGCAGACAAAGACTACAAAGA CCATGACGG

InvFP23Flag	GCGGCACATGCCAGCACTCTGGCCAAAAGAATATGTGTCTCATATGAATA TCCTCCTTAG
InvFP13FlagUP	GCGTATGGCGCAATCGCTGC
InvFP23FlagDOWN	CCCACTTCCCGTTACAGG
	Cloning
greASalmUP	GAATTCTCGCGCTAACACCCTGG
greASalmDOWN	GGATCCTCGCTGCCGCGTTAAGG
greBSalmUP	GAATTCGTACTCCCAAAGGTTCGC
greBSalmDOWN	GGATCCATGGGTTAGCTTCGTC
greASalIUP	GGGGTCGACGGAACTCCAGGGTAAAATGG
greABamHIDOWN	GGGGGATCCTCGCTGCCGCGTTAAGG
pBR-FW	CCATTATTATCATGAACATTAAC
pBR-RV	GATGCCGGCCACGATGCGTCC
hilDNcoI1	GGGCCATGGGGAGCGCGTTTACAACATTATA
UTRSalI3	GGGTCGACTTAAAATATTTTTTGAAACA
UTRSalI8	GGGTCGACGCAAATAGTTCTCAGAGGGAAC
UTRSalI6	GGGTCGACAAGGAGATAGTGTCATCTGGAC
hilDFw322	GGATCCTGTTAGCGATGTCTGTCG
hilDRv322A	GTCGACTGCCTGGCAGAAACTAAC
hilDBADFw	CGGAATTCTAACATCAACAAAGGGATAATATGGAA
hilDBADRv	GCTCTAGATTAATGGTTCGCCATTTTTATGAA
	* Restriction sites underlined
	Quantitative PCR (qPCR)
hilDRTFW	GCCAGAAGAG AGGTATTTG
hilDqPCRRV	CAGTAAGCAGGAACAGCAG
hilAqPCR1	GGATATTCTTGAGCTCATGG
hilAqPCR2	GAGAAGCGGGTTGGTGTTC
hilCqPCR1	CTCACCCGCAAATGGTCAC
hilCqPCR2	GCCTGATTCATACGAGCATC
rtsART-FW	GTATATTACGGCATCAGGGC
rtsART-RV	GCCTGTTTCTATTGGCGC
GAPDHqPCR1	GTCCGTCTAAAGACAACACC
GAPDHqPCR2	CATCAGACCTTCGATGATGC
	RT-PCR
SipAFor	GAACGGTGTGGAGGTATCTG
SipARev	GAGAATGTTAAAACCGATACC
SalI16S	CTACTGGAAACGGTGGCTAA
SalII16S	AAGCCTGCCAGTTTCGAATG