Departamento de Genética

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Genetic and molecular analysis of the *Salmonella* effector SrfJ and use of type III secretion effectors as carriers for heterologous vaccine design

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ABBREVIATIONS LIST

ABC	ATP-binding cassette
Ap	Ampicillin
APC	Antigen-presenting cells
bdH_2O	bi-distilled H_2O
BSA	Bovine serum albumin
CABIMER	Centro Andaluz de Biología Molecular y Medicina Regenerativa
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CFU	Colony-forming unit
CI	Competitive index
CIP	Calf Intestine Phosphatase
Cm	Chloramphenicol
Ct	Threshold-cycle of amplification
DMEM	Dulbeco's modified eagle's medium
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assays
ENP	Extracellular nucleation-precipitation
FBS	Fetal bovine serum
FRT	FLP recognition target
GAP	GTPase-activating protein
GEFs	Guanine nucleotide exchange factors
Gm	Gentamicin
GO	Gene Ontology
GST	Glutathione-S-transferase
IBiS	Instituto de Biomedicina de Sevilla
IL	Interleukin
IM	Inner membrane
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JNK	Jun kinase
Km	Kanamycin
LB	Bertani's Lysogenic Broth
LD50	Median lethal dose
LM	Lettuce medium
LPM	Low phosphate, low magnesium-containing medium
Lrp	Leucine-responsive regulatory protein
LTNs	LAMP-1 negative tubules
MAPK	Mitogen-activated protein kinase
MOI	Multiplicity of infection

mRNA	Messenger RNA
MS	Murashige and Skoog
NAPs	Nucleoid associated proteins
NB	Nutrient Broth
NCBI	National Center for Biotechnology Information
OD	Optical density
OM	Outer membrane
PAK	p21-activated kinase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
p.i.	Postinfection
PMN	Polymorphonuclear leukocytes
PTS	Phosphotransferase system
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RMA	Robust Multi-array Average
RppH	RNA 5'Pyrophosphohydrolase
RT-PCR	Reverse Transcription Polymerase chain reaction
RTX	Repeat-in-toxins
SCAMP3	Secretory carrier membrane protein 3
SCV	*Salmonella* containing vacuole
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sifs	*Salmonella*-induced filaments
SISTs	*Salmonella*-induced secretory carrier membrane protein 3 (SCAMP3) tubules
SNX	Sorting nexin
SPI	*Salmonella* pathogenicity island
spv	*Salmonella* plasmid virulence
srf	SsrB-regulated factors
Tc	Tetracycline
TM	Tomato medium
TNF	Tumor necrosis factor
TxSS	Type x secretion system
wt	Wild-type
XLD	Xylose Lysine Deoxycholate
6His	6 Histidine

SUMMARY

Salmonella enterica is a species of bacterial pathogens that can produce different diseases from gastroenteritis to typhoid fever. *Salmonella* possesses two different virulence-related type III secretion systems (T3SSs) that are key elements in the interaction with the host cell. These systems mediate the translocation of effector proteins into the cytosol of the host cell where they interfere with different cellular processes to allow the pathogen entry and its survival inside vacuoles.

We studied the *S. enterica* serovar Typhimurium SrfJ effector expression. We provide evidence for the existence of two distinct promoters that control the expression of *srfJ*. A proximal promoter, P*srfJ*, responds to intravacuolar signals inside mammalian cells and is positively regulated by SsrB and PhoP and negatively regulated by RcsB. A second distal promoter, P*iolE*, is negatively regulated by the *myo*-inositol island repressor IolR, whereas it is expressed upon *Salmonella* colonization of plants. Importantly, we also found that inappropriate expression of *srfJ* leads to reduced proliferation inside macrophages whereas lack of *srfJ* expression increases survival and decreases activation of defense responses in plants. These observations suggest that SrfJ is a relevant factor in the interplay between *Salmonella* and host of different kingdoms. Transcriptomic carried out in human epithelial HeLa cells and murine RAW264.7 macrophages detected 16 genes that are significantly down-regulated and 12 genes that are significantly up-regulated in response to the presence of SrfJ. Proteomic analysis revealed that SrfJ is involved in dephosphorylation of WNK1 and prevention of induction of HSP60.

The last part of this thesis was focused on the development of a live *Salmonella* vaccine against *Pseudomonas aeruginosa*. As T3SS-mediated translocation can be used for efficient delivery of heterologous antigens to the cytosol of antigen-presenting cells, we tested the possibility of using *Salmonella* effectors SseJ, SrfJ, SlrP, SteA and SseK1, as carriers in the design of this vaccine. We finally developed a vaccine delivering the *Pseudomonas* antigen PcrV in fusion with SseJ through the T3SS. This vaccine protected mice against lethal infections with *P. aeruginosa*.

RESUMEN

Salmonella enterica es una especie de bacterias patógenas que pueden producir diferentes enfermedades desde gastroenteritis a enfermedades sistémicas. *Salmonella* posee dos sistemas de secreción tipo III (T3SS) relacionados con la virulencia que son elementos clave en la interacción con la célula hospedadora. Estos sistemas median la translocación de proteínas efectoras al citosol de la célula hospedadora donde interfieren con diferentes procesos celulares para permitir la entrada del patógeno y su supervivencia dentro de vacuolas.

Se estudió la expresión del efector SrfJ de *S. enterica* serovar Typhimurium. Se averiguó la existencia de dos promotores distintos que controlan la expresión de *srfJ*. Un promotor proximal, P*srfJ*, que responde a las señales intravacuolares dentro de las células de mamífero y está regulado positivamente por SsrB y PhoP y negativamente por RcsB. Un segundo promotor distal, P*iolE*, que está regulado negativamente por el represor de la isla de utilización del *mio*-inositol, IolR, mientras que se expresa tras la colonización de plantas por *Salmonella*. A su vez, se estableció que la expresión inapropiada de *srfJ* conduce a una reducción de la proliferación en macrófagos, mientras que la falta de expresión de *srfJ* aumenta la supervivencia y disminuye la activación de las respuestas de defensa en plantas. Estas observaciones sugieren que SrfJ es un factor relevante en la interacción entre *Salmonella* y hospedadores de diferentes reinos.

A través del análisis transcriptómico llevado a cabo en células epiteliales humanas HeLa y macrófagos de ratón RAW264.7 se detectaron 16 genes con expresión significativamente reducida y 12 genes con expresión significativamente aumentada en respuesta a la presencia de SrfJ. Un análisis proteómico indicó que SrfJ está implicado en la desfosforilación de WNK1 y en la prevención de la inducción de HSP60.

La última parte de esta tesis se centró en el desarrollo de una vacuna viva de *Salmonella* contra *Pseudomonas aeruginosa*. Dado que la translocación mediada por T3SS puede usarse para la administración eficiente de antígenos heterólogos al citosol de células presentadoras de antígeno, se probó el uso de los efectores de *Salmonella* SseJ, SrfJ, SlrP, SteA y SseK1, como portadores en el diseño de la vacuna. Finalmente desarrollamos una vacuna en la que el antígeno PcrV de *Pseudomonas* en fusión con SseJ se secreta a través de un T3SS. Esta vacuna protegió a los ratones contra una infección letal con *P. aeruginosa*.

INTRODUCTION

1. THE GENUS *Salmonella*

The genus *Salmonella* belongs to the family *Enterobacteriaceae* and includes facultative anaerobic, non-spore forming, rod-shaped Gram negative bacteria. *Salmonella* is a close relative of *Escherichia*, *Shigella* and *Citrobacter*. The genomic organization of *Salmonella* and *Escherichia coli* are similar (Groisman and Ochman, 1994; Sanderson *et al*., 1995). Non-pathogenic *E. coli* and *S. enterica* serovar Typhimurium genomes share 80% similarity in their sequences (Blattner *et al*., 1997; McClelland *et al*., 2001), although *Salmonella* contains specific regions that are not found in related genera.

The genus *Salmonella* is currently divided into two species, *Salmonella enterica* and *Salmonella bongori* (Tindall *et al*., 2005). *S. enterica* includes 6 subspecies (Grimont and Weill, 2008): *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI) (Desai *et al*., 2013; Porwollik *et al*., 2004; Tindall *et al*., 2005). Historically, *S. enterica* subsp. V was *bongori*, which nowadays is considered a different species. The subspecies of *S. enterica* II, IIIa, IIIb, IV and VI, as well as *S. bongori* are associated with cold-blooded vertebrates; whereas the members of subspecies I of *S. enterica* are more frequently associated to birds and mammals (Bäumler *et al*., 1998; Boyd *et al*., 1996). *Salmonella* subspecies are classified into serovars (or serotypes) based on the White-Kuffman classification scheme (Popoff *et al*., 2004), which relies on antisera that recognize two highly variable surface antigens, O (lipopolysaccharide O-antigen) and H (flagellar protein) (Grimont *et al*., 2007; McQuiston *et al*., 2004). There are more than 2500 *Salmonella* serovars, more of which belongs to the subspecies I (Porwollik *et al*., 2004).

2. EVOLUTION OF *Salmonella* PATHOGENICITY

The genera *Salmonella* and *Escherichia* diverged about 120-160 million years ago (Ochman and Wilson, 1987). Almost 25% of the *Salmonella* genome consists of genetic material that is absent in *E. coli* (McClelland *et al.*, 2001; Porwollik and McClelland, 2003). The evolution of *Salmonella* pathogenicity is related to the acquisition of virulence genes, many of them clustered in the *Salmonella* pathogenicity islands (SPIs) (Groisman and Ochman, 1997; Kelly *et al*., 2009). The fact that these regions are absent in the chromosome of other *Enterobacteriaceae* and have different G+C contents than the average of the *Salmonella* chromosome suggest that they have been acquired by

horizontal gene transfer (**Figure I.1**) (Kelly *et al.*, 2009; Porwollik and McClelland, 2003).

Figure I.1. Phylogeny of the genus *Salmonella*. The acquisition of SPI1, SPI2, and the ability to infect warm-blooded vertebrates is indicated. Modified from (Dworkin and Falkow, 2006).

The best-characterized SPIs are *Salmonella* pathogenicity island 1 (SPI1) and *Salmonella* pathogenicity island 2 (SPI2). SPI1 was acquired first by the common ancestor of the two *Salmonella* species, and is involved in the invasion of intestinal epithelial cells in the animal host (Que *et al.*, 2013). SPI1 acquisition likely allowed *Salmonella* to become an intracellular pathogen associated with cold-blooded vertebrates (Dworkin and Falkow, 2006). SPI2 allows *Salmonella* to survive in macrophages and to colonize deeper tissues (Wood *et al.*, 1998), and its acquisition marked the split of the two *Salmonella* species (Dworkin and Falkow, 2006). Hence, only members of *S. enterica* have the ability to reach deep tissues and organs to produce systemic infections.

The ancestors of subsp. *enterica* acquired the capacity to infect warm-blooded vertebrates, by a mechanism that remains a mystery. Different lineages subsequently evolved to colonize a variety of hosts. Even though the mechanisms of host specificity are not fully understood, the presence of a virulence plasmid in some serovars of subsp. *enterica* has suggested the potential involvement of plasmid functions (Bäumler *et al.*, 1998). Another factor that may be involved in host specificity is the presence of different sets of fimbrial operons in different serovars (Bäumler *et al.*, 1998; Humphries *et al.*, 2001).

3. *Salmonella* INFECTION

According to reports from the World Health Organization (2013), salmonellosis is one of the most common and widely distributed foodborne diseases, with millions of human cases (from which more than a hundred thousand result in death) occurring worldwide every year. In addition, since the beginning of the 1990s, *Salmonella* strains that are resistant to a range of antimicrobials have emerged and are now a serious public health concern. Furthermore, *Salmonella* are ubiquitous and resilient bacteria that can survive several weeks in dry environments and several months in water (WHO, Fact sheet Nº139, 2013).

The *Salmonella* infection process usually begins with the ingestion of contaminated water or food. **Figure I.2** depicts the biology of the infection by *Salmonella* in humans. *Salmonella* is able to survive the acidic pH in the stomach, produced by gastric acids, and to evade the defense systems that it encounters in the small intestine thus reaching the intestinal epithelium. Invasion of this epithelium occurs generally through M cells, allowing the bacteria to reach lymphocytes B and T, which are below Peyer patches (Haraga *et al*., 2008). This invasion is mediated by the virulence associated type 3 secretion system (T3SS) encoded by SPI1 (Zhou and Galán, 2001).

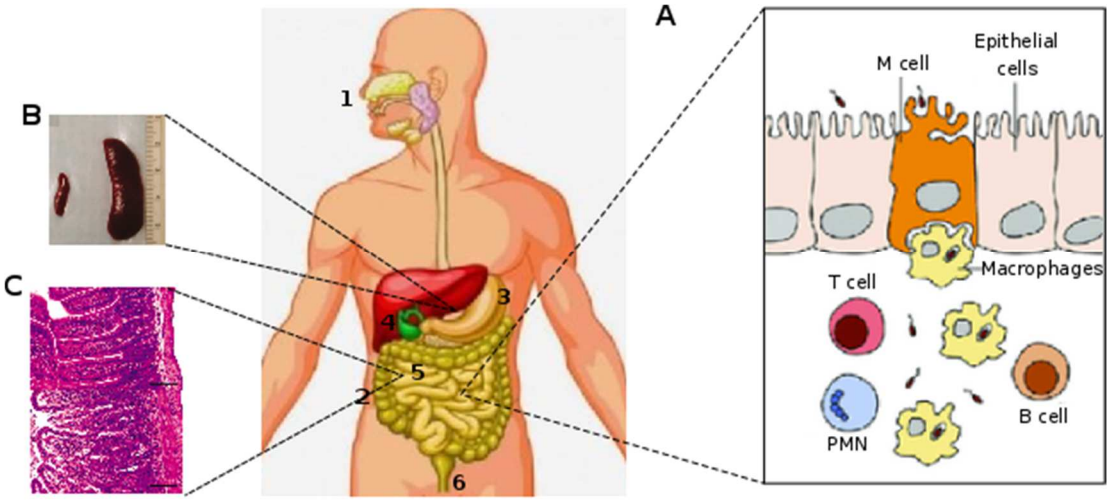


Figure I.2. *S. enterica* infection pathogenesis. (A) In humans, typhoid fever is caused by ingestion of food or water contaminated with *S. enterica* serovar Typhi: (1) the surviving bacteria go through the acid pH in the stomach, invade the intestine epitelial cells and migrate to the lamina propia. (2) In the intestinal mucosa, *S. enterica* serovar Typhi is phagocytized by macrophages and survives within these cells due to virulence factors, which interfere with the host cell functions. After the invasion, the bacteria expresses other factors that inhibit detection by the host's innate immune system. This allows the systemic dissemination of the bacteria, colonizing macrophages in the liver, spleen and bone marrow. (3) From the liver, the bacteria can reach the gall-bladder, this infection can give rise to a state of asymptomatic carrier. (4) The *S. enterica* serovar Typhi carriers are continuosly secreting bacteria from the gallbladder to the small intestine with the secretion of bile, (5) excreting viable bacteria in their feces, (6) consequently infecting other hosts. Image adapted from Tischler and Mckinney, 2010 (Tischler and McKinney, 2010). **(B)** Spleen of a non-infected 129Sv mouse (left) and spleen of a 129Sv mouse infected with *S. enterica* serovar Typhimurium SL1344 60 days p.i. (right). Image adapted from Monack *et al.*, 2004 (Monack *et al.*, 2004). **(C)** Histological sections of BALB/c mice ileum stained with hematoxylin and eosin (scale: 100 µm). The upper image corresponds to a non-infected control, the lower image shows the ileum of a mouse 5 days p.i. of a lethal dose of *S. enterica* serovar Typhimurium ATCC 14028 (Santos *et al.*, 2001).

Once in the epithelium, two main outcomes are possible: (i) the serovars that produce systemic disease crosses the epithelial barrier, enter into macrophages and spread throughout the body; (ii) the non-typhoid serovars trigger an early and localized inflammatory response that cause polymorphonuclear leukocytes (PMNs) infiltration to the intestinal lumen leading to diarrhea (Haraga *et al.*, 2008).

In humans, serovar Typhi and Paratyphi produce a systemic disease called typhoid fever whose clinical manifestations include fever, abdominal pain, transient diarrhea or constipation and, in some cases, maculopapular rash. It is estimated that typhoid fever affects 22 million people worldwide, causing around 200000 deaths per year (Crump *et al.*, 2004). Systemic disease is mainly produced in underdeveloped or developing countries due to sanitary deficiencies (eg, lack of drinking water) and food handling and preservation in unsanitary conditions. Systemic infection occurs when bacterial serotypes invade intestinal macrophages and disseminate inside the organism through the lymphatic system, allowing colonization of internal organs (liver, spleen, bone marrow, and gallbladder) (**Figure I.2**). *S. enterica* activate virulence mechanisms within the

macrophages to survive and replicate in the intracellular environment. In this phase, the T3SS encoded by SPI2 plays a crucial role (Bäumler *et al.*, 2011; Haraga *et al.*, 2008).

Serovar Typhi is established asymptomatically in 1-4% of typhoid patients due to persistent infection of the gallbladder (Parry *et al.*, 2002). These patients, despite not suffering the symptoms of the disease, release a large amount of bacteria in their droppings, being this related to the long-term maintenance of *S. enterica* serovar Typhi (*S.* Typhi) in human populations (Tischler and McKinney, 2010).

The mesenteric lymph nodes, the liver and the gallbladder have been proposed as potential *Salmonella* reservoirs (Crawford *et al.*, 2010; Watson and Holden, 2010). Particularly, colonization of the gallbladder by *Salmonella* in asymptomatic carriers allows constant shedding of bacteria into the medium since *Salmonell*a cells are released into the small intestine each time the gallbladder contracts. In most cases antibiotics are not effective to eliminate chronic typhoid infection (Dutta *et al.*, 2000; Lai *et al.*, 1992), and removal of gallbladder is usually necessary.

Other serovars, such as Enteritidis and Typhimurium, are responsible for millions of non-typhoidal salmonellosis that occur annually throughout the world. These serovars, which in humans cause transient gastroenteritis of variable severity (but, which usually does not require treatment for remission), also infect a wide range of domestic animals (eg, birds, cattle and pigs) where they can produce various types of infections from asymptomatic to systemic. Non-typhoidal salmonellosis complicated by bacteremia is frequent in sub-Saharan Africa (De Wit *et al.*, 1988; Morpeth *et al.*, 2009). Certain non-typhoidal serovars of *Salmonella* can cause persistent or chronic cholecystitis (Lalitha and John, 1994). These persistent symptomatic infections are also difficult to eradicate, requiring the removal of the gallbladder.

4. MODELS FOR THE STUDY OF *Salmonella* VIRULENCE

S. enterica serovar Typhimurium (*S.* Typhimurium) is the most widely used model for the study of *Salmonella* infections. This serovar infects a wide variety of hosts. There are several models for the study of virulence.

4.1. Mouse model

In certain strains of domestic mice, *Mus musculus*, *S.* Typhimurium produces a disease similar to typhoid fever, which serves as a model to study systemic infections caused by serovar Typhi in humans.

BALB/c mice are particularly susceptible to *Salmonella* infection because they carry a mutation at the locus encoding Nramp1, a protein, involved in the innate defense to infections with intracellular parasites (Canonne-Hergaux *et al*., 1999). The expression of this locus is restricted to monocytes and it is necessary to control the intracellular replication of microorganisms such as *Salmonella*, *Mycobacterium* and *Leishmania* (Bellamy, 1999; Govoni and Gros, 1998; Gruenheid *et a*l., 1997). *Salmonella* replicates in the mice gut and reaches the distal end of the small intestine, the ileum, where it adheres preferentially to M cells (Jones *et al*., 1994). After invasion, *Salmonella* binds the intestinal epithelium and is phagocytized by macrophages, where it survives and replicates evading its phagocytic functions. Subsequently, bacteria spread to other organs through the bloodstream.

The classical method to identify an attenuated mutant of *Salmonella* in the mouse model is the calculation of its median lethal dose (LD50) compared to the LD50 of the wild-type (wt) strain (Reed and Muench, 1938). However, a more sensitive procedure that requires a smaller number of mice, consists of infecting the same mouse with a mixture of two strains, generally, a mutant strain and a wt strain, to obtain a competitive index (CI) (Freter *et al*., 1981; Taylor *et al*., 1987). This model also allows the study of gene interactions *in vivo* through the use of mixed infections with double and single mutants (Beuzón and Holden, 2001).

A limitation of this model is that serovar Typhimurium does not cause colitis in this organism. However, mice previously treated with antibiotics develop acute intestinal inflammation in response to oral infection with *S.* Typhimurium (Barthel *et al*., 2003). These studies have opened the possibility of using mice as a model for gastroenteritis studies (Hapfelmeier and Hardt, 2005).

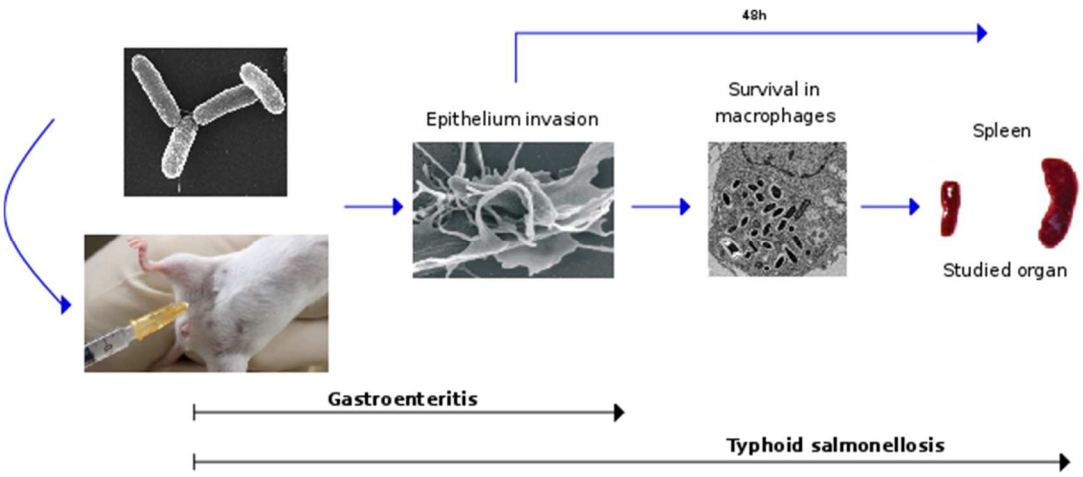


Figure I.3. Animal model for the study of the pathogenesis of *Salmonella*. An intraperitoneal infection is shown in which, the organs required for the analysis of bacteria, usually the spleen, are removed 48 h after the infection.

4.2. Mammalian cell lines

In addition to the *in vivo* models, a large variety of mammalian cell lines have been used as *in vitro* models for the study of invasion (epithelial cell lines) or intracellular survival and proliferation (phagocytic cell lines, epithelial and fibroblast cell lines). **Table I.1** summarizes the main cell lines used for the study of *Salmonella* infection.

Table I.1. Cell lines used as a model for the study of *Salmonella* infection.

Cell type	Cell culture model	Internalization mode
M cells	Mixed culture of CaCo-2 and Raji B cells.	Caveolae-mediated endocytosis
Dendritic cells	Primary cells	Phagocytosis
Macrophages	RAW264.7, J774, etc. and primary cells	Phagocytosis
Epithelial cells	HeLa, CaCo-2, HT-29, INT407, etc. and primary cells	Macropinocytosis
Fibroblasts	NRK-49F, 3T3, etc. and primary cells	Multiple ways

*Adapted from (Garai *et al.*, 2012).

The most used method for these *in vitro* studies is the gentamicin protection assay. This method is based on the inability of gentamicin to cross the eukaryotic cell membrane (Elsinghorst, 1994). After *in vitro* infection, the extracellular bacteria are killed by a gentamicin treatment and the intracellular bacteria are plated using appropriate dilutions to count colonies and perform calculation of percent invasion relative to the initial inoculum. On the other hand, the intracellular proliferation rate is calculated counting the number of viable intracellular bacteria present at 24 h relative to those present at 2 h

postinfection (p.i.) (Cano *et al*., 2001). Given the variability shown by the results of these experiments *in vitro*, a modification of the gentamicin protection method has been proposed, which incorporates the advantages of mixed infections and allows the calculation of invasion and proliferation competitive index (Segura *et al*., 2004).

4.3. Plant model

S. Typhimurium and other serovars can enter the agricultural production chain at different levels, e.g., via animal feces used for soil amendments or as post-harvest contamination. *Salmonella* is able to adhere to plant surface, colonize plant organs, and suppress the plant immune system (Neumann *et al*., 2014; Schikora *et al*., 2012). Therefore, plants are considered as alternative hosts for these pathogens, and fresh fruits and vegetables are recognized as an important source of food-borne disease (Holden *et al*., 2015; Wiedemann *et al*., 2015). *Arabidopsis* can be colonized by different human pathogens like: *S. enterica*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *E. coli* and *Listeria monocytogenes* (Neumann *et al*., 2014). It has been shown that *S.* Typhimurium is pathogenic for plant hosts, like *Arabidopsis* (Hernández-Reyes and Schikora, 2013). Whether these bacteria use the same or different effectors in order to survive in different hosts is not yet clear. It seems however to be acceptable to conclude that *Salmonella* requires T3SSs during interaction with plants. Although several *Salmonella* effectors have homologues in phytopathogenic bacteria, the function of *Salmonella* proteins during the inactivation of the plant immune system remains elusive (García and Hirt, 2014; Hernández-Reyes and Schikora, 2013; Wiedemann *et al*., 2015). For instance, the SpvC effector from *Salmonella spp.*, that encodes a phosphothreonine lyase that dephosphorylates and therefore deactivates the ERK1/2 kinases, key regulators of animal immune system, is known to be functional also in plants (Neumann *et al*., 2014).

5. VIRULENCE FACTORS

Four genetic mechanisms have been proposed to explain the phenotypic differences between related bacterial species, such as *E. coli* and *S. enterica*, which differ in their pathogenic potential: (i) presence of specific virulence genes; (ii) absence in pathogenic species of a virulence suppressor gene that inhibits or interferes with the activity of virulence factors, as occurs in the case of the loss of the *lac* operon in *Salmonella*

(Eswarappa *et al.*, 2009); (iii) allele differences between homologous genes; and (iv) differential regulation of homologous sequences (Groisman and Ochman, 1994).

As mentioned above, *Salmonella* contains different SPIs that provide the ability to invade epithelial cells and survive inside the phagocytic cells. These islands are absent in commensal *E. coli*, which neither invades nor can survive in mammalian cells. At least 21 SPIs have been identified in several serovars of *S. enterica* (Sabbagh *et al.*, 2010). There are several indications that suggest that these regions have been acquired by horizontal transfer processes: (i) they have different G+C content from the rest of the genome; (ii) they are flanked by genes that are contiguous in evolutionarily related, non-pathogenic species; and (iii) in some cases, they carry genes encoding mobility factors from transposons or prophages, suggesting a possible acquisition mechanism (Groisman and Ochman, 1997; Hacker and Kaper, 2000).

In addition to the SPIs, other smaller regions can be found, sometimes referred as "islets of pathogenicity", related to virulence and specific of *Salmonella* (Groisman and Ochman, 1997). There are also virulence genes associated with prophages (Figueroa-Bossi *et al.*, 2001). In addition, some members of the genus *Salmonella* carry a plasmid required for systemic infection, called "virulence plasmids", whose size ranges from 50 to 90 kb, although only a 7.8 kb region called *Salmonella* plasmid virulence (*spv*) is important for virulence in mice (Casadesús, 1999; Gulig *et al.*, 1993).

5.1. *Salmonella* Pathogenicity Island 1 (SPI1)

SPI1 plays a crucial role in both gastroenteritis and systemic infection caused by *Salmonella* (Hansen-Wester and Hensel, 2001). Among the proteins encoded in this island, there are the necessary elements to build the T3SS1, an important virulence device through which the pathogen injects effector proteins directly into the host cell cytoplasm, as well as some effectors secreted by the T3SS1 (**Figure I.4**). SPI1 is involved in multiple processes, such as cytotoxicity in macrophages (Chen *et al.*, 1996), epithelial cell invasion (Galán, 1999), inflammation and fluid secretion in the ileum (Hobbie *et al.*, 1997), cytokine secretion and apoptosis prevention in epithelial cells (Knodler *et al.*, 2005).

5.2. *Salmonella* Pathogenicity Island 2 (SPI2)

SPI2 encodes another important T3SS for *Salmonella* virulence (T3SS2). Proteins encoded in SPI2 (**Figure I.4**) are essential for systemic infection because they allow the intracellular survival of *Salmonella* within the host cells, but not for gastroenteritis. The formation and maintenance of the *Salmonella* containing vacuole (SCV) involve sequential events controlled by this pathogenicity island (Haraga *et al.*, 2008). SPI2 confers protection against reactive oxygen species (Janssen *et al.*, 2003), as well as reactive nitrogen intermediates (Chakravortty *et al.*, 2002) within macrophages. The tetrathionate reductase encoded in SPI2 acts on tetrathionate to generate thiosulfate, which acts as an alternative electron donor for *Salmonella* in environments containing tetrathionate, such as human intestine, soil or decaying corpses (Hensel *et al.*, 1999; Winter *et al.*, 2011).

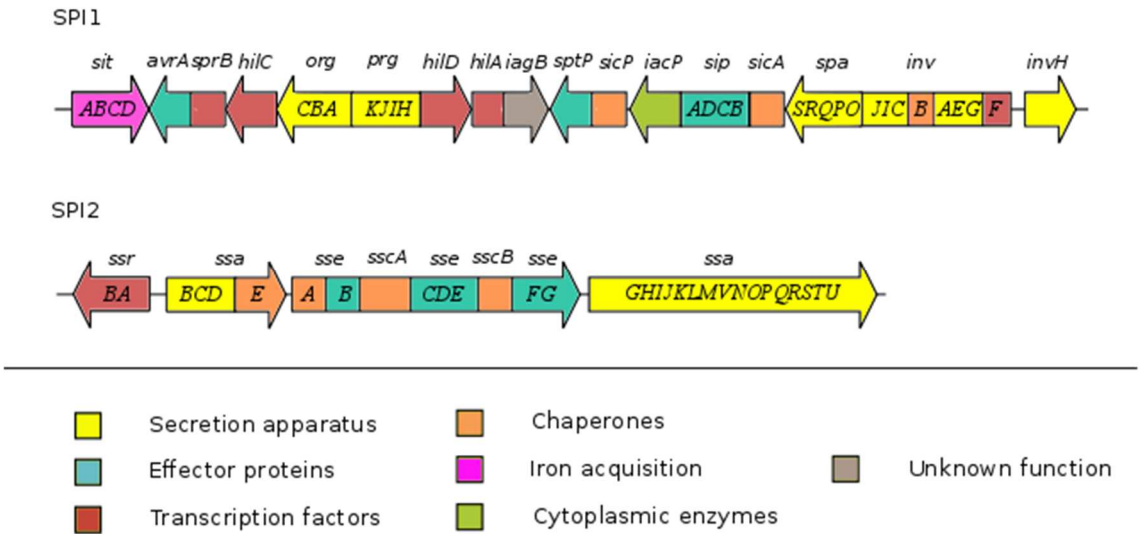


Figure I.4. Schematic representation of genes encoded in SPI1 and SPI2 and their associated functions. [Adapted from (Fàbrega and Vila, 2013; Hensel, 2000)].

5.3. Other pathogenicity islands

Twenty-one SPIs have been identified in *S. enterica*. The *S.* Typhi and *S.* Typhimurium genomes contain 11 common islands (SPI1 to SPI6, SPI9, SPI11, SPI12, SPI13 and SPI16); 6 are only in serovar Typhi (SPI7, SPI8, SPI10, SPI15, SPI17 and SPI18; although SPI8 and SPI10 have equivalent regions in Typhimurium with completely different genes); and only one, SPI14, is specific for *S.* Typhimurium (Sabbagh *et al.*, 2010).

Some features of most of the islands, aside form SPI1 and SPI2, are summarized here.

SPI3 encodes a magnesium transporter that allows *Salmonella* to survive in low Mg^{2+} conditions, and is required for survival in macrophages, as well as for systemic infection in the mouse model (Blanc-Potard *et al.*, 1999).

SPI4 encodes a type I secretion system (T1SS) that secretes a protein of 600 kDa called SiiE (Morgan *et al.*, 2007) and contributes to virulence (Kiss *et al.*, 2007).

SPI5 encodes SopB and PipB, two effector proteins translocated by T3SS1 and T3SS2, respectively (Knodler *et al.*, 2002).

SPI6 encodes a type VI secretion system (T6SS), a fimbriae and an invasin (Lambert and Smith, 2008; Townsend *et al.*, 2001). The T6SS is repressed by H-NS and is involved in competition between subpopulations of *S. enterica* during infection (Brunet *et al.*, 2015).

SPI7 is the largest island identified so far, with 134 kb. It encodes the Vi antigen, the effector SopE and a type IVB pilus (Seth-Smith, 2008). This island is found in serovars Typhi, Paratyphi C and some strains of serovar Dublin. This is an example of a pathogenicity island that can excise from the bacterial chromosome by site-specific recombination (Nieto *et al.*, 2016).

SPI8 encodes two bacteriocins immunity proteins in *S.* Typhi (Parkhill *et al.*, 2001).

SPI9 encodes a T1SS similar to that of SPI4 (Latasa *et al.*, 2005).

SPI10 in *S.* Typhi contains a prophage called ST46 (Parkhill *et al.*, 2001) which encodes genes of Ser/Thr kinases and phosphatases of eukaryotic type that are involved in the survival of the pathogen in macrophages (Faucher *et al.*, 2008).

SPI11 includes the PhoP-activated genes *pagD* and *pagC*, involved in intramacrophage survival (Gunn *et al.*, 1995).

SPI12 encodes the effector SspH2 (Miao *et al.*, 1999).

SPI14 contains genes upregulated in macrophages (Eriksson *et al.*, 2002).

SPI15, SPI16 and SPI17 were identified by bioinformatics work (Vernikos and Parkhill, 2006).

SPI18 contains the gene *hlyE* that encodes a hemolysin that is involved in invasion of epithelial cells and colonization of deep organs in mice (Fuentes *et al.*, 2008).

SPI19, SPI20 and SPI21 encode T6SSs and are present in serovars different from Typhi and Typhimurium (Blondel *et al.*, 2009).

5.4. Virulence plasmid

Some members of the genus *Salmonella* carry a low-copy plasmid that contains virulence genes. These plasmids are, 50 to 90 kb in size, and are required for systemic infection (Rotger and Casadesús, 1999). Conjugal transfer of the virulence plasmid pSLT in *S.* Typhimurium is regulated by adenine methylation and the leucine-responsive regulatory protein (Lrp protein) (Camacho and Casadesús, 2002) through the regulators of the *tra* operon TraJ and FinP (Camacho *et al.*, 2005; Camacho and Casadesús, 2005). This plasmid has a 7.8 kb region known as *spv*, which is necessary to enable systemic infection in animal models (Gulig *et al.*, 1993; Rotger and Casadesús, 1999). Proteins encoded by the *spv* operon are secreted through the T3SSs and are essential for virulence (Browne *et al.*, 2008). The SpvB effector plays an important role in autophagy inhibition (Chu *et al.*, 2016; Li *et al.*, 2016) and SpvD participates in inflammatory response inhibition (Rolhion *et al.*, 2016). On the other hand, Pef fimbriae, also encoded in the virulence plasmid, mediates adhesion to intestinal epithelial cells and induce a proinflammatory response (Bäumler *et al.*, 1996b; Chessa *et al.*, 2008). A small RNA encoded in the plasmid, named IesR-1, is expressed when *Salmonella* is inside fibroblasts and is necessary for virulence in mice (Gonzalo-Asensio *et al.*, 2013).

5.5. Adhesins

The binding of bacteria to target cells requires several steps mediated by adhesins (Wagner and Hensel, 2011). The adhesiome of *S. enterica* includes up to 20 adhesins (Hansmeier *et al.*, 2017) encoded by fimbrial genes such as *fim* (Lockman and Curtiss, 1992), *pef* (Bäumler *et al.*, 1996), *lpf* (Bäumler and Heffro, 1995) and *agf* (Grund and Weber, 1988); or non-fimbrial genes such as *misL* (Dorsey *et al.*, 2005) and *shdA* (Kingsley *et al.*, 2002), that encode autotransporters, or *siiE* (Barlag and Hensel, 2015; Gerlach and Hensel, 2007) and *bapA* (Latasa *et al.*, 2005), which encode large adhesins secreted by T1SSs. Each adhesin mediates adhesion to a particular cell type, depending on the receptors present on the surface of the target cells (Misselwitz *et al.*, 2011). The same cell type can be targeted by different adhesins during the adhesion process (Misselwitz *et al.*, 2011). In addition, flagella are also involved in efficient adhesion and invasion of the host cells (Ibarra *et al.*, 2010; Dibb-Fuller *et al.*, 1999; Schmitt *et al.*, 2001).

6. BACTERIAL SECRETION SYSTEMS

Gram-negative bacteria rely on dedicated secretion systems to transport virulence proteins outside of the cell and, in some cases, directly into the cytoplasm of a eukaryotic or prokaryotic target cell (Green and Mecsas, 2016). Extracellular protein secretion can be a challenge for Gram-negative bacteria because these secreted proteins must cross two (and, in some cases, three) phospholipid membranes in order to reach their final destination. Approximately, 3% of the total proteome of *Salmonella* constitutes secretion proteins, which are known as secretome (Arnold *et al.*, 2009).

In Gram-negative bacteria we can find at least nine different secretion systems: the T1SS-T6SS, T8SS, T9SS, and the *chaperone-usher* system (CU system) (Abby *et al.*, 2016; Costa *et al.*, 2015; Filloux *et al.*, 2008; Fronzes *et al.*, 2009; Gerlach *et al.*, 2007; Lasica *et al.*, 2017; Rêgo *et al.*, 2010; Saier, 2006). They use two main translocation mechanisms: (i) single-step or Sec-independent, where the proteins are directly exported from the cytoplasm to outside the cell, and (ii) two-step or Sec-dependent (or Tat-dependent), where the proteins are first exported through the inner membrane to the periplasm and then through the outer membrane (**Figure I.5**).

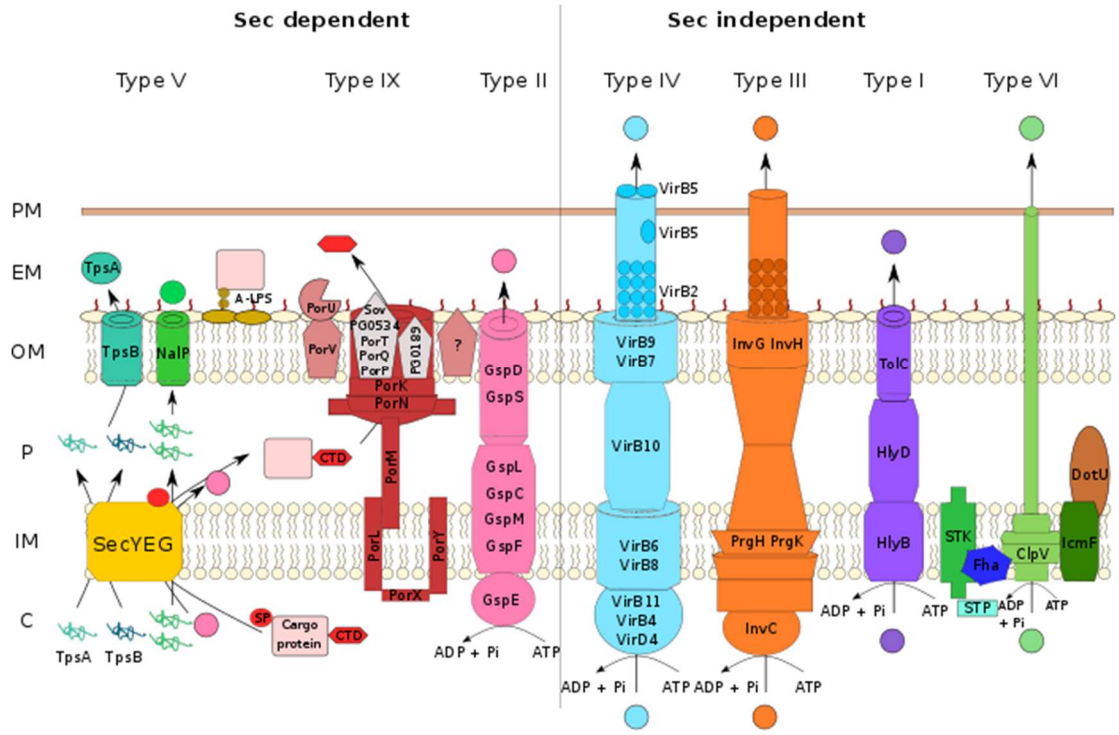


Figure I. 5. Schematic overview of the main secretion systems in Gram negative bacteria. T2SS, T5SS and T9SS are Sec-dependent, so the secretion of their substrates is carried out in two steps. On the other hand, T1SS, T3SS, T4SS and T6SS are Sec independent, translocating their substrates in a single step. PM: Plasma membrane; EM: extracellular media; OM: outer membrane; P: periplasm; IM: inner membrane; C: cytosol [adapted from (Filloux *et al.*, 2008; Fronzes *et al.*, 2009; Green and Mecsas, 2016; Lasica *et al.*, 2017)].

6.1. T1SS

T1SSs have been found in a large number of Gram-negative bacteria, including pathogens of plants and animals, where they transport their substrates in a one-step process (Koronakis *et al*., 1991). T1SSs have three essential structural components: an ABC transporter protein in the inner membrane (*ATP-binding cassette*), that provides the energy for protein secretion; a membrane fusion protein that crosses the inner membrane (IM) and bridges it to the outer membrane; and the outer membrane factor in the outer membrane (OM) (Thomas *et al*., 2014).

T1SSs are involved in the secretion of proteases, lipases, adhesins, heme-binding proteins, and proteins with repeat-in-toxins (RTX) (Omori and Idei, 2003). As an example, *Salmonella* SPI4 encodes a T1SS and the cognate substrate protein SiiE, a 600 kDa adhesin that mediates the first contact to the apical membrane of the intestinal cells microvilli and collaborates with T3SS1, helping bacteria to cross the epithelial barrier (Gerlach *et al*., 2007, 2008). This protein contains Ca^{2+}-binding sites that are critical for supporting its secretion (Peters *et al*., 2017). SiiA and SiiB proteins are involved in a mechanism of controlling SPI4-T1SS-dependent adhesion (Wille *et al*., 2014). BapA is another *Samonella* protein, with adhesion function, which is secreted by a T1SS. It contributes to biofilm formation and invasion of *S.* Enteriditis (Latasa *et al*., 2005).

6.2. T2SS

T2SSs are multicomponent machineries that use a two-step mechanism for translocation: (i) the precursor effector protein is translocated through the inner membrane by the Sec translocon (Gold *et al*., 2007) or via Tat (Voulhoux *et al*., 2001); (ii) the effector is translocated from the periplasm by T2SS through the outer membrane. There are 12 core components of the T2SS that are essential for biogenesis and secretion, that are distributed in the outer membrane secretin, the inner-membrane platform, the cytosolic ATPase, the pseudopilus, and the prepilin peptidase (Gu *et al*., 2017).

A number of bacterial pathogens employ T2SSs to transport virulence factors or enzymes that help them adapt to their environment outside of the cell. This system is found in pathogens such as *Erwinia*, *Legionella*, *Vibrio* or *Yersinia* (Korotkov *et al*., 2012); however, it is not found in others such as *Salmonella* or *Shigella*.

6.3. T3SS

T3SSs resembles molecular syringes that use a mechanism of secretion of a single step, independent of Sec, found in many symbionts and Gram-negative pathogens of animals and plants, including members of the genera *Salmonella*, *Shigella*, *Yersinia*, *Rhizobium*, *Escherichia* and *Pseudomonas* (Buttner, 2012). These systems are genetically, structurally and functionally related to bacterial flagella (Cornelis, 2000; Diepold and Armitage, 2015; Galán *et al.*, 2015; Macnab, 2003; Tampakaki *et al.*, 2004). They transport effectors from the cytoplasm of the bacteria to the host cell cytoplasm, crossing the bacterial envelope and the host cell membrane (Cornelis, 2006; Galán and Wolf-Watz, 2006). Although the T3SSs of different genera of pathogens have a different effector repertoire, they maintain functional similarities, so that a T3SS of a bacterial genus can secrete and translocate effectors of another bacterial genus (Ho and Starnbach, 2005).

The assembly of a T3SS requires more than 20 proteins and comprise several substructures including a cytosolic ATPase complex, a cytoplasmic ring, an inner membrane export apparatus, a basal body (spanning the bacterial inner and outer membranes and encircling an inner rod and a needle), and a translocation pore that is in the host cell membrane (Deng *et al.*, 2017). The basal body contains several rings structures embedded in the bacterial membranes. In most systems it includes at least 15 proteins (Abrusci *et al.*, 2014; Burkinshaw and Strynadka, 2014). The inner rod may help to anchor the needle to the basal body. The needle protrudes from the bacterial surface and is capped externally by the tip complex in some T3SSs. The tip complex facilitates the assembly of translocation pores in host cell membranes. The T3SS needle has an inner hollow core that is wide enough to permit an unfolded effector to traverse (Deane *et al.*, 2006; Demers *et al.*, 2014). Excitingly, recent work has visualized a 'trapped' effector protein by electron microscopy and single particle analysis, supporting the model that substrates are unfolded and secreted through the basal body and needle channel (Dohlich *et al.*, 2014; Radics *et al.*, 2014). The secretion is promoted by an ATPase (Akeda and Galán, 2005), although the proton motive force could be the primary source of energy for secretion (Lee *et al.*, 2016).

The typical translocation process occurs from bacterium to the host cytosol. However, there is evidence that effectors located on the bacterial surface can also be translocated. This transport, demonstrated for the YopH effector of *Y. pseudotuberculosis*, also occurs in *trans* through T3SS1 (Akopyan *et al.*, 2011). (**Figure I.6**)

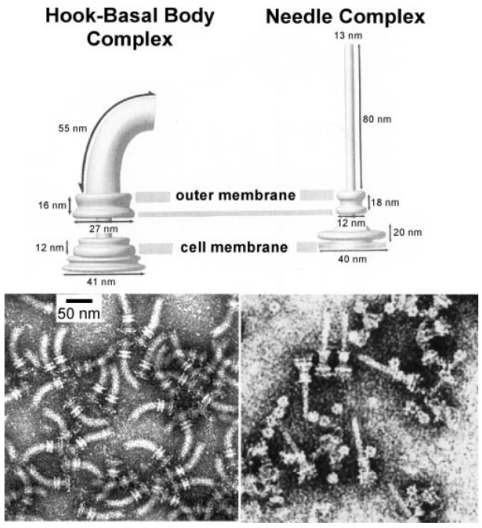


Figure I.6. Comparison of the flagellar hook-basal body complex and the needle complex used for the secretion of virulence factors. The T3SS are structures related to the flagella and share regulatory mechanisms (Lin *et al.*, 2008; Pallen *et al.*, 2005; Wang *et al.*, 2007)[adapted from (Macnab, 2003)].

6.4. T4SS

T4SSs represent a highly diverse superfamily of secretion systems found in many Gram-negative and Gram-positive bacteria (Grohmann *et al.*, 2018). They are ancestrally related to bacterial DNA conjugation systems and can secrete a variety of substrates, including single proteins and protein-protein and DNA-protein complexes (Alvarez-Martinez and Christie, 2009; Llosa *et al.*, 2009). Three families of T4SS can be defined, according to their function: (i) conjugative systems that transfer plasmids and transposons from donor to host bacterium; (ii) DNA uptake or release systems; and (iii) translocation systems that transport DNA or proteins into eukaryotic cells which are involved in virulence of many Gram-negative pathogens (Fronzes *et al.*, 2009). Due to functional overlap between certain T4SSs, a simpler classification has recently been proposed: type IVa, exemplified by the VirB/D4 system of *Agrobacterium tumefaciens*, and type IVb, exemplified by the Dot/Icm *of Legionella pneumophila* (Chandran Darbari and Waksman, 2015; Kubori and Nagai, 2016).

6.5. T5SS

These systems translocate the substrate in two steps and include five subcategories called Va to Ve. All, except the Vb type, are autotransporters that insert a β-barrel domain, into the outer membrane to form a channel for the secretion of a passenger domain that exerts

biological activity in the extracellular space (Gawarzewski *et al*., 2013; Leyton *et al*., 2012; Pohlner *et al*., 1987). Type Vb works in a similar way but the passenger and the transporter are two different polypeptides (Fan *et al*., 2016). Because protein secretion by T5SSs only occurs in the outer membrane, these proteins must be first translocated across the inner membrane into the periplasm in an unfolded state by the Sec apparatus. Approximately 700 proteins, with functions that include adhesion, auto-aggregation, cytotoxicity, invasion, resistance to serum, cell-to-cell dissemination and proteolysis, use these secretion systems to cross the outer membrane of Gram-negative bacteria (Henderson and Nataro, 2001; Mazar and Cotter, 2007). Among well-studied autotransporter of *Salmonella*, there are ShdA (Kingsley *et al*., 2000), MisL (Blanc-Potard *et al*., 1999) and SadA (Raghunathan *et al*., 2011) proteins. ShdA is expressed when bacteria are in the host intestine and it has a domain that mediates adhesion to fibronectin. This protein is important for *Salmonella* persistence and its long-term presence in mouse feces (Kingsley *et al*., 2002). MisL, which is encoded in SPI3, is an adhesin able to bind to fibronectin and it is involved in intestine colonization (Dorsey *et al*., 2005). SadA is a trimeric autotransporter (type Vc), which is another adhesin whose expression contributes to cell aggregation, biofilm formation and adhesion to intestine CaCo-2 cells (Raghunathan *et al*., 2011).

6.6. T6SS

They are one-step secretion complexes (Pukatzki *et al*., 2006) which are widely distributed among proteobacteria (Boyer *et al*., 2009) including pathogens such as *P. aeruginosa*, enteroaggregative *E. coli*, *S.* Typhimurium, *Vibrio cholerae* and *Yersinia pestis*. These systems are made of 13 essential units and several accessory components (Cascales, 2008; Nguyen *et al*., 2018; Pukatzki *et al*., 2009). They are evolutionary related to myophages (like T4 or Mu) and could be some kind of tamed phage (Leiman *et al*., 2009). These systems can translocate proteins to both eukaryotic and prokaryotic cells, so they have a role in virulence and interactions with other bacteria (Ho *et al*., 2014). The first report for a T6SS in *Salmonella* correspond to the genetic characterization of the SPI6 (Folkesson *et al*., 2002), formerly known as SCI (*Salmonella enterica* centisome 7 genomic island). There are conflicting results about the contribution of this system to virulence. Whereas one study suggested that it limits the intracellular growth in macrophages, and decreases virulence in mice (Parsons and Heffron, 2005), another

report showed that this T6SS was necessary for full proliferation in macrophages and virulence in mice (Liu *et al*., 2013). It also contributes to gastrointestinal colonization and systemic dissemination in chickens (Pezoa *et al*., 2013). In addition, The SPI6-T6SS antibacterial activity is essential for *Salmonella* to establish infection within the host gut (Sana *et al*., 2016). *In silico* analysis identified three additional T6SS in several serovars of *S. enterica* (Blondel *et al*., 2009), found in SPI19, SPI20 and SPI21. Most serovars works only with one T6SS, encoded in SPI6 or in SPI19, although some can host two.

6.7. T8SS

Also known as the extracellular nucleation-precipitation (ENP) pathway. This system is involved in the secretion and assembly of curli. Curli are extracellular amyloid fibers produced by many enteric bacteria including *Salmonella*. These are components of biofilms and are important for surface colonization and interaction with host factors and the host immune system (Desvaux *et al*., 2009; Evans and Chapman, 2014).

6.8. T9SS

This is a recently described secretion system that appears to be present in over 1000 sequenced species/strains of the Fibrobacteres-Chlorobi-Bacteroidetes superphylum (Veith *et al*., 2017). T9SSs are involved in the generation of bacterial coating necessary for virulence, gliding motility, and degradation of complex biopolymers. Among the many substrates of these systems, there are proteinases, glycosidases, nucleases, lipases, adhesins, hemagglutinins, and leucine-rich proteins.

6.9. CU systems

They are two-step translocation systems that only require two proteins: (i) the usher protein, which forms the β-barrel channel in the outer membrane; and (ii) the chaperone, a periplasmic protein that facilitates folding of the secreted protein prior to delivery to the channel (Waksman and Hultgren, 2009). Chaperone-usher systems are commonly used to assemble *pili* or fimbrias on the surface of Gram-negative bacteria. They act as virulence factors in (i) recognition; (ii) host adhesion and invasion; and, (iii) biofilm formation (Hospenthal *et al*., 2017). These systems have been proposed to be referred as

T7SSs (Desvaux *et al.*, 2009), but misleadingly this name is also being used for another system that is specific of Gram-positive bacteria (Abdallah *et al.*, 2007).

7. *Salmonella* T3SSs

The T3SS encoded in SPI1 (T3SS1) is present in both species of the genus *Salmonella*, whereas, an additional T3SS (T3SS2) encoded in a different pathogenicity island, SPI2, is specific of *S. enterica* (Bäumler, 1997). Both secretion systems play an important role in host interaction during pathogenesis. T3SS1 facilitates invasion of non-phagocytic cells and contributes to cross the intestinal epithelium. T3SS2 is required for bacterial replication within many eukaryotic cell types (Aussel *et al.*, 2011; Geddes *et al.*, 2007) of the multiple organs reached during systemic infection (Carter and Collins, 1974).

7.1. T3SS1

SPI1 has been the subject of decades of research, which has shown to encode a T3SS, a molecular syringe that directly delivers a cohort of virulence effector proteins (encoded both within SPI1 and elsewhere in the *Salmonella* genome) into host cells (Deng *et al.*, 2017). There are 10 to 100 complexes of this type per cell (Kubori, 1998) evenly spread out across the bacterium. This allows the extracellular bacteria to increase the probability of stablishing host cell contact for injection of effector proteins (Diepold and Wagner, 2014). The substructures of T3SS1 are homo- or heteromultimeric protein complexes (**Fig I.6** and **I.7**). The basal body is composed of concentric rings spanning the inner and outer membranes. The inner-membrane ring consists of 24 subunits of PrgK and PrgH and the outer-membrane ring and neck region is made of 15 copies of InvG (24:24:15) (Schraidt *et al.*, 2010; Schraidt and Marlovits, 2011; Worrall *et al.*, 2016). The needle is helically assembled from approximately 120 copies of PrgI, with a very similar arrangement to flagellin subunits in flagella. The axial lumen of the needle, through which the effectors are secreted, has a diameter of 25 Å (Loquet *et al.*, 2012). The needle is connected to the inner rod, which is formed by PrgJ. This substructure traverses the basal body and controls the needle length (Marlovits *et al.*, 2006). The needle tip is formed by several SipD molecules that interact with PrgI through conformational changes of both proteins (Lunelli *et al.*, 2011; Rathinavelan *et al.*, 2011, 2014). Finally, SipB and SipC are

translocator proteins that are inserted into host membranes and form a channel, known as the translocon pore, that translocates effectors into the host cell (Myeni *et al.*, 2013).

7.2. T3SS2

In contrast to the T3SS1, the translocon encoded by SPI2 is only present singly or in few copies at one pole of the bacterium (Chakravortty *et al.*, 2005). This system is activated by bacteria tightly surrounded by the vacuolar membrane, where a single injectisome seems to be enough to stablish contact and translocate the corresponding effectors. SseB, SseC and SseD have been identified as secreted proteins with translocon functions for the T3SS2 (Ruiz-Albert *et al.*, 2003; Nikolaus *et al.*, 2001). Mutants *ssaG*, *ssaH* and *ssaI* are unable to translocate effector proteins and to form surface structures, suggesting a role for SsaG, SsaH and SsaI in the formation of a functional T3SS2 and surface structures (Chakravortty *et al.*, 2005). The selective secretion of this system is controlled by a regulatory complex formed by three proteins (SsaL, SsaM and SipC), which is located in the bacterial cytosol, probably in contact with the basal body (Yu *et al.*, 2002, 2004, 2010). SsaQ constitutes a cytoplasmic platform (C-ring) connected to the base of the secretion system (Yu *et al.*, 2011).

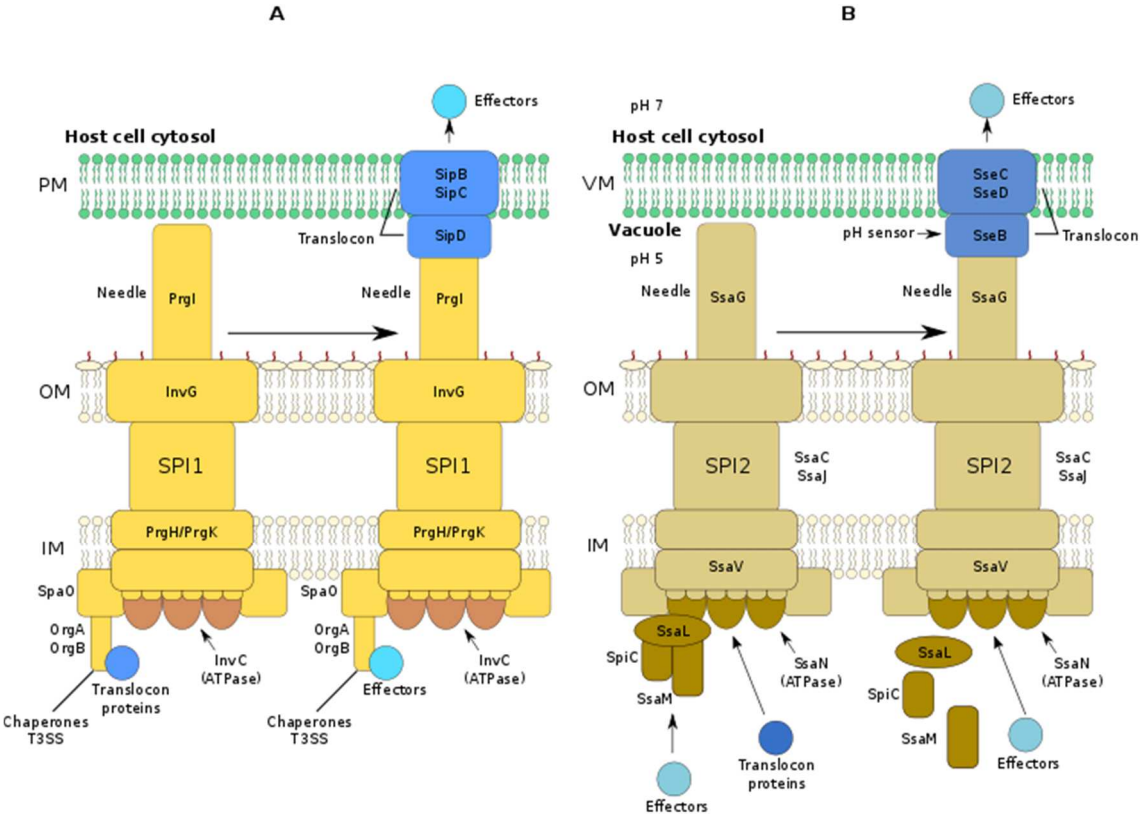


Figure I. 7. Schematic representation of the structure and composition of the *Salmonella* T3SSs. (A) Control of secretion mediated by T3SS1. The SpaO-OrgA-OrgB complex serves as a docking platform for translocon and effector proteins that are targeted to the complex by T3SS chaperones. Effectors are hierarchically secreted after secretion of translocon proteins. **(B)** Control of secretion mediated by T3SS2. The SpiC, SsaL and SsaM complex controls the secretion of translocon and effector proteins in response to differences in external pH. At pH 5 (within the SCV), the complex blocks the secretion of effector proteins, while translocon proteins are secreted. At pH 7 (in the cytosol) the SpiC-SsaL-SsaM complex is dissociated and the secretion of effector proteins is induced. Changes in pH could be sensed by the extracellular components of the T3SS. Note: the architecture of T3SS2 is not well detailed and it is proposed according to amino acid sequence similarities between predicted components of T3SS2 and what is known of other T3SSs. PM: plasma membrane; OM: outer membrane; IM: inner membrane; VM: vacuolar membrane [adapted from (Buttner, 2012)].

7.3. T3SS regulation

The adaptation to different environmental conditions requires a rapid and accurate regulation of the expression of the *Salmonella* virulence genes during the infection process. T3SSs activities are controlled by a complex system of activating and inhibitory signals acting with different strength on different levels, ranging from transcription and translation to protein-protein interactions and secretion (Fass and Groisman, 2009; Golubeva *et al.*, 2012; Moest and Méresse, 2013). SPI1 genes are expressed during the early stages of infection in the intestine. Once inside the epithelial cells, SPI2 expression increases and shows a predominant expression once the bacterium has crossed the epithelium and resides in the SCV (Hautefort *et al.*, 2008). Despite this seemingly

reciprocal expression pattern throughout invasion, both islands are co-regulated by the same global regulatory proteins. They are strongly repressed by the H-NS protein (Lucchini *et al.*, 2006; Oshima *et al.*, 2006) and activated by IHF, FIS and OmpR (Ellermeier and Slauch, 2007; Feng *et al.*, 2003; Kelly *et al.*, 2004; Mangan *et al.*, 2006; Osborne and Coombes, 2011). In addition, there is a relationship between both islands by the induction of the expression of *hilA* (SPI1) and *ssrAB* (SPI2) by HilD, which mitigates the repressive action of H-NS (Bustamante *et al.*, 2008). The regulatory pathways of the two *Salmonella* secretion systems are schematized in **Figure I.8**.

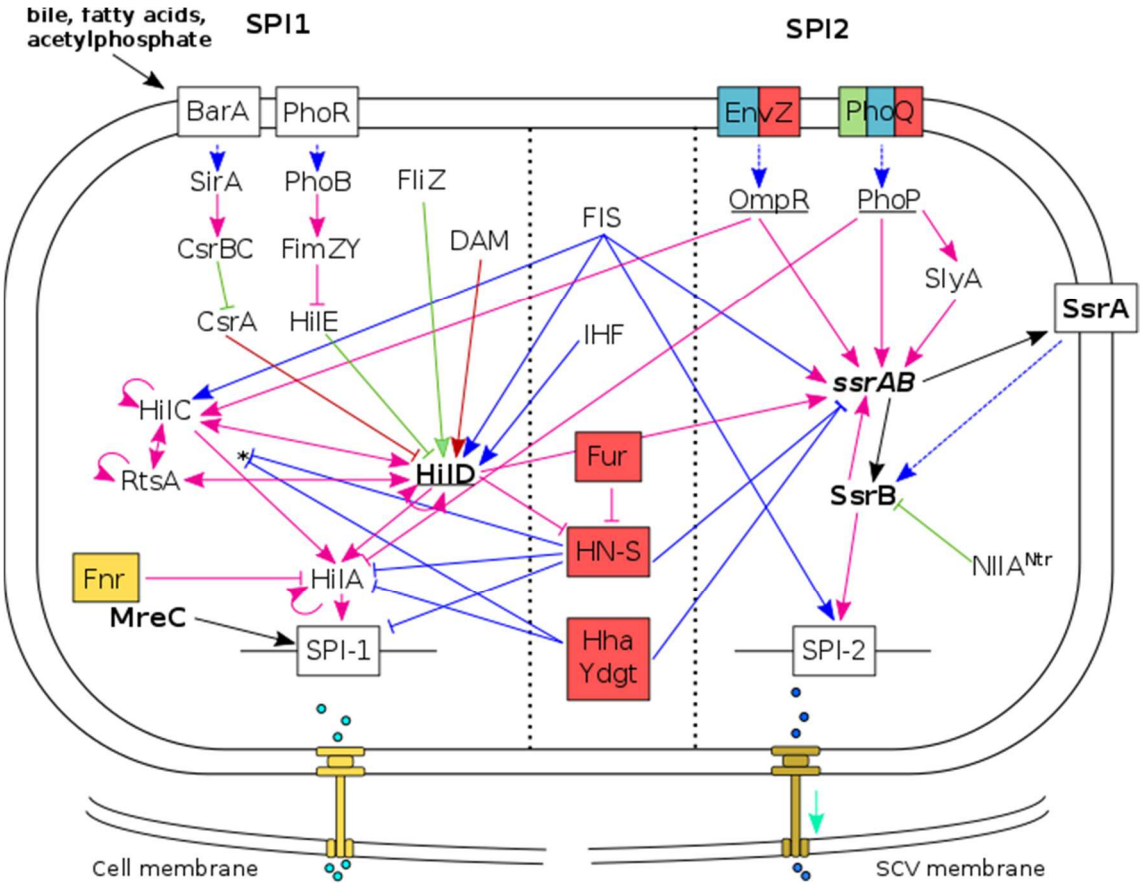


Figure I.8. *Salmonella* T3SSs regulation. (Left) SPI1 regulation. (Middle part) DNA structuring elements with impact on both pathogenicity islands. (Right) SPI2 regulation. Key regulators are in bold and main players in cross-regulation are underlined. The nature of the impact on regulation is indicated with a color code. Environmental factors: osmolarity (red), pH (light blue), oxygen (yellow) and antimicrobial peptides (light green). Protein synthesis: promoter binding/transcription (pink), DNA structuring/transcription (dark blue), posttranscriptional regulation on mRNA (dark red) and translational regulation (orange). Protein interactions: direct binding (dark green), phosphorylation (dashed dark blue). *Regulation mechanisms on the triumvirate of HilD, HilC and RtsA [adapted from (Moest and Méresse, 2013)].

7.3.1. T3SS1 regulation

T3SS1 activation is stimulated by several intestinal environmental factors such as osmolarity, oxygen tension, pH (Altier, 2005), short chain fatty acids (acetate, formate, propionate and butyrate) (Golubeva *et al*., 2012) and long chain fatty acids (Golubeva *et al*., 2016). It is likely that bacteria begin to secrete effectors to the intestinal lumen after colonization. There are studies that support this idea, showing that a high proportion of the effectors secreted by the T3SS1 are found associated with non-adherent bacteria or in the infection media, while only about 10% are translocated to the host cell cytosol (Collazo and Galán, 1997).

SPI1 expression is controlled by four AraC-like transcriptional activators encoded on the island: HilA, HilC, HilD and InvF. Like HilC and HilD, RtsA activates expression of SPI1 genes by binding upstream of the master regulatory gene *hilA*. HilA activates the T3SS1 structural genes (Ellermeier *et al*., 2005; Olekhnovich and Kadner, 2002; Schechter and Lee, 2001). Neither HilC nor RtsA, can activate *hilA* by themselves, but they act by amplifying activation mediated by HilD (Cott Chubiz *et al*., 2010; Golubeva *et al*., 2012). HilA activates *invF* and the genes that encode T3SS1 and its effector proteins (Bajaj *et al*., 1995). The network formed by these regulators also incorporates signals from global regulators. For example, Lrp protein represses transcription of key virulence regulator genes (*hilA*, *invF*) in SPI1, by binding directly to their promoter regions (Baek *et al*., 2009). H-NS and Hha, nucleoid associated proteins (NAPs), repress *hilA* by binding to regions located upstream and downstream of its promoter (Olekhnovich and Kadner, 2006; Queiroz *et al*., 2011). HilC and HilD are substrates of the ATP-dependent protease Lon (Takaya *et al*., 2005), which contributes to the repression of SPI1 after the invasion of epithelial cells (Boddicker and Jones, 2004). HilE is a negative regulator of SPI1 (Fahlen *et al*., 2000) that may interfere with the function of HilD by direct protein-protein interaction (Baxter *et al*., 2003). The *hilE* transcript is activated by the fimbrial regulator FimYZ (Baxter and Jones, 2005) and is repressed by the phosphotransferase system (PTS)-dependent regulator Mlc (Lim *et al*., 2007), and this is transmitted to SPI1 through HilD. The two-component systems PhoQ/PhoP and PhoR/PhoB can activate the expression of *hilE* (Ellermeier and Slauch, 2007; Jones, 2005) and repress the expression of *hilA* (Golubeva *et al*., 2012; Lucas *et al*., 2000). The Csr system represses the expression of SPI1 through *csrA* overexpression (Altier *et al*., 2000; Martínez *et al*., 2011). The two-component system BarA/SirA activates SPI1

expression through Csr system, activating transcription of *csrB* and *csrC*, which encode CsrA antagonist (Fortune *et al*., 2006). SPI1 is also activated through the iron absorption regulator Fur (Ellermeier and Slauch, 2008; Teixidó *et al*., 2011; Troxell *et al*., 2011). In *S.* Enteriditis, the expression of *hilA* is repressed in aerobic conditions through Fnr (Immerseel *et al*., 2008), which activates many genes of SPI1 in *S.* Typhimurium under anaerobic conditions (Fink *et al*., 2007). The two-component system EnvZ/OmpR activates SPI1 by controlling the expression of *hilD* at posttranscriptional level (Ellermeier *et al*., 2005; Ellermeier and Slauch, 2007). FliZ, an inhibitor of RpoS (Pesavento *et al*., 2008), activates SPI1 expression by controlling HilD activity (Cott Chubiz *et al*., 2010). SPI1 regulation by DNA adenine methylation (Dam) was also described (Balbontín *et al*., 2006; Garcia-Del Portillo *et al*., 1999; López-Garrido and Casadesús, 2010). Dam activates synthesis of HilD but this regulation is posttranscriptional and is mediated by products of another horizontally acquired element, the *std* fimbrial operon, which encodes an appendix assembled by a CU system (López-Garrido and Casadesús, 2012). The bacterial cytoskeleton is also important for the regulation of this system, but not for its assembly and activity (Bulmer *et al*., 2012). SPI1 repression by LeuO has also been described. This LysR-type transcriptional regulator acts through two pathways: a main pathway through *hilE* activation, since HilE is a HilD inhibitor; and a secondary HilE- and HilD-independent pathway (Bustamante and Calva, 2014; Dillon *et al*., 2012; Espinosa and Casadesús, 2014).

7.3.2. T3SS2 regulation

T3SS2 expression depends mainly on three two-component regulatory systems: SsrA/SsrB, PhoQ/ PhoP and EnvZ/OmpR.

SsrA/SsrB system is the central regulator of T3SS2 functions (Fass and Groisman, 2009). SsrA is the integral membrane sensor and SsrB is the response regulator. The system is activated when *Salmonella* is inside macrophages, and also in vitro when the bacteria are incubated in minimal medium with an acidic pH (Miao *et al*., 2002). Phosphorylated SsrB binds to the promoters of all the functional gene clusters of SPI2 (Walthers *et al*., 2007), and it is essential for the expression of T3SS2 and its effectors regardless of whether they are encoded within or outside SPI2 (Worley *et al*., 2000). In addition, SsrB activates its own transcription and that of *ssrA* (Feng *et al*., 2003). This is negatively controlled by $EIIA^{Nrt}$, which directly interacts with SsrB thereby preventing it from binding its target

promoters and avoiding undesirable effects due to overexpression of SPI2 genes (Choi *et al*., 2010). SsrB directly activates the transcription of genes encoding T3SS2 substrates (SifA, SifB, SseJ, PipB, etc) and also promotes the expression of the T3SS2 apparatus itself by displacing the DNA-binding protein H-NS from the promoter regions of SPI2 (Walthers *et al*., 2011). In addition, H-NS represses the expression of *ssrA* through direct interaction with its promoter (Bustamante *et al*., 2008). The NAPs YdgT and Hha repress SPI2 gene transcription in a SsrB-dependent manner (Coombes *et al*., 2005; Silphaduang *et al*., 2007), whereas HF and Fis are necessary for the activation of SPI2 expression (Kelly *et al*., 2004; Lim *et al*., 2006b; Yoon *et al*., 2003).

Fis can regulate SPI2 expression through the expression of other genes such as *phoP*. PhoP is an important regulator of SPI2 (Bijlsma and Groisman, 2005; Deiwick *et al*., 1999; Lee *et al*., 2000a; Worley *et al*., 2000), although dispensable (Miao *et al*., 2002). The PhoQ/PhoP system is an ancestral regulatory system conserved in *Salmonella* and other related species, which is required for virulence and survival of *Salmonella* within macrophages (Groisman, 2001; Miller *et al*., 1989). PhoP binds directly to the *ssrB* promoter and controls SsrA at posttranscriptional level (Bijlsma and Groisman, 2005). PhoP also regulates the expression or activation of the SlyA protein (Cano *et al*., 2001; Navarre *et al*., 2005), which binds to the *ssrA* promoter, regulating SPI2 expression. The role of SlyA seems to be limited to prevent silencing by H-NS (Perez *et al*., 2008).

EnvZ/OmpR plays an important role in SPI2 expression. OmpR acts as a response regulator and binds to the *ssrA* promoter (Lee *et al*., 2000a) and the *ssrB* promoter (Feng *et al*., 2003) activating their transcription. Although the binding site of OmpR to the *ssrA* promoter overlaps with that of SsrB, it does not antagonizes the silencing promoted by H-NS (Bustamante *et al*., 2008).

The T3SS2 is also regulated at the secretion level by an unknown pH sensor that reacts to the neutral pH of the eukaryotic cytoplasm. The T3SS2 is assembled in low pH conditions in the vacuolar environment and secretes translocon proteins and insignificant amount of effectors. The translocon assembly allows the detection of neutral pH of the eukaryotic cytoplasm, and this causes the dissociation and degradation of the regulatory complex SsaL-SsaM-SpiC, allowing the secretion of approximately 25 effectors, which are translocated through the vacuolar membrane (Yu *et al*., 2010).

Interestingly, there is a transcriptional crosstalk between SPI1 and SPI2 that helps *Salmonella* transition to the intracellular lifestyle. One element in this crosstalk is the SPI1-regulator, HilD, that also favors the expression of SPI2 genes by displacing H-NS

from a region downstream of the *ssrAB* promoter (Martínez *et al*., 2014). In addition, SsrB acts as a dual regulator that positively controls SPI2 and represses expression of SPI1 during intracellular stages of infection (Pérez-Morales *et al*., 2017). Other factors involved in the regulation of expression of SPI1 and SPI2 are the bacterial alarmone, ppGpp, the alternative sigma factor, RpoS, and the RNA polymerase accessory protein, DskA (Rice *et al*., 2015).

7.4. Effectors of *Salmonella* T3SS and processes in which they are involved

T3SSs substrates can be encoded in both SPI1 and SPI2 as well as out of them, normally within gene fragments with horizontal acquisition characteristics. Bacterium-host cell surface contact activates the T3SS1-mediated effector translocation (Galán, 2001; Hayward *et al*., 2005). Some of these effectors are involved in induction of local membrane ruffles and in bacterial invasion (Galán, 1996). T3SS1 effectors are also involved in proinflammatory cytokine production, in MAPK (*mitogen-activated protein kinase*) pathways activation, in PMN recruitment and in acute intestinal inflammation induction. T3SS2 is expressed intracellularly in response to acidic pH and nutrient limitation found in the SCV lumen. It translocates effectors from the vacuole that are required for modulation of the intracellular environment (Jennings *et al*., 2017; Malik-Kale *et al*., 2011). **Table I.2**, summarizes some features of known *Salmonella* effectors.

Table I.2. *Salmonella* T3SSs effectors. Adapted from (Ramos-Morales, 2012) and expanded from (Habyarimana *et al*., 2014; Jaslow *et al*., 2018; Jennings *et al*., 2017; Sun *et al*., 2016; Yang *et al*., 2015).

Effector	Localization	T3SS	Activity	Cell target	Function
AvrA	SPI1	1	Acetyltransferase Deubiquitinase	B-catenin, ERK2, IκBα, MKK4, MKK7, p53	Antiapoptotic, anti-inflammatory
CigR	SPI3	2			
GogA	Gifsy-1	2	Protease		
GogB	Gifsy-1	2		FBXO22, SKP1	Inhibits NF-κB signaling
GtgA	Gisfy-2	2	Zinc metalloprotease	p65,RelB	Inhibits NF-κB signaling
GtgE	Gifsy-2	1 and 2	Cysteine protease	Rab29, Rab32, Rab38	Prevents accumulation of Rab29, Rab32 and Rab38 on SCV and SITs
PipB	SPI5	2			
PipB2		1 and 2		Kinesin-1 light chain	Sifs extension, recruits kinesin-1 to the SCV

Effector	Localization	T3SS	Activity	Cell target	Function
SifA		2		Rab7, RhoA, SKIP, PLEKHM1, PLEKHM2, GDP-Rho	Sifs, SIST, SCV formation, GEF, detoxifies lysosomes, recruits late endosomes and lysosomes to the SCV, mantains vacuolar membrane stability, SIT formation
SifB		2			
SipA/SspA	SPI1	1		Caspase-3, F-actin, T-plastin	Actin assembly disruption of tight junctions, PMN migration, SCV positioning
SipB	SPI1	1		Caspase-1	Pyroptosis
SipC/SspC	SPI1	1		Cytokeratin-8, cytokeratin-18, Exo70, F-actin, syntaxin-6	Actin nucleation, SCV maturation
SipD/SspD	SPI1	1			
SlrP		1 and 2	E3 ubiquitin ligase	ERdj3, Trx1	Apoptosis, inhibits release of IL-1β
SopA		1	E3 ubiquitin ligase	Caspase-3, HsRMA1, UcH7	Invasion, escape from SCV, PMN migration
SopB/SigD	SPI5	1	Phosphoinositide phosphatase/ guanine nucleotide dissociation inhibitor	Cdc24, Cdc42	Invasion, nuclear responses, SCV maturation, fluid secretion
SopD		1 and 2			Invasion, inflammation, fluid secretion
SopD2		2	GAP	Rab7, Rab32	Inhibition of LNT formation, SIT formation, prevents accumulation of Rab32 on SCV and SITs
SopE	SopEϕ	1	GEF	Cdc42, Rab5, Rac1	Actin remodeling, inflammation
SopE2	Phage remnant	1	GEF	Cdc42, Rac1	Actin remodeling, inflammation
SpiC/SsaB	SPI2	2		Hook3, TassC	Inhibition of fusion of SVC with lysosomes
SptP	SPI1	1	GAP, tyrosine phosphatase	Cdc42, Rac1, VCP, vimentin	Reversion of actin reorganization, inhibition of ERK activation
SpvB	Plasmid	2	ADP-rybosyl transferase	G-actin	Actin depolymerization, cytotoxicity delay, autophagy inhibition, inhibits F-actin polymerization, promotes macrophages apoptosis and P-body disassembly
SpvC	Plasmid	1 and 2	Phosphothreonine lyase	ERK2, p-ERK, p-p38, pJNK	MAPK inactivation

Effector	Localization	T3SS	Activity	Cell target	Function
SpvD	Plasmid	1 and 2	Cysteine hydrolase	Xpo2	Inflammatory response inhibition, inhibits NF-kB signaling
SrfJ	*myo*-inositol island	2			
SrgE		2			
SseB	SPI2	2			
SseC	SPI2	2			
SseD	SPI2	2			
SseF	SPI2	2		Plakoglobin, TIP60, SseG, ACBD3	Sifs formation, SCV positioning
SseG	SPI2	2		Caprin-1, desmoplakin, SseF, ACBD3	Sifs formation, SCV positioning
SseI/SrfH	Gifsy-2	2	Cysteine hydrolase	Filamin A, IQGAP1, TRIP6	Modulation of host cell migration
SseJ		2	Acyltransfer ase	GTP-RhoA, Cholesterol	Cholesterol esterification
SseK1		1 and 2	Glycosyltran sferase	FADD, TRADD	Inhibits TNFα-stimulated NF-κB signaling and necroptosis
SseK2		2	Putative glycosyltranf erase		Inhibits TNFα-stimulated NF-κB signaling
SseK3	ST64B phage	2	Glycosyltran sferase	TRIM32, TRADD	Inhibits TNFa-stimulated NF-κB signaling and necroptosis
SseL		2	Deubiquitina se	IκBα, OSBP-1, talin, ubiquitin	Prevents accumulation of lipid droplets, inhibits autophagic clearance of cytosolic aggregates, induces late macrophage cell death
SspH1	Gifsy-3	1 and 2	E3 ubiquitin ligase	PKN1	Inhibits androgen steroid receptor signaling
SspH2	Phage remnant	2	E3 ubiquitin ligase	14-3-3γ, AIP, BAG2, Bub3, Filamin A, Profilin-1, Sgt1, UbcH5-Ub, NOD1	Activates NOD1 signaling
SteA		1 and 2		PI(4)P	SIT formation, vacuolar membrane partitioning
SteB		1 and 2			
SteC		2	Kinase	MEK1, HSP27	Induces assembly of F-actin meshwork around SCV
SteD		2		mMHCII, MARCH8	Inhibits antigen presentation and T cell activation
SteE/SarA	Gifsy-1	1 and 2			Activates transcription of STAT3 and production of IL-10

The previously discussed idea, that many effectors of *S. enterica* are secreted into the intestinal environment (Collazo and Galán, 1997) suggests that *Salmonella* effectors could functionally interact with the host intra- and extracellularly. An example of this is the SipA effector that promotes gastroenteritis by means of two functional motifs that trigger, individually, mechanisms of bacterial entry or inflammation (Wall *et al.*, 2007). SipA undergoes a processing, through the host caspase-3 on the surface of the intestinal epithelium. This suggests that SipA needs to be cleaved before the interaction with the apical surface of the host cell (Srikanth *et al.*, 2010).

Despite differences between T3SS1 and T3SS2, there are effectors of both systems that are needed at the same time (Brawn *et al.*, 2007; Lawley *et al.*, 2006). In **Figure I.9** are presented these virulence proteins and their secretion system pathways.

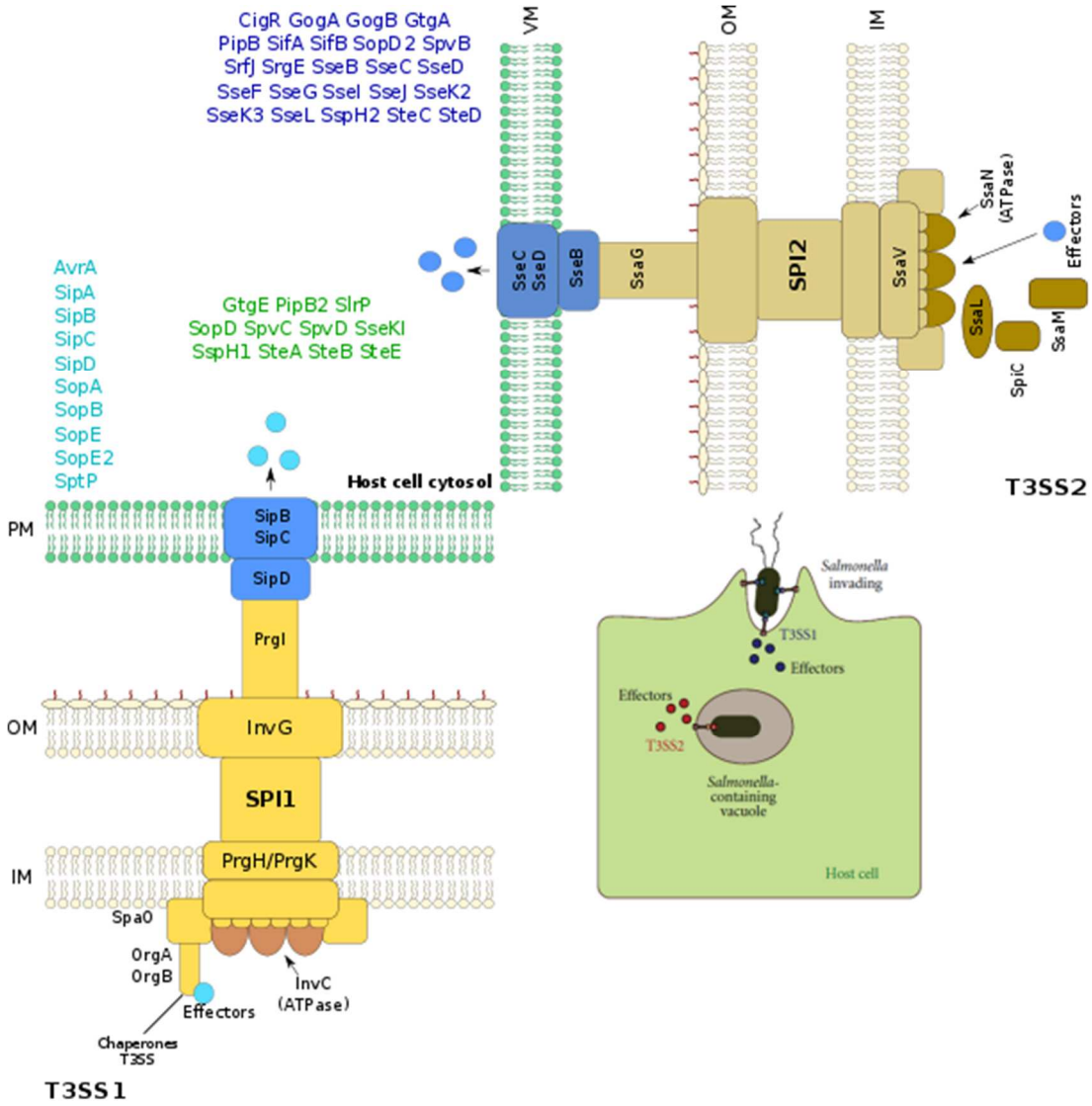


Figure I.9. Schematic representation of the *Salmonella* T3SS1 and T3SS2. The effectors of T3SS1 appear in light blue; the effectors of the T3SS2 appear in dark blue. The effectors translocated by both secretion systems appear in green. PM: plasma membrane; OM: outer membrane; IM: inner membrane; VM: vacuolar membrane [adapted from (Jennings *et al.*, 2017; Ramos-Morales, 2012)].

7.4.1. Salmonella-mediated endocytosis

Central to the pathogenesis of *Salmonella* is its ability to invade intestinal cells: M-cells, epithelial cells, and dendritic cells. Invasion usually occurs in M cells, although it can also occur in enterocytes (**Figure I.10**). M cells are specialized epithelial cells that transport intestinal antigens by pinocytosis to lymphoid cells located under the intestinal epithelium, in Peyer's patches (Brandtzaeg, 1989).

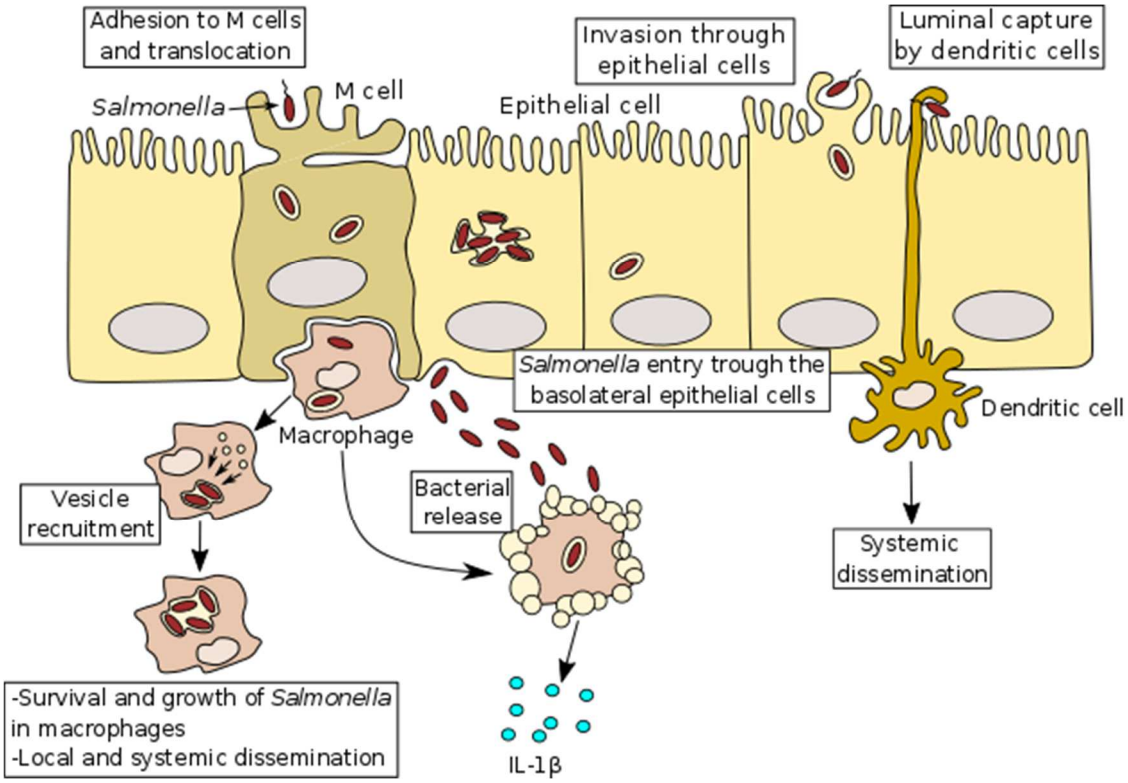


Figure I.10. Strategies that allow *Salmonella* spp. to cross the intestinal barrier, survive in intestinal tissues and spread systemically. The way in which *Salmonella* crosses the intestinal epithelium varies according to the type of cell found in the epithelium. M cells capture the bacteria through receptor mediated endocytosis, while dendritic cells engulf them by phagocytosis. Epithelial cell membranes are modified by the action of SPI1 to facilitate the entry of the bacteria. Once the intestinal barrier has been crossed, *Salmonella* is captured by macrophages, T cells, B cells, neutrophils, etc., [reviewed in (Garai *et al.*, 2012)] and can infect epithelial cells from the basolateral surface [(Criss and Casanova, 2003)]. IL: interleukin [adapted from (Sansonetti, 2004)].

Epithelial cell invasion by *Salmonella*-mediated endocytosis is characterized by a deep reorganization of the host cells actin cytoskeleton leading to the production of large lamellipodia-like surface protrusions termed membrane ruffles, which eventually engulf the pathogen in a large vesicle (Francis *et al.*, 1993; Galán, 2001). This process is similar to macropinocytosis induced by growth factors and is morphologically and functionally different from receptor-mediated endocytosis, a mechanism by which other pathogens enter non-phagocytic cells. This is the best characterized *Salmonella* invasion

mechanism, known as the "trigger" mechanism, and involves several T3SS1 effectors (SipA, SipC, SopB, SopD, SopE, and SopE2).

SipA enhances *Salmonella* entry but is not strictly required (Jepson *et al.*, 2001). SipA potentiates the actin nucleating and bundling activity of SipC and T-plastin (fimbrin) (Haraga and Miller, 2006; McGhie *et al.*, 2001; Zhou *et al.*, 1999). SipA binds with high affinity and stabilizes actin filaments, both mechanically and by preventing their depolymerization by host proteins such as ADF/cofilin and gelsolin (McGhie *et al.*, 2004).

SipC, together with SipB, is essential for the formation of the translocon in the host cell membrane (Myeni and Zhou, 2010). The C-terminal region of SipC is also involved in the process of actin nucleation and F-actin boundling (McGhie *et al.*, 2001; Myeni and Zhou, 2010). SipC also interacts with Exo70, a component of the exocyst complex, which mediates docking and fusion of exocytic vesicles with the plasma membrane (Nichols and Casanova, 2010). It has been recently described that SipC is involved in the apical accummulation of PERP, a host factor that is key to the inflammatory response induced by *Salmonella* (Hallstrom and McCormick, 2016).

SopB (also known as SigD) is a lipid phosphatase which can remove phosphates from the 4′ and 5′ position of various phosphatidylinositol species and these activities are required for SopB-mediated actin remodeling and ruffle formation (Marcus *et al.*, 2001; Piscatelli *et al.*, 2016). SopB could mediate its function in invasion through interaction with the GTPase Cdc42 (Alemán *et al.*, 2005) activation of SGEF, an exchange factor for the GTPase RhoG (Patel and Galán, 2006), recruitment of Annexin A2, p11 and AHNAK (Jolly *et al.*, 2014), and recruitment of sorting nexin (SNX) 9 (Piscatelli *et al.*, 2016) and SNX18 (Liebl *et al.*, 2017).

SopD is translocated through both T3SSs of *S. enterica*. It acts in cooperation with SopB to promote inflammatory responses and fluid secretion in *Salmonella*-infected intestines (Jones *et al.*, 1998) and macropinosome formation during host cell invasion (Bakowski *et al.*, 2007).

SopE and SopE2, functionally mimic host guanine nucleotide exchange factors (GEFs)(Buchwald *et al.*, 2002; Orchard and Alto, 2012; Schlumberger *et al.*, 2003), the endogenous activators of Rho-family GTPases. Through this mechanism, SopE and SopE2 activate Rac1 and Cdc42 GTPases (Hardt *et al.*, 1998; Stender *et al.*, 2000). SopE can activate both Cdc42 and Rac1 while SopE2 only activates Cdc42 (Friebel *et al.*, 2001). Rac1 activation by SopE, but not Cdc2, is required for bacteria internalization

(Patel and Galán, 2006) through the recruitment of the WAVE regulatory complex and the cooperation of a host GEF called ARNO (Humphreys *et al.*, 2012). SopE also mediates recruitment to the membrane of MYO6, a member of the myosin superfamily with important roles in membrane dynamics and membrane ruffle formation, via Rho GTPase activation of p21-activated kinase (PAK) (Brooks *et al.*, 2017). Moreover, SopE also activates RalA, a GTPase required for exocyst assembly (Nichols and Casanova, 2010).

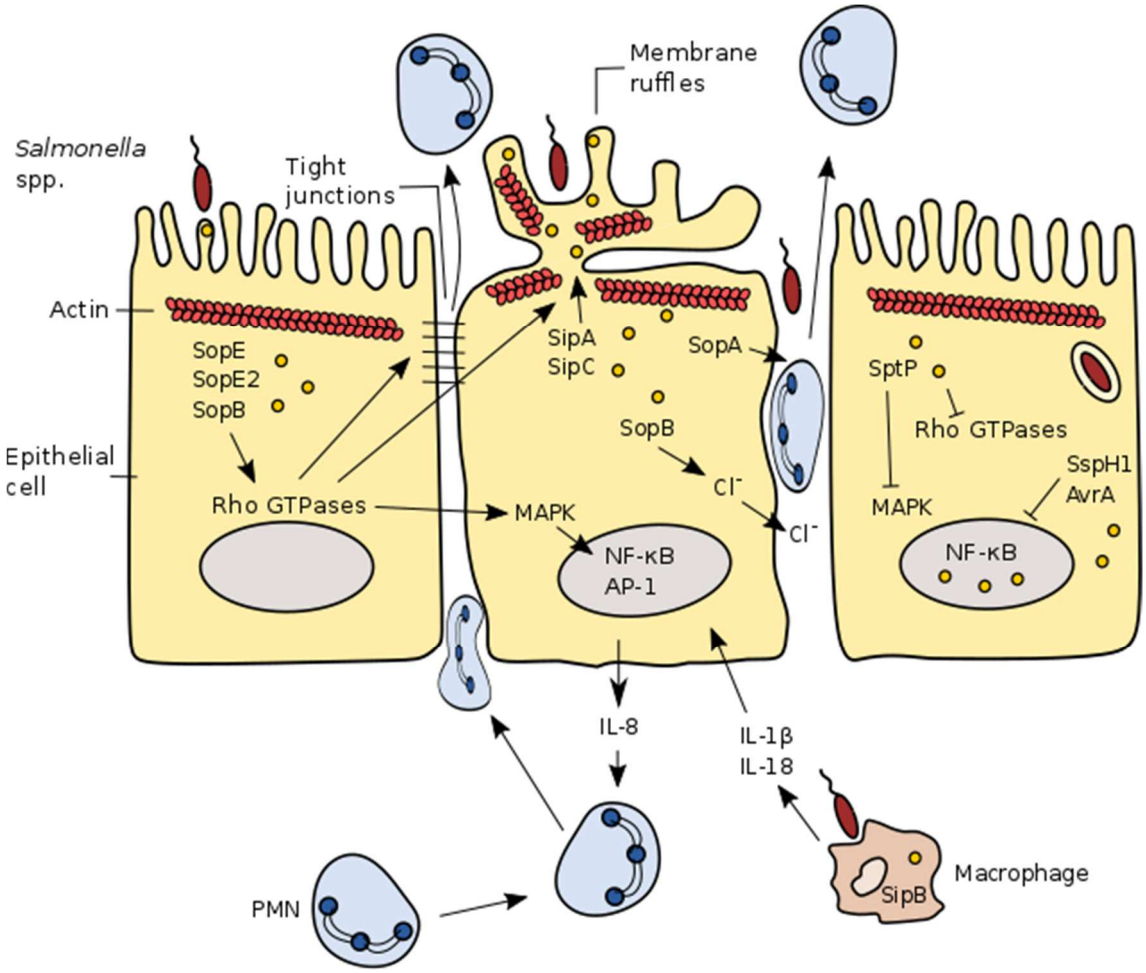


Figure I.11. Changes induced by T3SS1 in the host cells. *Salmonella* directs its internalization in host cells by a mechanism characterized by the appearance of membrane ruffles where bacteria interacts with the host cell. T3SS1 effectors (yellow spheres) SipA, SipC, SopB, SopE and SopE2 induce this process, which is reversed by the action of SptP effector. Moreover, SipA, SopB, SopE and SopE2 contribute to the tight junction disruption. On the other hand, AvrA stabilizes them [adapted from (Haraga *et al.*, 2008)].

After bacterial entry, SptP intervenes. This effector possesses GAP (GTPase-activating protein) activity antagonist to SopE and SopE2, inactivating Rac1 and Cdc42 and reverting the actin cytoskeleton to its basal state after 3 h.p.i. (Fu and Galán, 1999; Galán and Zhou, 2000). SptP possesses a second activity as a protein tyrosine phosphatase that

contributes to the evasion of innate immune response by mediating the suppression of degranulation of local mast cells (Choi *et al.*, 2013).

In addition to phagocytosis, other T3SS1-independent mechanism have been proposed for *Salmonella* entry into the host cell. Two outer membrane proteins, Rck and PagN, have been identified as invasins, and Rck mediates a "zipper" entry mechanism, typical of other pathogens like *Listeria* (Velge *et al.*, 2012). Additional factors that can contribute to invasion are the SPI4-encoded protein SiiE, the outer membrane channel TolC, and the pore-forming toxin HylE (Hume *et al.*, 2017).

After invasion by serovars that produce gastroenteritis, an induction of the secretory response occurs in the intestinal epithelium and neutrophils migrate into the intestinal mucosa and the gut lumen (Galyov *et al.*, 1997). This inflammatory reaction, together with the disruption of tight junctions (see below), probably contributes to the induction of diarrhea.

As mentioned above, *Salmonella* that enter M-cells can be transported to lymphoid cells in the underlying Peyer's patches. Alternatively, they can cross the intestinal epithelium after uptake by dendritic cells. These macrophage-like cells are antigen-presenting cells that phagocytose the bacteria and present antigens to specific $CD4^+$ and $CD8^+$ T cells. Although they do not provide a proper environment for pathogen proliferation, they act as carriers of *Salmonella* for their passive dissemination to systemic sites. Bacteria also induces caspase-1-mediated cytotoxicity in this cell type, dependent on T3SS1 assembly and on SipB SPI1 effector expression (Haraga *et al.*, 2008).

Once across the epithelium *S.* Typhimurium can efficiently invade further epithelial cells from the basolateral side (Criss and Casanova, 2003). *Salmonella* serotypes that cause systemic infection, are phagocytosed by macrophages and activate virulence mechanisms that allow them to evade macrophages microbicidal functions. This allows them to survive and even replicate within the phagocytic cells. Subsequently, infected macrophages migrate to other organs of the reticulo-endothelial system, facilitating bacterial spread throughout the host (Ohl and Miller, 2001). Transport and presentation of *Salmonella* antigens in the lymph is carried out mainly by monocytes and granulocytes, instead of dendritic cells (Bonneau *et al.*, 2006).

7.4.2. Tight junction alterations

The epithelial barrier is a critical border that prevents luminal material from entering tissues. Essential components of this epithelial fence are physical intercellular structures termed tight junctions. These junctions use a variety of transmembrane proteins coupled with cytoplasmic adaptors, and the actin cytoskeleton, to attach adjacent cells together thereby forming intercellular seals. They are composed of claudin and occludin transmembrane proteins, peripheral intracellular membrane proteins (ZO proteins), and some other associated proteins. *Salmonella* modifies tight junctions (**Figure I.11**) through four T3SS1 effectors: SipA, SopB, SopE and SopE2 (Boyle *et al*., 2006), thereby increasing epithelial barrier permeability. Destabilization of these intercellular junctions allows PMNs transmigration from the basolateral to the apical surface, cellular fluid leakage and bacteria access to the basolateral surface. This transmigration, however, can also occur without altering the tight junctions, and this is achieved by SopA, which acts as an ubiquitin ligase HECT3 (Zhang *et al*., 2006). Interestingly, the AvrA effector, another T3SS1 substrate, acts as a tight junction stabilizer (Liao *et al*., 2008), blocking the Jun kinase (JNK) pathway (Lin *et al*., 2016; Zhang *et al*., 2015).

7.4.3. Nuclear responses

Salmonella infection induces global changes in the transcriptome of host cells (Eckmann *et al*., 2000). T3SS effectors contribute to this transcriptional changes. The transcriptional profiles of epithelial cells infected with an *invA* mutant strain, which lacks a functional T3SS1, or with an "effectorless" mutant strain, lacking genes encoding T3SS1 effectors AvrA, SlrP, SopA, SopB, SopE, SopE2, SptP, and SspH1, were similar to that of uninfected cells demostrating that the transcriptional reprogramming triggered by the wt strain depended on one or more T3SS1 effectors (Bruno *et al*., 2009). A *sipA sopA sopB sopD sopE sopE2* mutant shows a different pattern of cellular response in the regulation of actin cytoskeleton and within phosphatidylinositol, CCR3, Wnt and TFG-β signaling pathways, when compared to a wt strain in bovine Peyer's patches infection (Lawhon *et al*., 2011). SopB, SopE and SopE2 activate Rho family GTPases leading to innate immune response stimulation in epithelial cells by the activation of MAPK (ERK, P38, and JNK) and NF-κB signaling pathways (Hobbie *et al*., 1997; Bruno *et al*., 2009; Patel and Galán, 2006).

SspH1 inhibits NF-κB-dependent gene expression, and, together with SptP, participates in interleukin (IL) 8 release inhibition.

Additionally, GogB, SseL and AvrA present deubiquitinase activity, blocking IκBα degradation and inhibiting the NF-κB pathway (Collier-Hyams *et al.*, 2002; Le Negrate *et al.*, 2008; Ye *et al.*, 2007). AvrA targets several host pathways in vivo including mTOR, NF-κB, platelet-derived growth factor, vascular endothelial growth factor oxidative phosphorylation and MAPK signaling (Liu *et al.*, 2010). An *in vitro* study suggested a role of AvrA in *Salmonella*-induced p53 acetylation in epithelial cells (Wu *et al.*, 2010). Studies performed in chicken macrophages infected with *S.* Enteritidis revealed that the T3SS1 effector SipA, and the T3SS2 effector PipB, suppressed host innate response by altering the levels of certain chemokines and RhoA (Zhang *et al.*, 2008). Finally, expression of SteA, an effector secreted by T3SS1 and T3SS2, in epithelial cells up-regulated the expression of genes related to extracellular matrix organization, regulation of cell proliferation and serine/threonine kinase signaling pathways, and down-regulated the expression of genes related to immune processes, regulation of purine nucleotide synthesis and pathway-restricted SMAD protein phosphorylation (Cardenal-Muñoz *et al.*, 2014).

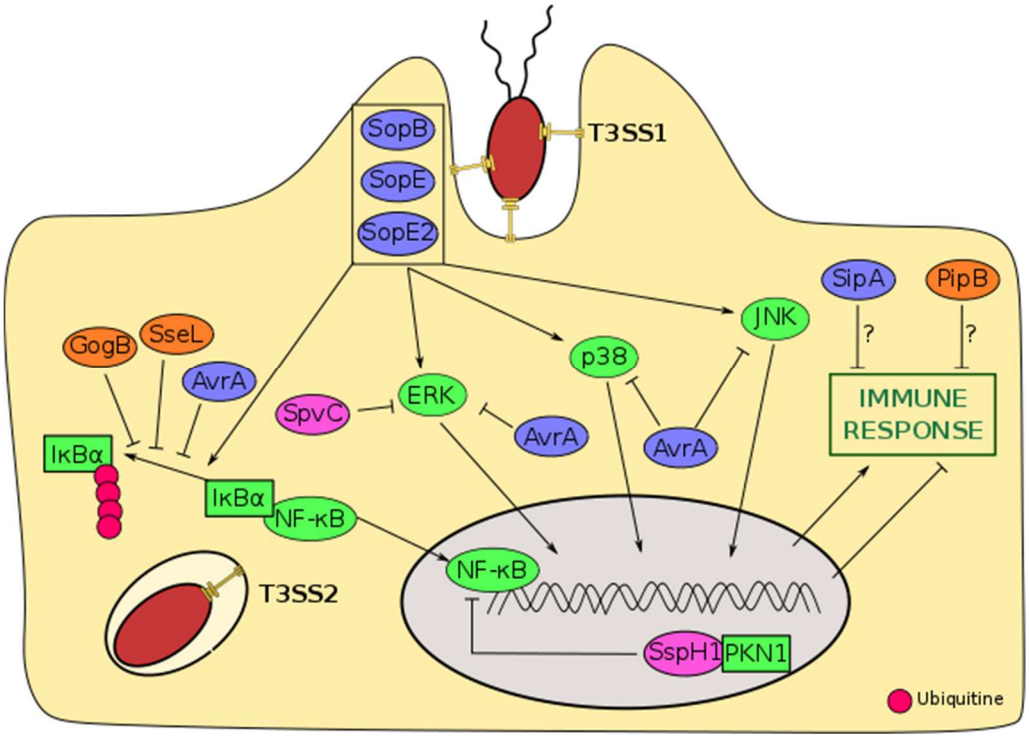


Figure I.12. Nuclear responses induced by *Salmonella* effectors. Some of the effects of *Salmonella* T3SS effectors on host signal transduction pathways are represented, leading to the induction or inhibition of host immune responses [adapted from (Ramos-Morales, 2012)].

7.4.4. Biogenesis of the SVC and intracellular proliferation of Salmonella

Once internalized, *Salmonella* establishes its intracellular niche in a modified phagosome, the SCV, which may persist from a few hours to days (Bakowski *et al.*, 2008). The SCV biogenesis and maturation are characteristically accompanied by the formation of different tubules originated in and connected to the SCV (Schroeder *et al.*, 2011). The SCV moves from the plasma membrane to a perinuclear position (Ramsden *et al.*, 2007), recruits several members of the Rab family of GTPases (Smith *et al.*, 2007) and interacts with endo- and exocytic pathways.

Effectors that are important during the early stage of SCV biogenesis are SopB and SptP. SopB, despite being translocated by T3SS1, persists at least 12 hours after infection (Giacomodonato *et al.*, 2007; Lawley *et al.*, 2006). It is ubiquitinated by TRAF6 within the host cell, which allows it to carry out its biological activity (Ruan *et al.*, 2014). The role of SopB involves direct and indirect manipulation of the host membrane phosphoinositide contents (Steele-Mortimer *et al.*, 2000; Terebiznik *et al.*, 2002). This causes Rab exclusion from the SCV and may serve to delay its fusion with the lysosome (Bakowski *et al.*, 2008). In addition, SopB recruits the GTPase Rab5 to the SCV membrane (Mallo *et al.*, 2008), which will attract important regulators of membrane trafficking that contribute to the recruitment of Rab7 and LAMP1 to the SCV (Braun *et al.*, 2010; Bujny *et al.*, 2008). The function of SptP in the biogenesis of the SCV involves its GAP activity, that downregulates Cdc42 and Rac1 and reverts membrane ruffling (Fu and Galán, 1998), and its phosphatase activity, with dephosphorylates VCP (Humphreys *et al.*, 2009).

During the intermediate stage of development, the SCV reaches a juxtanuclear position adjacent to the microtubule organizing center, close to the Golgi apparatus, a location where the bacteria are able to acquire nutrients and membrane components (Salcedo and Holden, 2003). To reach it, it moves along the microtubules with a dynein-mediated movement (Ramsden *et al.*, 2007). This process requires the participation of the T3SS1 effectors SipA and SopB, and the T3SS2 effectors SifA, SseF and SseG (Boucrot *et al.*, 2005; Deiwick *et al.*, 2006). The actin-based motor nonmuscle myosin II appears to contribute to the SCV positioning in a process involving the phosphatase activity of SopB (Wasylnka *et al.*, 2008). The SCV accumulates cholesterol during its maturation (**Figure I.13**). This accumulation is linked to the bacterial replication and seems to be dependent

on SPI2 (Catron *et al*., 2002). Cholesterol esterification through SseJ at the SCV is also important for intracellular survival of *Salmonella* (Nawabi *et al*., 2008).

Several hours after *Salmonella* entry, a meshwork of F-actin filaments can be observed around the SCV, and this is important for bacterial replication (Méresse *et al*., 2001). SseI and SspH2 co-localize with actin cytoskeleton by interacting with the host actin binding protein filamin (Miao *et al*., 2003). Toxin SpvC, secreted by T3SS2 induces actin depolymerization associated with the vacuole (Browne *et al*., 2002; Lesnick *et al*., 2001), which has a negative effect on Sifs formation.

During the late stage of SCV maturation, three types of tubular networks are generated: Sifs (*Salmonella*-induced filaments), SISTs (*Salmonella*-induced secretory carrier membrane protein 3 (SCAMP3) tubules) and LTNs (LAMP-1 negative tubules), that are observed more easily in cultured epithelial cells.

(i) Sifs are long tubules extending from the SCV and necessary for the correct positioning of the vacuole. They appear at the beginning of bacterial replication, hours after invasion. Derived from late endocytic compartments, they have the same composition as SCV membranes and contain cholesterol, vATPase, LAMPs, Rab7, lysobisphosphatidic acid and cathepsin D (Drecktrah *et al*., 2008; Rajashekar *et al*., 2008; Steele-mortimer, 2009). Sifs are also positive for SCAMP3 which indicates that they can incorporate membrane from the secretory pathway (Mota *et al*., 2009).

The effectors PipB2, SifA, SopD2, SseF, SseG and SseJ are involved in Sifs formation, whereas SpvB seems to have a negative effect on their formation. SifA activates RhoA GTPase and binds to the SKIP eukaryotic protein (SifA and kinesin-interacting protein), regulating the level of the microtubule motor protein kinesin-1 in the SCV (Boucrot *et al*., 2005). PipB2 also recruits kinesin-1 (Henry *et al*., 2006), which together with the SifA-SKIP complex, promotes the elongation of tubules along microtubules. Sif formation requires an intact microtubule network (Brumell *et al*., 2002b; García-del Portillo *et al*., 1993).

SopD2 is associated to late endosome and it may contribute to Sif formation by targeting the endocytic vesicles to the SCV and nascent tubules (Schroeder *et al*., 2010). SseF and SseG mediate the bundling of microtubules near the SCV, that can promote fusion of aggregated vesicles into tubules (Deiwick *et al*., 2006; Schroeder *et al*., 2010).

(ii) SISTs also contain SCAMP3 and T3SS2 effectors, but do not possess either LAMP1 or other late endocytic markers (Mota *et al*., 2009). The formation of SISTs requires effectors PipB2, SifA, SopD2, SseF and SseG.

(iii): LNTs lack LAMP1 and SCAMP3 but are enriched in T3SS2 effectors. They are also formed along microtubules in a kinesin-1 dependent manner, but lack Sifs and SISTs markers except the vATPase. It has been suggested that SopD2 and SifA exert negative and positive roles, respectively, in their formation, and that PipB2 is involved in their centrifugal extension (Schroeder *et al.*, 2010).

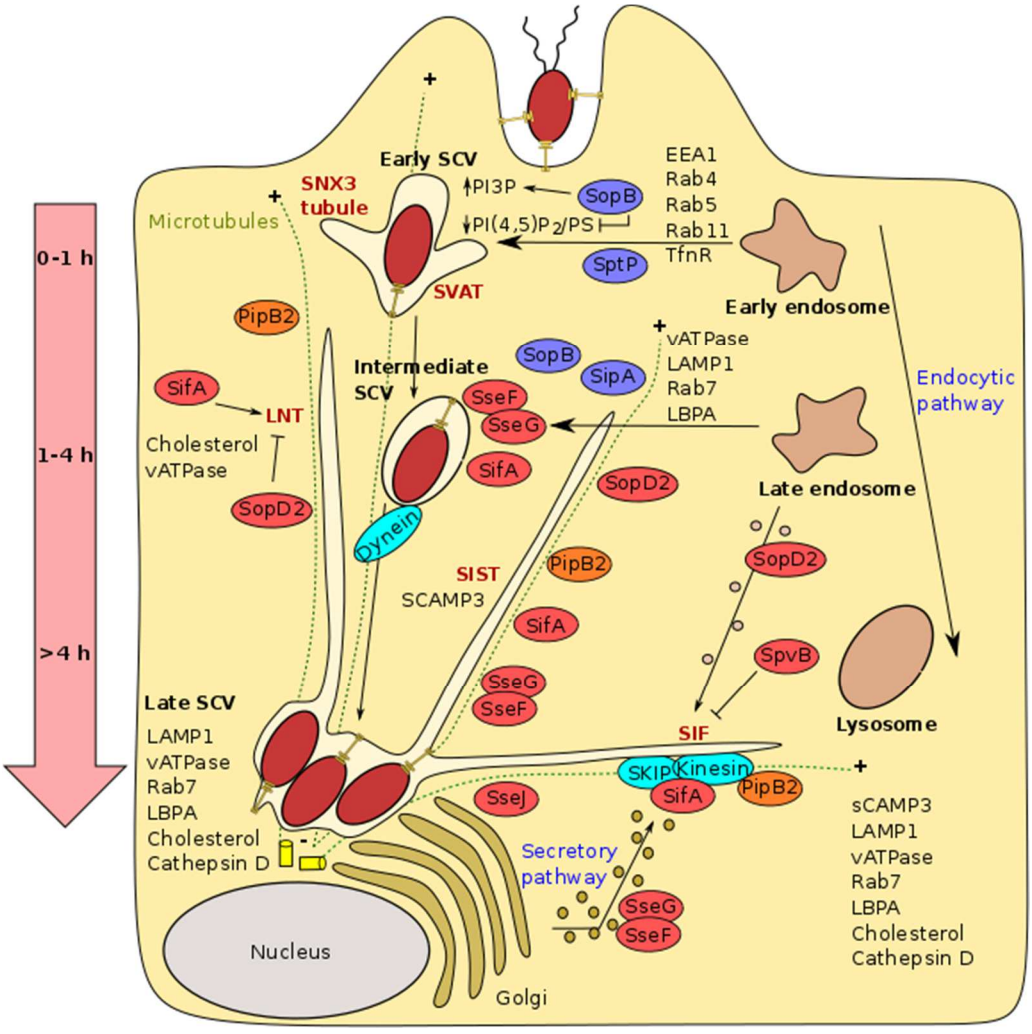


Figure I.13. Biogenesis of the *Salmonella*-containing vacuole. After internalization, *Salmonella* establishes an intracellular niche inside a modified phagosome known as *Salmonella*-containing vacuole (SCV). The initial step in SCV biogenesis (0-1h) is governed by T3SS1 effectors SopB and SptP (dark blue) and is characterized by the formation of SVATs and SNX3 tubules. The movement of the SCV to a juxtanuclear position during the intermediate stage of development (1-4h) requires the participation of the T3SS1 effectors SipA and SopB, and the T3SS2 effector SifA, SseF and SseG (red). Many effectors are involved in the final stage of maturation and maintenance of the SCV. Replication starts 4-6 h postinvasion and is accompanied by the formation of different types of tubules known as the Sifs, SISTs and LNTs. The effectors involved in the formation of these tubules are represented in red (T3SS2 effectors) and orange (effectors of both systems). The maturation process is also characterized by the interaction with the host endocytic and secretory pathways [adapted from (Ramos-Morales, 2012)].

It should be noted that although the typical intracellular niche of *S.* Typhimurium is the SCV, epithelial cells contain two subpopulations of *Salmonella*, one vacuolar and another cytosolic. The escape from the SCV leads to a bacterial transcriptional reprogramming

and their replication in the cytosol (Malik-Kale *et al.*, 2012). Finally, the epithelial cell dies by pyroptosis and the bacteria reaches the extracellular space (Knodler, 2015). In addition, in epithelial cells, a subset of SCVs migrate towards the host cell periphery, in a centrifugal movement dependent on microtubules, kinesin and the T3SS2 effector PipB2 (Knodler and Steele-Mortimer, 2005), that is associated with a decrease of SipA and SopB levels. This centrifugal displacement, together with the bacterial ability to escape from the vacuole, have been associated with the ability of the bacteria to move from one cell to another in order to repeat the infection cycle (Knodler *et al.*, 2010; Szeto *et al.*, 2009).

7.4.5. Cell death

T3SSs are involved in the induction of three different cell death mechanisms in host cells (Guiney, 2005): epithelial cell apoptosis, rapid T3SS1-dependent macrophage pyroptosis, and delayed T3SS2-dependent macrophage pyroptosis (Fink and Cookson, 2007)(**Figure I.14**).

(i) Apoptosis is a programmed cell death that can be initiated by internal or external stimuli. Different caspases are involved in its development, such as caspase-3, which leads to features associated with apoptosis such as mitochondrial membrane potential reduction, cytokeratin cleavage, nuclear condensation, DNA fragmentation, surface exposure of phosphatidylserine and plasma membrane maintenance. The two *Salmonella* T3SSs are required for triggering apoptosis in epithelial cells. SlrP, which can be translocated by both T3SS, seems to contribute to epithelial cell death through its interaction with Trx-1, to which ubiquitinates, and through its interaction with ERdj3, an endoplasmic reticulum chaperone (Bernal-Bayard *et al.*, 2010; Bernal-Bayard and Ramos-Morales, 2009). The T3SS2 effector SpvB, is also required for apoptosis (Paesold *et al.*, 2002). AvrA and SopB effectors may have antiapoptotic activity by repressing the JNK apoptotic pathway (Wu *et al.*, 2012) and activating Akt, respectively (Steele-Mortimer *et al.*, 2000). The action of these effectors could explain the apoptosis delay of 12-18 h after *Salmonella* entry (Kim *et al.*, 1998), allowing bacteria to establish a stable intracellular niche and avoid adaptive immunity (Wu *et al.*, 2012). Later, phagocytosis of apoptotic bodies, together with *Salmonella*, by incoming macrophages could contribute to bacterial systemic dissemination (Guiney, 2005).

(ii) Rapid pyroptosis is triggered in macrophages by *Salmonella* expressing T3SS1. This form of cell death is detected in the first 1-2 h p.i. and has also been observed in dendritic cells (Monack *et al.*, 2001; van der Velden *et al.*, 2003). Pyroptosis requires the assembly of a molecular complex termed the inflammasome. This complex provides a signaling platform that activate specific inflammatory caspases. The best characterized inflammatory caspase is caspase-1, a central mediator of innate immunity that is not activated in apoptosis. Its activity leads to IL-1β and IL-18 production, rapid cell lysis and release of proinflammatory intracellular contents (Bierschenk *et al.*, 2017; Cookson and Brennan, 2001; Fink and Cookson, 2007). Different proteins released by or related to the T3SS1 have been shown to participate in the activation of inflammasome and caspase-1: (i) flagellin may act as an inducer of the NLRC4 inflammasome after its injection into the host cell cytosol through T3SS1 (Franchi *et al.*, 2006; Miao *et al.*, 2006; Sun *et al.*, 2007); (ii) PrjG, a component of the basal body inner rod of T3SS1, is also a stimulator of the NLRC4 inflammasome (Miao *et al.*, 2010); (iii) the T3SS1 effector SopE activates caspase-1 through its GEF activity (Hoffmann *et al.*, 2010; Müller *et al.*, 2009); (iv) the translocase SipB was shown to interact with caspase-1 (Hersh *et al.*, 1999), but it is not clear how translocon insertion results in caspase-1 activation (Müller *et al.*, 2009).

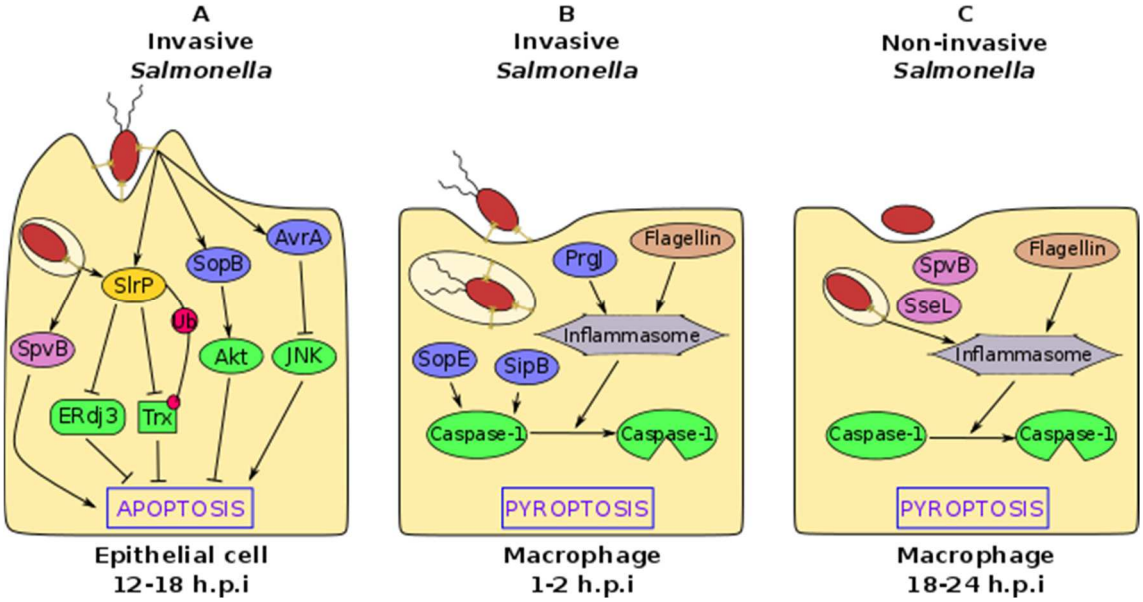


Figure I.14. *Salmonella*-induced host cell death. (A) *Salmonella* induces apoptosis in epithelial cells 12-18 h p.i.. It has been suggested that effectors AvrA and SopB (secreted through T3SS1), SpvB (secreted through T3SS2), and SlrP (secreted through T3SS1 and T3SS2) participate in this process through different mechanisms. Ub: ubiquitin. **(B)** *Salmonella* expressing T3SS1 induces rapid pyroptosis in macrophages. Pyroptosis is a proinflammatory form of programmed cell death that depends on caspase-1 activation. The T3SS1 effector SopB T3SS1, the T3SS1 rod protein PrgJ, and flagellin secreted through T3SS1 are involved in rapid pyroptosis induction. **(C)** Non-invasive *Salmonella* induces delayed pyroptosis in infected macrophages. T3SS2 effectors SpvB and SseI, and flagellin are involved in this cell death mechanism [adapted from (Ramos-Morales, 2012)].

(iii) During systemic bacterial infection, T3SS1 and flagellin expression are repressed, and rapid cell death does not occur (Schlumberger and Hardt, 2006; Stewart *et al.*, 2011). However, T3SS2-dependent delayed pyroptosis is induced 18-24 h p.i. (Monack *et al.*, 2001; van der Velden *et al.*, 2003). During this form of cell death *Salmonella* activates two inflammasomes, NLRC4 and NLRP3. Activation of NLRC4 requires the T3SS2 and flagellin, whereas the signal that activates NLRP3 is not determined (Broz *et al.*, 2010; Libby *et al.*, 2000; Rytkönen *et al.*, 2007), although the process where these effectors are involved could be similar to the apoptotic process in epithelial cells (Guiney and Fierer, 2011).

8. ANALYSIS OF THE TRANSLOCATION OF T3SS EFFECTORS

For the study of *Salmonella* effectors, infection of different cultured cell lines can be carried out. Invasive bacteria are used for the infection of non-phagocytic cell lines, such as HeLa or fibroblasts. Macrophages can be used in the same conditions for short-term infections (Fink and Cookson, 2007), but for infections of several hours it is necessary to use non-invasive bacteria in order to avoid the rapid pyroptosis described above. The expression of the T3SS1 and T3SS2 and the translocation of the effectors depend on the conditions used during the culture of the bacteria before the infection, the time p.i., and the host cell line (Hautefort *et al.*, 2008).

The translocation of effectors can be studied by fractionation of infected cells and immunodetection with antibodies against the effectors or against appropriate tags. An alternative, is the generation of fusions with a fragment of the *cyaA* gene of *Bordetella pertussis*, which encodes the catalytic domain of a calmodulin-dependent adenylate cyclase. This adenylate cyclase converts cellular ATP into cyclic adenosine monophosphate (cAMP) in the presence of calmodulin. Because calmodulin is present in host eukaryotic cells, but not in bacteria, the translocation of one of these fusions would be detected as an increase in cAMP levels in a cell culture infected with *Salmonella* (Sory and Cornelis, 1994).

To mimic, to some extent, in vivo environments, synthetic culture medium can be used, secretion of the effectors to the culture medium is analyzed by immuno-detection. High expression of T3SS1 and its effectors is obtained in rich medium with high concentration of NaCl and low aeration (García-Calderón *et al.*, 2007; Lee and Falkow, 1990), that mimics the conditions found in the lumen of the intestine. A medium with low

concentration of phosphate and magnesium and acidic pH is used to mimic the SCV environment and to induce the expression of the T3SS2 and its effectors (Deiwick *et al.*, 1998; García-Calderón *et al.*, 2007).

8.1. Secretion and translocation sequences of T3SS effectors

The N-terminal 100 amino acids of T3SS effectors contain two relevant signals: a secretion signal in the first 20 amino and a chaperone-binding region (Notti and Stebbins, 2016; Sory *et al.*, 1995) (**Figure I.15**). The sequences in the N-terminal extreme are not conserved, but they share a specific composition, which makes them functionally interchangeable (Anderson *et al.*, 1999) and allows the identification of putative effectors using computational approaches (McDermott *et al.*, 2011; Wang *et al.*, 2013b). These sequences may be sufficient for secretion, but some studies have shown that chaperone-substrate interactions are necessary, at least in certain cases, for the specific targeting of effectors, since in the absence of a chaperone-binding domain the substrate can be exported through the flagellar apparatus (Lee and Galán, 2004). Additional functions described for these chaperones are the prevention of premature degradation of T3SS substrates (Fu and Galán, 1998), the contribution to the establishment of a hierarchy of effector protein translocation (Lara-Tejero *et al.*, 2011) and acting as coactivators of T3SSs transcriptional regulators (Buttner, 2012).

The C-terminal region of some effectors, such as *Salmonella* SipB, is also important for specific secretion through a T3SS. SipB N-terminal domain (160 residues) is secreted through the flagellar system but fusion of the C-terminal domain restore secretion via T3SS1 (Kim *et al.*, 2007). Regions outside the N-terminus are also required for translocation of SifA (Brown *et al.*, 2006).

Some effectors require the action of the T3SS ATPase for its translocation. SptP, together with its chaperone SicP, interacts with the T3SS1 ATPase, InvC, which dissociates SptP-SicP allowing SptP access to the needle of the secretion system (Akeda and Galán, 2005). There are also examples of direct binding of the effector to the ATPase, like in the case of SopD, which interacts with InvC through its C-terminal region (Boonyom *et al.*, 2010). Finally, a controversial issue is the identification of RNA signals with a role in the translocation of certain effectors, including *Salmonella* effectors GtgA, CigR, GogB, SseL and SteD (Ghosh, 2004; Lloyd *et al.*, 2001; Niemann *et al.*, 2013; Sorg *et al.*, 2005).

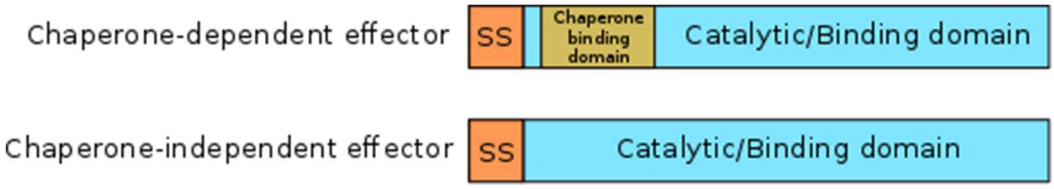


Figure I.15. Signal sequences for T3SS effectors translocation. Effectors have a secretion signal (SS) within their first mRNA codons, their first amino acids, or both. Translocation of many effectors depends on the action of T3SS chaperones, which generally bind to a chaperone binding region following the secretion signal. The activities of the effectors, either catalytic or binding to host cell targets, are encoded in domains that are normally found following the chaperone binding region. Some effectors, apparently, do not have related chaperones and they are translocated independently of the chaperone action [adapted from (Ghosh, 2004)].

9. SrfJ

SsrB-regulated factors (*srf)* genes were identified in a screen for genes outside SPI2 that were activated by SsrB (Worley *et al.*, 2000). The fact that they were part of the SsrB regulon suggested that they may be coding for T3SS2 effectors. In fact, one of these genes, strongly regulated by SsrB, *srfH* encodes the effector protein SrfH (also known as SseI) (Miao and Miller, 2000), which is a T3SS2 effector that is secreted by *Salmonella* in infected phagocytes and manipulates their migration (Thornbrough and Worley, 2012). More recently, our laboratory identified the region in SrfJ necessary for T3SS2-dependent secretion (Cordero-Alba *et al.*, 2012).

SrfJ shares 30% amino acid sequence identity with human lysosomal glucosylceramidase, a peripheral membrane protein that catalyzes hydrolysis of glucosylceramide, a membrane sphingolipid, into β-glucose and ceramide (Grabowski *et al.*, 1990; Worley *et al.*, 2000). Inherited mutations in the gene encoding this enzyme result in Gaucher's disease, a lysosomal storage disease, and in strong risk for Parkinson's disease (Pitcairn *et al.*, 2018). The SrfJ structure is very similar to the structure of glucosylceramide and is composed of two domains: a catalytic domain (residues 46-379) and a β-sandwich domain (residues 1-45 and 380-477) (Kim *et al.*, 2009). High structural similarities are observed in the active site, and the substrate binding model, suggesting that SrfJ may act as a glucosyl hydrolase to modify SCV membrane lipids to enhance bacterial virulence. Sphingolipids, including ceramides, are structural components of membranes and important secondary messenger molecules that regulate cellular processes such as cellular proliferation, differentiation, cell death, oxidative stress and inflammation (Ilan, 2016). Interestingly, ceramides play an important role in the process of infection of host cells by some bacterial pathogens including *S.* Typhimurium (Grassmé and Becker, 2013).

Previous studies carried out in our laboratory showed that transcription of *srfJ* was positively regulated by the PhoQ/PhoP two-component system in an SsrB-dependent manner, and by the Rcs system in an SsrB-independent manner. Interestingly, a screening for additional regulators identified IolR, the regulator of genes involved in *myo*-inositol utilization, as a repressor of *srfJ* transcription (Cordero-Alba *et al*., 2012). These results suggested that SrfJ was synthetized inside the host cells in response to intravacuolar signals but also in *myo*-inositol-rich environment. However, the molecular basis and the biological meaning of this dual regulation remained elusive.

10. T3SSs AS PROTEIN DELIVERY TOOLS FOR BIOMEDICAL APPLICATIONS

T3SSs have emerged as promising tools for delivery of proteins directly into target cells without protein purification (Bai *et al*., 2018). Interesting applications include the following:

(i) Delivery of reporter proteins such as adenylate cyclase (Sory and Cornelis, 1994), dihydrofolate reductase (Feldman *et al*., 2002) and glycogen synthase kinase tag (García *et al*., 2006), that were mainly used for the study of translocation and functions of effectors themselves. Although GFP is not a good substrate for the T3SS, a split-GFP method has been successfully used to track *Salmonella* effectors like PipB2, SteA, or SteC, after translocation into the host cell (Van Engelenburg and Palmer, 2010).

(ii) Signalling protein delivery was used to investigate apoptosis by injecting a truncated form of a proapoptotic member of the Bcl-2 protein family into HeLa cells using *Yersinia* T3SS (Ittig *et al*., 2015).

(iii) Delivery of transcription factors using *P. aeruginosa* T3SS can induce cell type conversion, cellular reprogramming (Berthoin *et al*., 2016; Bichsel *et al*., 2013) or the novo differentiation from pluripotent stem cells (Bai *et al*., 2015).

(iv) Delivery of nucleases such as Cre recombinase and TALENs as genome editing tools has been explored using *P. aeruginosa* T3SS (Bichsel *et al*., 2011; Jia *et al*., 2014, 2015).

(v) Finally, T3SSs have been exploited to deliver antigenic peptides and proteins to elicit immune response (vaccination) (Rüssmann *et al*., 1998) or cancer immunotherapy (Panthel *et al*., 2006). Heterologous antigens in fusion with effector proteins were efficiently injected into the cytosol of antigen-presenting cells (APC) (Hegazy *et al*., 2012; Panthel *et al*., 2008; Rüssmann, 2003; Xiong *et al*., 2010). The *Y. enterocolitica*

T3SS was used to deliver the model antigens listeriolysin O and p60 of *L. monocytogenes* using the N-terminal domain of YopE as carriers (Rüssmann *et al.*, 2000; Trülzsch *et al.*, 2005). The *P. aeruginosa* T3SS has also been used for delivery of heterologous antigens fused to the N-terminal 54 amino acids of the effector ExoS (Derouazi *et al.*, 2010; Wang *et al.*, 2012). *S.* Typhimurium is among the first bacteria used for this purpose. Antiviral, antibacterial and anti-tumor immunotherapy have been tested by delivering antigens such as the influenza virus nucleoprotein, Gag proteins of simian and human immunodeficiency viruses, listeriolysin O and p60 of *L. monocytogenes*, NY-ESO1, hepatitis B virus x, surviving, tyrosine-related protein 2, and vascular endothelial growth factor receptor 2. *Salmonella* T3SS effectors used as carriers included SptP, SopE, SspH2, and SseF [reviewed in (Bai *et al.*, 2018)]. The live-attenuated *Salmonella* vectors for antigen delivery have the advantages of low cost of production, absence of animal products, safety, and elicitation of efficient humoral and cellular immune responses via stimulation of innate and adaptive immunity (Cheminay and Hensel, 2008).

OBJECTIVES

Based on the previous work carried out in our laboratory, the main purpose of this thesis was the study of the *Salmonella* T3SS effector SrfJ. We wanted to understand the dual expression of this effector in response to unrelated signals, such as the intravacuolar environment and the sugar *myo*-inositol, and to analyze the effects of SrfJ on the host cell. Additionally, we were interested in exploring the possibility of using this and other T3SS effectors as carriers in the development of live vaccines against a relevant human bacterial pathogen such as *P. aeruginosa*.

The specific objectives were:

1. Study of the environmental and genetic factors that regulate the expression of SrfJ during the infectious process in different model organisms for *S. enterica*.

2. Analysis of the effects of *srfJ* expression on mammalian cell lines.

3. Evaluation of *Salmonella* T3SS effectors as carriers for heterologous antigen delivery in vaccine design.

MATERIALS AND METHODS

1. BIOLOGICAL MATERIAL

1.1. Bacterial strains

S. Typhimurium, *E.coli* strains and *P. aeruginosa* strains used in this thesis are listed in **Table M.1**. All the strains from *S. enterica* are derivatives of the virulent strain for mice ATCCC 14028, known also as ATCC14028 or 14028.

Table M.1. Bacterial strains and plasmids used in this study.

Strain	Relevant characteristics	Source/reference
E. coli strains		
DH5α	*supE44 ΔlacU169* (*Δ80 lacΔ*M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	(Hanahan, 1983)
BL21(DE3)	F^- *ompT gal dcm lon* $hsdS_B$ (r^- m^-; *E. coli* B strain), with DE3, a λ prophage carrying the T7 RNA *pol* gene	Stratagene
P. aeruginosa		
PA01	Reference strain	(Holloway, 1995)
S. enterica serovar Typhimurium strains		
14028	Wild type	ATCC
55130	*pho-24* (PhoP constitutive)	(Groisman *et al*., 1989)
SV4608	*trg::*Mu*d*J Km^R	(Segura *et al*., 2004)
SV4699	*phoP7953::*Tn*10*	Laboratory stock
SV4338	*aroA551::*Tn*10*	Laboratory stock
SV4758	*rcsC55*	(García-Calderón *et al*., 2005)
SV5049	*ΔrcsB::*Cm^R	Laboratory stock
SV5373	*ΔhilA*	Laboratory stock
SV5452	*ΔssrB::*Cm^R	(García-Calderón *et al*., 2005)
SV5559	*ΔsrfJ::lacZ* Km^R	Laboratory stock
SV5599	*srfJ::*3xFLAG Km^R	(Cordero-Alba *et al*., 2012)
SV6891	*ΔiolR::*Cm^R	(Cordero-Alba *et al*., 2012)
SV8462	*ΔaroB::*Km	Laboratory stock
SV9416	*ΔPiolE::*Cm^R	This study

1.1.1. Freezing and thawing of bacterial strains

Seventy µl of dimethyl sulfoxide were added to 1 ml of an overnight bacterial culture in logarithmic phase. The vial was stored at -80ºC. Vials were thawed slowly on ice.

1.2. Bacteriophages

P22 HT105/1 *int-201*(Schmieger, 1972) was employed as transducing bacteriophage. For P22 sensitivity assays, the clear-plaque H5 derivative, which harbors a mutation in the *c2* gene (Smith and Levine, 1964), was used. The P22 *c2* gene is an equivalent of *cI* gene in phage λ.

1.3. Mammalian cell lines

Different mammalian cell lines has been used in this study: (i) HeLa (human epithelial; ECAC no. 93021013), and derivatives of this cell line transiently transfected with plasmids pcDNA3 or pcDNA3-SrfJ-3xFLAG; (ii) RAW264.7 (murine macrophages; ECACC no.91062702).

1.3.1. Freezing and thawing of mammalian cell lines

Cells ($5x10^6$ to 10^7) were suspended in 1.5 ml of freezing medium[1] and placed in a 2 ml cryovial. The vial was incubated 2h at -20ºC, 24h at -80ºC and finally stored in liquid nitrogen.

For thawing, vials were immersed in water at room temperature for a few minutes (until there was just a small bit of ice left in the vial). Thawed cells were diluted in 10 ml of pre-warmed growth medium and centrifuged 10 min at 1000 rpm at room temperature. Finally, cells in the pellet were resuspended in fresh medium and transferred to a culture plate.

[1]**Freezing medium for mammalian cell lines:** 10% DMSO, 90% fetal bovine serum (FBS).

1.4. Mice

For immunization assays, 6 to 8-week old female C57BL/6 mice were used (*Charles River Laboratories*).

1.5. Plants

Two different plants have been used in this thesis: Tomato (*Solanum lycopersicum 'Moneymaker'*) and Lettuce (*Lactuca sativa* L. cv. *Tizian*).

2. CULTURE MEDIA, SOLUTIONS AND GROWTH

2.1. Bacteria

2.1.1. Growth conditions for bacteria

Bacteria were routinely grown at 37ºC, and exceptionally at 30ºC. Cultures were shaken at 200 rpm. For microaerophilic conditions, 5 ml of bacteria were incubated at 37ºC without shaking in 10 ml plastic tubes.

2.1.2. Solutions and media for bacteria

Bertani's Lysogenic Broth (LB)[1] was used as standard liquid medium (Maloy, 1990). LPM (low phosphate, low magnesium-containing medium)[2], was used to mimic the SCV environment (Coombes *et al.*, 2004). LB with 0.3 M of NaCl in microaerophilic conditions was used to obtain the conditions of maximum invasiveness of *S. enterica*. TM[3] (Tomato Medium) or LM[4] (Lettuce Medium) were used to simulate the plant environment. Agar was added at a concentration of 18 g/l for solid media.

EBU[5] agar was used to identify phage-free isolates after transduction.

XLD-agar[6] was used to determine the colony-forming unit (CFU) of *Salmonella* in inoculated leaves.

When necessary, antibiotics and other chemicals were added to the medium at the final concentrations shown in **Table M.2**.

[1]**LB**: 10 g tryptone, 5 g yeast extract and 5 g NaCl for 1 L of distilled water.
[2]**LPM**: 5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM KSO_4, 0.1% casaminoacids, 38 mM glycerol, 80 mM MES, 337.5 µM K_2HPO_4/KH_2PO_4, 8 µM $MgCl_2$. Adjusted to pH 5,8 with KOH.
[3]**TM:** 25% tomato plant extract (previously sterilized with 0.22 µm filter), 20% M9-Minimal Salts (Sigma) (Fornefeld *et al.*, 2017).
[4]**LM:** 25% tomato plant extract (previously sterilized with 0.22 µm filter), 20% M9-Minimal Salts (Sigma).
[5]**EBU**: 0.5 ml of 50% glucose, 1 ml of 25% K_2HPO_4, 125 µl of 1% Evans Blue and 250 µl of 1% uranine (called fluorescein) in 100 ml of LB (Maloy *et al.*, 1996).
[6]**XLD-agar:** 55.25 g XLD-agar (Xylose Lysine Deoxycholate Agar) (Sigma) for 1 l of distilled water.

Table M.2. Final concentration of antibiotics and other chemicals used in the different bacterial culture media.

Chemicals	Mechanism of action	Preparation	Final concentration (µg/ml)
Antibiotics			
Ampicillin (Ap)	Inhibition of cell wall synthesis	100 mg/ml in bi-distilled H_2O (bd-H_2O). Sterilized by filtration. Storage at 4ºC.	100
Chloramphenicol (Cm)	Inhibition of protein synthesis	100 mg/ml in ethanol. Storage at -20ºC.	20
Kanamycin (Km)	Inhibition of protein synthesis	50 mg/ml in bd-H_2O. Sterilized by filtration. Storage at 4ºC.	50
Tetracycline (Tc)	Decoupling of oxidative phosphorylation, inhibition of protein synthesis and disruption of plasma membrane	12.5 mg/ml in ethanol 50%. Sterilized by filtration. Storage at 4ºC protected from light.	20
Other chemicals			
EGTA	Calcium chelator	1 M in bd-H_2O. Storage at room temperature.	0.004
X-Gal	Hydrolyzed by β-galactosidase to galactose and 5-bromo-4-choro-3-hydroxy indole, which when oxidized generates an insoluble blue compound.	20 mg/ml in N, N-Dimethylformamide. Storage at -20ºC protected from light.	40

2.2. Mammalian cell lines

2.2.1. Growth conditions of mammalian cell lines

Mammalian cell lines were cultured in a *Biotech Galaxy* incubator, at 37ºC in a humid atmosphere of 5% CO_2. Cell culture vessels used were 10-cm-diameter plates (with 10 ml of culture media), 15-cm-diameter plates (with 20 ml of culture media) or 6-well (3 ml per well), 24-well (1 ml per well) or 96-well (100 µl per well) dishes. Cells were subcultured twice per week (HeLa cells), or three times per week (RAW macrophages). The adherent HeLa cells were detached with trypsin, after removing all medium and washing twice with phosphate-buffered saline (PBS[1]). Cells were incubated at 37ºC and 5% CO_2 for 10 min before collecting them in fresh medium. Semi-adherent RAW264.7 cells where detached with a cell scraper (1.3 cm, SLP Life Sciences).

[1]**PBS 10x:** 1.37 M NaCl, 27 mM KCL, 43 mM $Na_2HPO_4 \cdot 7H_2O$ 14 mM, KH_2PO_4. Adjust pH to 7.3.

2.2.2. Solutions and media for mammalian cell lines

Cells were cultured in DMEM (*Dulbeco's modified eagle's medium*, Biowest) supplemented with 10% FBS previously heat inactivated[1], 2 mM L-glutamine, 60 µg/ml penicillin and 100 µg/ml streptomycin. During infection assays, antibiotics were not added to the medium.

[1]**FBS inactivation:** serum was incubated at 56ºC for 30 min, thus complement proteins were inactivated to prevent cell lysis.

2.3. Plants

2.3.1. Growth conditions for plants

Non-sterile *S. lycopersicum* and *L. sativa* seeds were cultivated in soil under controlled conditions: 8 h light/ 16h dark at 20ºC, 40-60% humidity, ~120 µE/m^2s light intensity.
S. lycopersicum sterile seeds were germinated in Petri dishes for 1 week. Three seedlings were transferred to a sterile glass pot and incubated for 2 further weeks in a cabinet with a light intensity of 150 µE/m^2s (16 h photoperiod) at 22ºC.

2.3.2. Seeds sterilization

Tomato (*S. lycopersicum*) seeds were surface sterilized in 2% natrium hypochlorite solution (10 ml) for 10 min. The seeds were then washed vigorously six times with sterile distilled water.

2.3.3. Solutions and media for plants

For *S. lycopersicum* germination in Petri dishes 0.5xMurashige and Skoog (MS)[1] medium was used. Seedlings were grown in sterile conditions in 0.25xMurashige and Skoog (MS)[2] medium.

[1]**0.5 x MS**: 2.2 g Murashige and Skoog (MS) (Sigma), 10 g D+ Saccharose, 0.5 g MES-KOH, 6 g Agar-agar (*KOBE-I*) for 1 l of distilled water. Adjust to pH 6.4 with KOH.

[2]**0.25 x MS:** 1.1 g Murashige and Skoog (MS) (Sigma), 5 g D+ Saccharose, 6 g Agar-agar (*KOBE-I*) for 1 l of distilled water. Adjust to pH 6.4 with KOH.

2.4. Mice

Mice were maintained in the Instituto de Biomedicina de Sevilla (IBiS) facility and their care was in accordance with institutional guidelines.

3. DNA MANIPULATION AND GENE TRANSFER

3.1. Plasmids

Plasmids used in this thesis are listed in **Table M.3**.

Table M.3. Plasmids used in this study.

Plasmid	Description	Reference
pcDNA3	Vector for transient or stable transfection, Ap^r	Invitrogen
pET15b	6His fusion vector, Ap^r	Novagen
pET15b-OprF/I	OprF/I cloned with NdeI and BamHI	This study
pGEM-T Easy	Vector for cloning PCR products	Promega
pGEX-4T-2	GST fusion vector, Ap^r	GE Healthcare
pIZ1855	pcDNA3-SrfJ-3xFLAG	Laboratory stock
pIZ2132	pWSK29-P*steA*-SlrP-OprF/I-FLAG	This study
pIZ2160	pWSK29-P*sseA*-SseJ-OprF/I-FLAG	This study
pIZ2162	pWSK29-P*sseA*-SteA-OprF/I-FLAG	This study
pIZ2182	pSB377-P*iolG1*	This study
pIZ2183	pSB377-P*iolI1*	This study
pIZ2184	pSB377-P*srfJ*	This study
pIZ2185	pSB377-P*iolE*	This study
pIZ2186	pWSK49-P*sseKI*-SlrP-OprF/I-FLAG	This study
pIZ2196	pWSK29-P*srfJ*-SlrP-OprF/I-FLAG	This study
pIZ2306	pSB377-P*iolE*-P*srfJ*	This study
pIZ2267	pWSK29-P*sseA*-SseJ-PcrV-FLAG	This study
pIZ2338	pGEX-4T-2-PcrV	This study
pKD3	*bla* FRT *cat* FRT PS1 PS2 oriR6K	(Datsenko and Wanner, 2000)
pKD46	*bla* P_{BAD} *gam bet exo* pSC101 oriTS	(Datsenko and Wanner, 2000)
pSB377	Parent for *luxCDABE* transcriptional fusions, Ap^R	(Winson *et al.*, 1998)
pSUB11	3xFLAG fusion vector, Km^r	(Uzzau *et al.*, 2001)

Plasmid	Description	Reference
pWSK29	Low-copy-number vector, Ap^r	(Wang and Kushner, 1991)

pIZ is a plasmid prefix registered by J. Casadesús in the Plasmid Reference Center, Stanford University, California (Lederberg, 1986).

3.2. Plasmid extraction from *E.coli*

For DNA plasmid extraction, two different commercial kits where used: *GeneElute™ Plasmid Miniprep Kit*, provided by Sigma Aldrich Co; and *DNA-spin™ Plasmid DNA Purification Kit*, provided by iNtRON Biotechnology.

3.3. Digestion, modification and ligation of DNA fragments

Restriction endonucleases were supplied by Roche Diagnostics GmbH, Takara, New England Biolabs and Promega Biotec. In each case, enzymes were used following the manufacturer's instructions.

To dephosphorylate plasmids the CIP phosphatase was used (*Calf Intestine Phosphatase,* Roche). Between 1 and 20 µg of cut DNA and 1 U of CIP were mixed in the phosphatase alkaline buffer and were incubated at 37ºC for 60 min.

For ligation of DNA fragments, 1 U of T4 DNA ligase (1 U/µl, Roche Diagnostics) was used in the buffer supplied by the manufacturer. Routinely, the mixture was incubated at 16º C for 12 hours at least.

3.4. Agarose DNA gel electrophoresis

Electrophoresis in an agarose gel was used to test the quality of DNA extraction, to visualize DNA fragments after plasmid restriction, to estimate the efficiency of endonuclease restriction, to confirm PCR amplification, etc. The agarose gel was submerged in TAE[1] buffer.

Low Electro Endosmosis Agarose (Pronadisa) was employed. Its concentration varied between 0.7 and 1.5% depending on the size of the fragment to be separated. The loading buffer used was a solution of bromophenol blue (0.125%) and Ficoll 400 (12.5%).

The *1 kb DNA ladder RTU* (Nippon Genetics Europe GmbH) was used as a molecular weight marker. Samples were mixed with 1/10 of loading buffer (Takara). Ethidium

bromide (0.5 µg/ml final concentration) was added to the gels to make bands visible. The system *Molecular Imager Gel Doc™ XR+* with *Image Lab™ Software* (BioRad) was used for imaging, documentation, and analysis of gels.

[1]**TAE:** 40 mM Tris-acetate, 10 mM EDTA, pH 8.2-8.4.

3.5. Isolation of DNA fragments from agarose gels

The commercial system *MEGAquick-spin™ Total Fragment DNA Purification Kit* provided by iNtRON Biotechnology was used for the isolation of DNA fragments from agarose gels,

3.6. Bacterial transformation

3.6.1. Chemical E. coli transformation

Competent cells were prepared using the rubidium chloride protocol. An overnight culture of *E. coli* was diluted 1:100 in 100 ml of LB in a 250 ml flask, and incubated at 37ºC and 200 rpm until an OD_{600} of 0.5 was reached. The culture was chilled quickly on ice for 5 min. Cells were harvested by centrifugation at 4000 g and 4ºC for 5 min. The pellet was resuspended gently in cold TFB1[1] (30 ml for a 100 ml culture) and the suspension was kept on ice for 90 min. Cells were collected by centrifugation (5 min, 4000 g, 4ºC), the supernatant was carefully discarded and the cells were resuspended in 4 ml ice-cold TFB2[2]. Aliquots of 0.2 ml were prepared in sterile microcentrifuge tubes, frozen in liquid nitrogen, and stored at -80ºC.

For transformation, an aliquot of competent cells was slowly thawed on ice and mixed with the plasmid. The mixture was incubated on ice for 20 min, transferred to a 42ºC heating block for 90 s, and 500 µl of Psi broth[3] was added. The cells were incubated at 37ºC for 1h, and plated on selective medium.

[1]**TFB1:** 100 mM RbCl, 50 mM MnCl2, 30 mM potassium acetate, 10 mM CaCl2, 15% glycerol, pH 5.8, sterile filter.
[2]**TFB2:** 10 mM MOPS, 10 mM RbCl, 75 mM CaCl2, 15% glycerol, adjust to pH 6.8 with KOH, sterile filter.
[3]**Psi broth:** LB medium, 4 mM MgSO4, 10 mM KCl

3.7. Bacterial electroporation

An overnight culture, was diluted 1:100 in LB and, depending on the strains, was grown at 37ºC or 30ºC until OD_{600} of 0.6-0.8 was reached. The culture was chilled on ice for 5 min. Twenty-five ml were transferred to a tube, and the cells were harvested by centrifugation at 4000 g for 5 min at 4ºC. The supernatant was discarded, and the bacterial pellet was softly suspended in 1 ml of cold bd H_2O. Afterwards, 24 ml of cold water were added and cells were collected by centrifugation. This wash was repeated a second time. Finally, cells were harvested and resuspended in 250 µl of water.

Electroporation was performed by mixing 1µl of plasmid DNA or 5 µl of PCR product with 40 µl of competent cells. The mixture was transferred to a cooled cuvette with a gap between the electrodes of 2 mm. The cuvette was exposed to an electric discharge in the electroporator (2.5 kV, 200 Ω and 25 µF). The electroporator employed was a *BTX Electrocell Manipullator 600* (Harvard Apparatus). After the discharge, 1 ml of LB was added to the cells, which were then transferred to a 10 ml plastic tube and incubated at 37ºC with shaking (200 rpm) for 1h. Finally, cells were concentrated in 100 µl and spread on selective medium.

3.8. Bacterial transduction

3.8.1. P22 lysates

To prepare P22 lysates, 1 ml of P22 broth[1] was mixed with 0.2 ml of the donor strain. The mixture was incubated at 37ºC and 200 rpm for 8-16 h. Bacterial debris was removed by centrifugation for 2 min at 13000 rpm. The supernatant was transferred to a fresh tube, 100 µl of chloroform were added, and the mix was vortexed. The lysates were maintained at room temperature for a few hours and then stored at 4ºC; under these conditions the lysates are stable for months or years (Maloy, 1990).

[1]**P22 broth:** 100 ml of NB[2] (Nutrient Broth), 2 ml of 50x E salts[3], 1 ml of glucose 20%, and 0.1 ml of a phage lysate obtained from wt *S. enterica*.

[2]**NB:** 3 g beef extract, 5 g peptone for 1 l.

[3]**50x E salts**: Add and dissolve components in 1 L of heated distilled H_2O (d H_2O) in the following order: 300 g citric acid monohydrate ($H_3C_6H_5O_7 \cdot H_2O$), 14.1 g of $MgSO_4$, 1965 g of $K_2HPO_4 \cdot 3\ H_2O$ and 525 g of $NaNH_4HPO_4 \cdot 4H_2O$. Bring volume to 3 l with d H_2O, aliquot and sterilize with chloroform.

3.8.2. P22 transduction in liquid medium

To carry out transductions in liquid medium, 100 µl of an overnight culture of the recipient strain and 10 µl of the lysate of the donor strain were mixed in a sterile 1.5 ml tube. This mix was incubated at 37ºC for 30-45 min (depending on the marker of transduction). The mix was then spread on selective medium plates that were incubated at 37ºC until colonies appeared.
This method does not yield independent transductants, but incubations shorter than 30 min do not permit transductants to divide, so the proportion of siblings is minimal.

3.8.3. Detection of lysogenic transductants

Transductants harbouring a selective marker could have been infected by P22 phage and become pseudolysogenic (the *int* mutation prevents integration, and delays the formation of true lysogens). Pseudolysogens become resistant or immune to new P22 infections and cannot be lysed or transduced again. To avoid pseudolysogeny, transductant colonies were isolated in EBU plates (with antibiotics if necessary). When streaked in these plates, that contain a pH indicator, nonlysogens and true lysogens form light-colored colonies. However, in a colony containing pseudolysogens many cells are undergoing lysis which lowers the pH of the medium resulting in dark blue colonies. It is possible to obtain phage-free segregants by streaking for isolated colonies in EBU plates. A transductant was considered P22-free when streaking did not give rise to any dark colony.

3.8.4. P22 sensitivity assay

In EBU plates, isolates that forms light color colonies could be stable lysogens that do not undergo visible lysis. These isolates are P22-resistant and are not very useful for genetic studies. To avoid this situation, an assay to detect P22-sensitive strain is advisable. A streak with a P22 H5 lysate is done on a LB or EBU plate, and air-dried. The test strain is then streaked in a perpendicular way to the H5 streak. P22-sensitive strains grow until they reach the H5 streak, while P22-resistant strains grow over the streak.

3.8.5. Construction of bacterial strains

Genetic markers were transferred from one strain to another by P22 transduction. All of them were selectable markers, so the transduced bacteria were directly cultured in selective medium. Usually, a new mutation was checked by PCR in the new transductant bacteria.

3.9. HeLa cells transfection

3.9.1. Transient transfections

Subconfluent cell cultures were obtained in 10 or 15 cm diameter plates to get several millions of HeLa cells. Cells were detached with trypsin and counted in a hemocytometer. They were centrifuged at 1000 rpm for 10 min and suspended in electroporation media (DMEM supplemented with 15 mM HEPES, pH 7.4) to a final concentration of 10^7 cells /200 µl.

Five µg of DNA (pcDNA3, pcDNA3-SrfJ-3xFLAG) were mixed with NaCl (final concentration 200 mM) and bd H_2O to a volume of 50 µl. The DNA mixture was mixed with 200 µl of HeLa cells. The mixture was transferred to a cooled cuvette with a gap between the electrodes of 4 mm. The cuvette was subjected to an electric discharge in the electroporator (240 V, ∞ Ω and 950 µF). The electroporator employed was a *BTX Electrocell Manipullator 600* (Harvard Apparatus). After the discharge, 1 ml of electroporation media was added to the cells, which were then transferred to a 10 ml plastic tube with 9 ml of electroporation media. The sample was centrifuged at 1000 rpm for 10 min, and once the supernatant was discarded, the pellet was suspended in 10 ml of fresh DMEM media and cultured in a 10 cm diameter plate for 24 h before analysis.

3.10. Oligonucleotides

Oligonucleotides used in amplification and sequencing were provided by StabVida and are listed in **Table M.4.**

Table M.4. Oligonucleotides used in this study.

Oligonucleotide	Sequence 5'-3'
Amplification of *pcrV* with FLAG tag for cloning in pWSK29	
pcrVfwBam	ATCGGGATCCGAAGTCAGAAACCTTAATGC
pcrVFLAGrevNot	GCATGCGGCCGCCTATTTATCGTCGTCATCTTTGTAGTCGATC GCGCTGAGAATGTCGC
Amplification and fusion of *oprF* and *oprI* with FLAG tag	
oprFfwBam	ATCGGGATCCGCTCCGGCTCCGGAACCGGTTGCCGAC
oprFrev	TTCAACGCGACGGTTGATAGCGCG
oprF/oprIfw	GAAGGCCGCGCTATCAACCGTCGCGTTGAAAGCAGCCACTCC AAAGAAAC CGAAGCT
oprIFLAGrevNot	GCATGCGGCCGCCTATTTATCGTCGTCATCTTTGTAGTCCTTGC GGCTGGCTTTTTCCAG
Amplification of *sseJ*	
sseJfwEco	CCTAGAATTCGTAAGGAGGACACTATGCC
sseJrevBam	ACGTGGATCCTTCAGTGGAATAATGATGAG
Amplification of *slrP*	
slrPfwEco	CCTAGAATTCGATCAGGTAGGGAAAATATG
slrPrevBam	ACGTGGATCCCTGCTCGCTTTCTGTCACAC
Amplification of *steA*	
steAfwEco2	GAATGAATTCAGGAGGTAGGATATGCCATATACATCAGTTTC
steArevBam	CAAGGGATCCATAATTGTCCAAATAGTTATG
Amplification of *sseKI*	
sseKIfwEco	GACTGAATTCAGGAGGTTTGTTATGATCCCACCATTAAATAG
sseKIrevBam	GATCGGATCCCTGCACATGCCTCGCCCATG
Amplification of *srfJ*	
srfJfwEco	GAATGAATTCAGGAGGTTCCCTATGAAAGGCAGACTC
srfJrevBam	CAAGGGATCCTAACGCGTGGCGCGGTAAGAC
Amplification of *sseA* promoter	
PsseAfwKpn	GCTAGGTACCAGAAGAGAACAACGGCAAG
PsseArevEco	CACTGAATTCACGATAGATAATTAACGTGC
Amplification of *slrP* promoter	
PslrPfwKpn	CATGGGTACCCGATCGCCAGCGAGTCATCG
PslrPrevEco	GATCGAATTCATTTTCCCTACCTGATCTG
Amplification of *steA* promoter	
PsteAfwKpn	GATCGGTACCAAGCAGCATAAGATCAGGCC
PsteArevEco	GATCGAATTCCTCTCATTATGACGATATG
Amplification of *sseKI* promoter	
PsseKIfwKpn	AGTCGGTACCTTGGGACAATTACATTATG
PsseKIrevEco	AGTCGAATTCACATGATGATTATTAGCAC
Amplification of *srfJ* promoter	
PsrfJfwKpn	ATGCGGTACCTCACTGCGATGTTACCGGCG
PsrfJrevEco	TGCAGAATTCAGGGAAGTTCCGGATAAAAGAAG
Amplification of *pcrV* for cloning in pGEX-4T-2	
pcrVfwBam	As above
pcrVrevEco	ATCGGAATTCCTAGATCGCGCTGAGAATGT
Construction of pIZ2182	
PiolG1ecofw	GTTCGAATTCCATGCCGCTACTGAGTAAAC
PiolG1ecorev	ATGCGAATTCTTAAAGTCATTTTCTGTTTCC
Construction of pIZ2183	
PiolI1ecofw	CTGAGAATTCTGACATGATTGGTAATTTCAAATC
PiolI1ecorev	ATGCGAATTCTCAGATCGACTCCTGCCGCC

Oligonucleotide	Sequence 5'-3'
Construction of pIZ2184	
PsrfJecofw	ATGCGAATTCTCACTGCGATGTTACCGGCG
PsrfJecorev	TGCAGAATTCAGGGAAGTTCCGGATAAAAGAAG
Construction of pIZ2185	
PiolEecofw	GTCAGAATTCTCAATATCGCAAGGACTATC
PiolEecorev	CTGAGAATTCTGGCTCCCACTTAATGAAAC
Construction of pIZ2306	
PiolEecofw	GTCAGAATTCTCAATATCGCAAGGACTATC
PsrfJecorev	TGCAGAATTCAGGGAAGTTCCGGATAAAAGAAG
Construction of pIZ2338	
PcrVfw	ATCGGGATCCGAAGTCAGAAACCTTAATGC
PcrVEcoRIrev	ATCGGAATTCCTAGATCGCGCTGAAGAATGT
Deletion of P*iolE*	
PiolEH1P1fw	TTCAGAATTACTTCAAAAATAAAGTAGGGAAAACGCCCGGGT GTAGGCTGGAGCTGCTTC
PiolEH2P2rev	ATCCCCAACTTAATGCTCTTTTTTACATTGTACATATTGCCATA TGAATATCCTCCTTAG
RT-PCR	
Efw	GGGCATCAATATTCTGGCTG
G1fw	ACCCTCAAAACCTGATTTCTAC
G1rev	TTAAGTGATCGGAGCCGATC
Jrev2	GACGATGCGAAAAAGAGACC
Jfw	CAGACTCATCTCTTCCGATC
Jrev	CATGCTGTTGAATACCACGC
Irev	AAACGTTCCGCCAACACAAC
Jfw2	GATGTCCAGGAAAGGCGTTG
5'RACE	
5RaceNested	GACACTGACATGGACTGAAGGA
Erev	GAAACAACGGCGACATATGC
Erev2	ATCATTGCGCCAACCGATAG
Jrev	CATGCTGTTGAATACCACGC
Jrev2	GACGATGCGAAAAAGAGACC
Gene Racer RNA Oligo	UGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA

3.11. Polymerase chain reaction (PCR)

For PCR reactions, a *T100™ Thermal Cycler* (BioRad) was used. PCR reactions were carried out with 1 ng of DNA, 300 µM of dNTPs (final concentration each), 0.3 µM oligonucleotides, 1.5 mM of $MgCl_2$, and 1U of polymerase per reaction in a final volume of 100 µl. The thermal program included the following steps: (i) initial denaturation 3 min at 95ºC; (ii) 25 cycles of denaturation (98ºC, 20 s), annealing (55ºC, 15s), and extension (72ºC, 30 s/kb); and (iii) final incubation 7 min at 72ºC to complete the extension. The polymerase used in these reactions was *KAPA HiFi DNA polymerase* supplied by Kapa Biosystems.

To confirm clones, colony PCR was performed *using MyTaq™ Red DNA polymerase*, supplied by Bioline. In these cases, a mixture with 5 µl of 5x reaction buffer (containing 5 mM dNTPs and 15 mM $MgCl_2$), 0.2 µM of oligonucleotides, and 1U of polymerase per reaction in a final volume of 25 µl was prepared. A colony was suspended in this mixture and was used as DNA template. The thermal program included the following steps: (i) initial denaturation 3 min at 95ºC; (ii) 30 cycles of denaturation (95ºC, 15 s), annealing (55ºC, 15s), and extension (72ºC, 30 s/kb); and (iii) final incubation of 7 min at 72ºC to complete the extension.

Before using any PCR product obtained for further molecular techniques, the enzyme, oligonucleotides and dNTPs were removed using the commercial *MEGAquick-spin dual agarose gels and purification kit*, which was supplied by iNtRON Biotechnology.

3.12. Chromosomal gene disruption using PCR products

To obtain knockout mutants of chromosomal genes, the Datsenko and Wanner method was used (Datsenko and Wanner, 2000). This method is based in the λ Red recombination system. One of the reasons why *E. coli* and *Salmonella* are not transformable with linear DNA is due to the presence of intracellular exonucleases that degrade it. The λ Red systems harbors *α*, *β* and *exo* genes that encodes the proteins Gam, Bet and Exo, respectively. Gam inhibits host exonuclease V, allowing the Bet and Exo proteins to carry out recombination of the DNA. The strategy consists in replacing the chromosomal sequence (for example gene B in **Figure M.1**) by an antibiotic resistance marker that is generated by PCR using oligonucleotides that harbor 40 nucleotides of homology with the sequence to be replaced (H1 and H2 in **Figure M.1**). λ Red recombination gene expression is carried out under an inducible promoter in a thermosensitive low copy number plasmid (pKD46). After selection, gene resistance marker can be removed using a different plasmid (pCP20) that harbors the FLP recombinase of the 2 µ plasmid from *Saccharomyces cerevisiae*. FLP system acts over FRT repetitions ("*FLP recognition target*") that flank the sequence (**Figure M.1**). Plasmids that harbor Red and FLP systems are thermosensitive and can be cured easily by growing the cells at 37ºC.

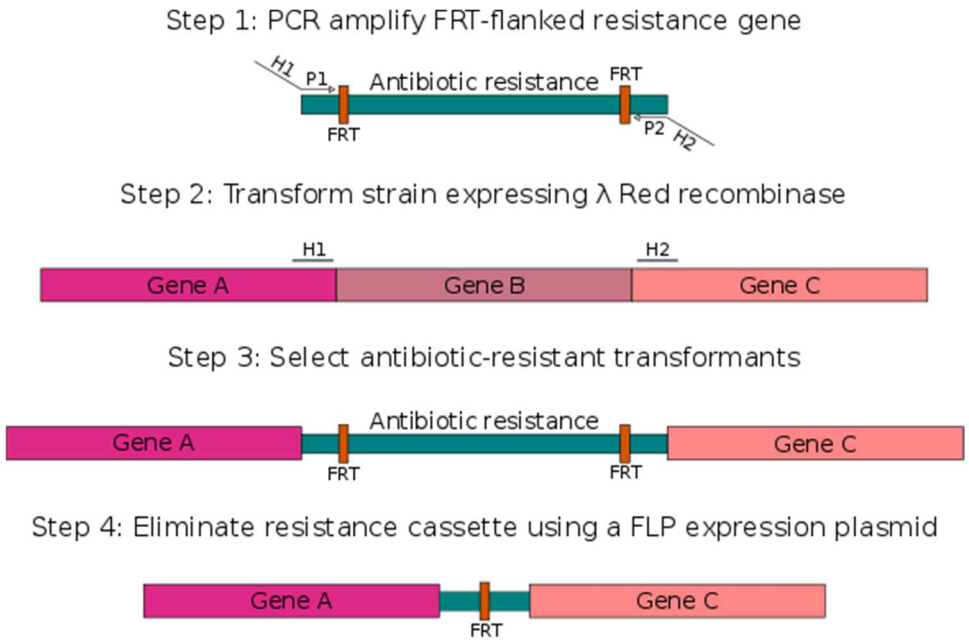


Figure M.1. A simple gene disruption strategy. H1 and H2 refer to the homology extensions or regions. P1 and P2 refer to priming sites. [Adapted from (Datsenko and Wanner, 2000)].

3.12.1. Preparation of DNA for substitution

The plasmids used as templates in PCR reactions were pKD3 (Cm^R), pKD4 (Km^R) and pKD13 (Km^R). The oligonucleotides used had 40 nucleotides that were homologous to the genomic DNA and 20 nucleotides that were homologous to pKD3, pKD4 and pKD13 (**Table M.4**). The PCR product obtained was gel-purified using a commercial kit.

3.12.2. Cell transformation

Competent cells of the wt strain, which harboured the pKD46 plasmid, were prepared. This plasmid expresses the λ Red system from the *araB* promoter, which is inducible by arabinose. Cultures grown in LB with ampicillin and arabinose (1 mM) were incubated in a shaker at 30ºC to an OD_{600} of 0.5. The competent cells were prepared and electroporation was done as described previously. Bacteria were cultured in selective medium, and the mutation was confirmed by PCR using external oligonucleotides.

3.12.3. Excision of the antibiotic resistance gene

After the substitution of the genomic genes with antibiotic resistance cassettes (Km or Cm), mutations were transferred to different genomic backgrounds by transduction with P22 and selection in the appropriate media. When it was necessary, the resistance marker

of the host was excised by transducing de plasmid pCP20 with the P22 HT phage (**Figure M.1**). This transduction was incubated at 30ºC for 1 h and was spread with LB with ampicillin. To eliminate the plasmid, EBU plates were prepared without antibiotic and incubated at 37ºC. To confirm the excision of the marker, the strains were streaked in plates of LB with Ap and plates of LB with Cm or Km. The excision of the antibiotic marker was also observed by colony PCR with external oligonucleotides.

3.13. Construction of 3xFLAG fusions

The method described by Uzzau *et al*. (Uzzau *et al*., 2001) was used for tagging proteins with the 3xFLAG epitope. This method is an adaptation of the Datsenko and Wanner recombineering procedure (Datsenko and Wanner, 2000). The objective is to manipulate the chromosomal sequence adding a DNA fragment that contains the sequence of the 3xFLAG epitope and a selectable marker (Km^R). The construction is made by PCR and transformation (**Figure M.2**). As a rule, one of the oligonucleotides used for amplification has a sequence of roughly 40 nucleotides (P1 in **Figure M.2**) that corresponds to the downstream nucleotides of the gene to be tagged without its stop codon. The other oligonucleotide (P2 in **Figure M.2**) contains a sequence of 40 nucleotides homologous to the sequence next to the stop codon (but in the complementary DNA strand). The plasmid used as template is pSUB11 (**Table M.3**) which harbors three copies of the FLAG epitope next to a Km resistance gene. The PCR product is used to transform a strain that contains the pKD46 plasmid, which expresses the λ Red system. Correct insertion of the epitope can be tested by PCR using external oligonucleotides, and by Western blot using anti-FLAG monoclonal antibodies. If necessary, the Km marker can be excised by FLP recombination.

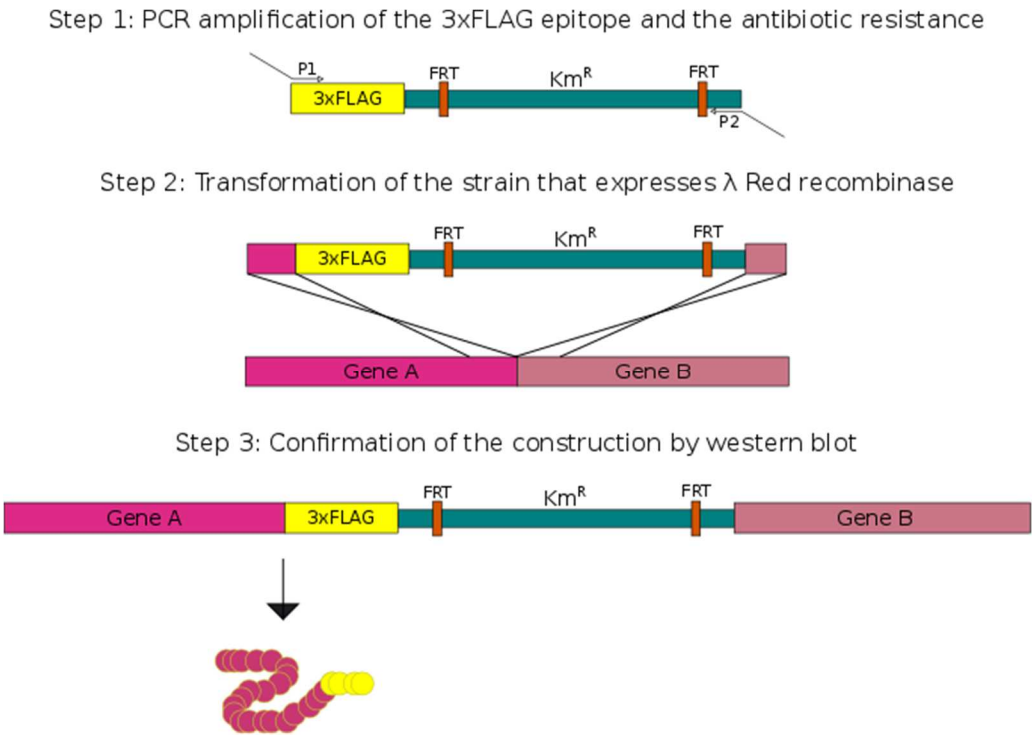


Figure M.2. Construction of fusion proteins with the 3xFLAG epitope. A DNA fragment, which starts with the epitope coding sequence and includes a resistance gene flanked by FRT sites, is amplified with oligonucleotides that have a sequence (36-40 bp) homologous to the region immediately preceding the stop codon of the target gene and to a region downstream of this. The amplified fragment is introduced into a strain expressing the *red* operon of the bacteriophage λ and antibiotic-resistant recombinants are selected. The recombinant bacterium synthesizes the target protein with the epitope fused to its C-terminal end. [Adapted from (Uzzau *et al.*, 2001)].

3.14. Splicing by overlap extension

The hybrid gene *oprF-oprI* was generated using the gene splicing by overlap extension approach (Horton *et al.*, 1989) as previously described (Massarrat *et al.*, 1995) with some modifications. The mechanism of splicing by overlap extension is illustrated in **Figure M.3**. The segments to be joined were amplified in separate PCRs. An *oprF* fragment was amplified using oligonucleotides oprFfwBam and oprFrev and a colony of *P. aeruginosa* PA01. An *oprI* fragment was amplified using oligonucleotides oprF/Ifw and oprIFLAGrevNot and a colony of *P. aeruginosa*. Primers oprFrev and oprF/Ifw are made complementary to one another by including nucleotides at their 5' ends that are complementary to the 3' portion of the other primer.

The products of both amplifications were used as templates in a third PCR with primers oprFfwBam and oprFIFLAGrevNot. These products overlap because they share homologous sequences at the ends to be joined. One strand from each fragment contains

the overlap sequence at the 3' end, and these strands can serve as primers from one another. Extension of this overlap by DNA polymerase yields the recombinant product.

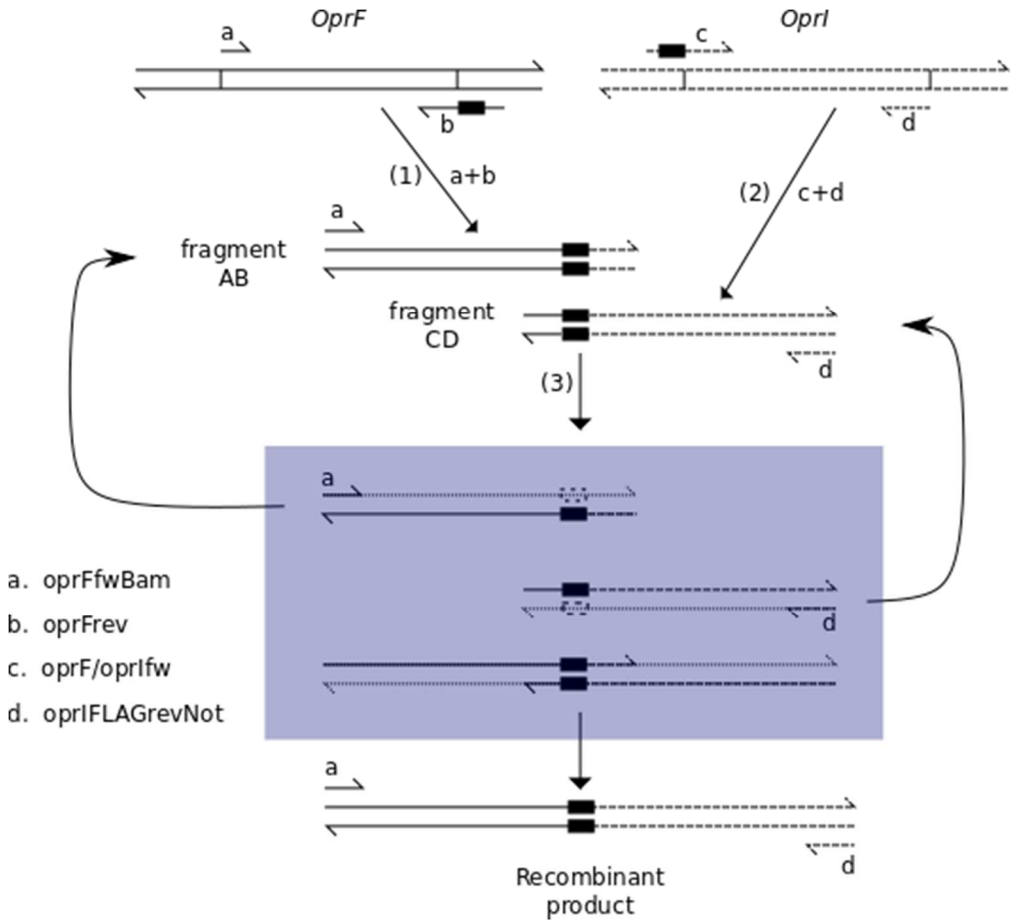


Figure M.3. Mechanism of the gene splicing by overlap extension. Gene *oprF* is shown as solid lines and gene *oprI* as dashes. The 5'-3' direction of each strand is shown by half arrowheads. Oligos are labelled with single lower case letters and PCR products are labelled using the two corresponding upper case letters. Product AB and product CD are generated in separated reactions (reactions **1** and **2**). The two PCR products are shown with the homologous segments aligned. In reaction **3**, the segments are mixed along with excess primers "a" and "d", denaturated, reannealed, and primer-extended by DNA polymerase. The intermediates in this reaction are shown in the purple box. The end of one strand of each product is capable of hybridizing with the complementary end from the other product. The strands having this overlap at their 3' ends can act as primers for one another and be extended by the polymerase to form the full-length recombinant product. DNA segments synthesized by the polymerase are shown as dotted lines. The recombinant product is PCR-amplified in the presence of "a" and "d". [Adapted from (Horton *et al.*, 1989)].

3.15. DNA sequencing

Plasmid DNA and chromosomal DNA obtained by PCR were sequenced in the sequencing service of Stab Vida.

3.1. DNA sequence analysis

Bioinformatics analysis of DNA sequences was performed using the algorithms of molecular biology of the *National Center for Biotechnology Information* (NCBI) at www.ncbi.nlm.nih.gov.

4. RNA MANIPULATION

4.1. RNA extraction with *TRIzol*®

This method is a modification of the RNA extraction protocol of *TRIzol® Reagent* supplied by Invitrogen Life Technology.

4.1.1. Eukaryotic RNA extraction

4.1.1.1. Mammalian cells RNA extraction

Total RNA of transfected HeLa cells, or HeLa or RAW cells infected with *Salmonella*, was obtained from three different biological replicates. Growth medium was removed from subconfluent cell cultures in 6 well plates, and 1 ml of TRIzol was added directly to each well to avoid any degradation. The content of each well was mixed thoroughly to achieve complete cell lysis. The homogenized sample was collected and stored at -80ºC (it can be stored that way at least one month). Sample was thawed and incubated for 5 minutes at room temperature. Total RNA from mammalian cells was extracted with *TRIZOL reagent* (Ambion) (see **Bacterial RNA isolation** section) and purified by phenolization (see **RNA phenol extraction** section).

4.1.1.2. *S. lycopersicum* RNA extraction

Total RNA of 3-week old *S. lycopersicum* leaves infected with *Salmonella* was obtained from three different biological replicates for each case. Leaf samples were collected 6 and 12 hours post inoculation, and they were frozen in liquid N_2 and stored at -80ºC. Frozen leaf samples were homogenized in 2 ml tubes with 4 glass beads in a *Retsch TissueLyzer II Ball Mill Homogenizer* (Retsch). Total RNA from plant leaves was extracted with

TRIzol® reagent (Ambion) (see **Bacterial RNA isolation** section) and purified by phenolization (see **RNA phenol extraction** section).

4.1.2. Bacterial RNA isolation

To prepare cells for RNA extraction, 3 ml of fresh LB was inoculated with a 1:100 dilution from an overnight bacterial culture, and incubated with shaking at 200 rpm at 37ºC. A 2 ml aliquot from a stationary culture (OD_{600}~2) was centrifuged at 13000 rpm, 4ºC, for 5 min. The pellet was suspended in 100 µl of a solution of lysozyme (3 mg/ml in water; Sigma Chemical Co.). Cell lysis was facilitated by a freeze-thaw cycle. After lysis, RNA was extracted using 1 ml of *TRIzol® reagent*, and the preparation was incubated for 5 min at room temperature. Samples were centrifuged at 4ºC at 13000 rpm for 10 min. The supernatants were recovered and poured out in clean tubes (eliminating the genomic DNA in this step). Two hundred µl of chloroform were added, and the samples were vortexed for 15 s and centrifuged for 15 min at 4ºC and 13000 rpm. The supernatants were carefully recovered, avoiding recovering the interphase, and transferred to clean tubes. Five hundred µl of isopropanol were added. Samples were mixed by inversion 2-3 times and they were incubated at room temperature for 10 min, followed by a 10 min centrifugation. The supernatants were discarded and the pellets were washed with cold ethanol 70%, air-dried and then stored at -20ºC until use. The pellets were suspended in 30 µl of RNAse-free water. To obtain a homogenous mixture, the samples were incubated at 65ºC for a few minutes. The quantity and quality of the RNA was determined using a *ND-1000 spectrophotometer* (NanoDrop Technologies).

4.2. RNA phenol extraction

Whenever RNA samples were not clean enough, they were subjected to phenol treatment. First, bd H_2O was added until a volume of 150 µl. Samples were subsequently cleaned by an extraction with acidic phenol, followed by a second extraction with chloroform:isoamilic alcohol (24:1). After extraction, RNA was precipitated with ethanol in the presence of 0.3 M sodium acetate (pH 5.2), and the dried pellet was suspended in RNAse-free water. The quantity and quality of the RNA was determined using a *ND-1000 spectrophotometer* (NanoDrop Technologies).

4.3. RNA cleaning by a commercial kit

As an alternative of RNA phenol extraction, RNA samples were cleaned with the kit *Direct-zol™ RNA MiniPrep Plus* (Zymo Research) following the system indications to obtain pure samples.

4.4. Real Time-PCR (RT-PCR)

Bacterial strains were grown overnight in LPM or LB. RNA was isolated from 4 ml culture as described in **Bacterial RNA isolation** section and purified by phenolization or the kit *Direct-zol™ RNA Miniprep Plus* (Zymo Research). RNA (~1 µg) was reverse transcribed into complementary cDNA (cDNA) with the *Quantitect Reverse Transcriptase* (Qiagen) following the manufacturers' instructions. PCR was performed later with appropriate primers. (**Table M.4**)

4.5. Quantitative RT-PCR (qRT-PCR)

qRT-PCR was used to perform the analysis of gene expression. Previously frozen leaf samples were homogenized in 2 ml tubes with 4 glass beads in a *Retsch TissueLyzer II Ball Mill Homogenizer* (Retsch). Total RNA from plant leaves was extracted with *TRIzol® reagent* (Ambion) and was purified by phenolization. Two µg of RNA were treated with *DNase I* (Quanta BioScience) following the suppliers' protocols. Poly A-tailed RNA (1 µg) was converted to cDNA using the *qScript cDNA synthesis kit* (Quanta BioScience) and oligo-dT primers. qRT-PCR reaction was performed in triplicated using 5 µl per sample with the *Maxtra SYBR Green Master Mix* (Fermentas) and run on a *BioRad iCycle* (BioRad) according to the manufacturer's instructions. Each reaction was carried out in a total volume of 25 µl on a *480-well optical reaction plate* (Roche). Real-time cycling conditions were: (i) holding stage 95ºC, 2 min; (ii) 40 cycles at 95ºC, 30 s; 56ºC, 30s and 72ºC, 30 s; (iii) final elongation 72ºC, 5 min. The primers used for the qRT-PCR are shown in **Table M.4** Relative gene expression was normalized to the expression of actin transcript. Expression levels were compared to the control (10 mM $MgCl_2$).

4.5.1. Quantification of qRT-PCR results

Quantitative RT-PCR data were analysed using the "$2^{-\Delta\Delta Ct}$" method, in which the amount of mRNA of a target gene in an experimental sample is normalized to a reference gene and relative to a control sample. This method is based in the comparison of the threshold cycle of amplification (Ct) of a target gene against a reference gene, obtaining the ΔCt value ($Ct_{target\ gene} - Ct_{reference\ gene}$). The ΔCt experimental sample value is compared to the ΔCt control sample, obtaining the ΔΔCt value ($\Delta Ct_{experimental\ sample} - \Delta Ct_{control\ sample}$). The $2^{-\Delta\Delta Ct}$ represents the fold-change of the target gene in the experimental sample relative to the control sample.

Previously to the qRT-PCR, a survey of the efficiency of amplification of the oligonucleotides was carried out. Serial dilutions of cDNA were used as templates of the PCR. A standard curve was graphically represented as a semi-long regression line plot of Ct values against log of input nucleic acid. Efficiency of the qRT-PCR was calculated using the slope of the regression line following the equation:

$$Efficiency\ (\%) = \left[10^{\left(\frac{1}{slope}\right)} - 1\right] x\ 100$$

4.6. Microarray gene expression analysis

Gene expression profiles were obtained in the following conditions: (i) non infected human HeLa cells, (ii) HeLa cells infected for 8 h with *S.* Typhimurium 14028 (wt strain), (iii) HeLa cells infected for 8 h with *S.* Typhimurium SV5559 (*srfJ* mutant), (iv) HeLa cells transiently transfected with pcDNA3 (empty vector), (v) HeLa cells transiently transfected with pIZ1855 (pcDNA3-SrfJ-3xFLAG), (vi) non-infected murine RAW macrophages, (vii) RAW cells infected for 8 h with *S.* Typhimurium 14028, (viii) RAW cells infected for 8 h with *S.* Typhimurium SV5559 (*srfJ* mutant). *Microarrays Clariom™ S Assay, Mouse* (Affymetrix) were used for samples from RAW cells, and *microarrays Clariom™ S Assay, HUMAN* (Affymetrix) were used for samples from HeLa cells.

To carry out the analysis, RNA was extracted from three independent cultures for each condition. The quantification and integrity of the isolated RNA was analysed with *ND-1000 Spectrophotometer NanoDrop®* (NanoDrop Technologies, Inc.). Biotinylated single-stranded cDNA was prepared from 100 ng per sample of total intact RNA. Labeled

cDNA was hybridized to microarrays following the manufacturer's instructions. The arrays were scanned in a *3000 7G Scanner* from Affymetrix. Image analysis, fluorescent data quantification and quality control was carried out with Affymetrix software. All procedures and preliminary data analysis, including fluorescent data processing, normalization using Robust Multi-array Average (RMA) algorithms, and annotations, were performed at the Genomics Unit of the Andalusian *Center for Molecular Biology and Regenerative Medicine* (CABIMER, Seville, Spain). Fold change was calculated for different comparisons as explained in the **Results** section. Statistical significance (*p* value) was calculated by empirical Bayes moderated t-test based on the results of three arrays per condition. Genes whose expression changed with a *p* value higher than 0.05 were removed from subsequent analysis.

4.6.1. Gene ontology analysis

Gene ontology (GO) analysis is based on the description of functional attributes of a gene product in three categories: molecular function, biological process and cellular component (The GO consortium, 2006). GO enrichment analysis was carried out on the sets of genes with differential expression using the *Gene Ontology Consortium* tools (http://www.geneontology.org/).

4.7. Identification of the transcriptional start site by 5'RACE

Fifteen µg of RNA were used to determine the cDNA 5' end of *srfJ* and *iolE*, using a similar protocol as the one described previously (Argaman *et al*., 2001; Bensing *et al*., 1996). RNAs were prepared either with or without *RNA 5'Pyrophosphohydrolase (RppH)* (New England BioLabs) to distinguish primary transcript 5' ends from internal 5' processing sites (**Figure M.4**). They were incubated at 37ºC for 1 h and phenolized later to obtain pure RNA samples (**RNA phenol extraction** section). A volume of 2.5 µl of the RNA adapter (*GeneRacer^{TM} RNA oligo*) were added to RNA samples in 14.5 µl of bd H_2O. Samples were heated at 95ºC for 2 min, chilled on ice, and incubated overnight at 16ºC with *T4 RNA Ligase* (New England BioLabs®) before phenolization and suspension in 20 µl bd H_2O. For reverse transcription, 10 µl of RNA sample were mixed with 1 µl of dNTP mix (10 mM each), and 2 µl of the specific primer Jrev or Erev (Jrev is 189 nucleotides downstream *srfJ* translation start site; Erev is 326 nucleotides downstream

iolE translation start site) at 1µM, and was heated at 65ºC for 5 min, and incubated on ice for 1 min before adding 4 µl of reverse transcriptase buffer (5x), 1 µl of dithiothreitol (DTT) 0.1 M, 1 µl of RNAse inhibitor, and 1 µl of reverse transcriptase (*SuperScript III Reverse Transcriptase* (Invitrogen)). The total 20 µl volume samples were heated at 50ºC for 1 h, at 70ºC for 15 min and finally stored at -20ºC.

A PCR reaction was performed with a homologous primer of the *GeneRacer™ RNA oligo* (GeneRacer™ 5'Nested primer) and the specific gene primers Jrev2 or Erev2 (Jrev2 is 59 nucleotides downstream *srfJ* translation start site; Erev2 is 40 nucleotides downstream *iolE* translation start site). PCR products were purified using a commercial DNA purification kit, were cloned using the commercial kit *pGEM®-T Easy* (Promega), and plasmids were transformed into *E. coli* DH5α competent cells. Transformants were selected in LB plates supplemented with Ap and X-Gal. White colonies were checked by PCR, DNA was extracted from positive colonies, and three clones of each candidate were sequenced. Sequences obtained were aligned upstream of the translational start site of each gene to look for the transcriptional start site. The transcriptional start site was defined as the first nucleotide after the sequence corresponding to the *GeneRacer™ RNA oligonucleotide*.

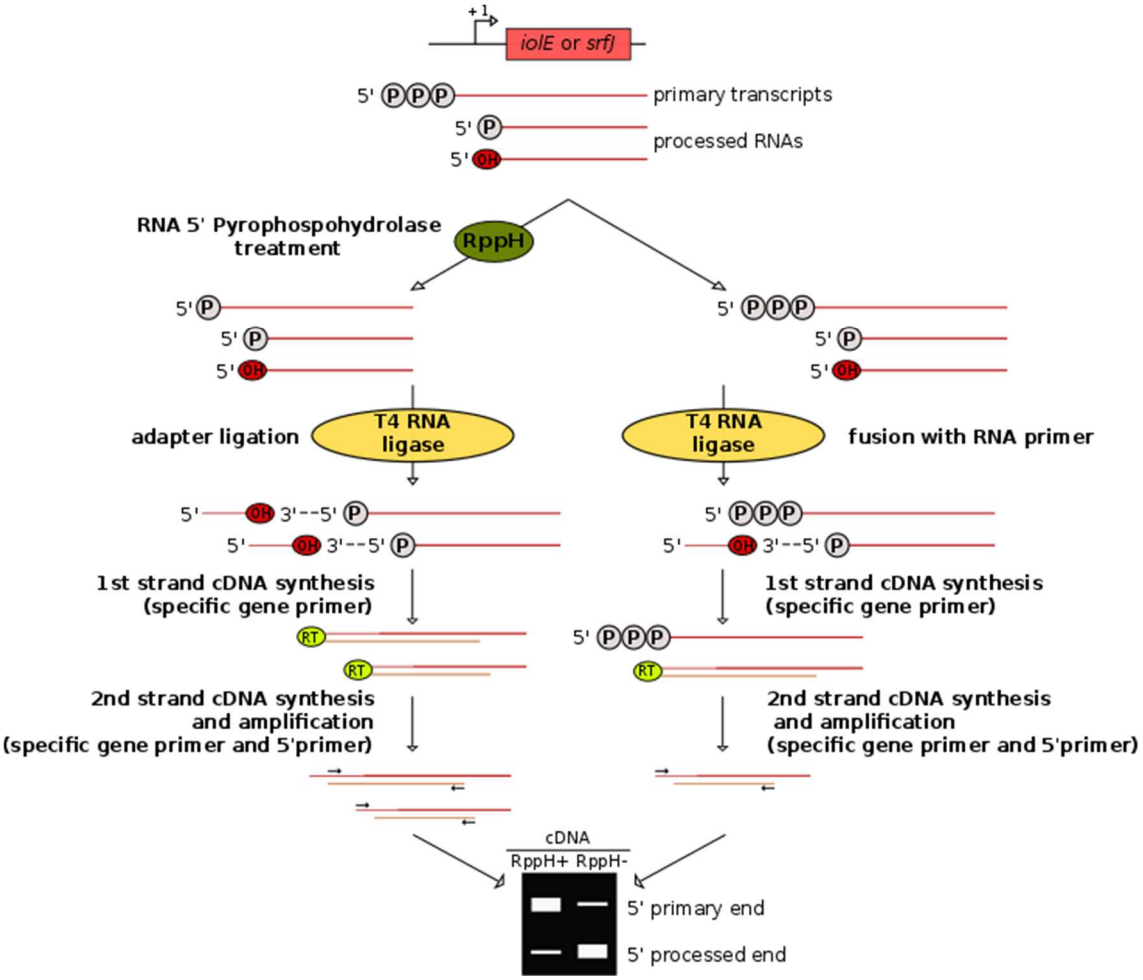


Figure M.4. Identification of the transcriptional start site by 5'RACE. Bacterial primary transcripts have a 5'triphospate end (5'PPP), which has to be hidrolyzed by RppH between the first and second phosphate group. RNAs with 5'monophosphate end (5'P) are ligated to the 3'OH end of and RNA adapter (5'adapter). Retrotranscription is performed using a specific primer of the gene of interest. Subsequently, PCR amplification is performed with a specific primer of the gene of interest and a primer homologous to the RNA 5'adapter. A specific or enriched band for the primary transcript is expected in the sample previously treated with RppH, compared with the samples that have not been treated with RppH.

5. PROTEIN ANALYSIS

5.1. Preparation of protein extracts

5.1.1. Bacterial protein extracts for analysis in polyacrylamide gels

Bacteria from a liquid culture were collected by centrifugation, resuspended in Laemmli buffer[1], and heated to 95ºC for 5 min. After centrifugation, samples were loaded in a polyacrylamide gel.

[1]**Laemmli buffer:** 2 % SDS, 10% (v/v) glycerol, 50 mM Tris-HCl, 5 % β-mercaptoethanol, 0.01 % bromophenol blue, pH 6.8.

5.1.2. Preparation of protein extracts from mammalian cells

Cells were detached with trypsin (adherent cells) or a cell scraper (semiadherent cells) and counted in a hemocytometer. They were centrifuged at 1000 rpm for 10 min at room temperature and washed twice with PBS. The cell pellet was incubated on ice for 20 min with NP40 lysis buffer[1] containing protease inhibitors[2]. Phosphatase inhibitors[3] were also added for certain experiments. After lysis, the sample was centrifuged at 13000 rpm at 4ºC for 15 min, and the supernatant was transferred to a new tube for storage at -80ºC. For analysis in polyacrylamide gels, a certain volume of the sample was mixed with Laemmli buffer and heated to 95ºC for 5 min.

[1]**NP40 lysis buffer:** 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP40.
[2]**Protease inhibitors:** 5 µl/ml protease inhibitor cocktail, 10 µl/ml of 0.1 M PMSF.
[3]**Phosphatase inhibitors:** 50 µl/ml of 100 mM NaF (sodium fluoride), 100 µl/ml of 200 mM PPi (pyrophosphate), and 10 µl/ml of 100 mM Na_3VO_4 (sodium orthovanadate).

5.2. Polyacrylamide gel electrophoresis

Proteins were separated according to their molecular weight by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) (Laemmli, 1970), using a *Mini Protean® Tetra Cell* vertical system (Bio-Rad). Proteins were packed in a staking gel[1] 1 cm long, and separated in a resolving gel[2] 5 cm long. The concentration of acrylamide was 4 % for the stacking gel, and 10 or 12% for the resolving gel, depending on the size of the proteins to be separated. The electrophoresis was carried out at 175 V for 45-60 min in running buffer[3].

[1]**Stacking gel:** 125 mM Tris-HCl pH 6.8, SDS 0.1% (p/v), 4% acrylamide:bisacrylamide (*Solu Gel 29:1 Ultra Pura*, Pronadisa), 0.1% (v/v) TEMED, 0.05% (p/v) ammonium persulfate.
[2]**Resolving gel:** 375 mM Tris-HCl pH 8.8, 0.1% (p/v) SDS, 10-12% acrylamide:bisacrylamide (*Solu Gel 29:1 Ultra Pura*, Pronadisa), 0.05% TEMED, 0.05% (p/v) ammonium persulfate.
[3]**Running buffer 10x**: Glycine 144.1 g/l, 10 g/l SDS, 30.3 g/l Trizma base. Adjust to pH 8.8.

5.3. Molecular weight markers

As molecular weight ladders, two different commercial markers were used: *SDS-PAGE Molecular Weight Standards Low Range* (Bio-Rad) or *Precision Plus Protein Standards Dual Color* (Bio-Rad).

5.4. Coomassie Blue Staining

Coomassie Brilliant Blue R-250 (Sigma-Aldrich) was used to stain SDS-PAGE gels. For staining, gels were soaked in staining solution[1] for 30 min. The staining solution was then removed, the gel was briefly rinsed with d H_2O, and covered with destaining solution[2] until proteins bands were visible.

[1]**Staining solution:** 0.25% (p/v) *Coomassie brilliant Blue R-250*, 10% acetic acid and 10% ethanol.
[2]**Destaining solution:** 10% acetic acid and 40% ethanol.

5.5. Immunodetection of proteins by Western Blot

5.5.1. Nitrocellulose membrane transfer

Proteins resolved by SDS-PAGE were transferred to a nitrocellulose filter (*Amersham Hybond-ECL*, GE Healthcare) using the *Trans-Blot® Turbo™ Transfer System* (Bio-Rad). The gel was equilibrated in transfer buffer for 10 min and the nitrocellulose membrane and 6 pieces of *Whatman 3MM* filter paper were soaked on the same buffer. The transfer sandwich was assembled on the cassette base by placing three pieces of wet filter paper on the bottom, then the membrane, the gel, and finally the remainder of the wet filter paper on top. The cassette was introduced into the instrument bay and proteins were transferred for 30 min at 25 V and 1A.

[1]**Transfer buffer 10x:** 58.2 g/l Tris, 29.3 g /l glycine, 39.5 ml/l SDS (10%). Transfer buffer 1x contains 20% ethanol (v/v).

5.5.2. Ponceau staining

After transfer, the membrane was washed twice with d H_2O and was stained with Ponceau S staining solution[1] for 5 min. The membrane was washed again with d H_2O until bands were visible. This provided a loading control if necessary.

[1]**Ponceau S staining solution:** 0.5% (p/v) Ponceau S in 1% acetic acid.

5.5.3. Nitrocellulose membrane blocking

Previously to the incubation with antibodies, the nitrocellulose membrane was incubated with blocking buffer[1] for at least 1 h at room temperature with gentle shaking.

[1]**Blocking buffer:** Odyssey® Blocking Buffer (TBS) (LI-COR), or 5% non-fat milk in TBS-Tween.

5.5.4. Incubation with primary antibody

After membrane blocking, the primary antibody, which was appropriately diluted in blocking buffer, was added. The preparation was incubated for 2 h at room temperature with gentle shaking or at 4ºC overnight with gentle shaking. Primary antibodies used in this thesis are listed in **Table M.5**.

5.5.5. Incubation with secondary antibody

After the incubation with the primary antibody, membranes were washed three times with TBS-Tween[1] buffer for 10 min and incubated with secondary antibody (**Table M.5**) diluted in TBS-Tween for 1 h at room temperature. Secondary antibodies were conjugated to peroxidase (HRP) or a fluorescent dye. After this step, membranes were washed six times with TBS-Tween for 5 min.

[1]**TBS-Tween 10x:** 24.2 g/l Trizma base, 80 g/l NaCl, 10 ml/l Tween-20. Adjust to pH 7.6.

Table M.5. List of antibodies used in this study.

Antibody	Type	Source	Company	Dilution
Anti-FLAG M2	Primary, monoclonal	Mouse	Sigma	1:5000
Anti-GroEL	Primary, plyclonal	Rabbit	Sigma	1:30000
IRDye® 800CW anti-Mouse	Secondary, polyclonal	Goat	LI-COR	1:15000
IRDye® 680 RD anti-Rabbit	Secondary, polyclonal	Goat	LI-COR	1:15000
IgG-peroxidase anti-Mouse	Secondary, polyclonal	Goat	Bio-Rad	1:5000

5.5.6. Signal detection

The immunofluorescence or luminescence signal detection was performed using the system *Odyssey® Fc Imaging System* (LI-COR). Digital images were taken and processed with the *LI-COR Image Studio Software* for accurate data analysis.

5.6. Protein precipitation by TCA/DOC

To concentrate the proteins from a cell lysate, it was centrifuged at 13000 rpm for 20 min at 4ºC to separate the soluble fraction, consisting of the host cytosol and translocated bacterial proteins, from the insoluble fraction, containing the internalized bacteria. The soluble fraction was filtered through a 0.22 µm pore-size filter (*Millex® Syringe Filter Units 4 mm*, Millipore) and incubated 30 min on ice with 0.02% sodium deoxycholate. Proteins were then precipitated by adding trichloroacetic at a final concentration of 10% v/v, followed by incubation at -20ºC for 5 min, on ice for 15 min, and centrifugation (13000 rpm, 4ºC for 15 min). The supernatant was discarded, and the pellet was incubated with 300 µl of cold acetone, 15 min on ice. After a new centrifugation step, the pellet was processed for electrophoresis and Western blot.

5.7. Protein purification by affinity chromatography

Two systems were employed: glutathione-S-transferase (GST) system and 6 histidine (6His) system. In both cases, the fusion protein is purified by affinity to an agarose matrix which contains glutathione in the GST system, and nickel ions in the 6His systems.
An overnight culture of *E. coli* BL21 (DE3) containing pGEX-4T-2 or its derivatives (for expression of GST fusion proteins), or pET15b or its derivatives (for expression of 6His

proteins) was diluted 100 fold in 250 ml of LB supplemented with Ap, and was incubated at 37ºC for 1 h. Expression of the fused protein was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubation at 30ºC for 3 h with shaking. Cells were collected by centrifugation at 10000 rpm for 10 min at 4ºC, and lysed by sonication (50% amplitude, 5 min, on ice) in 10 ml of NP40 lysis buffer supplemented with protease inhibitors or lysis buffer for 6His proteins[1]. Soluble (supernatant) and insoluble fractions (pellet) were pulled apart by centrifugation at 10000 rpm for 30 min at 4ºC. One ml of the soluble fraction was incubated for 2 h at 4ºC in an orbital shaker with 100 µl of *glutathione agarose beads* (Sigma-Aldrich) or *Ni-NTA agarose beads* (Qiagen), previously equilibrated with lysis buffer. After incubation, beads were washed 5 times with NP40 lysis buffer (GST proteins) or washing buffer[2] (6His proteins) and was suspended in 100 µl of the same buffer. Five µl were used for protein detection in a polyacrylamide gel electrophoresis followed by *Comassie Blue* staining.

For elution, a fraction of the purified protein immobilized on the beads was incubated for 2 h at 4ºC in an orbital shaker with an appropriate volume of elution buffer for GST proteins[3] or for 6His proteins[4]. Eluted protein fraction was separated by centrifugation.

[1]**Lysis buffer for 6His proteins:** 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole. Adjust to pH 8 with NaOH.
[2]**Washing buffer:** 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole. Adjust to pH 8 with NaOH.
[3]**Elution buffer for GST proteins:** 10 mM reduced glutathione in 50 mM Tris HCl pH 8.
[4]**Elution buffer for 6His proteins:** 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole. Adjust to pH 8 with NaOH.

5.8. Protein concentration quantification

Concentration of total protein in extracts was measured with the reactive *Protein Assay Dye Reagent Concentrate* (BioRad), following the manufacturer instructions. This protocol is based on the Bradford method (Bradford, 1976). Concentration of a purified protein was calculated with the use of a polyacrylamide gel electrophoresis followed by *Comassie Blue* staining. Sample was compared with a calibration curve done with BSA (*bovine serum albumin*, New England Biolabs). This quantification was processed with the system *Molecular Imager Gel Doc™ XR+* with *Image Lab™ Software* (BioRad).

5.9. *Human Phospho-Kinase Antibody Array* (R&D Systems)

This kit is used to analyze the phosphorylation of 43 kinases and 2 related proteins (**Figure M.5**). Three different protein conditions were analyzed: non-infected RAW264.7 cels, RAW264.7 cells infected with wild-type *Salmonella*, and RAW264.7 cells infected with a *srfJ* mutant. Proteins lysates were prepared in lysis buffer containing phosphatase inhibitors (see **Preparation of protein extracts from mammalian cells**) and 600 µg of proteins were used per array in a maximum volume of 334 µl following the manufacturer instructions. Signal detection was obtained using the *Odyssey® Fc Imaging System* (LI-COR). Digital images were taken and processed with the *LI-COR Image Studio Software* for accurate data analysis.

A

Membrane/Coordinate	Target/Control	Phosphorilation site
A-A1, A2	Reference spot	----
A-A3, A4	p38α	T180/Y182
A-A5, A6	ERK1/2	T202/Y204, T185/Y187
A-A7, A8	JNK 1/2/3	T183/Y185, T221/Y223
A-A9, A10	GSK-3α/β	S21/S9
B-A13, A14	p53	S392
B-A17, A18	Reference spot	----
A-B3, B4	EGF R	Y1086
A-B5, B6	MSK1/2	S376/S360
A-B7, B8	AMPKα1	T183
A-B9, B10	Akt 1/2/3	S473
B-B11, B12	Akt 1/2/3	T308
B-B13, B14	p53	S46
A-C1, C2	TOR	S2448
A-C3, C4	CREB	S133
A-C5, C6	HSP27	S78/S82
A-C7, C8	AMPKα2	T172
A-C9, C10	β-Catenin	----
B-C11, C12	p70 S6 Kinase	T389
B-C13, C14	p53	S15
B-C15, C16	c-Jun	S63
A-D1, D2	Src	Y419
A-D3, D4	Lyn	Y397
A-D5, D6	Lck	Y394
A-D7, D8	STAT2	Y689
A-D9, D10	STAT5a	Y694
B-D11, D12	p70 S6 Kinase	T421/S424
B-D13, D14	RSK1/2/3	S380/S386/S377
B-D15, D16	eNOS	S1177
A-E1, E2	Fyn	Y420
A-E3, E4	Yes	Y426
A-E5, E6	Fgr	Y412
A-E7, E8	STAT6	Y641
A-E9, E10	STAT5b	Y699
B-E11, E12	STAT3	Y705
B-E13, E14	p27	T198
B-E15, E16	PLC-γ1	Y783

Membrane/Coordinate	Target/Control	Phosphorilation site
A-F1, F2	Hck	Y411
A-F3, F4	Chk-2	T68
A-F5, F6	FAK	Y397
A-F7, F8	PDGF Rβ	Y751
A-F9, F10	STAT5a/b	Y694/Y699
B-F11, F12	STAT3	S727
B-F13, F14	WNK1	T60
B-F15, F16	PYK2	Y402
A-G1, G2	Reference spot	----
A-G3, G4	PRAS40	T246
A-G9, G10	PBS (negative control)	----
B-G11, G12	HSP60	----
B-G17, G18	PBS (negative control)	----

B

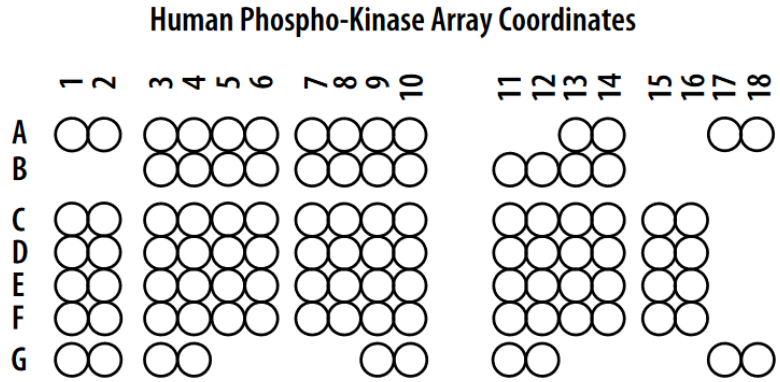


Figure M.5. ***Human Phospho-Kinase Antibody Array*** **(R&D Systems).** (A) List of kinases with the tested phosphorylation sites and their distribution in the membranes. (B) *Human Phospho-Kinase* membranes Part A and Part B with protein localization.

6. CELL BIOLOGY TECHNIQUES

6.1. Luminescence measurements

6.1.1. Bacterial culture

Salmonella strains were grown in triplicate in media described above and samples of 150 µl of each culture were used to measure luminescence and OD_{600}. Luminescence was read in white, clear bottom 96-well plates (Corning) using a *Synergy™ HT microplate reader* (Bio Tek) or a *Sunrise reade*r (Tecan).

6.1.2. Bacterial infection of macrophages RAW264.7

To measure luminescence of intracellular bacteria, RAW264.7 cells were plated into white, clear bottom, 96-well plates at 3 x 10^4 cells per well, and were infected 24 h later with non-invasive bacteria. For that purpose, bacteria were grown in LB medium for 24 h at 37ºC with shaking and were added at a multiplicity of infection (MOI) of 500. Bacteria were centrifuged onto the cell monolayer at 200 g for 5 min and then incubated at 37ºC with 5% CO_2. The cell culture was washed twice with PBS 30 min p.i., overlaid with DMEM containing 100 µg/ml gentamicin (Gm), and incubated for 1 h and 30 min. The culture was washed twice with PBS, covered with DMEM with Gm (16 µg/ml), and incubated for 24 h. Luminescence was measured 2, 4, 8 and 24 h p.i. using a *Synergy™ HT microplate reader* (Bio Tek); and the number of CFU per well were calculated after incubation with 1% Triton X-100 in PBS for 10 min a 37ºC to release bacteria, plating appropriate dilutions in LB with Ap, and counting colonies after 24 h of incubation at 37ºC.

6.1.3. Bacterial colonization of tomato plants

S. Typhimurium 14028 carrying derivatives of plasmids pBS377 (empty, P*iolE*, P*srfJ*, P*iolE*-P*srfJ*) were used to spray 3-week old tomato plants grown in sterile conditions. Bacteria were grown 1 day before the infection on LM plates. Three tomato plants were spray-inoculated with bacteria suspended in 10 mM $MgCl_2$ at OD_{600} 0.1. Tomato plants were imaged 2 days p.i. with an X-ray film exposed for 48 h.

6.2. Infection of mammalian cells

6.2.1. Single infections

6.2.1.1. HeLa infection assay

HeLa cells were plated 24 h before infection in 6 well plates at 8x10^5 cells per well and incubated 24 h at 37ºC with 5% CO_2 in media without antibiotics. Bacteria were grown under invasive conditions (LB 0.3 M NaCl medium without aeration for 15 h) and were added to the cell monolayer at a MOI of 50. The cell cultures were washed twice with PBS 1 h p.i., overlaid with DMEM containing 100 µg/ml gentamicin, and incubated for

another hour. The culture was then washed twice with PBS, covered with DMEM with Gm (16 µg/ml) and incubated for 6 additional hours.

6.2.1.2. RAW264.7 macrophages infection assay

RAW 264.7 cells were plated 24 h before infection in 6 well plates at $8x10^5$ cells per well, or 10 cm diameter plates at $2,7x10^6$ cells per plate, and incubated 24 h at 37ºC with 5% CO_2 in media without antibiotics. Bacteria were grown in LB medium (plus specific antibiotic if needed) for 24 h at 37ºC with shaking and were added to the cell monolayers at a MOI of 50 (for RNA or protein extraction) or 250 (for protein translocation assays). The cell culture was washed twice with PBS 1 h p.i., overlaid with DMEM containing 100 µg/ml Gm, and incubated for another hour. The culture was then washed twice with PBS, covered with DMEM with Gm (16 µg/ml) and incubated for 6 additional hours.

6.2.2. Mixed infections

For proliferation comparison between two *S. enterica* strains, mixed infection assays and competitive index (CI) were performed as described (Segura *et al*., 2004). Strains 14028 (wt) or SV4608 (14028 *trg::*Mu*d*J) were used as controls. SV4608 carries a *lacZ* insertion in a gene non-related with virulence. Strains tested were SV5559, which carries a *srfJ:lacZ* translational fusion, and SV6891 which is a null mutant for *iolR*. Using the appropriate control strain in each case, we could distinguish each strain in media with X-Gal.

6.3. Protein translocation into mammalian cells

To study translocation of fusion proteins SseJ-OprF/I-FLAG, SlrP-OprF/I-FLAG, SteA-OprF/I-FLAG, SseKI-OprF/I-FLAG, SrfJ-OprF/I-FLAG and SseJ-PcrV-FLAG, SlrP-PcrV-FLAG, SteA-PcrV-FLAG, SseKI-PcrV-FLAG, SrfJ-PcrV-FLAG under the control of different promoter regions, mammalian cells were infected and lysed with 0.1% Triton X-100 in PBS to separate soluble fraction from insoluble fraction. Soluble fraction contains the cytosol and bacterial translocated proteins, while insoluble fraction contains the internalized bacteria. Soluble fraction was filtered with 0.22 µm filters (*Millex*® *Syringe Filter Units 4 mm*, Millipore) and precipitated using the TCA/DOC protocol (see

Protein precipitation by TCA/DOC). Samples were analyzed by SDS-PAGE and immunodetection with anti-FLAG antibodies.

7. IMMUNIZATION ASSAYS IN MICE

7.1. Mouse immunization

Mice were maintained in the IBiS facility and their care was in accordance with institutional guidelines. Attenuated *S.* Typhimurium with appropriate plasmids were grown overnight at 37ºC with shaking in LB with Ap, diluted in fresh medium (1:100), and grown to an OD_{600} of 0.3 to 0.6. Vaccination was carried out in 6 to 8-week-old, female C57BL/6 mice (Charles River Laboratories) by a single intraperitoneal injection with 0.2 ml of PBS containing $2x10^5$ CFU of *Salmonella*. Control mice were injected similarly with PBS. Mice were infected with *P. aeruginosa* strain PA01 on day 21 post-immunization by intraperitoneal injection with $9x10^6$ bacteria in 0.2 ml of PBS and survival was monitored for 7 days. Mice that received the same infection were housed together with up to 5 mice per cage. Mice were monitored twice daily, and culled using thiopental at the end of the experiments. The procedures (intraperitoneal injection) made unnecessary the use of analgesics. To minimize animal suffering, euthanasia using thiopental was carried out immediately when detecting severe clinical signs: hunching, labored breathing, severe weight loss, inactivity or lethargy.

7.2. Infection of mice with *P. aeruginosa*

Mice were infected with *P. aeruginosa* strain PA01 on day 21 post-immunization by intraperitoneal injection with $9x10^6$ bacteria in 0.2 ml of PBS and survival was monitored for 7 days.

7.3. Spleen and lung bacterial loads and serum cytokine levels

Post-infection bacterial loads were determined in vaccinated and control mice 12 h after infection. Mice were euthanized with an overdose of thiopental (0.2 ml with 25G needle). From these mice two different samples were obtained: (i) blood samples from the retro-orbital sinus to measure the serum cytokine levels; (ii) spleens and lungs for bacterial loads.

7.4. Blood samples from the retro-orbital sinus to measure the serum cytokine levels

Blood samples of at least 0.5 ml were obtained from the retro-orbital sinus of euthanized mice at 12 hours p.i.. Serum was obtained after centrifugation at 4ºC and samples were stored at -80ºC. Serum levels of tumor necrosis factor alpha (TNF-α), and IL-6 were determined using *R&D DouSet ELISA kits* (R&D Systems).

7.5. Spleens and lungs for bacterial loads

Spleens and lungs were aseptically removed, weighed and homogenized in 2 ml of physiological saline. Each organ was introduced in a plastic bag and mixed with 2 ml of PBS. First homogenization step was performed with the help of the base of a 50 ml flask to speed up the process. The second homogenization step was performed with a *Stomacher® 400 Circulator* (Seward). Serial log dilutions were plated on agar plates for bacterial quantification. CFUs were relativized to each tissue weight.

7.6. Enzyme-linked immunosorbent assays (ELISAs)

For indirect ELISAs, *Costar® Assay Plate, 96 Well Clear, Flat Bottom High Binding* (Corning) were coated with 1 µg/ml of purified 6His-OprF/I or GST-PcrV by incubating at 4ºC overnight protected from light.

Next day, wells were washed twice with 0.2 ml of PBST[1] and the content of each well was discarded inverting the plate and blotting it against clean paper towels. Proteins were blocked with 0.2 ml PBSTM[2] at room temperature for 30 min. Wells were washed again twice with PBST. ELISAs were performed using sera collected on day 21 as described previously (McConnell *et al.*, 2006). Serial dilutions of each serum were prepared in PBST (0.1 ml total volume) and they were transferred to the 96 well plate. Proteins were incubated with the serum dilutions for 1 h at room temperature. Each well was washed three times with PBST. A total volume of 0.1 ml of streptavidin-HRP conjugated with IgG mouse was added to each well and the plate was incubated for 1 h at room temperature. Each well was washed three times with PBST and once with PBS. A volume of 0.1 ml of peroxidase substrate (*33'55'-Tetramethyl-benzidine Liquid Substrate, Supersensitive for ELISA*, Sigma) was added, and it was incubated for 15 min at room temperature, until wells turned blue. Reaction was stopped adding 50 µl of 1 M HCl and

OD_{450} was measured using a *Synergy™ HT microplate reader* (Bio Tek). Antibody titers were measured against the OprF/I or PcrV antigens, and were defined as the dilution in which spectrophotometric readings were at least 0.1 units above background wells (wells containing no serum).

[1]**PBST:** 0.1% Tween® 20 in PBS
[2]**PBSTM:** 0.1% Tween® 20, 5% milk in PBS

8. BACTERIAL SURVIVAL IN PLANTS (INFILTRATION ASSAY)

To prepare the bacterial inoculum, bacteria were grown on solid LM ant then suspended in 10 mM $MgCl_2$ and diluted to an OD_{600} of 0.01. Leaves of tomato (*S. lycopersicum*) and lettuce (*L. sativa*) were syringe infiltrated with bacterial solutions, the inoculated areas were sampled 3 h (day 0), 7 days and 14 days after the inoculation. Four excised leaf discs of 0.7 cm^2 (one sample) were homogenized in 10 mM $MgCl_2$. Appropriate serial dilutions of each sample were plated on XLD agar to determine the CFU numbers. The experiments were repeated three times with six plants per experiment.

9. STATISTICAL ANALYSIS

Student's *t*-test was used to analyse every competitive index against the null hypothesis that the mean is not significantly different from 1. This test was also used to compare mean survival of mutants and wt *Salmonella* strains in plants, as wells as expression levels of defense response genes after colonization with different *Salmonella* strains. *P* values of 0.05 or less were considered significant.

Antibody titters, bacterial loads, and cytokine levels were compared using the Kruskal-Wallis test and the Mann-Whitney U test. Survival data were compared using the log-rank test. Statistics were performed using *SPSS version 24.0 software* (SPSS Inc.). *P* values of 0.05 or less were considered significant.

For microarray analysis, differences with ANOVA p-value < 0.05 were considered significant. This value represents the probability that a change in the expression of a gene was due to chance.

10. ETHICS STATEMENT

All the experiments involving *S.* Typhimurium were carried out using the standard biosecurity procedures that included containment level 2 practices, and safety equipment and facilities.

Experiments involving the use of animals were approved by the Committee on Ethics and Experimentation of the Consejería de Agricultura, Pesca y Desarrollo Rural (Junta de Andalucía, Spain) (permit number 18-01-16-005) and followed the EU Directive 2010/63/EU for animal experiments. Efforts were made to minimize suffering, and any animal appearing moribund during the course of experimentation was immediately euthanized using thiopental.

RESULTS

Chapter 1

Dual expression of the *Salmonella* effector SrfJ in mammalian cells and plants

Genes *srf* were identified in a screening of genes regulated by the SsrA/SsrB two-component system and they are localized out of SPI2 (Worley *et al*., 2000). The fact that they were regulated by the SsrA/SsrB system suggested that they could be coding for effector proteins of the T3SS2. Previously, the study of *srfABC* genes was initiated in our laboratory (García-Calderón *et al*., 2007). Our interest became higher and the rest of the other *srf* genes were investigated, trying to elucidate its regulation before studying their possible function during infection. In particular, the study of *srfJ* went further. Several conclusions about the regulation of *srfJ* were achieved: (i) *srfJ* was expressed and translocated to the culture media under SPI2-inducing conditions; (ii) expression of *srfJ* was positively regulated by SsrB and PhoP and negatively regulated by RcsB; and (iii) IolR was identified as a negative regulator of transcription in a T-POP-based screen and *myo*-inositol was described as an inducer of *srfJ* expression (Cordero-Alba *et al*., 2012). But several questions remained unanswered. The molecular bases of this regulation and the biological meaning of the *myo*-inositol-dependent expression were not completely understood. In this chapter we study these issues and their relevance in the interaction between *Salmonella* and different hots. This work has been done in Departamento de Genética, Facultad de Biología, Universidad de Sevilla (Sevilla, Spain) with the collaboration of Dr. Adam Schikora and Azhar A. Zarkani in Julius Kühn-Institut-Bundesforschungsinstitut für Kulturpflanzen, Federal Research centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics (Brunswick, Germany). This work has been recently published in Frontiers in Microbiology (Aguilera-Herce *et al*., 2017).

1.1. Identification of promoter regions driving the expression of *srfJ*

Previous data showed expression of *srfJ* under two disparate conditions: culture medium imitating the intravacuolar environment (LPM) and culture medium supplemented with *myo*-inositol (Cordero-Alba *et al*., 2012). To understand this dual expression at molecular level, we explored the genomic region around the *srfJ* gene. As shown in **Figure R.1.1**, this gene resides inside the *myo*-inositol utilization island (Kröger and Fuchs, 2009), with *iolE* and *iolG1* upstream and *iolI1* downstream of *srfJ*. Promoter activities for regions upstream of these genes (putatively called P*iolE*, P*iolEG1*, P*srfJ* and P*iolI1*) were tested using plasmid pSB377 (Winson *et al*., 1998) that carries a promoterless version of the *luxCDABE* operon of *Photorhabdus luminescens* that encode the luciferase LuxAB

subunits and fatty acid reductase complex involved in synthesis of the fatty aldehyde substrate for the luminescence reaction (Meighen, 1991).

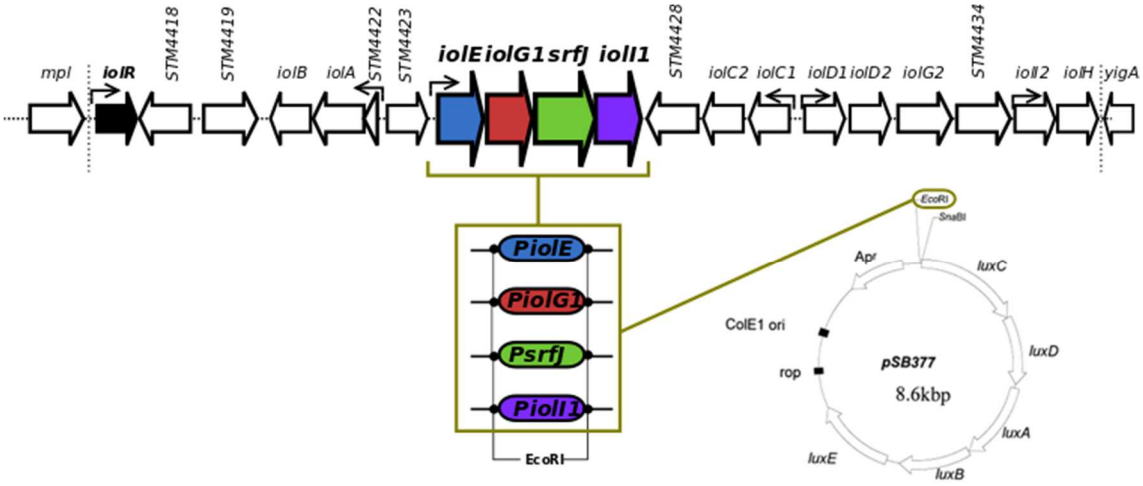


Figure R.1.1. Representation of the coding regions of *srfJ* and surrounding genes in *S.* Typhimurium strain 14028. Fragments of DNA upstream of the coding regions of genes *iolE*, *iolG1*, *srfJ* and *iolI1* were cloned into plasmid pSB377 to generate *luxCDABE* transcriptional fusions. These plasmids were introduced into *S.* Typhimurium strain 14028.

This reporter system allows continuous monitoring of light production without disrupting the bacteria or the infected host. Plasmids were introduced in wt *S.* Typhimurium strain 14028 and the luminescence was measured after growth in three different culture conditions: LPM at pH 5.8 with high aeration for SPI2-inducing conditions, LB with 0.3 M NaCl without aeration for SPI1 inducing conditions and the later medium supplemented with *myo*-inositol to induce expression of the *iol* genes. Only DNA fragments upstream of *iolE* and *srfJ* coding regions showed promoter activity (**Figure R.1.2).**

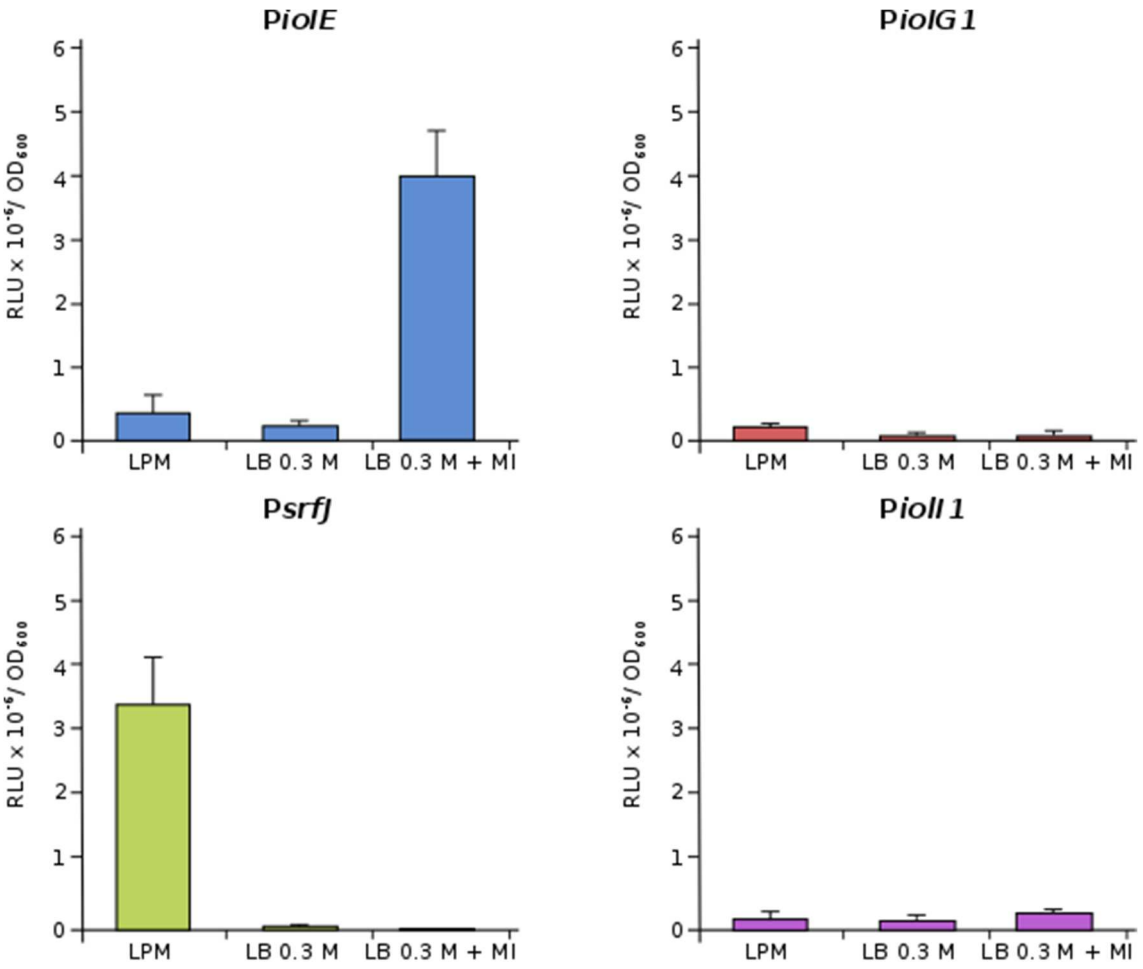


Figure R.1.2. Activity of the putative promoter regions in response to LPM and *myo*-inositol. *S.* Typhimurium strain 14028 with plasmid pSB377 carrying different promoter constructions were cultured and grown until stationary phase in LPM, LB 0.3 M NaCl and LB 0.3 M NaCl with *myo*-inositol. Luminescence signal was measured. RLU: relative light units.

Interestingly, P*iolE* was specifically active in the presence of *myo*-inositol whereas P*srfJ* was only active upon SPI2-inducing conditions. These results suggest that expression of *srfJ* is driven by two promoters: a distal promoter, P*iolE*, and a proximal promoter, P*srfJ,* depending on the environmental conditions.

Additional support for these conclusions was obtained studying the production of a chromosomically tagged version of the protein SrfJ by immunoblot. As show in **Figure R.1.3**, SrfJ-3xFLAG was detected in extracts from bacteria grown in minimal LPM medium and in rich LB medium supplemented with *myo*-inositol. In a ΔP*iolE* background, however, the protein was detected only in LPM, confirming that the distal promoter, P*iolE*, is specifically necessary for *myo*-inositol-dependent induction of *srfJ*.

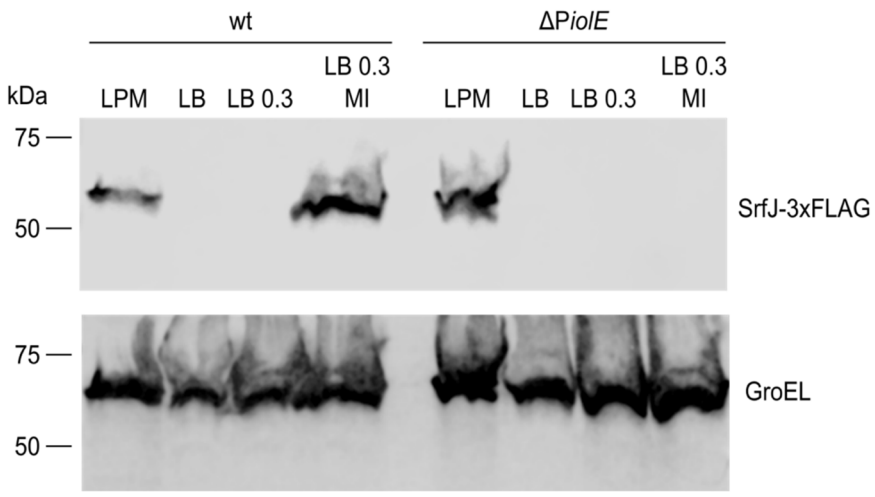


Figure R.1.3. Activity of the putative promoter regions in response to LPM and *myo*-inositol. Extracts of a derivative of *S.* Typhimurium 14028 expressing 3xFLAG-tagged SrfJ were resolved by 12% SDS-PAGE. Immunoblotting was performed with monoclonal anti-FLAG antibodies. Anti-GroEL antibodies were used as loading control. Medium tested were LPM, LB, LB with 0.3 M NaCl (LB 0.3) and LB 0.3 M NaCl with *myo*-inositol (LB 0.3 MI). Molecular mass markers are indicated on the left.

1.2. Differential regulation of P*iolE* and P*srfJ*

In order to study the regulation of both promoters, the corresponding plasmids were transferred into different genetic backgrounds. We tested the effect of null mutations in genes encoding relevant regulators: IolR, SsrB, PhoP and RcsB. IolR is the negative regulator of the *myo*-inositol utilization island (Kröger and Fuchs, 2009). SsrB is encoded in SPI2 and is the main positive regulator of the island (Cirillo *et al*., 1998). PhoP positively regulates SPI2 through SsrB (Bijlsma and Groisman, 2005). RcsB is the response regulator of the Rcs phosphorelay system (Chen *et al*., 2001; Stout and Gottesman, 1990). In *Salmonella*, it positively or negatively regulates genes in SPI1 and SPI2 depending on the level of activation (Wang *et al*., 2007; Wang and Harshey, 2009). We also used the allele *rcsC55*, which causes constitutive activation of the Rcs system (García-Calderón *et al*., 2005). Analysis of the expression patterns in the different genetic backgrounds (**Figure R.1.4 A and C**) indicates that IolR negatively regulates P*iolE*, whereas P*srfJ* is positively regulated by PhoP and SsrB. In addition, P*srfJ* is also negatively regulated by the Rcs global regulatory system, since the activating mutation *rcsC55* abrogates expression of the *lux* reporter from this promoter. Interestingly, a transcriptional *lux* fusion with 2357 bp upstream of *srfJ*, containing both promoters and the intervening genes (P*iolE*-P*srfJ*) is regulated by IolR, PhoP, SsrB and Rcs, recapitulating the regulation patterns observed with the isolated promoters (**Figure R.1.4 B and D**).

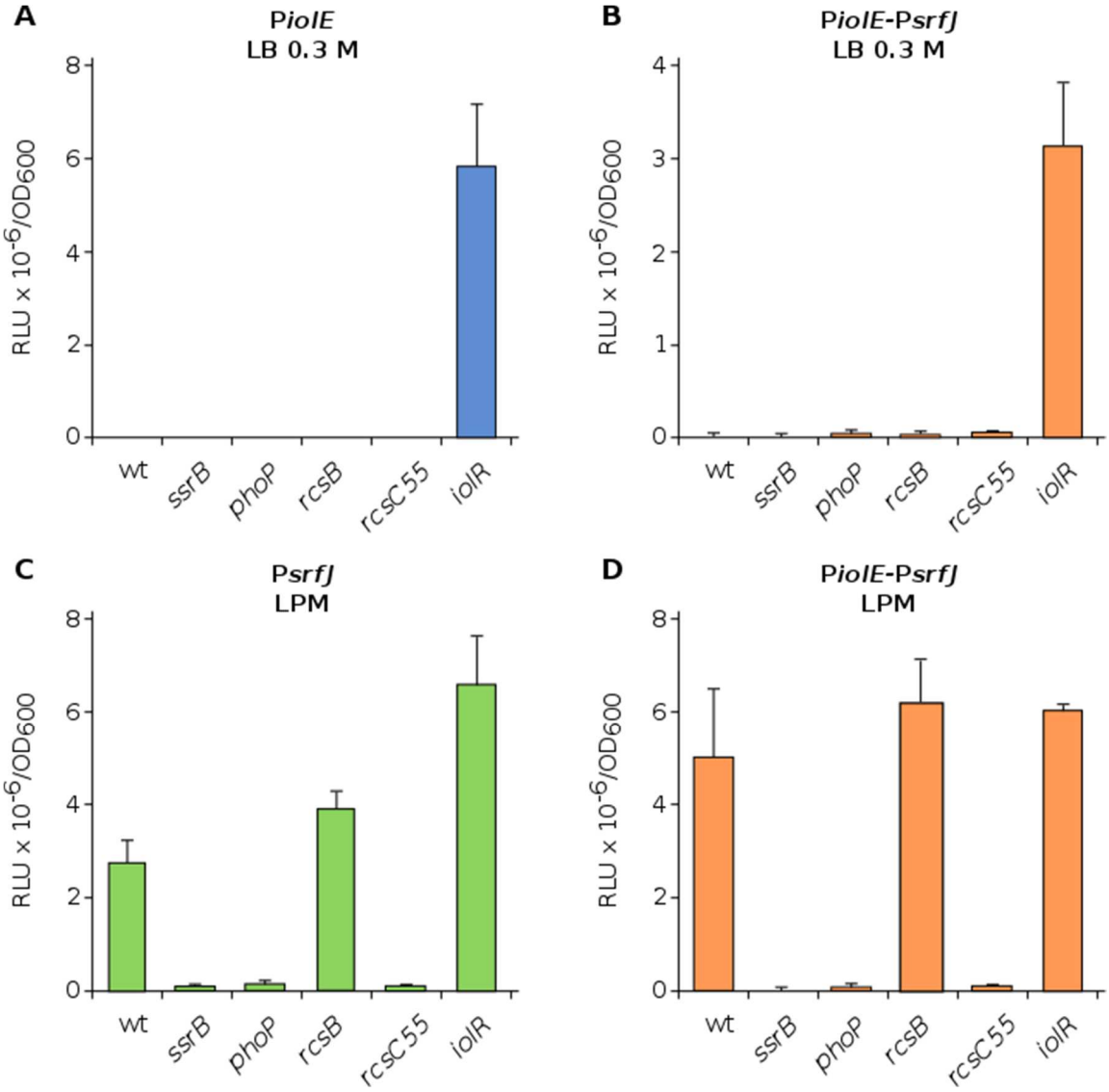


Figure R.1.4. Regulation of P*iolE* and P*srfJ* promoters. Fragments of DNA containing the promoters of *iolE* (P*iolE*) (A) or *srfJ* (P*srfJ*) (C) or the region containing from the promoter of *iolE* to the promoter of *srfJ*, including genes *iolE* and *iolG1* (P*iolE*-P*srfJ*) (B and D) were cloned into plasmid pSB377 to generate *luxCDABE* transcriptional fusions. These plasmids were introduced into *S.* Typhimurium strain 14028 or derivatives with null mutations in *iolR*, *ssrB*, *phoP*, *rcsB*, or a point mutation in *rcsC* (*rcsC55*) that confers constitutive activation to the Rcs system. Luminescence was measured in cultures grown until stationary phase in LB 0.3 M NaCl (A and B) and LPM (C and D). RLU: relative light units.

1.3. Characterization of transcriptional units containing *srfJ*

Results presented above suggest that two different promoters can initiate the expression of *srfJ*. This would result in RNAs of different lengths. To test this hypothesis, RT-PCR was performed using primers designed to amplify different fragments in the *srfJ* region (**Figure R.1.5.A**). RNA was obtained from two sources: (i) wt *S.* Typhimurium incubated in LPM, where P*srfJ* is expected to be active, and (ii) *iolR* mutant strain incubated in LB,

where the absence of IolR repressor should lead to constitutive expression from P*iolE*. Positive and negative controls were carried out using genomic DNA and non-retrotranscribed RNA, respectively. A seen in **Figure R.1.5.B**, RT-PCR carried out on RNA from wt bacteria incubated in LPM yielded only an internal fragment of *srfJ*. In contrast, fragments partially expanding *iolE-iolG1* and *iolG1-srfJ* were obtained when RT-PCR was carried out using RNA from the *iolR* mutant, indicating that these genes are transcriptionally linked when P*iolE* is derepressed.

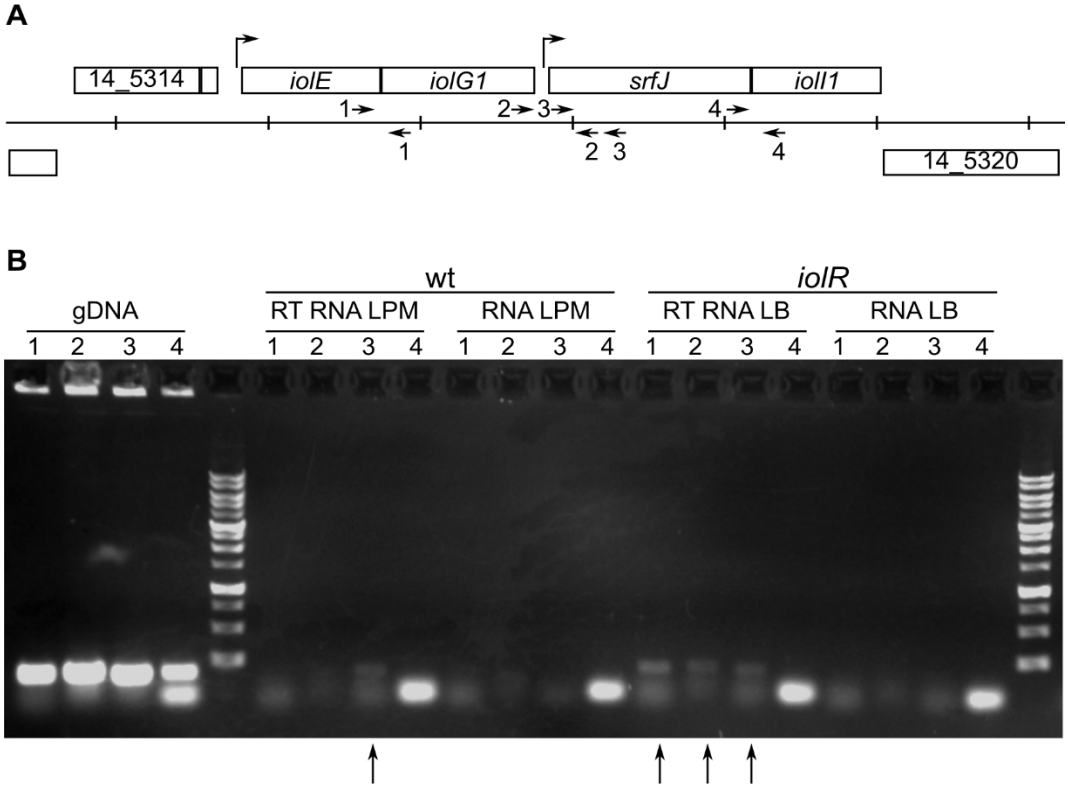


Figure R.1.5. Transcriptional organization of the *srfJ* region. (**A**) Organization of the chromosomal region containing the *srfJ* gene in *S.* Typhimurium strain 14028. Vertical lines are separated by 1 kb. The arrows indicate the positions and orientations of the primers that were used for RT-PCR. (**B**) Agarose gel of the products obtained with the following primers: 1, Efw and G1rev; 2, G1fw and Jrev2; 3, Jfw and Jrev; 4, Jfw2 and Jrev. RT-PCR was carried out on RNA isolated from cultures on LPM of the wild-type strain (wt, RT RNA LPM) and cultures in LB of the *iolR* mutant strain (*iolR*, RT RNA LB). PCR were also carried out on genomic DNA (gDNA) as positive control and non-retrotranscribed RNA as negative control (RNA LPM and RNA LB). Vertical arrows indicated lanes with amplified products after retrotranscription. The molecular weight marker is the 1 kb DNA ladder (NIPPON Genetics).

5'RACE was used for the determination of both transcriptional start sites. They were located 99 bp and 33 bp upstream of the coding regions of *iolE* and *srfJ*, respectively (**Figure R.1.6**). These results confirm that *srfJ* belongs to two different transcriptional units: a short transcriptional unit with P*srfJ* as a promoter and an operon including genes *iolE*, *iolG1* and *srfJ* with P*iolE* as promoter.

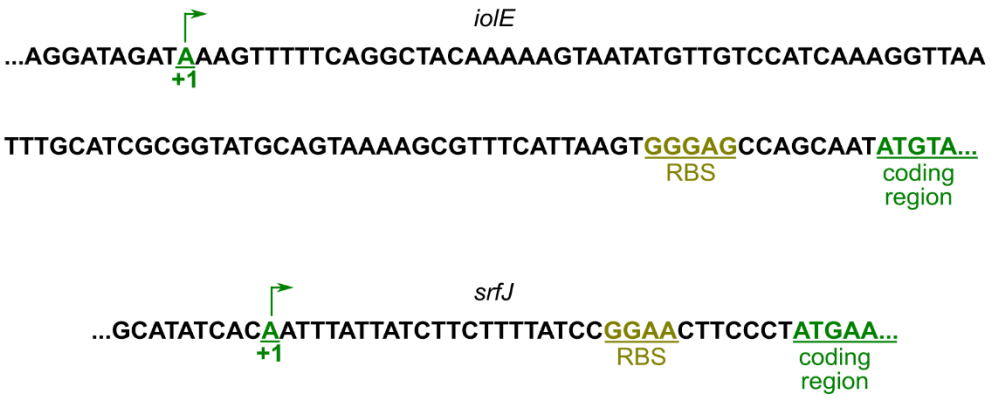


Figure R.1.6. Transcriptional start sites for *srfJ*. 5'RACE was carried out on RNA isolated from cultures in LB of the *iolR* mutant to map the transcriptional start site of *iolE* and from cultures in LPM of the wt strain to map the transcriptional start site of *srfJ*. The sequences surrounding the transcriptional start sites (+1) and the start of the coding regions are shown. RBS: ribosomal binding site.

1.4. Expression of *srfJ* inside macrophages

S. Typhimurium is known to infect macrophages and express the T3SS2 several hours p.i. (Drecktrah *et al*., 2006). Since SrfJ is an effector of this secretion system, it is expected to be produced inside macrophages. To ascertain the relevance in this context of the two promoters that drive the expression of *srfJ*, *Salmonella* strains carrying *PiolE::lux* or P*srfJ::lux* transcriptional fusions were used to infect RAW264.7 macrophages. The luminescence resulting from the activity of the *lux* operon driven by P*srfJ* increased over time during the infection (**Figure R.1.7**). In these conditions, the P*iolE* promoter was not active.

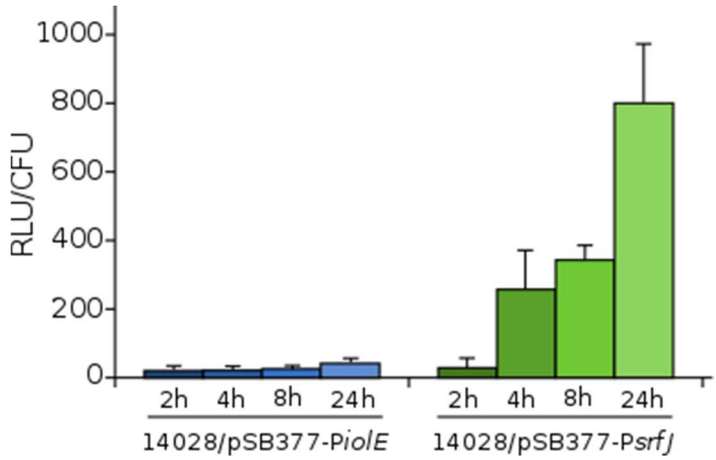


Figure R.1.7. Activity of P*iolE* and P*srfJ* during macrophage infection. The wt strain of *S.* Typhimurium carrying a plasmid expressing P*iolE::luxCDABE* or P*srfJ::luxCDABE* transcriptional fusions was grown for 24 h in LB at 37°C with aeration (non-invasive conditions). These bacteria were used to infect RAW264.7 murine macrophage-like cells and luminescence produced by intracellular bacteria was measured 2, 4, 8 and 24 h p.i. RLU: relative light units.

Expression of *srfJ* in internalized bacteria was also studied by immunoblot using a strain of *Salmonella* that expresses a chromosomally 3xFLAG-tagged version of SrfJ. Intracellular expression was detected both in wt background and in a strain lacking the distal promoter P*iolE* (**Figure R.1.8**). These results suggest that *srfJ* expression is induced in response to intravacuolar signals and that the induction depends specifically on the proximal promoter.

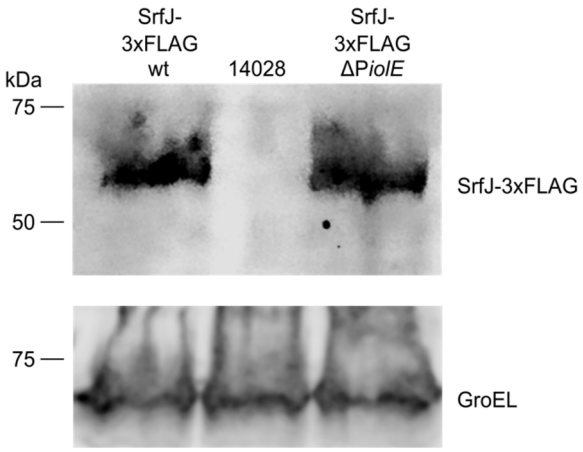


Figure R.1.8. Activity of P*iolE* and P*srfJ* during macrophage infection. The wt strain of *S.* Typhimurium (14028) and derivatives expressing a 3xFLAG-tagged form of SrfJ in a wt background or in a ΔP*iolE* background were grown under non-invasive conditions and used to infect RAW264.7 cells. Expression of *srfJ* was measured 8 h p.i by immunoblot using anti-FLAG antibodies. Anti-GroEL antibodies were used as loading control. Molecular mass markers, in kDa, are indicated on the left.

1.5. Contribution of SrfJ to proliferation of *Salmonella* in macrophages

Since the *srfJ* mutant is attenuated in mice (Ruiz-Albert *et al.*, 2002), we decided to explore the possibility that this mutant could also have a defect in survival and proliferation inside macrophages. This was assessed calculating the competitive index in RAW264.7 macrophages of the *srfJ* mutant against the *trg*::Mu*d*J strain, which is wt for intracellular proliferation (Segura *et al.*, 2004). No significant defect was detected for this mutant ($P>0.05$; **Figure R.1.9**). We also tested the effect of a null mutation in *iolR* and we found a very significant defect in intracellular proliferation (**Figure R.1.9**). Since the *iolR* mutation leads to derepression of *srfJ* transcription (**Figure R.1.4**), we then measured the intracellular proliferation of the double null mutant *iolR srfJ*. Interestingly, the *srfJ* mutation suppressed the effect of the *iolR* mutation on macrophages (**Figure R.1.9**), suggesting that the proper regulation of the expression of *srfJ* is essential for survival and/or proliferation of *S.* Typhimurium inside murine macrophages.

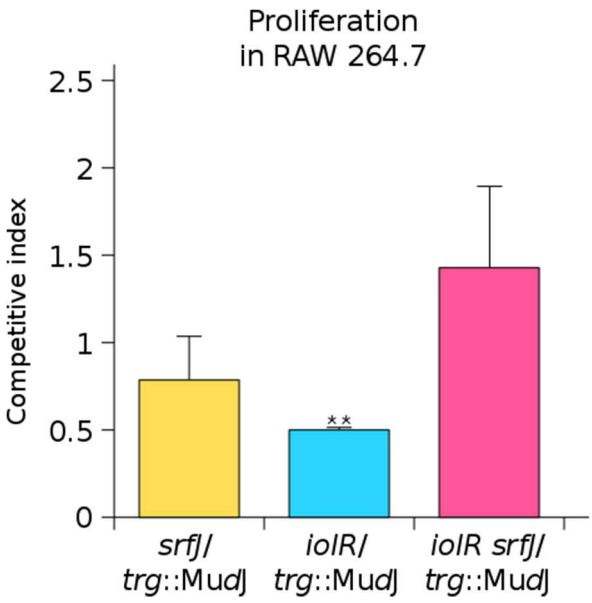


Figure R.1.9. Effect of the expression of *srfJ* in intracellular proliferation. Analysis of intracellular proliferation of *srfJ*, *iolR* and *srfJ iolR* mutants in mixed infections with a *trg*::Mu*d*J mutant or the 14028 strain (wt) used as control strains. The competitive indices are the mean from three infections. Error bars represent the standard deviations. Asterisks denote that the indices are significantly different from 1 for a *t*-test: * *P* value <0.05, ***P* value <0.01.

1.6. Expression of *srfJ* in the presence of plant extracts

The results obtained in macrophages confirmed our hypothesis and proved that SrfJ, as a T3SS2 effector, depends on P*srfJ*, the promoter that is induced in medium imitating intravacuolar conditions. In contrast, it is more difficult to understand the physiological role of the expression of *srfJ* from the distal promoter, P*iolE*. In order to investigate the significance of the double regulation we analysed different environments known to host *Salmonella*, one of them are plants. *Salmonella* is able to thrive and proliferate in plants, including crop plants designated for direct consumption, e.g., lettuce or tomatoes (Hernández-Reyes and Schikora, 2013). Thus, we reasoned that since most plants produce *myo*-inositol (Loewus and Murthy, 2000), P*iolE* could be relevant in allowing transcription of *srfJ* in response to plants signal. *Salmonella* with *lux* transcriptional fusions were grown in LB or in media supplemented with lettuce (LM) or tomato (TM) extracts. We detected high level of luminescence 24 hours after the inoculation of LM or TM media with *Salmonella* carrying the long fusion P*iolE-srfJ::lux* or the P*iolE::lux* fusion. Luminescence was not detected after inoculation with a strain carrying the P*srfJ::lux* fusion (**Figure R.1.10**).

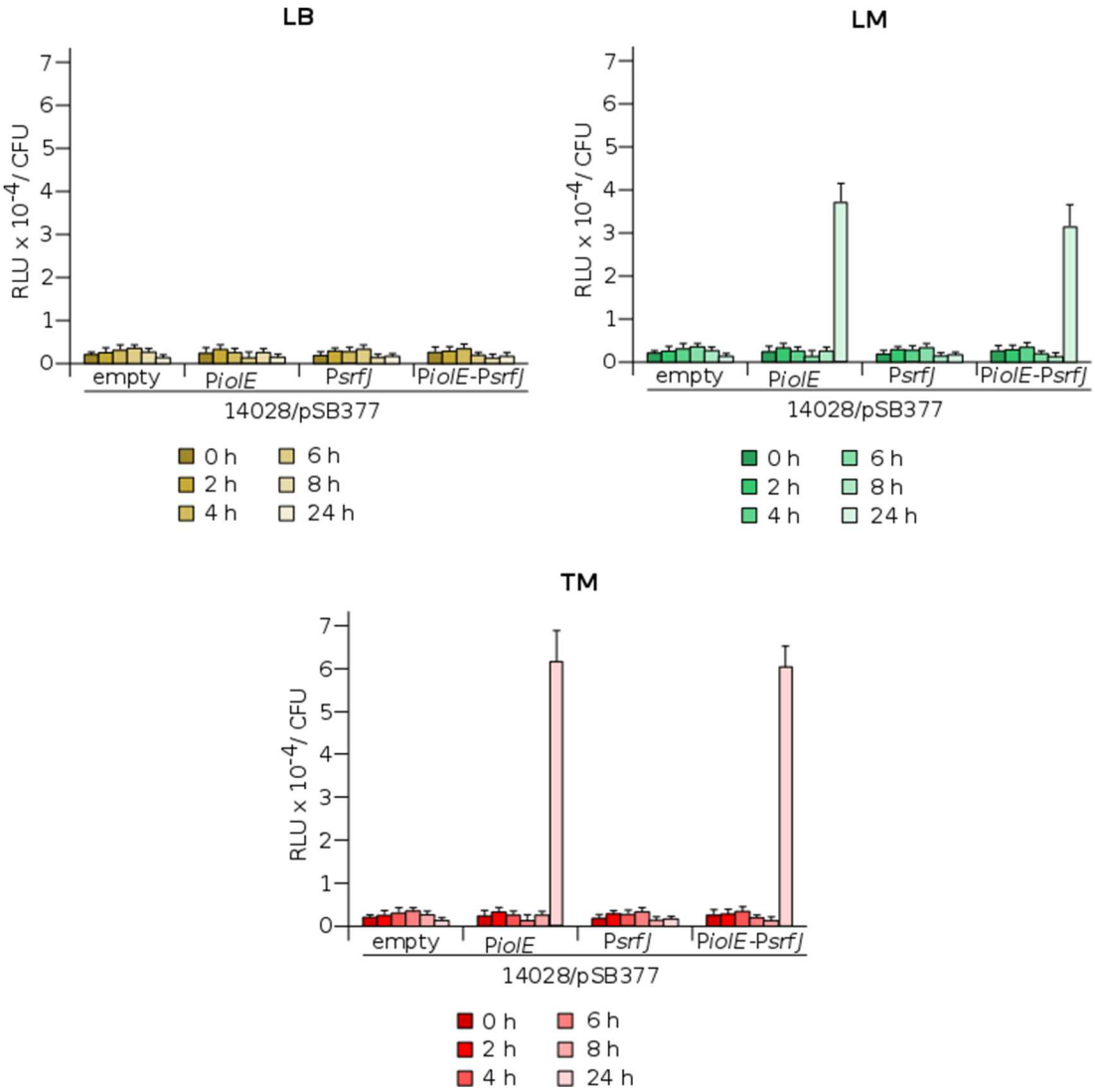


Figure R.1.10. Activity of P*iolE* and P*srfJ* in media with plant extracts. Fragments of DNA containing the promoters of *iolE* (P*iolE*) or *srfJ* (P*srfJ*) or the region containing from the promoter of *iolE* to the promoter of *srfJ*, including genes *iolE* and *iolG1* (P*iolE*-P*srfJ*) were cloned into plasmid pSB377 to generate *luxCDABE* transcriptional fusions. These plasmids as well as the empty plasmid were introduced into *S.* Typhimurium strain 14028. Luminescence was measured at different time points (0, 2, 4, 6, 8, 10 and 24 h) in cultures grown in LB, Lettuce Medium (LM) and Tomato Medium (TM). Bacteria were grown overnight in LB and diluted to OD_{600} 0.1 in the different test media before 0 h time point. RLU: relative light units.

Expression of *srfJ* was also studied at the protein level taking advantage of the chromosomal SrfJ-3xFLAG fusion. As shown in **Figure R.1.11**, SrfJ-3xFLAG was detected by immunoblot in extracts from bacteria grown in LM or TM for 24 h. However, the protein was not produced if P*iolE* was deleted. These results show that P*iolE* can drive expression of *srfJ* in response to plant extracts.

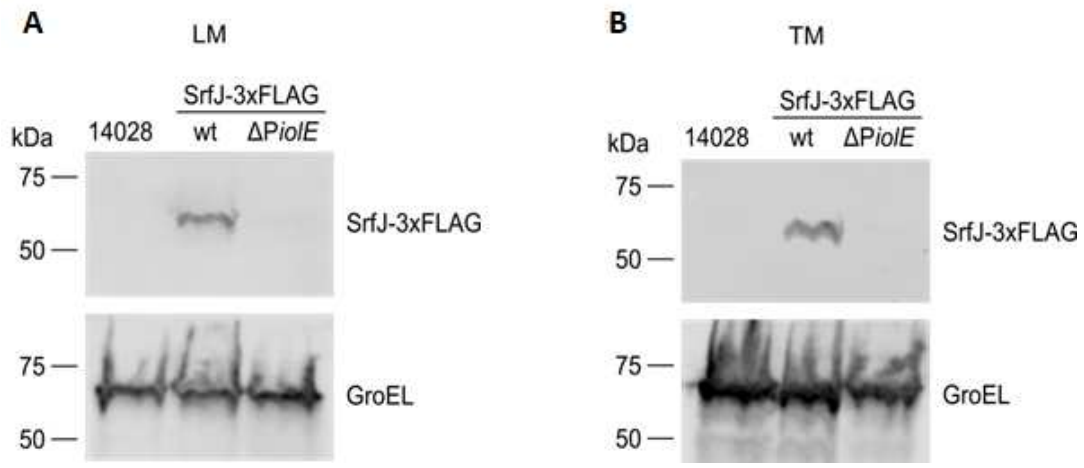


Figure R.1.11. Synthesis of SrfJ in media with plant extracts. The wt strain of *S.* Typhimurium (14028) and derivatives expressing a 3xFLAG-tagged form of SrfJ in a wt background or in a Δ*PiolE* background were grown in LM (A) or TM (B). Expression of *srfJ* was measured 8 h p.i. by immunoblot using anti-FLAG antibodies. Anti-GroEL antibodies were used as loading control. Molecular mass markers, in kDa, are indicated on the left.

1.7. Expression of *srfJ* in plants

The results presented above suggest that *srfJ* could be expressed during *Salmonella* colonization of plants. To evaluate this hypothesis, three-week-old tomato plants were spray-irrigated with suspensions of wt *S.* Typhimurium carrying derivatives of plasmids pSB377 to generate transcriptional *luxCDABE* fusions with P*iolE*, P*srfJ* or P*iolE-srfJ*. Tomato leaves were imaged two days post-inoculation using an X-ray film. Luminescence was detected in plants colonized with bacteria carrying P*iolE::lux* and P*iolE-srfJ::lux* fusions but not with P*srfJ::lux* or the empty vector (**Figure R.1.12**).

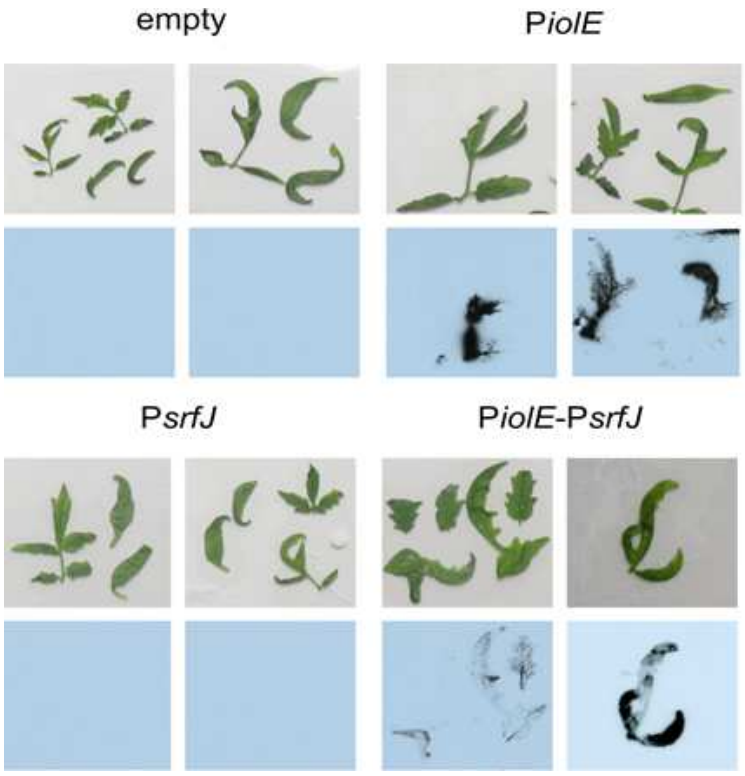


Figure R.1.12. Expression in plants. *S.* Typhimurium strain 14028 carrying derivatives of plasmid pSB377 (empty, P*iolE*, P*srfJ*, P*iolE*-P*srfJ*) were used to spray 3 week-old tomato plants grown in sterile conditions. Plants were germinated in ½ MS for 1 week and grown in ¼ MS media during 2 weeks. Bacteria were grown 1 day before the infection in LM plates. Three tomato plants were spray-inoculated with bacteria suspended in 10 mM $MgCl_2$ at OD_{600} 0.1. Tomato plants were imaged 2 days p.i. with an X-ray film exposed for 48 hours.

Promoters of genes encoding effectors SlrP and SteA were also tested in this system but their expression was not detected (data not shown). These results reveal that *Salmonella* expresses *srfJ* together with the *myo*-inositol utilization island, during colonization of a plant host.

1.8. Contribution of SrfJ to survival of *Salmonella* in plants

The expression of SrfJ in plants suggested that the product of this gene could be relevant during *Salmonella* colonization of these alternative hosts. To test this hypothesis, we compared the survival of wt *S.* Typhimurium with the survival of the *srfJ* mutants in leaves of lettuce and tomato. Leaves were syringe infiltrated with bacterial suspensions and the CFU were counted at different time points. Interestingly, the *srfJ* null mutant showed a significantly improved survival in leaves of both plants 14 days post-inoculation (**Figure R.13**). Since expression of *srfJ* in plants depends specifically on the P*iolE* promoter, we also tested survival in plant leaves of a *S.* Typhimurium mutant with an

intact coding sequence of *srfJ* but with a deletion of P*iolE*. As shown in **Figure R.13**, this mutant confirmed the results obtained with the *srfJ* mutant.

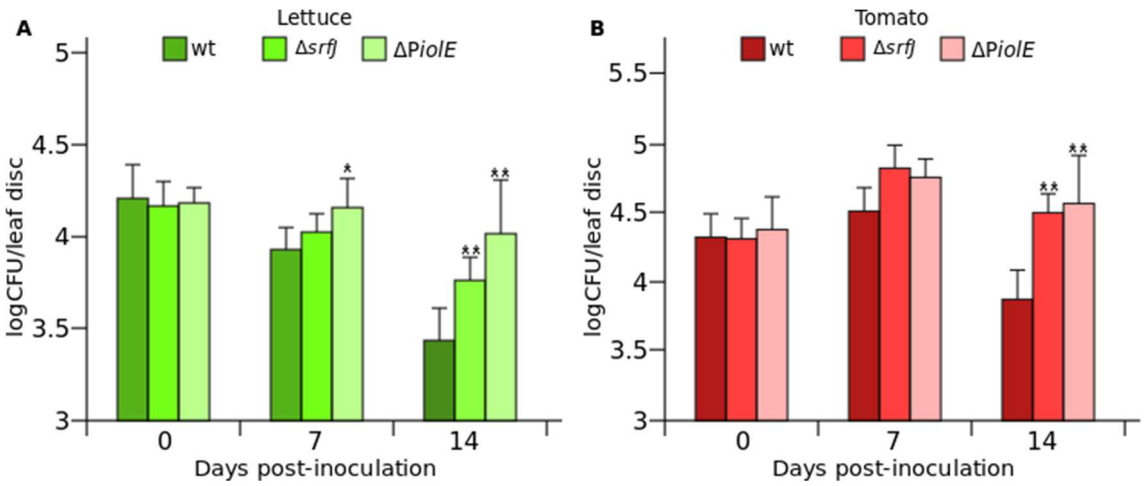


Figure R.1.13. Survival of *Salmonella srfJ* mutant in plants. *Salmonella* wt strain 14028 (wt), *srfJ* mutant and P*iolE* mutant were syringe infiltrated onto leaves of lettuce (A) or tomato (B). The infiltrated leaves were sampled 0, 7, and 14 days after infiltration to determine the number of CFU of *Salmonella*. The results shown are the means from 6 experiments. Error bars represent standard deviations. Asterisk indicate significant differences of mutants compared to wt the same day post-inoculation by Student's *t*-test (**P*<0.05, ** *P*<0.01).

These results suggest that SrfJ could be involved in the modulation of plant defense responses that could limit bacterial growth. To test this hypothesis, the expression of five genes known to be involved in tomato defense responses was studied after inoculation of tomato plants with *Salmonella* wt of *srfJ* mutant using qRT-PCR. Monitored genes encode an acidic extracellular chitinase (*CHI3*), a basic intracellular chitinase (*CHI9*), an acidic extracellular β-1,3-glucanase (*GLUA*), a basic intracellular β-1,3-glucanase (*GLUB*), and a PR-1 protein isoform PR-P6 (*PR-1a*) (Enkerli *et al.*, 1993; Joosten *et al.*, 1989; Uehara *et al.*, 2010). Interestingly, the expression of these genes was significantly lower 6 hours and/or 12 hours after inoculation with the *srfJ* mutant compared to the wt (**Figure R.1.14**).

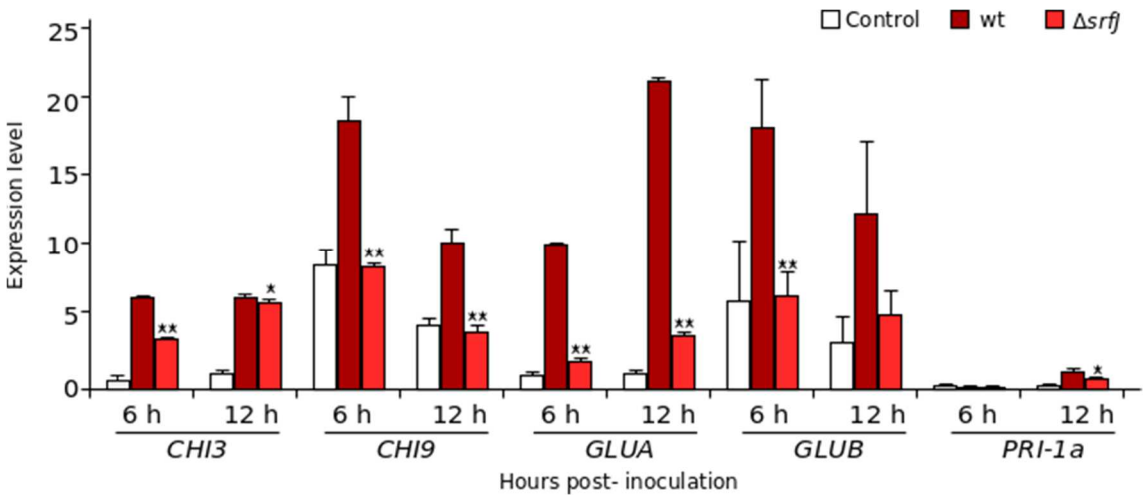


Figure R.1.14. Activation of defense response genes in plants. *Salmonella* wt, and *srfJ* mutant were used to spray 3-week-old tomato plants grown in sterile conditions. Plants were sampled 6 and 12 hours post-inoculation. Relative expression levels of *CHI3*, *CHI9*, *GLUA*, *GLUB*, and *PR-1a* were assessed using qRT-PCR and normalized to the expression of the house-keeping gene for actin. Data are presented as mean values + standard deviations of three replicates. Asterisks indicate significant differences of mutants compared to wt by Student's *t*-test (**P*<0.05, ***P*<0.01).

Chapter 2

Transcriptomic effect of SrfJ in mammalian cell lines

Our aim in this chapter was to explore the transcriptome changes induced by *S.* Typhimurium in the mammalian host cell, and specifically the role of SrfJ in these changes. We used two different mammalian cell lines: (i) HeLa (ECAC no. 93021013), epithelial human cell line; and (ii) RAW264.7 (ECACC no.91062702), macrophage cell line established from a mice tumor induced by the Abelson murine leukemia virus.

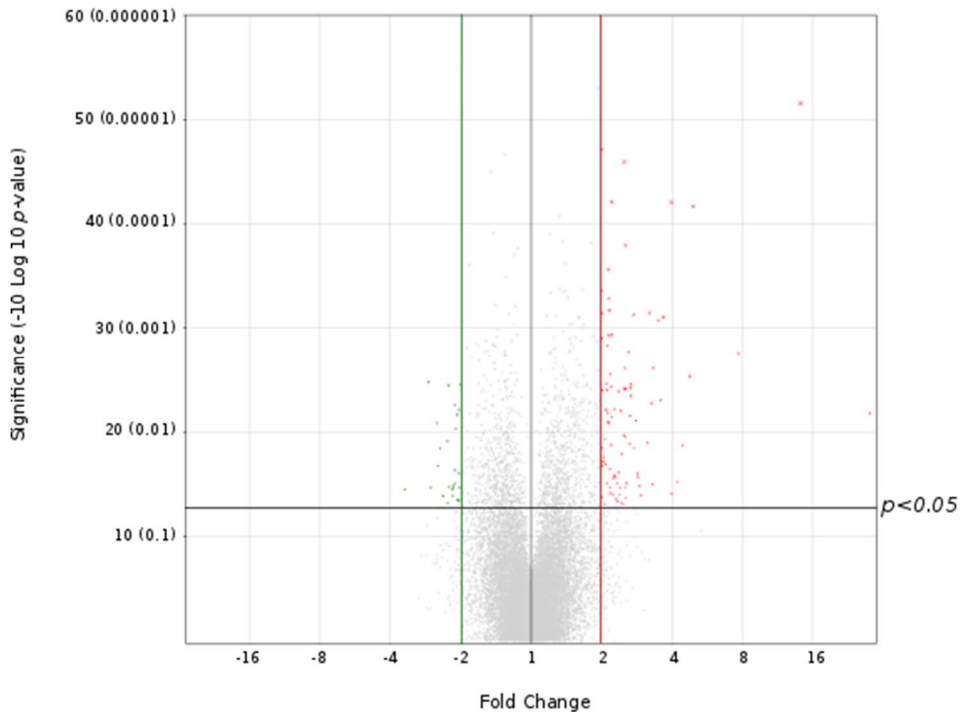
The RNA was analyzed using Affymetrix microarrays: *Clariom*^{TM} *S Assay, Mouse* for RAW264.7; and *Clariom*^{TM} *S Assay, Human* for HeLa cells. First, we analysed the changes induced by wt *Salmonella* infections in both cell types. Then, we studied the specific role of SrfJ in these changes using two strategies: (i) comparison of the effects of wt *S.* Typhimurium and a *srfJ* null mutant on RAW264.7 macrophages, and (ii) analysis of the effects of transfection of *srfJ* in HeLa cells.

2.1. Global analysis of gene expression in HeLa cells in response to *Salmonella* infection

RNA was obtained from HeLa cultures infected or not with *S.* Typhimurium (MOI 50:1) for 8 hours, and analysed using *Clariom*^{TM} *S Assay, Human* (Affymetrix), which has 21448 human genes represented.

Figure R.2.1. represents the number of genes with statistically significant (p<0.05) differential expression in infected vs non-infected cells applying or not a fold change threshold of 2.

A



B

Salmonella-infected HeLa cells vs non-infected HeLa cells		
	$p<0.05$	$p<0.05$. Fold change >2 or <-2
Total genes	1469	121
Down-regulated genes	675	27
Up-regulated genes	794	94

Figure R.2.1. Number of genes with differential expression in 14028-infected cells compared to non-infected HeLa cells. (**A**) Volcano plot of RNA abundance in infected and non-infected cells showing differential expression with a fold change thereshold of 2. Depicted in the plot is the comparison of infected/non-infected ratios versus *p*-values. Red dots: up-regulated genes. Green dots: down-regulated genes. Grey dots: genes not significantly differentially expressed for a $p<0.05$ or that do not reach the fold-change threshold. (**B**) Table showing the number of up-regulated or down-regulated genes with statistically significant ($p<0.05$) differential expression applying or not a fold change threshold of 2.

The 121 differentially expressed genes with a fold change > 2 are described in **Table R.2.1**.

Table R.2.1. Differentially expressed genes in infected HeLa cells vs non-infected HeLa cells (Fold change > 2 or < -2, $p < 0.05$).

***Salmonella*-infected HeLa cells vs non-infected HeLa cells**				
	Gene symbol	**Description**	**Fold change (linear)**	**ANOVA *p*-value**
Down- regulated	*YEATS4*	YEATS Domain Containing 4	-3.47	0.035401
	UGT2A3	UDP glucuronosyltransferase 2 family, polypeptide A3	-2.75	0.003302
	GULP1	GULP, engulfment adaptor PTB domain containing 1	-2.69	0.033907
	LONRF1	LON peptidase N-terminal domain and ring finger 1	-2.53	0.008158
	ARHGAP15	Rho GTPase activating protein 15	-2.51	0.020891
	DEPTOR	DEP domain containing MTOR-interacting protein	-2.45	0.014287
	SETD3	Histone-lysine N-methyltransferase	-2.38	0.040855
	FAM69A	Family with sequence similarity 69, member A	-2.28	0.012125
	RNF32	Ring finger protein 32	-2.28	0.048057
	TBC1D9	TBC1 domain family, member 9 (with GRAM domain)	-2.26	0.003577
	ANKK1	Ankyrin repeat and kinase domain containing 1	-2.25	0.033153
	ZSWIM2	Zinc finger, SWIM-type containing 2	-2.17	0.040709
	TMSB15A	Thymosin beta 15a	-2.17	0.035282
	GRIN1	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	-2.16	0.034287
	ZNF219	Zinc finger protein 219	-2.16	0.0324
	EDNRA	Endothelin receptor type A	-2.12	0.005493
	EFNA2	Ephrin-A2	-2.12	0.031318
	MEI1	Meiotic double-stranded break formation protein 1	-2.12	0.023082
	PSG1	Pregnancy specific beta-1-glycoprotein 1	-2.1	0.009287
	NMRK1	Nicotinamide riboside kinase 1	-2.07	0.044469
	OMD	Osteomodulin	-2.07	0.006807
	MSN	Moesin	-2.05	0.043753
	BMP4	Bone morphogenetic protein 4	-2.04	0.006078

	Gene symbol	Description	Fold change (linear)	ANOVA *p*-value
Down-regulated	*THRA*	Thyroid hormone receptor, alpha	-2.04	0.04498
	HOXB-AS3	HOXB cluster antisense RNA 3	-2.04	0.024674
	LOXL1	Lysyl oxidase-like 1	-2.03	0.033951
	TRAT1	T cell receptor associated transmembrane adaptor 1	-2.01	0.003476
Up-regulated	*PRG4*	Proteoglycan 4	2.01	0.000019
	SERPINE2	Serpin peptidase inhibitor, clade E (nexin. plasminogen activator inhibitor type 1), member 2	2.01	0.014185
	STEAP4	STEAP family member 4	2.01	0.000433
	SSX1	Synovial sarcoma, X breakpoint 1	2.01	0.00124
	LRRIQ3	Leucine-rich repeats and IQ motif containing 3	2.02	0.020916
	POGZ	Pogo transposable element withznf domain	2.02	0.041854
	PPP1R17	Protein phosphatase 1, regulatory subunit 17	2.02	0.041863
	PIP4K2A	Phosphatidylinositol-5-phosphate 4-kinase, type II, alpha	2.02	0.003976
	GPRC5A; MIR614	G protein-coupled receptor, class C, group 5, member A; microRNA 614	2.02	0.000718
	GDPD1	Glycerophosphodiester phosphodiesterase domain containing 1	2.02	0.019491
	KCNMB2	Potassium channel subfamily M regulatory beta subunit 2	2.03	0.018646
	WSB1	WD repeat and SOCS box containing 1	2.04	0.017284
	IL7R	Interleukin 7 receptor	2.07	0.011674
	CCDC84	Coiled-coil domain containing 84	2.07	0.048679
	ALKBH1	AlkB homolog 1, histone H2A dioxygenase	2.08	0.020377
	ZNF705CP	Zinc finger protein 705C, pseudogene	2.09	0.006046
	RUNX1	Runt-related transcription factor 1	2.09	0.00615
	RNF24	Ring finger protein 24	2.1	0.003441
	SCN8A	Sodium channel, voltage gated, type VIII alpha subunit	2.11	0.030855
	A2BP1	RNA binding protein, fox-1 homolog 1	2.11	0.003942
	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	2.12	0.001459
	UCN2	Urocortin 2	2.13	0.006572
	EFNA1	Ephrin-A1	2.14	0.000271
	HIST1H1A	Histone cluster 1, H1a	2.14	0.008203
	SNAPC4	Small nuclear RNA activating complex polypeptide 4	2.14	0.007974
	IFI16	Interferon gamma-inducible protein 16	2.15	0.000516
	EPAS1	Endothelial PAS domain protein 1	2.15	0.001162
	BCL3; MIR8085	B-cell CLL/lymphoma 3; microRNA 8085	2.16	0.000667
	TMEM106A	Transmembrane protein 106A	2.17	0.033622
	KLHL41	Kelch-like family member 41	2.18	0.038196
	MB21D2	Mab-21 domain containing 2	2.18	0.002763
	ICAM1	Intercellular adhesion molecule 1	2.18	0.022446
	CLDN1	Claudin 1	2.2	0.013152
	VPS11	Vacuolar protein sorting-associated protein 11 homolog	2.2	0.003656
	PLPP3	Phospholipid phosphatase 3	2.21	0.000061
	OR4F6	Olfactory receptor, family 4, subfamily F, member 6	2.21	0.007173
	TRAF4	TNF receptor-associated factor 4	2.21	0.001149
	IRF1	Interferon regulatory factor 1	2.24	0.039966

	Gene symbol	Description	Fold change (linear)	ANOVA *p*-value
Up-regulated	*DDX11*	DEAD/H-box helicase 11	2.25	0.027689
	PELI2	Pellino E3 ubiquitin protein ligase family member 2	2.26	0.025949
	TTC38	Tetratricopeptide repeat domain 38	2.27	0.006046
	PFKFB4; MIR6823	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4; microRNA 6823	2.28	0.026913
	ERGIC1	Endoplasmic reticulum-golgi intermediate compartment 1	2.31	0.043221
	AOAH	Acyloxyacyl hydrolase (neutrophil)	2.33	0.030804
	CTSS	Cathepsin S	2.34	0.045653
	ZNF705D	Zinc finger protein 705D	2.37	0.004079
	CCDC34	Coiled-coil domain containing 34	2.37	0.024375
	CREB5	cAMP responsive element binding protein 5	2.39	0.039885
	PTPRE	Protein tyrosine phosphatase, receptor type, E	2.4	0.034089
	KAT6B	K(lysine) acetyltransferase 6B	2.42	0.006272
	RELB	V-rel avian reticuloendotheliosis viral oncogene homolog B	2.43	0.047394
	MME	Membrane metallo-endopeptidase	2.44	0.016251
	ASMTL	Acetylserotonin O-methyltransferase-like	2.47	0.048418
	ASMTL	Acetylserotonin O-methyltransferase-like	2.47	0.048418
	KIAA0040	KIAA0040	2.49	0.010748
	SOD2	Superoxide dismutase 2, mitochondrial	2.5	0.000025
	PTPN2	Protein tyrosine phosphatase, non-receptor type-2	2.5	0.003839
	PDP1	Pyruvate dehyrogenase phosphatase catalytic subunit 1	2.52	0.002433
	LBP	Lipopolysaccharide binding protein	2.52	0.033594
	OCLM	Oculomedin	2.53	0.011058
	SNAI1	Snail family zinc finger 1	2.53	0.000161
	GRAMD1B	GRAM domain containing 1B	2.54	0.042753
	GADD45B	Growth arrest and DNA-damage-inducible, beta	2.54	0.003842
	TRIM15	Tripartite motif containing 15	2.55	0.030694
	HK2	Hexokinase 2	2.61	0.00167
	NFIL3	Nuclear factor, interleukin 3 regulated	2.64	0.012943
	TGM2	Transglutaminase 2	2.65	0.006944
	STRIP2	Striatin interacting protein 2	2.66	0.003754
	ZNF26	Zinc finger protein 26	2.67	0.003465
	IL4R	Interleukin 4 receptor	2.67	0.004484
	IER3	Immediate early response 3	2.75	0.000735
	CCDC84	Coiled-coil domain containing 84	2.76	0.014334
	PPAP2B	Phospholipid phosphatasen 3	2.81	0.007789
	SERPINB3	Serpin peptidase inhibitor, clade B (ovalbumin), member 3	2.85	0.024959
	C12ORF42	Chromosome 12 open reading frame 42	2.86	0.026905
	DEFB128	Defensin. beta 128	2.9	0.032736
	UBE2D3	Ubiquitin conjugating enzyme e2 d3	2.95	0.040277
	IL24	Interleukin 24	3.14	0.012747
	AGT	Angiotensinogen (serpin peptidase inhibitor. clade A. member 8)	3.21	0.000707
	FAP	Fibroblast activation protein alpha	3.27	0.005322
	ABCC3	ATP binding cassette subfamily C member 3	3.31	0.031503

	Gene symbol	Description	Fold change (linear)	ANOVA *p*-value
Up-regulated	*CFB*	Complement factor B	3.32	0.002421
	SAT1	Spermidine/spermine N1-acetyltransferase 1	3.5	0.000834
	ZFP36	ZFP36 ring finger protein	3.58	0.004936
	AIM1	Absent in melanoma 1	3.68	0.000773
	JUNB	Jun B proto-oncogene	3.97	0.000062
	ST5	Suppression of tumorigenicity	3.99	0.03885
	EHF	Ets homologous factor	4.21	0.029983
	DUSP5	Dual specificity phosphatase 5	4.44	0.013364
	FGA	Fibrinogen alpha chain	4.78	0.002936
	FGG	Fibrinogen gamma chain	4.93	0.000068
	FGB	Fibrinogen beta chain	7.69	0.001723
	CCL2	Chemokine (C-C motif) ligand 2	14.19	0.000007
	IL6	Interleukin 6	28.05	0.006588

To explore the main biological processes that are altered in HeLa cells during *Salmonella* infection, we carried out a GO enrichment analysis on the set of differentially expressed genes using the tool provided by the *Gene Ontology Consortium* (geneontology.org). The biological process significantly overrepresented in the set of up-regulated genes (false discovery rate < 0.05) are summarized in **Table R.2.2**. A similar analysis carried out on the set of down-regulated genes did not detect any significant overrepresentation of GO terms.

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Table R.2.2. Biological processes enriched in the set of genes up-regulated in HeLa cells 8 h after *Salmonella* infection.

Salmonella-infected HeLa cells vs non-infected HeLa cells							
			GO biological process	*Homo sapiens* total genes (21042)	Expected	Fold enrichment	Differentially up-regulated genes
Immune response	Innate immune response	General	Negative regulation of innate immune response	48	0.22	18.08	*MME, TNFAIP3, PTPN2, IFI16*
			Innate immune response	707	3.26	3.68	*LBP, CLDN1, FGA, CCL2, DEFB128, FGB, TRIM15, IFI16, ICAM1, RELB, CFB, IRF1*
		Signal transduction	Toll-like receptor signaling pathway	96	0.44	13.56	*CTSS, LBP, FGA, FGB, UBE2D3, FGG*
			Positive regulation of STAT cascade	78	0.36	11.12	*IL6, AGT, IL24, IL7R*
			Positive regulation of ERK1 and ERK2 cascade	195	0.90	6.67	*FGA, CCL2, FGB, ICAM1, ST5, FGG*
			Cellular response to LPS	145	0.67	10.47	*ZFP36, IL6, LBP, CCL2, TNFAIP3, IL24, ICAM1*
		Cytokines	Cellular response to IL-6	26	0.12	25.03	*IL6, ICAM1, FGG*
			Cellular response to IL-4	28	0.13	23.24	*NFIL3, IL24, IL4R*
			Regulation of IL-2 production	51	0.24	17.01	*ZFP36, TNFAIP3, RUNX1, FAP*
			Regulation of cytokine biosynthetic process	96	0.44	11.30	*ZFP36, IL6, LBP, IRF1, FAP*
			Positive regulation of protein secretion	237	1.09	6.41	*IL6, MME, FGA, FGB, IL4R, FGG, FAP*
			Positive regulation of exocytosis	80	0.37	10.85	*FGA, FGB, IL4R, FGG*
			Positive regulation of type I interferon production	73	0.34	11.89	*MME, TRIM15, IFI16, IRF1*
		Inflammation	Regulation of inflammatory response	383	1.77	5.10	*ZFP36, TGM2, IL6, LBP, AGT, TNFAIP3, PTPN2, CFB, AOAH*
			Inflammatory response	462	2.13	4.23	*IL6, LBP, CCL2, TNFAIP3, IFI16, IL4R, ICAM1, RELB, AOAH*
	Adaptive immune response		Regulation of adaptive immune response	133	0.61	8.16	*IL6, TNFAIP3, IL4R, IL7R, IRF1*
			Regulation of T cell differentiation	124	0.57	8.75	*PTPN2, IL4R, RUNX1, IL7R, IRF1*
			T cell differentiation	126	0.58	8.61	*IL6, PTPN2, RELB, IL7R, IRF1*
			Negative regulation of lymphocyte activation	128	0.59	8.47	*TNFAIP3, PTPN2, IL4R, IRF1, FAP*
Apoptosis			Negative regulation of endothelial cell apoptotic process	28	0.13	38.74	*FGA, FGB, TNFAIP3, ICAM1, FGG*
			Negative regulation of extrinsic apoptotic signaling pathway via death domain receptors	36	0.17	30.13	*FGA, FGB, TNFAIP3, ICAM1, FGG*

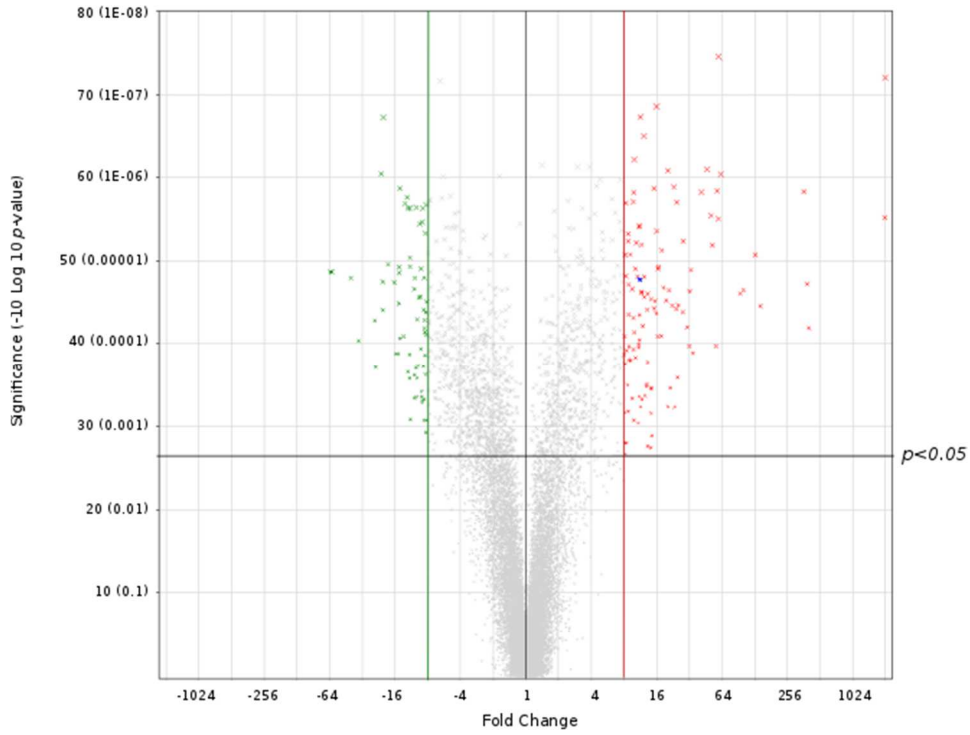
	GO biological process	*Homo sapiens* total genes (21042)	Expected	Fold enrichment	Differentially up-regulated genes
Cell adhesion	Positive regulation of heterotypic cell-cell adhesion	12	0.06	54.23	*FGA, FGB, FGG*
	Regulation of bicellular tight junction assembly	21	0.10	30.99	*SNAI1, CLDN1, RUNX1*
	Positive regulation of substrate adhesion-dependent cell spreading	34	0.16	19.14	*FGA, FGB, FGG*
	Homotypic cell-cell adhesion	52	0.24	16.69	*FGA, FGB, PPAP2B/PLPP3, FGG*
	Negative regulation of cell-cell adhesion	150	0.69	8.68	*SERPINE2, PTPN2, IL4R, FGG, IRF1, FAP*
Cell proliferation	Negative regulation of tissue remodeling	20	0.09	32.54	*SNAI1, CLDN1, RUNX1*
	Anatomical structure formation involved in morphogenesis	840	3.87	3.10	*JUNB TGM2, SNAI1, DUSP5, CCL2, KLHL41, TRIM15, EPAS1, SAT1, FAP, EFNA1, FAP*
	Regulation of cell proliferation	1588	7.32	2.60	*JUNB, ZFP36, SOD2, TGM2, IL6, MME, SERPINE2, KLHL41, AGT, TNFAIp3, PTPN2, SERPINB3, IL24, IL4R, SAT1, FAP, IL7R, IRF1, FAP*
	Positive regulation of biosynthetic process	1831	8.44	2.37	*JUNB, SOD2, IL6, SNAI1, MME, LBP, CCL2, AGT, DDX11, PTPN2, IFI16, RUNX1, ICAM1, RELB, EPAS1, CREB5, KAT6B, EHF, IRF1, FAP*
Circulatory system	Plasminogen activation	11	0.05	59.16	*FGA, FGB, FGG*
	Fibrinolysis	21	0.10	30.99	*FGA, FGB, FGG*
	Positive regulation of vasoconstriction	33	0.15	26.29	*FGA, FGB, ICAM1, FGG*
	Blood coagulation, fibrin clot formation	26	0.12	25.03	*FGA, FGB, FGG*
	Blood vessel remodeling	32	0.15	20.34	*TGM2, AGT, EPAS1*
	Negative regulation of hemopoiesis	128	0.59	8.47	*ZFP36, PTPN2, IL4R, RUNX1, IRF1*
Nervous system	Neuronal action potential	30	0.14	21.69	*SCN8A, KCNMB2, KCNMB2*
	Myeloid cell differentiation	197	0.91	7.71	*JUNB, PIP4K2A, PTPN2, IFI16, RUNX1, RELB, EPAS1*
Other signal transduction	Response to calcium ion	121	0.56	10.76	*JUNB, FGA, FGB, KCNMB2, FGG*
	Response to glucocorticoid	141	0.65	7.69	*ZFP36, IL6, CLDN1, AGT, ICAM1*

2.2. Global analysis of gene expression in RAW264.7 cells in response to *Salmonella* infection

RNA was obtained from RAW264.7 cultures infected or not with *S.* Typhimurium (MOI 50:1) for 8 h, and analysed using microarrays *Clariom*^{TM} *S Assay, MOUSE* (Affymetrix), which has 22206 mice genes represented.

Figure R.2.2. represents the number of genes with statistically significant (p<0.05) differential expression in infected vs non-infected cells applying or not a fold change threshold of 8.

A



B

Salmonella-infected RAW264.7 cells vs non-infected RAW264.7 cells		
	p<0.05	p<0.05. Fold change >8 or <-8
Total genes	5468	185
Down-regulated genes	3066	69
Up-genes genes	2402	116

Figure R.2.2. Number of genes with differential expression in 14028-infected RAW264.7 vs non-infected RAW264.7 cells. (**A**) Volcano plot of RNA abundance in infected and non-infected cells showing differential expression applying a fold change thereshold of 8. Depicted in the plot is the comparison of infected/non-infected ratios versus *p*-values. Red dots: up-regulated genes. Green dots: down-regulated genes. Grey dots: genes not significantly differentially expressed for a p<0.05 or that do not reach the fold-change threshold. (B) Table showing the number of up-regulated or down-regulated genes with statistivally significant (p<0.05) dfferential expression applying or not a fold change threshold of 8.

The 185 differentially expressed genes with a fold change threshold of 8 are described in **Table R.2.3**.

Table R.2.3. Differentially expressed genes in infected RAW cells vs non-infected RAW cells (Fold Change > 8 or < -8, $p < 0.05$).

Salmonella-infected RAW cells vs non-infected RAW cells				
	Gene Symbol	**Description**	**Fold Change (linear)**	**ANOVA *p*-value**
Down-regulated	*RGS2*	Regulator of G-protein signaling 2	-62.88	0.000013
	RASGRP3	RAS, guanyl releasing protein 3	-60.75	0.000014
	PTCHD1	Patched domain containing 1	-40.84	0.000016
	PTPN22	Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	-34.73	0.000091
	HIST1H1A	Histone cluster 1, H1a	-24.55	0.000052
	CD33	CD33 antigen	-24.09	0.000193
	LPL	Lipoprotein lipase	-21.47	8.95E-07
	RGS18	Regulator of G-protein signaling 18	-20.75	0.000018
	TCF7L2	Transcription factor 7 like 2, T cell specific. HMG box	-20.73	0.000039
	CELF2	CUGBP, Elav-like family member 2	-20.44	1.88E-07
	PLAU	Plasminogen activator. urokinase	-18.43	0.000011
	E2F8	E2F transcription factor 8	-16.26	0.000018
	CXCR4	Chemokine (C-X-C motif) receptor 4	-15.67	0.000131
	ALOX5	Arachidonate 5-lipoxygenase	-14.86	0.000131
	IL1RL1	Interleukin 1 receptor-like 1	-14.79	0.000033
	MEF2C	Myocyte enhancer factor 2C	-14.77	0.000012
	ANK; MIR7117	Progressive ankylosis; microRNA 7117	-14.62	0.000014
	CCND1; MIR3962	Cyclin D1; microRNA 3962	-14.43	0.000001
	NEIL3	Nei like 3 (*E. coli*)	-14.42	0.000086
	HIST1H1B	Histone cluster 1, H1b	-13.36	0.000081
	OIP5	Opa interacting protein 5	-12.9	0.000002
	RRM2	Ribonucleotide reductase M2	-12.43	0.000002
	9930111J21RIK1; 9930111J21RIK2	RIKEN cDNA 9930111J21 gene 1; RIKEN cDNA 9930111J21 gene 2	-12.21	0.000002
	HIST1H3G	Histone cluster 1, H3g	-12.13	0.000221
	9930111J21RIK2	RIKEN cDNA 9930111J21 gene 2	-11.73	0.000009
	RNF144B	Ring finger protein 144B	-11.73	0.000133
	RASGEF1B	RasGEF domain family, member 1B	-11.68	0.000263
	MCM6	Minichromosome maintenance deficient 6 (MIS5 homolog. *S. pombe*) (*S. cerevisiae*)	-11.64	0.000002
	DAGLB	Diacylglycerol lipase, beta	-11.61	0.000012
	TBXAS1	Thromboxane A synthase 1, platelet	-11.57	0.000828
	KITL	Kit ligand	-10.66	0.000457
	ST8SIA4	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4	-10.65	0.000022
	ITGA4	Integrin alpha 4	-10.62	0.00024
	FGD4	FYVE, RhoGEF and PH domain containing 4	-10.5	0.000016

	Gene Symbol	Description	Fold Change (linear)	ANOVA *p*-value
Down-regulated	*TCF19*	Transcription factor 19	-10.25	0.000197
	TLR7	Toll-like receptor 7	-10.19	0.000002
	PHKA2	Phosphorylase kinase alpha 2	-10.17	0.000441
	HIST1H2BF	Histone cluster 1, H2bf	-10.03	0.000259
	RCBTB2	Regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 2	-10.02	0.000051
	HIST1H2AE	Histone cluster 1, H2ae	-9.82	0.000184
	PLXNA2	Plexin A2	-9.66	0.000027
	ZFP36L2	Zinc finger protein 36, C3H type-like 2	-9.47	0.000004
	CHAF1B	Chromatin assembly factor 1, subunit B (p60)	-9.31	0.000027
	FAM49A	Family with sequence similarity 49, member A	-9.3	0.000114
	HIST1H3E	Histone cluster 1, H3e	-9.27	0.000012
	RCAN2	Regulator of calcineurin 2	-9.15	0.000379
	RASA3	RAS p21 protein activator 3	-9.09	0.000439
	TFDP2	Transcription factor Dp 2	-9.01	0.000484
	MYO1F	Myosin IF	-9.01	0.000522
	2810417H13RIK	RIKEN cDNA 2810417H13 gene	-8.98	0.000003
	1810011O10RIK	RIKEN cDNA 1810011O10 gene	-8.89	0.000002
	HIST1H2BM	Histone cluster 1, H2bm	-8.78	0.000186
	SLC14A1	Solute carrier family 14 (urea transporter), member 1	-8.67	0.000039
	HIST1H2AB	Histone cluster 1, H2ab	-8.65	0.000471
	HIST1H2AO; HIST1H2AP; HIST1H2AI; HIST1H2AH	Histone cluster 1. H2ao; histone cluster 1, H2ap; histone cluster 1. H2ai; histone cluster 1, H2ah	-8.63	0.000016
	HIST1H2AP	Histone cluster 1, H2ap	-8.63	0.000016
	HIST1H2BN	Histone cluster 1, H2bn	-8.6	0.000841
	SH2D3C	SH2 domain containing 3C	-8.57	0.000065
	SLC9A9	Solute carrier family 9 (sodium/hydrogen exchanger), member 9	-8.54	0.000071
	CD180	CD180 antigen	-8.52	0.000137
	FAM64A	Family with sequence similarity 64, member A	-8.51	0.000051
	HIST1H2BK	Histone cluster 1, H2bk	-8.44	0.000236
	HIST1H3H	Histone cluster 1, H3h	-8.42	0.000005
	HIST1H2BG	Histone cluster 1, H2bg	-8.38	0.000041
	HIST1H2BJ	Histone cluster 1, H2bj	-8.36	0.001177
	HIST1H3I	Histone cluster 1, H3i	-8.3	0.000002
	HIST1H2BH	Histone cluster 1, H2bh	-8.26	0.000077
	HIST1H2AI	Histone cluster 1, H2ai	-8.18	0.000031
	DGKG	Diacylglycerol kinase, gamma	-8.18	0.000844
Up-regulated	*CCR1*	Chemokine (C-C motif) receptor 1	8.06	0.000133
	GM10719	Predicted gene 10719	8.08	0.001553
	DPY19L3	Dpy-19-like 3 (*C. elegans*)	8.11	0.000683
	ERICH2	Glutamate rich 2	8.14	0.000081
	MNDA	Myeloid cell nuclear differentiation antigen	8.16	0.000008

	Gene Symbol	Description	Fold Change (linear)	ANOVA *p*-value
Up-regulated	*ZFP36*	Zinc finger protein 36	8.19	0.000174
	IGF2BP2	Insulin-like growth factor 2 mRNA binding protein 2	8.24	0.000009
	CSF2RB	Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	8.27	0.000002
	PIM2	Proviral integration site 2	8.28	0.002143
	IRF1	Interferon regulatory factor 1	8.36	0.000015
	GM10718; GM10722	Predicted gene 10718; predicted gene 10722	8.44	0.001572
	ANTXR1	Anthrax toxin receptor 1	8.47	0.00012
	DHX58	DEXH (Asp-Glu-X-His) box polypeptide 58	8.66	0.000316
	MS4A6D	Membrane-spanning 4-domains, subfamily A, member 6D	8.75	0.000005
	KDM5B	Lysine (K)-specific demethylase 5B	8.79	0.000655
	HIF1A	Hypoxia inducible factor 1, alpha subunit	8.79	0.000006
	IFITM6	Interferon induced transmembrane protein 6	8.81	0.000044
	TNFSF14	Tumor necrosis factor (ligand) superfamily, member 14	8.85	0.000019
	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	8.87	0.000156
	PROCR	Protein C receptor, endothelial	8.91	0.000107
	SLFN2	Schlafen 2	9.18	0.000008
	SLC9A3R1	Solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1	9.18	0.000157
	GM10197	Predicted gene 10197	9.56	0.00046
	JDP2	Jun dimerization protein 2	9.57	0.000022
	BNIP3	BCL2/adenovirus E1B interacting protein 3	9.74	0.000116
	BHLHE40	Basic helix-loop-helix family, member e40	9.75	0.000002
	AQP9	Aquaporin 9	9.81	0.000049
	TRIB3	Tribbles homolog 3 (*Drosophila*)	9.87	0.000001
	GM10801	Predicted gene 10801	9.91	0.00084
	JAK2	Janus kinase 2	9.96	6.09E-07
	SBNO2	Strawberry notch homolog 2 (*Drosophila*)	9.97	0.000073
	GGTA1	Glycoprotein galactosyltransferase alpha, 3	10.16	0.000012
	KLF2	Kruppel-like factor 2 (lung)	10.23	0.000147
	GYS1	Glycogen synthase 1, muscle	10.44	0.000006
	FCGR2B	Fc receptor, IgG, low affinity IIb	10.69	0.000015
	GM10720	Predicted gene 10720	10.88	0.000913
	BCAT1	Branched chain aminotransferase 1, cytosolic	10.97	0.0001
	IRF7	Interferon regulatory factor 7	11	0.000109
	P2RY2	Purinergic receptor P2Y, G-protein coupled 2	11.05	0.000004
	PLOD2	Procollagen lysine. 2-oxoglutarate 5-dioxygenase 2	11.07	0.000437
	ARC	Activity regulated cytoskeletal-associated protein	11.14	0.000045
	FZD7	Frizzled homolog 7 (*Drosophila*)	11.16	0.000089
	IL1A	Interleukin 1 alpha	11.2	0.000017
	SLC7A2	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 2	11.23	0.000004

	Gene Symbol	Description	Fold Change (linear)	ANOVA *p*-value
Up-regulated	*SRGN*	Serglycin	11.42	1.86E-07
	SMIM3	Small integral membrane protein 3	11.45	0.000581
	IL13RA1	Interleukin 13 receptor, alpha 1	11.58	0.000024
	SDC4	Syndecan 4	11.63	0.000006
	JUNB	Jun B proto-oncogene	11.65	0.000023
	ADORA2B	Adenosine A2b receptor	11.71	0.000473
	MCOLN2	Mucolipin 2	11.94	0.00006
	I830077J02RIK	RIKEN cDNA I830077J02 gene	12.27	0.000016
	BST1	Bone marrow stromal cell antigen 1	12.31	3.16E-07
	FCGR1	Fc receptor, IgG, high affinity I	12.34	0.000027
	CHPF	Chondroitin polymerizing factor	12.51	0.000425
	GJA1	Gap junction protein, alpha 1	12.91	0.000331
	CSRNP1	Cysteine-serine-rich nuclear protein 1	12.93	0.00031
	GPR35	G protein-coupled receptor 35	13.08	0.000038
	PPP1R3B	Protein phosphatase 1, regulatory (inhibitor) subunit 3B	13.24	0.001733
	GBP3	Guanylate binding protein 3	13.26	0.000166
	ZC3H12D	Zinc finger CCCH type containing 12D	13.31	0.000025
	KDM6B	KDM1 lysine (K)-specific demethylase 6B	14.1	0.000344
	GM10721	Predicted gene 10721	14.12	0.001798
	GCNT2	Glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	14.28	0.000691
	SERPINB2	Serine (or cysteine) peptidase inhibitor, clade B, member 2	14.34	0.000029
	IL1F6	Interleukin 1 family, member 6	14.35	0.000354
	GM17535	Predicted gene 17535	14.54	0.001291
	RND1	Rho family GTPase 1	15.15	0.000037
	SLPI; MIR7678	Secretory leukocyte peptidase inhibitor; microRNA 7678	15.22	0.000001
	CASP4	Caspase 4, apoptosis-related cysteine peptidase	15.48	0.00003
	OSBP2	Oxysterol binding protein 2	15.89	0.000042
	ODC1	Ornithine decarboxylase, structural 1	15.94	1.39E-07
	SOAT2	Sterol O-acyltransferase 2	16.04	0.000004
	CCL7	Chemokine (C-C motif) ligand 7	16.42	0.000081
	MXD1	MAX dimerization protein 1	16.5	0.000012
	NDRG1	N-myc downstream regulated gene 1	16.62	0.000012
	GBP7	Guanylate binding protein 7	17.74	0.000079
	STAT3	Signal transducer and activator of transcription 3	17.77	0.000007
	FCGR4	Fc receptor, IgG, low affinity IV	18.49	0.000021
	CHST11	Carbohydrate sulfotransferase 11	19.66	0.00003
	HMOX1	Heme oxygenase 1	20.33	8.14E-07
	GM10715	Predicted gene 10715	20.45	0.000577
	CTH	Cystathionase (cystathionine gamma-lyase)	20.75	0.000023
	IL19	Interleukin 19	21.42	0.000345
	ABCA1	ATP-binding cassette, sub-family A (ABC1). member 1	22.37	0.000035

	Gene Symbol	Description	Fold Change (linear)	ANOVA *p*-value
Up-regulated	*NFKBIZ*	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, zeta	23.01	0.000001
	MARCO	Macrophage receptor with collagenous structure	23.45	0.000584
	ZC3H12A	Zinc finger CCCH type containing 12A	24.36	0.000037
	SOD2	Superoxide dismutase 2, mitochondrial	24.53	0.000002
	IL6	Interleukin 6	25	0.000254
	IL1F9	Interleukin 1 family, member 9	25.37	0.000034
	DDIT4	DNA-damage-inducible transcript 4	27.89	0.000041
	NUPR1	Nuclear protein transcription regulator 1	28.24	0.000006
	ERO1L	ERO1-like (*S. cerevisiae*)	30.52	0.000063
	GLIPR2	GLI pathogenesis-related 2	32	0.000105
	CXCL2	Chemokine (C-X-C motif) ligand 2	32.39	0.000023
	IER3	Immediate early response 3	33.24	0.000013
	HILPDA	Hypoxia inducible lipid droplet associated	34.76	0.000128
	PTGS2	Prostaglandin-endoperoxide synthase 2	41.46	0.000001
	SERPINE1	Serine (or cysteine) peptidase inhibitor, clade E, member 1	46.74	7.96E-07
	MMP13	Matrix metallopeptidase 13	50.7	0.000003
	CD274	CD274 antigen	52.26	0.000007
	TGM1	Transglutaminase 1, K polypeptide	56.51	0.000106
	BCL3	B cell leukemia/lymphoma 3	57.83	0.000001
	PPBP	Pro-platelet basic protein	59.22	0.000003
	TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	59.52	3.52E-08
	SLC7A11	Solute carrier family 7 (cationic amino acid transporter (y+ system), member 11	62.68	9.15E-07
	IL4RA	Interleukin 4 receptor, alpha	94.6	0.000025
	CCL5	Chemokine (C-C motif) ligand 5	100.48	0.000022
	SOCS3	Suppressor of cytokine signaling 3	128.74	0.000008
	IL1B	Interleukin 1 beta	144.49	0.000035
	IL1RN	Interleukin 1 receptor antagonist	363.72	0.000001
	IRG1	Immunoresponsive gene 1	388.96	0.000019
	NOS2	Nitric oxide synthase 2, inducible	404.73	0.000064
	SAA3	Serum amyloid A 3	2031.49	0.000003
	LCN2	Lipocalin 2	2046.19	6.30E-08

A GO enrichment analysis carried out on these set of genes yielded significant results for up-regulated genes in the three categories provided by the *Gene Ontology Consortium*: cellular components, molecular functions and biological processes, and for the set of down-regulated genes in the categories of cellular components and biological processes. **Table R.2.4** shows the main enriched cellular components among the genes whose expression are modified during *Salmonella* infection.

Table R.2.4. Cellular components enriched in the set of genes up- or down-regulated in RAW264.7 after 8 h of infection (Fold change <8 or <-8, p<0.05).

GO cellular component	*Mus musculus* total genes (22262)	Expected	Fold Enrichment	Differentially up-regulated genes
Extracellular space	3739	19.31	1.97	*PROCR, LCN2, GBP3, HILPDA, TNFS14, PLOD2, SOD2, GLIPR2,IL19, SDC4, TGM1, PPNBP, MMP13, IL4RA, TNFRSF9, CCL7, TNFAIP3, IL1F9, FCGR4, NDRG1, IL1F6, IL1RN, SERPINE1, CTH, IL1B, IL1A, ABCA1, CCL5, IL6, NOS2, CXCL, SAA3, SLC9A3R1, BST1, SERPINB2, GJA1, ANTXR1, CD274*
GO cellular component	***Mus musculus* total genes (22262)**	**Expected**	**Fold Enrichment**	**Differentially expressed down-regulated genes**
Nucleosome	56	0.14	44.17	*HIST1H2BM, HIST1H2BK, HIST1H3G/HIST1H3H/HIST1H3I, HIST1H2BH, HIST1H1A, HIST1H1B*

Table R.2.5 summarizes the main enriched molecular functions in the set of up-regulated genes in RAW264.7 during *Salmonella* infection.

Table R.2.5. Molecular functions enriched in the set of genes that are up-regulated in RAW264.7 cells 8 h after infection with wt *Salmonella* (Fold change > 8 or <-8, p<0.05).

GO molecular function	*Mus musculus* total genes (22262)	Expected	Fold Enrichment	Differentially expressed up-regulated genes
IL-1 receptor binding	19	0.10	50.94	*IL1F9, IL1F6, IL1RN, IL1B, IL1A*
chemokine activity	41	0.21	18.89	*PPBP, CCL7, CCL5, CXCL2*
cytokine receptor activity	91	0.47	10.64	*IL13RA1, IL4RA, CCR1, GPR35, CSF2RB*

Table R.2.6 describes the main enriched biological processes among the genes that are down-regulated in RAW264.7 cells in response to *Salmonella* infection.

Table R.2.6. Biological processes that are overrepresented in the set of genes that are down-regulated in RAW264.7 mcrophages in response to *Salmonella* infection (Fold change > 8 or < -8, $p < 0.05$).

	GO biological process	*Mus musculus* (22262)	Expected	Fold Enrichment	Differentially down-regulated genes
Immune response	Innate immune response in mucosa	13	0.03	95.14	*HIST1H2BK, HIST1H2BG, HIST1H2BN/HIST1H2BN/HIST1H2BF*
	Antibacterial humoral response	28	0.07	44.17	*HIST1H2BK, HIST1H2BG, HISTH2BN/HIST1H2BJ/HIST1H2BF*
Nucleosome assembly		77	0.19	53.54	*HIST1H2BM, HIST1H2BK, CHAF1B, HIST1HBG, HIST1H2BN/HIST1H2BJ/HIST1H2BF, HIST1H3G/HIST1H3H/HIST1H3I, HIST1H2BH, HIST1H1A, HIST1H1B, OIP5*			

Finally, **Table R.2.7** shows the main enriched biological processes among the genes up-regulated in response to *Salmonella* infection.

Table R.2.7. Biological processes that are overrepresented in the set of genes that are up-regulated in RAW264.7 macrophages in response to *Salmonella* infection (Fold change > 8 or < -8, $p < 0.05$).

				GO biological process	*Mus musculus* total genes (22262)	Expected	Fold Enrichment	Differentially up-regulated genes
Immune response	Innate immune response		General	Negative regulation of innate immune response	51	0.26	15.18	*DHX58, TNFAIP3, CCR1, IRG1*
				Negative regulation of immune effector process	121	0.63	8.00	*IRF1, DHX58, IL4RA, TNFAIP3, HMOX1*
				Tolerance induction to LPS	2	0.01	> 100	*TNFAIP3, IRG1*
			Response to pathogens	Defense response to protozoan	32	0.17	30.25	*BCL3, GBP3, IL4RA, GBP7, IL6*
				Regulation of defense response to virus	41	0.21	14.16	*IL1B, CCL5, ZC3H12A*
				Defense response to virus	156	0.81	7.45	*IRF1, DHX58, IL6, BNIP3, DDIT4, IRF7,*
			Fever	Fever generation	6	0.03	96.79	*IL1RN, IL1B, IL1A*

				GO biological process	*Mus musculus* total genes (22262)	Expected	Fold Enrichment	Differentially up-regulated genes
Immune response	Innate immune response	Cytokines	Fever	Positive regulation of fever generation	9	0.05	64.53	*PTGS2, IL1B, CCL5*
			Signal transduction	Regulation of MyD88-dependent toll-like receptor signaling pathway	4	0.02	96.79	*IRF1, IRF7*
				JAK-STAT cascade involved in growth hormone signaling pathway	4	0.02	96.79	*STAT3, JAK2*
				I-κB kinase/NF-κB signaling	49	0.25	15.80	*BCL3, TNFSF14, IRF1, SAA3*
				Regulation of tyrosine phosphorylation of STAT protein	67	0.35	14.45	*IRF1, CCL5, IL6, JAK2, SOCS3*
				Positive regulation of I-κB kinase/NF-κB signaling	143	0.74	9.48	*TNFSF14, CTH, MOX1, IL1B, IL1A, PIM2, GJA1*
				Regulation of NF-κB import into nucleus	39	0.20	19.85	*TNFSF14, IL1B, ZC3H12A, ARC*
				Positive regulation of ERK1 and ERK2 cascade	201	1.04	8.67	*MARCO, GLIPR2, CCL7, CCR1, IL1B, IL1A, CCL5, IL6, GCNT2*
			General	Negative regulation of cytokine biosynthetic process	28	0.14	20.74	*BCL3, IL6, ZFP36*
				Negative regulation of cytokine secretion	57	0.29	13.58	*TNFRSF9, SRGN, IL6, ZC3H12A*
				Cytokine secretion	43	0.22	13.51	*MCOLN2, ABCA1, NOS2*
				Positive regulation of cytokine secretion	128	0.66	7.56	*CASP4, IL4RA, IL1B, IL1A, CD274*
			Interleukines	IL-6 production	8	0.04	72.59	*IL19, IL1B, NOS2*
				Regulation of IL-10 secretion	9	0.05	43.02	*TNFRSF9, CD274*
				IL-6-mediated signaling pathway	10	0.05	38.72	*STAT3, IL6*
				Positive regulation of IL-2 biosynthetic process	11	0.06	35.20	*IL1B, IL1A*
				Cellular response to IL-1	68	0.35	25.62	*IRF1, CCL7, SERPINE1, IL1A, CCL5, IL6, ZC3H12A, IRG1, SAA3*
				Regulation of IL-1 beta production	50	0.26	15.49	*CASP4, TNFAIP3, ZC3H12A, JAK2*

				GO biological process	*Mus musculus* total genes (22262)	Expected	Fold Enrichment	Differentially up-regulated genes
Immune response	Innate immune response	Cytokines	Interleukines	Positive regulation of IL-6 production	81	0.42	14.34	*IL1F9, IL1F6, ADORA2B, IL1B, IL1A, IL6*
				Negative regulation of IL-6 production	44	0.23	13.20	*KLF2, TNFAIP3, ZC3H12A*
				Negative regulation of IL-1-mediated signaling pathway	3	0.02	> 100	*IL1RN, IL6*
			Chemokines	Positive regulation of monocyte chemotactic protein-1 production	10	0.05	58.07	*MCOLN2, IL1B, IL1A*
				Positive regulation of chemokine (C-X-C motif) ligand 2 production	9	0.05	43.02	*MCOLN2, CCL5*
				Positive regulation of monocyte chemotaxis	18	0.09	32.26	*CCR1, SERPINE1, CCL5*
				Regulation of chemokine biosynthetic process	12	0.06	32.26	*IL1B, IL6*
				Chemokine-mediated signaling pathway	49	0.25	23.70	*PPBP, CCL7, CCR1, CCL5, GPR35, CXCL2*
				Chemokine-mediated signaling pathway	49	0.25	23.70	*PPBP, CCL7, CCR1, CCL5, GPR35, CXCL2*
				Positive regulation of neutrophil chemotaxis	29	0.15	20.03	*PPBP, IL1B, CXCL2*
				Neutrophil chemotaxis	62	0.32	12.49	*CCL7, IL1B, CCL5, CXCL2*
			Interferon	IFN-γ-mediated signaling pathway	6	0.03	64.53	*IRF1, JAK2*
				Negative regulation of type I interferon production	23	0.12	25.25	*GBP3, DHX58, IRG1*
				Cellular response to IFN-β	39	0.20	14.89	*GBP3, IRF1, IRG1*
				Positive regulation of type I interferon production	48	0.25	12.10	*IRF1, DHX58, IRF7*
				Regulation of IFN-γ production	100	0.52	7.74	*BCL3, IL1B, ZC3H12A, CD274*
			TNF	Negative regulation of TNF superfamily cytokine production	51	0.26	15.18	*BCL3, TNFAIP3, ZC3H12A, CD274*
				Regulation of TNF production	129	0.67	7.50	*BCL3, TNFAIP3, ZC3H12A, JAK2, ZFP36*
				Cellular response to TNF	111	0.57	13.95	*IRF1, CCL7, CCL5, IL6, ZC3H12A, IRG1, JAK2, ZFP36*
				Cellular response to TNF	111	0.57	13.95	*IRF1, CCL7, CCL5, IL6, ZC3H12A, IRG1, JAK2, ZFP36*

				GO biological process	*Mus musculus* total genes (22262)	Expected	Fold Enrichment	Differentially up-regulated genes
Immune response	Innate immune response	Cellular defense response	Reactive oxygen species	Oxygen homeostasis	5	0.03	77.43	*SOD2, HIF1A*
				Negative regulation of reactive oxygen species metabolic process	62	0.32	12.49	*HIF1A, STAT3, ZC3H12A, BNIP3*
				Regulation of transcription from RNA polymerase II promoter in response to oxidative stress	11	0.06	35.20	*HIF1A, HMOX1*
				Cellular response to hydrogen peroxide	49	0.25	19.75	*LCN2, TNFAIP3, KDM6B, IL6, BNIP3*
			NO	Nitric oxide biosynthetic process	10	0.05	38.72	*SLC7A2, NOS2*
				Positive regulation of nitric oxide biosynthetic process	46	0.24	25.25	*SOD2, PTGS2, KLF2, IL1B, IL6, JAK2*
		Inflammatory response		Connective tissue replacement involved in inflammatory response wound healing	4	0.02	96.79	*HIF1A, IL1A*
				Regulation of chronic inflammatory response	11	0.06	35.20	*TNFAIP3, ADORA2B*
				Negative regulation of inflammatory response	120	0.62	9.68	*IER3, TNFAIP3, ZC3H12A, IRG1, ZFP36, SOCS3*
				Positive regulation of macroautophagy	58	0.30	13.35	*HIF1A, HMOX1, PIM2, BNIP3*
				Response to granulocyte macrophage colony-stimulating factor	12	0.06	32.26	*JAK2, ZFP36*
				Regulation of mast cell degranulation	34	0.18	17.08	*IL4RA, ADORA2B, HMOX1*
	Adaptive immune response	T-helper		Positive regulation of T-helper 2 cell differentiation	7	0.04	55.31	*IL4RA, IL6*
				T-helper 17 cell lineage commitment	8	0.04	48.40	*STAT3, IL6*
				Positive regulation of T cell chemotaxis	11	0.06	35.20	*TNFSF14, CCL5*
				Regulation of T-helper 1 cell differentiation	12	0.06	32.26	*IRF1, IL4RA*
				Negative regulation of T cell activation	108	0.56	8.96	*IRF1, SDC4, IL4RA, ZC3H12D, CD274*
				Positive regulation of T cell proliferation	96	0.50	8.07	*IL1B, CCL5, IL6, CD274*

			GO biological process	*Mus musculus* total genes (22262)	Expected	Fold Enrichment	Differentially up-regulated genes
Immune response	Adaptive immune response	Leukocytes	Myeloid leukocyte activation	104	0.54	9.31	*SLC7A2, SBNO2, FCGR4, NDRG1, CCL5*
			Positive regulation of myeloid leukocyte mediated immunity	35	0.18	16.59	*IL4RA, FCGR1, ADORA2B*
			B-1 B cell homeostasis	5	0.03	77.43	*HIF1A, TNFAIP3*
			Positive regulation of myeloid cell differentiation	95	0.49	8.15	*HIF1A, CCR1, STAT3, CCL5*
Cell adhesion			Negative regulation of heterotypic cell-cell adhesion	10	0.05	38.72	*TNFAIP3, IL1RN*
			Positive regulation of heterotypic cell-cell adhesion	11	0.06	35.20	*IL1B, GCNT2*
			Regulation of cell-substrate adhesion	188	0.97	6.18	*SDC4, SERPINE1, FZD7, JAK2, GCNT2, BST1*
Cell proliferation			Positive regulation of growth factor dependent skeletal muscle satellite cell proliferation	2	0.01	> 100	*STAT3, JAK2*
			Positive regulation of smooth muscle cell proliferation	92	0.48	12.62	*PTGS2, HIF1A, HMOX1, CCL5, IL6, JAK2*
			Positive regulation of epithelial cell proliferation	181	0.94	7.49	*TGM1, KDM5B, TNFAIP3, FAZD7, STAT3, CCL5, IL6*
			Negative regulation of cell cycle	378	1.95	4.10	*IRF1, IER3, NUPR1, PTGS2, NOS2, TNFAIP3, ZC3H12D, SLC9A3R1*
			Negative regulation of cell proliferation	650	3.36	6.55	*SLFN2, IRF1, SOD2, SDC4, NUPR, PTGS2, TNFRSF9, TNFAIP3, NDRG1, STAT3, CTH, ZC3H12D, ADORA2B, HMOX1, IL1B, IL1A, PIM2, IL6, JAK2, SLC9A3R1, GJA1, CD274*
			Regeneration	81	0.42	9.56	*FZD7, HMOX1, JAK2, GJA1*
Cell differentiation			Brown fat cell differentiation	35	0.18	16.59	*PTGS2, ERO1L, BNIP3*

	GO biological process	*Mus musculus* total genes (22262)	Expected	Fold Enrichment	Differentially up-regulated genes
Cell differentiation	Positive regulation of epithelial to mesenchymal transition	44	0.23	13.20	*GLIPR2, IL1B, GCNT2*
	Regulation of fat cell differentiation	125	0.65	9.29	*SOD2, PTGS2, JDP2, ZC3H12A, TRIB3, ZFP36*
	Anatomical structure maturation	137	0.71	7.07	*PTGS2, HIF1A, KLF2, RND1, GJA1*
	Epithelial cell differentiation	498	2.57	3.50	*TGM1, PTGS2, HIF1A, KLF2, FZD7, IL1A, KDM6B, SLC9A3R1, GJA1*
	Negative regulation of cell differentiation	684	3.53	3.11	*IRF1, SOD2, IL4RA, JDP2, IL1F9, FZD7, STAT3, IL1B, IL1A, TRIB3, ZFP36*
	Anatomical structure morphogenesis	2049	10.58	2.08	*BLC3, CRNP1, SBNO2, CHST11, SDC4, TGM1, MMP13, PTGS2, KDM5B, HIF1A, KLF2, JUNB, FZD7, STAT3, HMOX1, KDM6B, IL6, ZC3H12A, SLC9A3R1, GJA1, ANTXR1, SOCS3*
	Regulation of erythrocyte differentiation	46	0.24	12.62	*HIF1A, STAT3, ZFP36*
	Marginal zone B cell differentiation	10	0.05	38.72	*BCL3, TNFAIP3*
Intracellular transport	Positive regulation of protein import into nucleus	103	0.53	11.28	*TNFSF14, PTGS2, IL1B, IL6, ZC3H12A, JAK2*
	Regulation of calcium ion transport	249	1.29	5.44	*PTGS2, CCR1, SERPINE1, CCL5, GPR35, ARC, GJA1,*
	Positive regulation of intracellular transport	231	1.19	5.03	*TNFSF14, IL4RA, ADORA2B, IL1B, IL6, JAK2*
	Organic anion transport	314	1.62	4.93	*SLC7A2, OSBP2, ABCA1, SLC7A11, NOS2, SLC9A3R1, GJA1, AQP9*

	GO biological process	*Mus musculus* total genes (22262)	Expected	Fold Enrichment	Differentially up-regulated genes
DNA/RNA	3'-UTR-mediated mRNA destabilization	13	0.07	44.67	*ZC3H12D, ZC3H12A, ZFP36*
	miRNA mediated inhibition of translation	12	0.06	32.26	*STAT3, ZFP36*
	Regulation of gene silencing by miRNA	35	0.18	16.59	*STAT3, ZC3H12A, ZFP36*
	Negative regulation of DNA binding transcription factor activity	145	0.75	6.68	*BHLHE40, TNFAIP3, HMOX1, ZC3H12A, IRG1*
	Regulation of transcription by RNA polymerase II	1848	9.55	2.62	*BCL3, CSRNP1, IRF1, SBNO2, HIF1A, KLF2, JUNB, STAT3, IL1B, IL1A, KDM6B, IL6, ZC3H12A, JAK2, IRF7*
Apoptosis	Ectopic germ cell programmed cell death	9	0.05	64.53	*CASP4, IL1B, IL1A*
	Cardiac muscle cell apoptotic process	6	0.03	64.53	*BNIP3, ARC*
	Negative regulation of extrinsic apoptotic signaling pathway via death domain receptors	28	0.14	27.65	*TNFAIP3, SERPINE1, HMOX1, ARC*
	Intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress	36	0.19	16.13	*CASP4, ERO1L, TRIB3*
	Negative regulation of muscle cell apoptotic process	51	0.26	15.18	*HMOX1, ZC3H12A, ARC, JAK2*
	Regulation of T cell apoptotic process	43	0.22	13.51	*HIF1A, CCL5, CD274*
	Negative regulation of epithelial cell apoptotic process	46	0.24	12.62	*TNFAIP3, SERPINE1, HMOX1*
	Regulation of cardiac muscle cell apoptotic process	48	0.25	12.10	*BNIP3, ARC, JAK2*
	Positive regulation of apoptotic process	591	3.05	4.26	*IL19, NUPR1, PTGS2, HIF1A, HMOX1, IL1B, CCL5, IL6, ZC3H12A, BNIP3, JAK2, SLC9A3R1, CD274*
	Extrinsic apoptotic signaling pathway in absence of ligand	39	0.20	14.89	*LCN2, IL1B, IL1A*
	Negative regulation of oxidative stress-induced intrinsic apoptotic signaling pathway	17	0.09	34.16	*SOD2, HIF1A, ARC*

		GO biological process	*Mus musculus* total genes (22262)	Expected	Fold Enrichment	Differentially up-regulated genes
Apoptosis		Negative regulation of mitochondrial membrane permeability involved in apoptotic process	4	0.02	96.79	*BNIP3, ARC*
		Intrinsic apoptotic signaling pathway in response to DNA damage by p53 class mediator	31	0.16	18.73	*BLC3, NUPR1, DDIT4*
External signals	Hormones	Positive regulation of prostaglandin secretion	12	0.06	48.40	*P2RY2, IL1B, IL1A*
		Cellular response to glucocorticoid stimulus	37	0.19	15.70	*JAK2, DDIT4, ZFP36*
		Regulation of insulin secretion	171	0.88	6.79	*HIF1A, IL1B, CCL5, JAK2, NOS2, GJA1*
		Gland development	392	2.02	3.95	*SOD2, KDM5B, HIF1A, HMOX1, IL6, JAK2, SLC9A3R1, GJA1*
		Positive regulation of steroid biosynthetic process	25	0.13	23.23	*ADORA2B, IL1B, IL1A*
	O_2	Regulation of aerobic respiration	8	0.04	48.40	*HIF1A, BNIP3*
		Response to hypoxia	179	0.92	6.49	*HIF1A, HMOX1, BNIP3, ARC, NOS2, DDIT4*
	Others	Response to antineoplastic agent	66	0.34	11.73	*HMOX1, ZC3H12A, JAK2, DDIT4*
		Sensory perception of chemical stimulus	1377	7.11	< 0.01	*GM17535*
		Response to herbicide	6	0.03	64.53	*LCN2, ZC3H12A*
		Cellular response to arsenic-containing substance	11	0.06	35.20	*HMOX1, ZC3H12A*
Metabolic process	Biosynthesis	Negative regulation of collagen biosynthetic process	11	0.06	35.20	*ADORA2B, IL6*
		Chondroitin sulfate biosynthetic process	9	0.05	43.02	*CHPF, CFST11*
		Protein oligomerization	527	2.72	3.67	*MCOLN2, LCN2, SOD2, TNFRSF9, CTH, HMOX1, CCL5, ZC3H12A, ARC, GJA1*
		Regulation of protein catabolic process	339	1.75	4.00	*ODC1, IER3, TNFAIP3, IL1B, NOS2, TRIB3, GJA1*
		Alpha-amino acid metabolic process	176	0.91	6.60	*ODC1, PLOD2, BCAT1, ERO1L, CTH, NOS2*
		Negative regulation of glycolytic process	12	0.06	48.40	*IER3, STAT3, DDIT4*

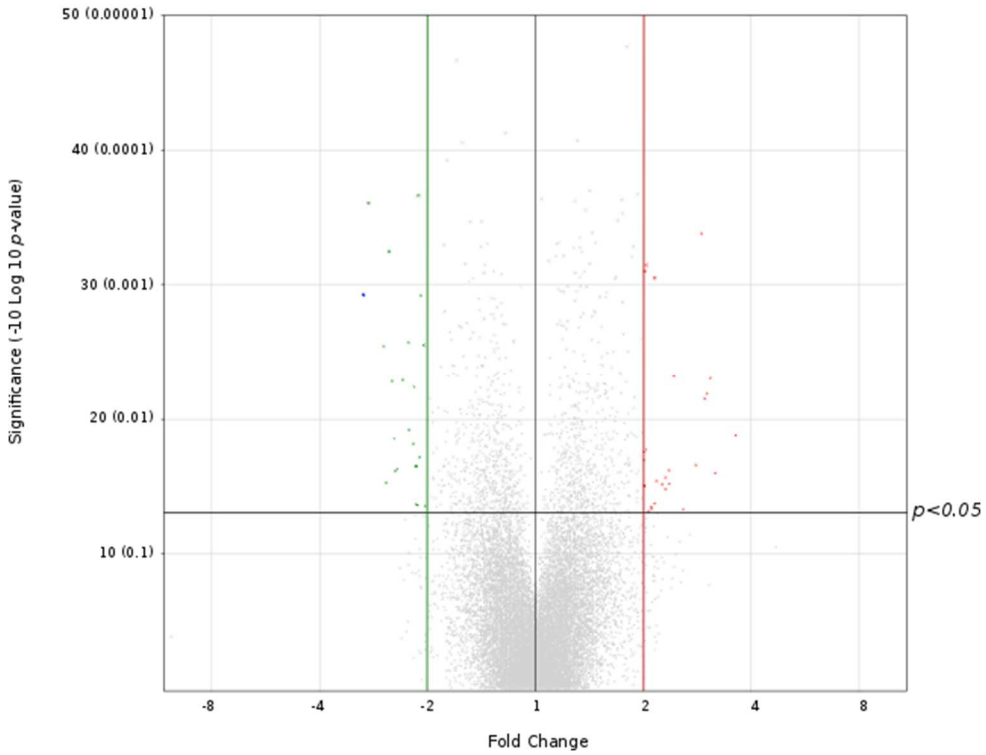
		GO biological process	*Mus musculus* total genes (22262)	Expected	Fold Enrichment	Differentially up-regulated genes
Metabolic process	Enzimatic activity	Negative regulation of protein phosphorylation	409	2.11	4.73	*GBP3, IRF1, TNFAIP3, IL1B, IL6, ZC3H12A, DDIT4, SLC9A3R1, TRIB3, SOCS3*
		Positive regulation of guanylate cyclase activity	5	0.03	77.43	*ADORA2B, NOS2*
		Positive regulation of metallopeptidase activity	7	0.04	55.31	*STAT3, ANTXR1*
	Others	Positive regulation of cytosolic calcium ion concentration	253	1.31	6.89	*P2RY2, CCR1, ERO1L, IL1B, GPR35, ARC, JAK2, CXCL2, GJA1*
		Iron ion homeostasis	85	0.44	9.11	*LCN2, SOD2, HIF1A, HMOX1*
		Negative regulation of glutamate secretion	9	0.05	43.02	*IL1RN, IL1B*
		Lipid localization	249	1.29	5.44	*SOAT2, OSBP2, OSBP2, ABCA1, NOS2, AQP9*
		Negative regulation of membrane potential	13	0.07	44.67	*IL1RN, IL6, BNIP3*
Tissue regulation	Circulatory system	Positive regulation of vascular endothelial growth factor production	24	0.12	40.33	*PTGS2, HIF1A, ADORA2B, IL1B, IL1A*
		Negative regulation of blood circulation	39	.20	14.89	*HIF1A, ZC3H12A, JAK2*
		Positive regulation of blood vessel diameter	75	0.39	12.91	*SOD2, HIF1A, ADORA2B, NOS2, GJA1.*
		Positive regulation of angiogenesis	150	0.77	11.61	*PTGS2, HIF1A, SERPINE1, STAT3, HMOX1, IL1B, IL1A, CCL5, ZC3H12A*
		Regulation of blood pressure	183	0.95	6.35	*IER3, SOD2, PTGS2, HMOX1, NOS2, GJA1*
		Circulatory system development	846	4.37	2.75	*SOD2, MMP13, PTGS2, HIF1A, GYS1, JUNB, HMOX1, KDM6B, ZC3H12A, GJA1, ANTXR1, SOCS3*
		Regulation of body fluid levels	331	1.71	4.09	*PROCR, P2RY2, MMP13, HIF1A, SERPINE1, SLC7A11, GJA1*

		GO biological process	*Mus musculus* total genes (22262)	Expected	Fold Enrichment	Differentially up-regulated genes
Tissue regulation	Nervous system	Regulation of neurological system process	92	0.48	8.42	*FCGR4, IL1A, IL6, GPR35*
		Negative regulation of neuron death	222	1.15	6.10	*SOD2, HIF1A, STAT3, HMOX1, CCL5, IL6, JAK2*
	Muscle cells	Regulation of myoblast fusion	24	0.12	24.20	*TNFSF14, IL4RA, IL1F9*
		Negative regulation of muscle contraction	30	0.15	19.36	*PTGS2, ADORA2B, ZC3H12A*
	Bone mineralization	Bone mineralization	34	0.18	17.08	*SBNO2, MMP13, PTGS2*
		Negative regulation of bone mineralization	18	0.09	32.26	*HIF1A, CCR1, SRGN*
	Embryo	Post-embryonic development	126	0.65	7.68	*CSRNP1, SOD2, CHST11, KDM5B, JAK2*
		Placenta development	165	0.85	5.87	*PTGS2, HIF1A, JUNB, SERPINE1, SOCS3*
		Positive regulation of epithelial cell migration	119	0.61	8.13	*GLIPR2, PTGS2, HIF1A, SERPINE1, ZC3H12A*

2.3. Specific effect of SrfJ in RAW264.7 macrophages infected with *S. enterica*

As a first approach to study the global effect of SrfJ on host cells, we compared the transcriptomes of RAW264.7 cells infected for 8 h with wt *S.* Typhimurium (strain 14028) or with and *srfJ* mutant (strain SV5559). RNA was analysed using microarrays *Clariom*^{TM} *S Assay, MOUSE* (Affymetrix). The number of genes with statistically significant (p<0.05) differential expression in this comparison are represented in **Figure R.2.3** applying or not a fold change threshold of 2.

A



B

Salmonella wild-type infected RAW264.7 vs *Salmonella srfJ*-infected RAW264.7 cells		
	$p<0.05$	$p<0.05$. Fold change >2 or <-2
Total genes	1188	33
Down-regulated genes	626	22
Up-genes genes	562	11

Figure R.2.3. Number of genes with differential expression in 14028-infected RAW264.7 cells vs *ΔsrfJ*-infected RAW264.7 cells. (A) Volcano plot of RNA abundance in wt and *srfJ* mutant infected cells showing differential expression with a fold change thereshold of 2. Depicted in the plot is the comparison of wt-infected/*srfJ*-infected ratios versus *p*-values. Red dots: up-regulated genes. Green dots: down-regulated genes. Grey dots: genes not significantly differentially expressed for a $p<0.05$ or that do not reach the fold-change threshold. **(B)** Table showing the number of up-regulated or down-regulated genes with statistically significant ($p<0.05$) differential expression applying or not a fold change threshold of 2.

The 33 differentially expressed genes with a fold change threshold of 2 are described in **Table R.2.8**.

Table R.2.8. RAW 14028 vs RAW 14028 *ΔsrfJ::lacZ* differentially expressed genes. (Fold change > 2 or < -2, $p < 0.05$).

Salmonella wt-infected RAW264.7 vs *Salmonella srfJ*-infected RAW264.7				
	Gene symbol	**Description**	**Fold Change (linear)**	**ANOVA *p*-value**
Down-regulated genes	*IL1A*	IL-1α	-3.02	0.001179
	XAF1	XIAP associated factor 1	-2.92	0.000247
	IL1B	IL-1β	-2.65	0.002849
	CD46	CD46 antigen, complement regulatory protein	-2.61	0.029687
	SERPINB2	Serine (or cysteine) peptidase inhibitor, clade B, member 2	-2.56	0.000565
	UPP1	Uridine phosphorylase 1	-2.51	0.005209
	IRF7	Interferon regulatory factor 7	-2.48	0.014064
	TM4SF1	Transmembrane 4 superfamily member 1	-2.46	0.024226
	OLFR391-PS	Olfactory receptor 391, pseudogene	-2.43	0.023512
	ISG20	Interferon-stimulated protein	-2.35	0.005126
	GBP2	Guanylate binding protein 2	-2.26	0.002655
	LRRC18	Leucine rich repeat containing 18	-2.25	0.012066
	TCF7L2	Transcription factor 7 like, T cell specific, HMG box	-2.19	0.015292
	GPR55	G protein-coupled receptor 55	-2.16	0.022381
	CCL5	Chemokine (C-C motif) ligand 5	-2.15	0.022401
	RMDN2	Regulator of microtubule dynamics 2	-2.15	0.042977
	CCRL2	Chemokine (C-C motif) receptor-like 2	-2.13	0.043322
	MMP8	Matrix metallopeptidase 8	-2.12	0.000216
	SULT1E1	Sulfotransferase family 1E, member 1	-2.1	0.019129
	U90926	cDNA sequence U90926	-2.09	0.001198
	CBLN1	Cerebellin 1 precursor protein	-2.05	0.002789
	CARMIL1	Capping protein regulator and myosin 1 linker 1	-2.03	0.044365
Up-regulated genes	*ABCC2*	ATP-binding cassette, sub-family C (CFTR/MRP). member 2	2.01	0.017454
	PRLH	Prolactin releasing hormone	2.04	0.016683
	GAL3ST2	Galactose-3-O-sulfotransferase 2	2.07	0.048187
	OLFR259	Olfactory receptor 259	2.11	0.04462
	GIMAP3	GTPase, IMAP family member 3	2.11	0.046479
	OLFR199	Olfactory receptor 199	2.18	0.028928
	CHRDL1	Chordin-like 1	2.26	0.030388
	VMN2R17	Vomeronasal 2, receptor 17	2.31	0.033174
	GM3685	Predicted gene 3685; predicted gene 3685	2.31	0.027304
	ATP2C2	ATPase, Ca^{2+} transporting, type 2C, member 2	2.36	0.024013
	RBBP9	Retinoblastoma binding protein 9	2.37	0.030248
	IPW; SNORD116	Imprinted gene in the Prader-Willi syndrome region; small nucleolar RNA, C/D box 116 cluster	2.97	0.007046

There are 22-down-regulated genes and 11 up-regulated genes due to SrfJ presence.

A GO analysis did not detect any enrichment when using the *Mus musculus* reference list. However, some biological processes were overrepresented in the set of down-regulated genes when using the reference list for *Homo sapiens* (**Table R.2.9**).

Table R.2.9. Biological processes that are overrepressed in the set of down-regulated genes in the comparison wt-infected RAW264.7 cells vs *ΔsrfJ*-infected RAW264.7 cells (Fold change > 2 or < -2, *p*<0.05).

	GO biological process	*Homo sapiens* (21042)	Expected	Fold Enrichment	Differentially down-regulated genes
Immune response	Fever generation	5	0.00	>100	*IL1A, IL1B*
	Heat generation	8	0.01	>100	*IL1A, IL1B*
	Response to type I interferon	71	0.07	59.27	*GBP2, IRF7, ISG20, XAF1*
Cell communication	Response to interleukin-1	196	0.19	21.47	*CCL5, GBP2, IL1A, IL1B*
	Response to cytokine	1069	1.02	8.86	*CCL5, CCRL2, GBP2, IL1A, IL1B, IRF7, ISG20, SERPINB2, XAF1*
Cell death	Regulation of macrophage apoptotic process	10	0.01	>100	*CCL5, IRF7*
	Programmed cell death involved in cell development	9	0.01	>100	*IL1A, IL1B*

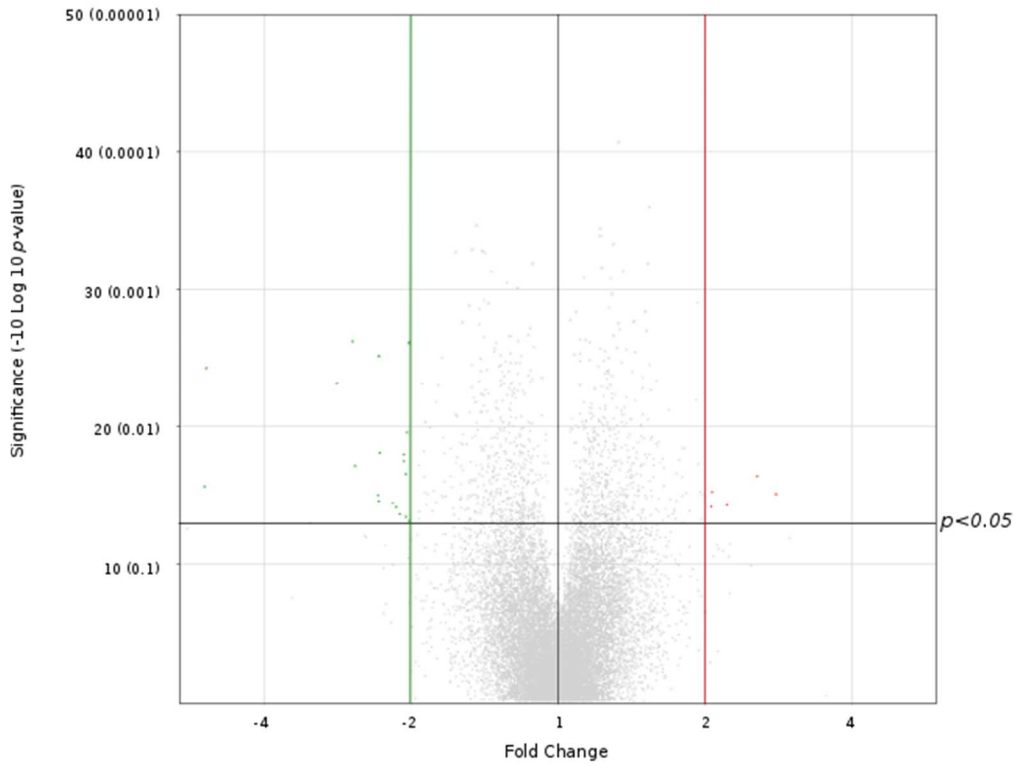
Interestingly, some of these processes are also overrepresented among the genes differentially expressed in the comparison between RAW264.7 cells infected with wt *Salmonella* and non-infected cells, suggesting that SrfJ is one of the factors involved in the alteration of these host biological processes.

2.4. Effect of the ectopic expression of SrfJ on HeLa cells

This transcriptomic analysis was carried out in order to analyze the specific effect of SrfJ study when it is present in the host cell without the interference and redundancies of other bacterial virulence factors. RNA was obtained from HeLa cultures transiently transfected with pcDNA3-SrfJ-3xFLAG or pcDNA3 and was analysed using microarrays *Clariom™ S Assay, HUMAN* (Affymetrix).

Figure R.2.4 represents the number of genes with statistically significant ($p<0.05$) differential expression in SrfJ-transfected vs empty vector-transfected HeLa cells applying or not a fold change threshold of 2.

A



B

HeLa pcDNA3-SrfJ-3xFLAG vs HeLa pcDNA3		
	$p<0.05$	$p<0.05$. Fold change >2 or <-2
Total genes	1083	24
Down-regulated genes	531	19
Up-genes genes	552	5

Figure R.2.4. Number of genes with differential expression in the comparison HeLa pcDNA3-SrfJ-3xFLAG vs HeLa pcDNA3. (A) Volcano plot of RNA abundance SrfJ- and empty vector-transfected cells showing differential expression applying a fold change thereshold of 2. Depicted in the plot is the comparison ratios versus *p*-values. Red dots: up-regulated genes. Green dots: down-regulated genes. Grey dots: genes not significantly differentially expressed for a $p<0.05$ or that do not reach the fold-change threshold. **(B)** Table showing the number of up-regulated or down-regulated genes with statistically significant ($p<0.05$) differential expression applying or not a fold change threshold of 2.

The 24 differentially expressed genes with a fold change threshold of 2 are described in **Table R.2.10**.

Table R.2.10. HeLa pcDNA3-SrfJ-3xFLAG vs HeLa pcDNA3 differentially expressed genes. (Fold Change > 2 or < -2, $p < 0.05$).

HeLa pcDNA3-SrfJ-3xFLAG vs HeLa pcDNA3				
	Gene Symbol	**Description**	**Fold Change (linear)**	**ANOVA *p*-value**
Down-regulated genes	*CREB5*	cAMP responsive element binding protein 5	-5.3	0.027482
	IFIT2	Interferon-induced protein with tetratricopeptide repeats 2	-5.26	0.003738
	IFIH1	Interferon induced, with helicase C domain 1	-2.84	0.004816
	CLGN	Calmegin	-2.64	0.002363
	CCL5	Chemokine (C-C motif) ligand 5	-2.61	0.019461
	CHAC1	ChaC glutathione-specific gamma-glutamylcyclotransferase 1	-2.34	0.032043
	CXCL8	Chemokine (C-X-C motif) ligand 8	-2.33	0.035133
	RELB	V-rel avian reticuloendotheliosis viral oncogene homolog B	-2.33	0.003044
	ABCC9	ATP Binding Cassette Subfamily C Member 9	-2.32	0.015548
	TMEM200A	Transmembrane protein 200A	-2.18	0.036452
	ANKRD6	Ankyrin repeat domain 6	-2.15	0.038811
	KLF4	Kruppel-like factor 4 (gut)	-2.11	0.04351
	NOCT	Nocturnin	-2.07	0.015994
	OR51B4	Olfactory receptor, family 51, subfamily B, member 4	-2.07	0.018028
	CXCL11	Chemokine (C-X-C motif) ligand 11	-2.05	0.022235
	BACH2	BTB Domain And CNC Homolog 2	-2.05	0.045715
	DDIT3	DNA-damage-inducible transcript 3	-2.04	0.01108
	OIT3	Oncoprotein induced transcript 3	-2.02	0.002428
	MMP19	Matrix metallopeptidase 19	-2.02	0.048979
Up-regulated genes	*PLD1*	Phospholipase D1, phosphatidylcholine-specific	2.06	0.038337
	ZKSCAN4	Zinc finger with KRAB and SCAN domains 4	2.07	0.030315
	HPGD	Hydroxyprostaglandin dehydrogenase 15-(NAD)	2.22	0.037286
	ABCA1	ATP binding cassette subfamily A member 1	2.56	0.023068
	ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	2.8	0.03128

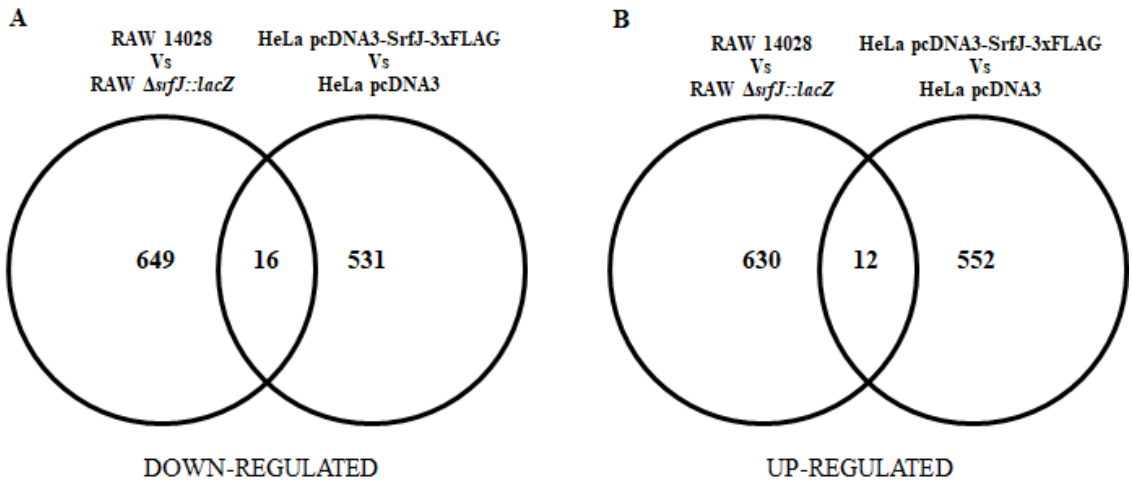
GO analysis revealed that biological processes related to defense response, response to chemical and response to biotic stimulus are overrepresented in the list of 24 genes (**Table R.2.11**).

Table R.2.11. Biological processes that are overrepresented in the set of differentially expressed genes in the comparison HeLa pcDNA3-SrfJ-3xFLAG vs HeLa pcDNA3 (Fold change > 2 or <-2, p<0.05).

GO biological process	*Mus musculus* (22262)	Expected	Fold Enrichment	Differentially expressed genes
Defense to biotic stimulus	907	0.90	10.04	*ABCA1, ABCC9, CCL5, CXCL11, DDIT3, IFIH1, IFIT2, NOCT, PLD1*
Defense response	1284	1.27	6.30	*ABCC9, CCL5, CXCL11, ELF3, IFIH1, IFIT2, PLD1, RELB*
Response to chemical	3259	13	4.04	*ABCA1, ABCC9, CCL5, CHAC1, CXCL11, DDIT3, HPGD, IFIH1, IFIT2, KLF4, NOCT, PLD1, RELB*

2.5. Comparison of the effect of SrfJ in RAW264.7 infections and HeLa transfections

In previous sections, we used two different approaches to explore the effect of SrfJ on host gene expression: infection of RAW264.7 cells with wt or *srfJ* mutant *Salmonella* and transfection of HeLa cells with a plasmid expressing SrfJ or with the empty vector. A direct comparison between the results obtained with these approaches revealed that 16 genes are significantly (p < 0.05) down-regulated and 12 genes are significantly up-regulated in the presence of SrfJ in both cases (**Figure R.2.5**).



C

Down-regulated genes		
	RAW wt vs RAW *ΔsrfJ:lacZ*	HeLa SrfJ vs HeLa pcDNA3
IL1A	-3.02	-1.42
CCL5	-2.15	-2.61
ZSCAN25	-1.72	-1.39

Up-regulated genes		
	RAW wt vs RAW *ΔsrfJ:lacZ*	HeLa SrfJ vs HeLa pcDNA3
TEMEM131	1.1	1.12
RPL9	1.1	1.12
DMKN	1.19	1.04

Down-regulated genes		
	RAW wt vs RAW *ΔsrfJ:lacZ*	HeLa SrfJ vs HeLa pcDNA3
GADD45A	-1.63	-1.34
TPM2	-1.59	-1.18
CYLD	-1.46	-1.93
TUFT1	-1.43	-1.39
LYRM5	-1.43	-1.45
SLC35A3	-1.37	-1.39
SLC7A11	-1.34	-1.25
MCM10	-1.32	-1.14
BIRC3	-1.26	-1.78
CD80	-1.23	-1.45
SOX3	-1.2	-1.37
HYOU1	-1.18	-1.13

Up-regulated genes		
	RAW wt vs RAW *ΔsrfJ:lacZ*	HeLa SrfJ vs HeLa pcDNA3
SDCBP	1.24	1.15
INTS10	1.24	1.31
PCDH11X	1.32	1.64
SELENBP1	1.33	1.98
NDRG1	1.33	1.48
KIDINS220	1.34	1.44
GLCCI1	1.5	1.4
KCNJ13	1.6	1.29

Figure R.2.5. Host genes differentially expressed in the presence of SrfJ. (**A**) Venn diagrams of down-regulated genes in RAW264.7 cells infected with wt *Salmonella* compared to RAW264.7 cells infected with *Salmonella ΔsrfJ* and HeLa cells transfected with pcDNA3-SrfJ-3xFLAG compared to HeLa cells transfected with the empty vector. (**B**) Venn diagrams of up-regulated genes in RAW264.7 cells infected with wt *Salmonella* compared to RAW264.7 cells infected with *Salmonella ΔsrfJ* and HeLa cells transfected with pcDNA3-SrfJ-3xFLAG compared to HeLa cells transfected with the empty vector. (**C**) Genes that are down- and up- regulated in both comparisons.

2.6. Effect of SrfJ on kinase activation

Transcriptome analyses carried out in this chapter suggested that the presence of SrfJ in the host cells has a significant impact on intracellular signal transduction. To complement these results, a phospho-kinase panel was used as a screening method to determine the effect of SrfJ on the phosphorylation levels of 43 protein kinases and the total amount of 2 related proteins (β-catenin and HSP60). Even though this kit (*Human Phospho-Kinase Array*, R&D Systems) is specifically recommended for human samples, it has also been successfully used with murine samples (Dreses-Werringloer *et al.*, 2013; Kuan *et al.*, 2017).

Protein extracts were obtained from RAW264.7 cultures infected or not with *S.* Typhimurium wt strain or *S.* Typhimurium *ΔsrfJ* (MOI 50:1) for 8 h. After the incubation with the cell lysates, the arrays were washed to remove unbound proteins and were incubated with a cocktail of biotinylated detection antibodies. Streptavidin-HRP and chemoluminiscent detection reagents were applied and a signal was produced at each capture spot corresponding to the amount of bound phosphorylated protein. The results are shown in **Figure R.2.6**.

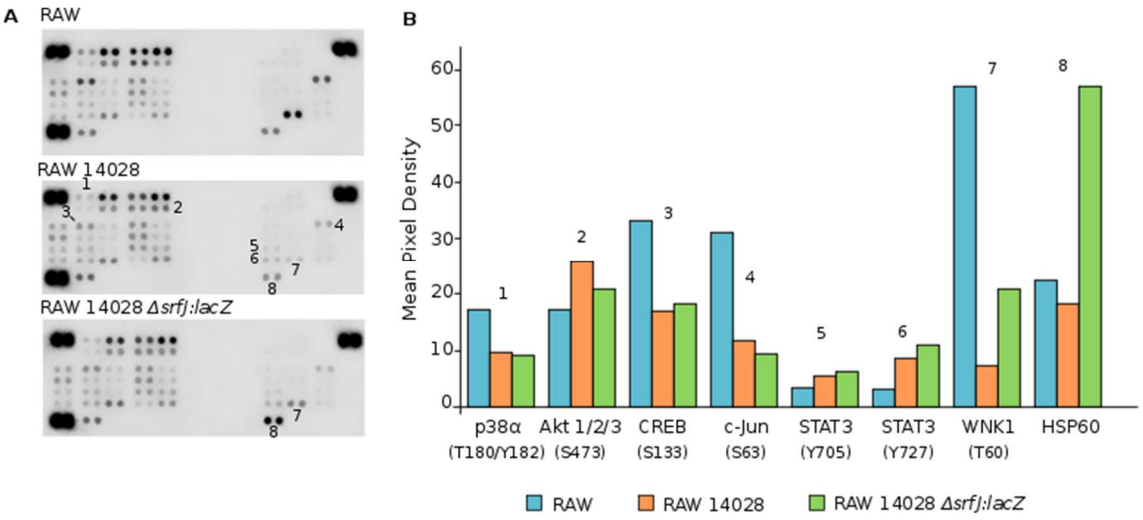


Figure R.2.6. Effect of *Salmonella* and SrfJ on kinase phosphorylation in RAW264.7 macrophages. (**A**) Cell lysates were obtained from RAW264.7 cells infected with wt *Salmonella* (RAW 14028) or a *srfJ* mutant *Salmonella* strain (RAW 14028 *srfJ*), and from non-infected cells (RAW). The lysates were analysed using the *Human Phospho-Kinase Array*. Data were obtained with an acquisition time of 2 min in an *Odyssey Fc system* (Li-Cor). Numbered dots are the kinases with apparent changes in their phosphorilation profiles. **(B)** Graphic representation of phosphorilation changes in kinases numbered in A after densitometric analysis.

We observed changes in the phosphorylation pattern of some proteins from RAW264.7 cells infected with *S.* Typhimurium wt compared to those that were not infected: p38α, CREB, c-Jun proteins are less phosphorylated during the infectious process, whereas WNK1, Akt, and STAT3 are more phosphorylated. Interestingly, the presence of SrfJ was important to determine the degree of phosphorylation of the kinase WNK1 during *Salmonella* infection, as well as the total amount of the heat shock protein HSP60. WNK1 is more phosphorylated and HSP60 is more expressed during infection with the *srfJ* mutant than during infection with wt bacteria. These results suggest that SrfJ contributes to dephosphorylation of WNK1 and prevents induction of HSP60.

Chapter 3

Evaluation of T3SS effectors as carriers for heterologous antigen vaccine design

As indicated in the **Introduction**, there are promising data about the use of attenuated *Salmonella* as live vaccine against heterologous antigens (Lin *et al*., 2015). Previous studies have shown that T3SS-mediated translocation can be used for efficient delivery of heterologous antigens in fusion with effector proteins to the cytosol of antigen-presenting cells (APC) (Hegazy et al., 2012b; Panthel et al., 2008b; Rüssmann, 2003b; Xiong et al., 2011). Effector SseJ has shown its usefulness as a carrier of model antigens in live vaccine design (Hegazy et al., 2012b). In this chapter, in collaboration with researchers from Vaxdyn, S.L. and the Instituto de Biomedicina de Sevilla (IBiS), we have tested the possibility of using SseJ and other effectors studied in our laboratory, SrfJ, SlrP, SteA and SseK1, as carriers in the design of a vaccine against *P. aeruginosa*, a clinically relevant Gram-negative opportunistic bacterial pathogen. Antigens used were the outer membrane proteins OprF and OprI (Baumann *et al*., 2004; Hassan *et al*., 2015; Gilleland *et al*., 1988; Lee *et al*., 2000b; Matthews-Greer and Gilleland, 1987; von Specht *et al*., 1996), and the *P. aeruginosa* V-antigen (PcrV), a component of the virulence-related T3SS of these bacteria (Le Moigne *et al*., 2015; Taylor *et al*., 2015).

3.1. Construction of *Salmonella* vectors for delivery of *P. aeruginosa* antigens

To generate a vaccine against *P. aeruginosa* using *Salmonella* T3SS effectors as carriers, we used different *Salmonella* promoters to direct the expression of fusion proteins between *Salmonella* effectors SlrP, SrfJ, SseJ, SseKI, and SteA, and *Pseudomonas* antigens OprF/I and PcrV.

Constructions of derivatives of the low-copy number plasmid pWSK29 for vaccine assays were carried out in three steps (**Figure R.3.1**). The first step was the cloning of *P. aeruginosa* DNA fragments, encoding antigens PcrV and OprFI (190-342)/OprI(21-83) in fusion with FLAG tag using BamHI and NotI restriction sites. The second step was the addition of *S.* Typhimurium DNA fragments encoding effectors SseJ, SlrP, SteA, SseKI and SrfJ (1-42) using EcoRI and BamHI restriction sites. Finally, the regions containing promoters P*sseA*, P*slrP*, P*steA*, P*sseKI* and P*srfJ* were added using KpnI and EcoRI.

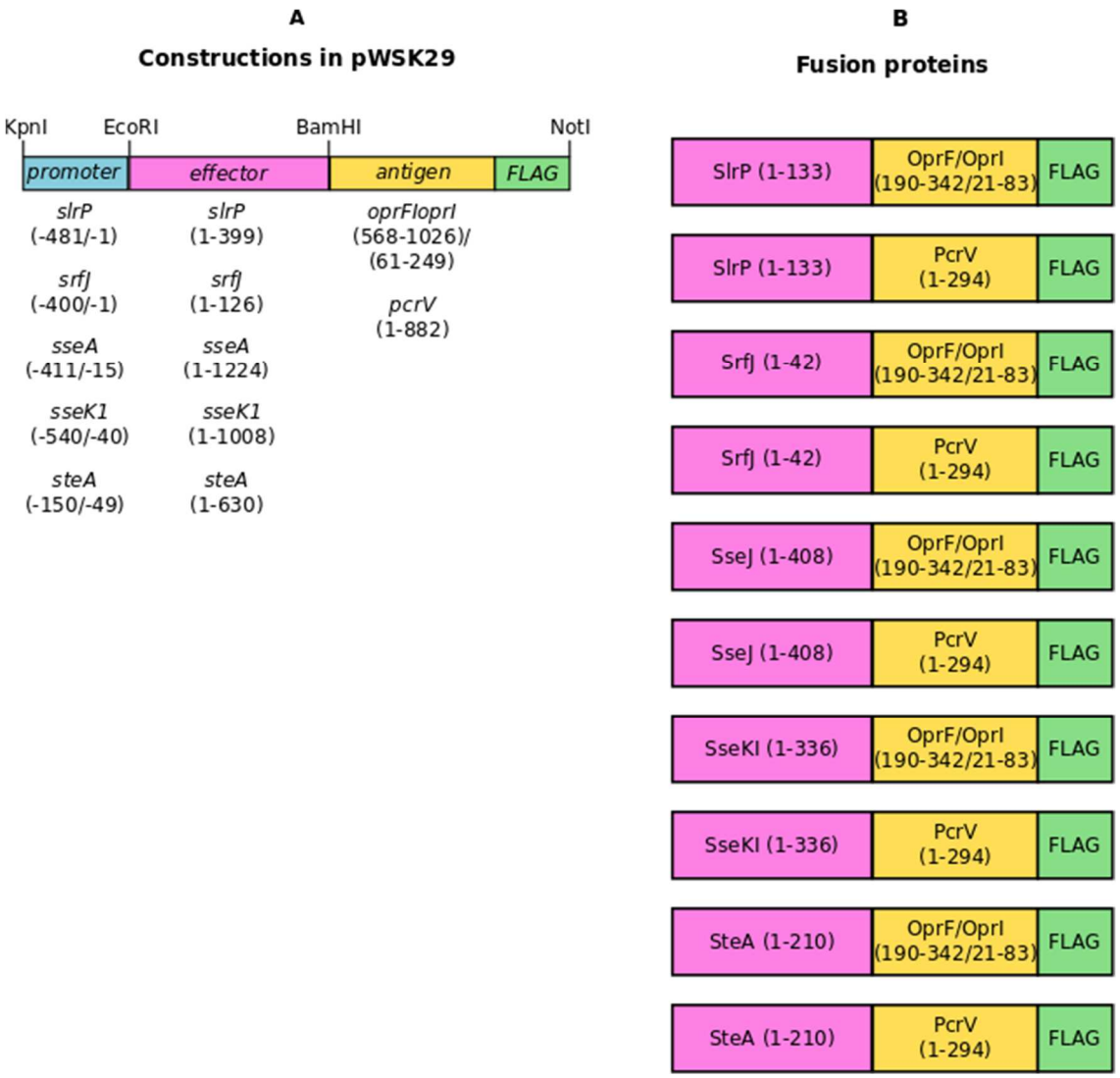


Figure R.3.1. Construction of plasmids for delivery of *P. aeruginosa* antigens. (**A**) Vector pWSK29 was used for the generation of 50 different plasmids in three steps. First, part of the coding region of antigens OprF/OprI or PcrV from *P. aeruginosa* fused to DNA encoding the FLAG epitope were cloned using BamHI and NotI endonucleases. Second, the coding region of genes *slrP*, *sseJ*, *sseKI*, *srfJ* or *steA* was added using EcoRI and BamHI enzymes. Finally, the promoter region of genes *slrP, sseA, sseK1, srfJ* or *steA* was added with KpnI and EcoRI. Numbers in parentheses indicate base pairs relative to the start of the coding region. (**B**) Fusion proteins that are expected to be produced under the appropriate conditions after the introduction of the plasmids described in (A) in *S.* Typhimurium. Expression of these proteins is controlled by 5 different promoters, depending on the plasmid. Numbers in parentheses refer to amino acids included in the fusions.

3.2. Evaluation of *Salmonella* vectors for delivery of *P. aeruginosa* antigens

SrfJ and SseJ are specifically secreted through the T3SS2 from inside the SCV (Cordero-Alba *et al.*, 2012; Miao and Miller, 2000), whereas SlrP, SseKI and SteA can be secreted through T3SS1 and T3SS2 (Baisón-Olmo *et al.*, 2015; Cardenal-Muñoz and Ramos-Morales, 2011; Choy *et al.*, 2004; Cordero-Alba and Ramos-Morales, 2014; Geddes *et al.*, 2005; Miao and Miller, 2000). Promoters used were from genes *sseA* (Cordero-Alba

et al., 2012; Hensel *et al*., 1998) and *srfJ*, that respond to intravacuolar signals and are induced several hours after invasion, and genes *slrP*, *sseKI* and *steA,* that are expressed under more varied conditions (Baisón-Olmo *et al*., 2015; Cardenal-Muñoz and Ramos-Morales, 2011; Cordero-Alba and Ramos-Morales, 2014). *S.* Typhimurium strain 14028 containing the plasmids with the hybrid genes were grown in LPM, a medium that imitates intravacuolar conditions, and expression of fusion proteins was monitored by western blot against the FLAG tag that was also added. Results shown in **Figure R.3.2** indicate that 13 plasmids (10 for OprF/I and 3 for PcrV) yielded a significant level of protein production.

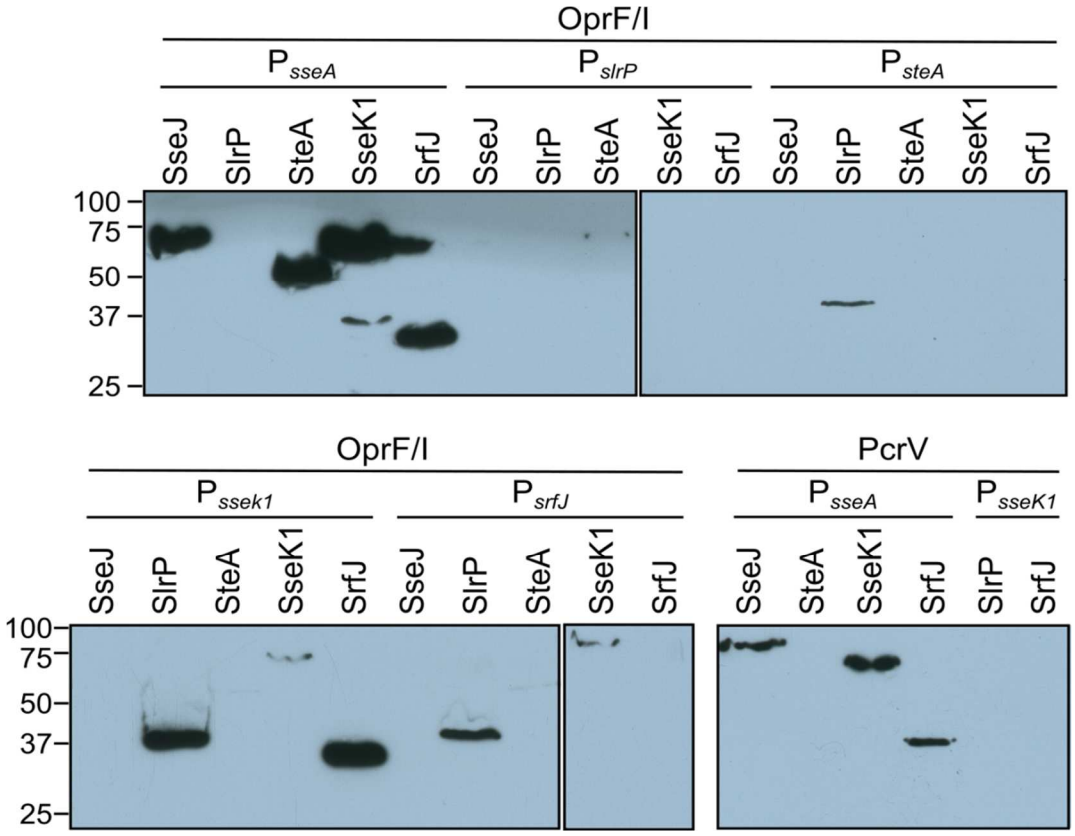


Figure R.3.2. Production of fusions proteins with *P. aeruginosa* antigens in *S. enterica*. Derivatives of *S.* Typhimurium strain 14028 carrying plasmids encoding fusion proteins were grown overnight, at 37ºC, with shaking in LPM medium. Expression of the fusions was driven by the indicated promoters (P*sseA*, P*slrP*, P*steA*, P*sseKI* or P*srfJ*). Fusions consisted of a *Salmonella* T3SS effector (SseJ, SlrP, SteA, SseKI; or SrfJ), a *Pseudomonas* antigen (OprF/I or PcrV), and the FLAG tag that was used for detection of the proteins by immunoblot. Molecular weights are indicated on the left. OprF/I:OprF (190-342)-OprI (21-83).

Strains containing these plasmids were tested for their capacity to translocate the fusion proteins into macrophages RAW264.7. Translocation was detected for 5 combinations with OprF/I: P*sseA*-SteA-OprF/I-FLAG, P*steA*-SlrP-OprF/I-FLAG, P*sseKI*-SlrP-OprF/I-

FLAG, P*sseA*-SseJ-OprF/I-FLAG, P*srfJ*-SlrP-OprF/I-FLAG, and one with PcrV: P*sseA*-SseJ-PcrV-FLAG (**Figure R.3.3**). These constructs were selected for further experiments.

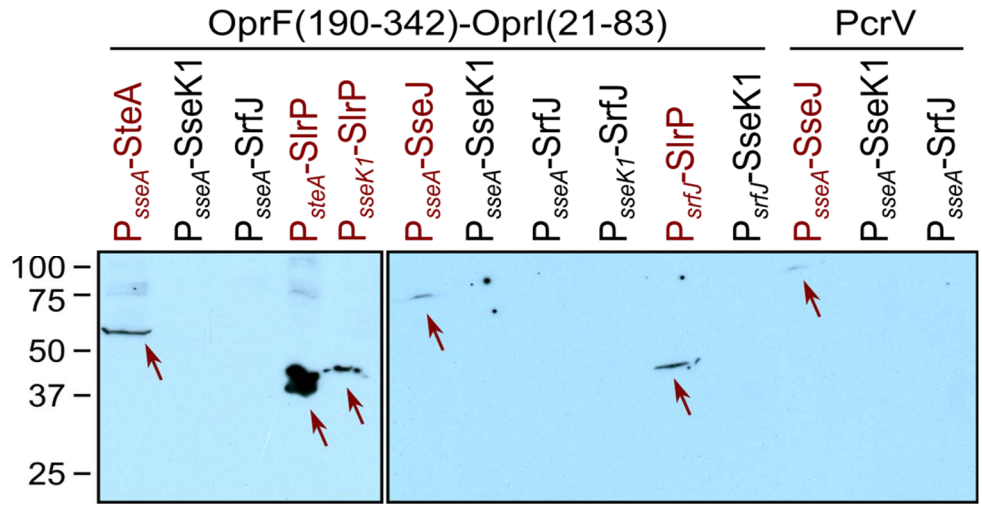


Figure R.3.3. Translocation of fusion proteins into RAW264.7 cells. *Salmonella* expressing the indicated combinations of effector and *Pseudomonas* antigens with a FLAG tag under the control of the indicated promoters were grown under non-invasive conditions. These bacteria were used to infect RAW 264.7 cells for 8 h. cells were lysed with 1% Triton X-100 in PBS and centrifuged 15 min at 15000 g. The filtered, concentrated supernatants were analysed by immunoblotting with anti-FLAG antibodies to detect translocation of fusion proteins into the host cytosols. Translocated bands are indicated with red arrows.

3.3. Antibody responses induced by candidate vaccines against *P. aeruginosa*

Before carrying out immunization studies in mice, the 6 plasmids selected above were introduced in an attenuated strain of *S.* Typhimurium with mutation in *aroA* and *aroB*. Expression of the fusion proteins was detected again in this background (not shown) and C57BL/6 mice were immunized by intraperitoneal injection with the attenuated strains carrying the plasmids. Mice immunized with *Salmonella aroA aroB* without pWSK29 derivatives or with PBS were used as controls. Indirect ELISAs were performed using sera collected 21 days after immunization. As shown in **Figure R.3.4**, immunization with SseJ-PcrV elicited detectable levels of antigen-specific total IgG in all mice, whereas the OprF/I constructs were not able to induce a specific response. As expected control mice had no detectable antigen-specific IgG.

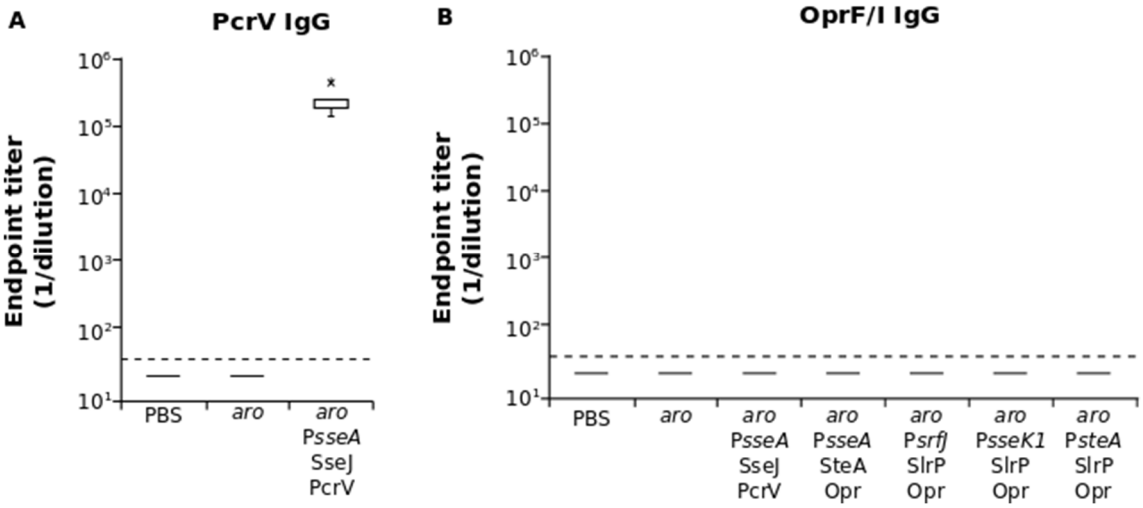


Figure R.3.4. Antibody responses to immunization with different *Salmonella* strains expressing *Pseudomonas* antigens. Mice were inoculated with PBS, with plasmid-less *S.* Typhimurium *aroA aroB* (*aro*), or with the same *Salmonella* strain carrying pWSK29 derivatives expressing PcrV or Opr fusion proteins as indicated. Serum samples were collected from vaccinated and control mice 21 days after immunization and levels of PcrV (**A**) or OprF/I (**B**) specific total IgG were measured by ELISA. Box and whisker plots represent the interquartile ranges and ranges, respectively, and horizontal lines represent median values. * $p<0.05$ compared to levels in control mice.

3.4. Protective responses induced by candidate vaccines

Vaccine efficacy was tested by infecting immunized and control mice with *P. aeruginosa* strain PA01 and survival was monitored over seven days (**Figure R.3.5**). Consistent with the antibody response results, only *Salmonella* expressing the SseJ-PcrV construct was able to provide significant protection.

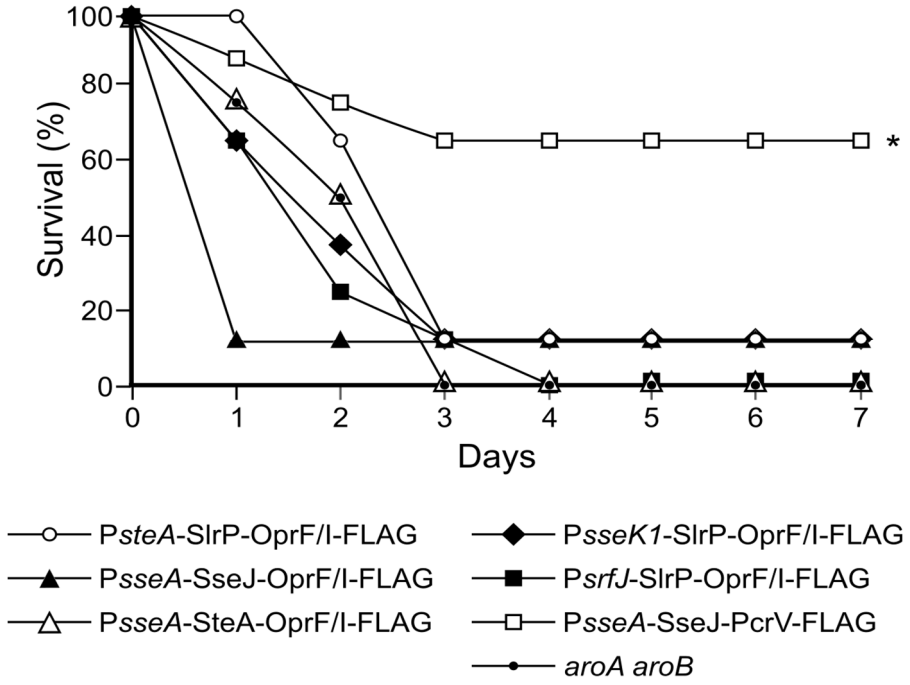


Figure R.3.5. Survival curve of challenged mice. Groups of 8 C57BL/6 mice were intraperitoneally vaccinated with an *aroA aroB* mutant of *S.* Typhimurium (*aroA aroB*) or with the same strain carrying the plasmids pIZ2132, pIZ2160, pIZ2162, pIZ2186, or pIZ2267 for expression of the fusion proteins SlrP-OprF/I-FLAG (from the P*steA* promoter), SseJ-OprF/I-FLAG (from the P*sseA* promoter), SteA-OprF/I-FLAG (from the P*sseA* promoter), SlrP-OprF/I-FLAG (from the P*sseKI* promoter), SlrP-OprF/I-FLAG (from the P*srfJ* promoter), or the SseJ-PcrV-FLAG (from the P*sseA* promoter), respectively. Twenty-one days after immunization, mice were infected with *P. aeruginosa* strain PA01 and the survival of the mice was registered for 7 days. * $p<0.05$ compared to levels in control mice inoculated with *Salmonella aroA aroB*.

3.5. Effect of vaccination on tissue bacterial loads, p.i. serum cytokine levels and survival

A new immunization experiment was carried out using the protective vaccine, or attenuated *Salmonella* without pWSK29 derivative. Immunized and control mice were challenged with *P. aeruginosa* PA01 21 days post-immunization. Twelve hours after infection, spleen and lung bacterial loads were determined (**Figure R.3.6**). Vaccination with *Salmonella* expressing the SseJ-PcrV fusion dramatically reduced the number of *P. aeruginosa* in spleen and lungs.

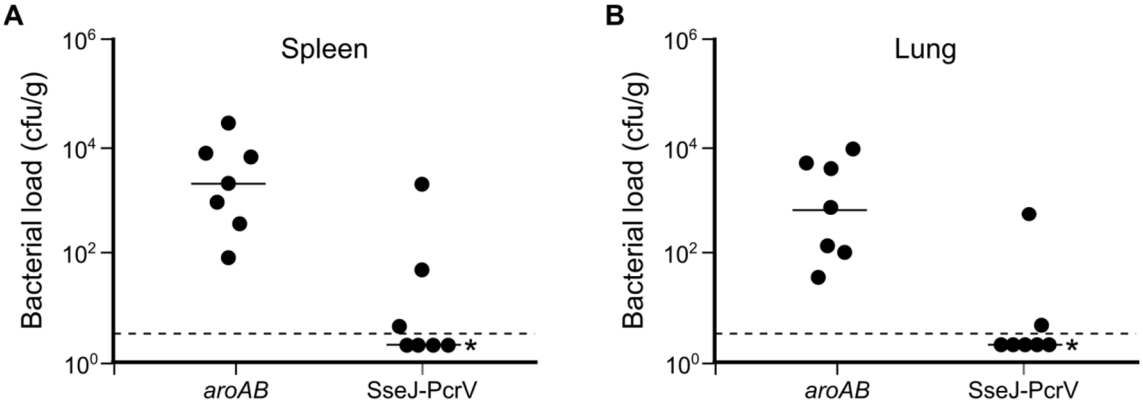


Figure R.3.6. Effect of vaccination on bacterial loads. C57BL6 mice were intraperitoneally vaccinated with an *aroA aroB* mutant of *S.* Typhimurium (*aroA aroB*), or with the same strain carrying the plasmid pIZ2267 for expression of the fusion SseJ-PcrV-FLAG from the P*sseA* promoter. Twenty one days after the immunization, mice were infected with *P. aeruginosa* strain PA01. Twelve h p.i. groups of 7 mice were euthanized to enumerate the spleen (A) and lung (B) bacterial loads. Data points represent bacterial loads levels from individual mice, and horizontal lines represent median values from groups of mice. **p*<0.05 compared to levels in control mice inoculated with *Salmonella aroA aroB*.

To characterize the effect of immunization on cytokine levels, sera were also collected 12 h after the infection with *P. aeruginosa* and the levels of TNF-α and IL-6 were determined (**Figure R.3.7**). Levels of cytokines were significantly lower in vaccinated mice compared to control mice.

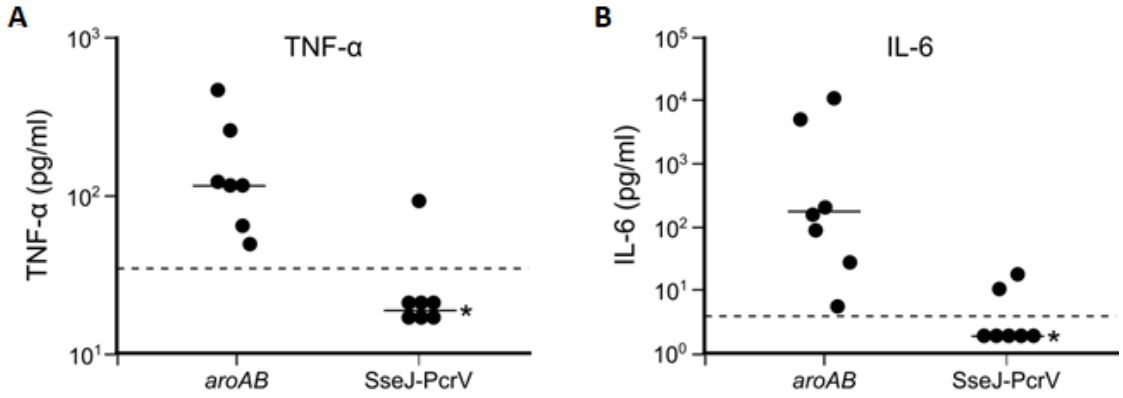


Figure R.3.7. Effect of vaccination on p.i. pro-inflammatory cytokine. C57BL6 mice were intraperitoneally vaccinated with an *aroA aroB* mutant of *S.* Typhimurium (*aroA aroB*), or with the same strain carrying the plasmid pIZ2267 for expression of the fusion SseJ-PcrV-FLAG from the P*sseA* promoter. Twenty-one days after the immunization, mice were infected with *P. aeruginosa* strain PA01. Twelve h p.i. groups of 7 mice were euthanized to determine serum levels of TNF-α (A) and IL-6 (B). Data points represent cytokine levels from individual mice, and horizontal lines represent median values from groups of mice. **p*<0.05 compared to levels in control mice inoculated with *Salmonella aroA aroB*.

Finally, the survival of vaccinated mice was monitored for 7 days after the challenge with *P. aeruginosa* (**Figure R.3.8**). All mice vaccinated with *Salmonella* expressing SseJ-PcrV were protected from challenge, whereas 6 out of 7 mice inoculated with control *Salmonella* died in less than 48 hours.

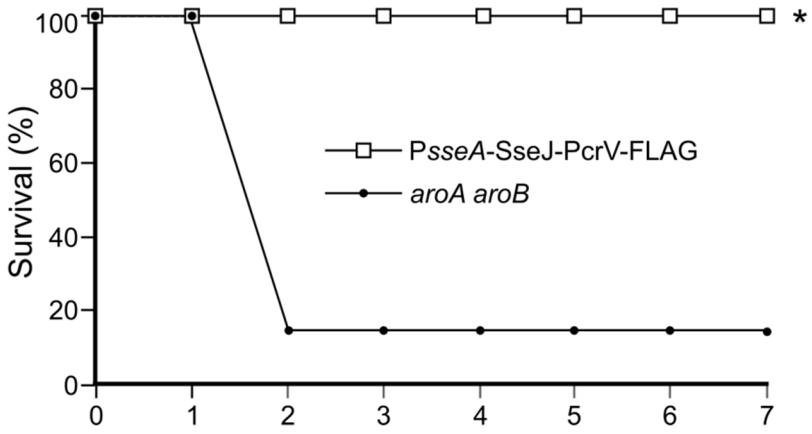


Figure R.3.8. Effect of vaccination on survival. C57BL6 mice were intraperitoneally vaccinated with an *aroA aroB* mutant of *S.* Typhimurium (*aroA aroB*), or with the same strain carrying the plasmid pIZ2267 for expression of the fusion SseJ-PcrV-FLAG from the P*sseA* promoter. Twenty-one days after the immunization, mice were infected with *P. aeruginosa* strain PA01. Survival of infected mice was registered for 7 days. **P*<0.05 compared to levels in control mice inoculated with *Salmonella aroA aroB*.

DISCUSSION

Previous work carried out in our laboratory showed that SrfJ can be secreted through the T3SS2 of *S.* Typhimurium and that its expression is positively regulated by PhoP and SsrB, and negatively regulated by RcsB and IolR (Cordero-Alba *et al.*, 2012). In **Chapter 1** we show that these regulators act through two different promoters: a proximal promoter, P*srfJ* that responds to PhoP, SsrB, and RcsB and a distal promoter, P*iolE*, that responds to IolR (**Figure R.1.4**).

The proximal promoter (P*srfJ*) initiate the transcription in an adenine located 33 bases upstream of the *srfJ* start codon (**Figure R.1.6**). The three regulators that control expression from P*srfJ* are well-known relevant regulators of *Salmonella* virulence. (i) SsrB is the response regulator of a *Salmonella*-specific two-component regulatory system where the kinase SsrA detects low pH in the host vacuole through a histidine-rich periplasmic sensor domain (Mulder *et al.*, 2015) and phosphorylates SsrB. Phosphorylated SsrB activates transcription of target genes (Deiwick *et al.*, 1999). Positive regulation by SsrB is a common feature of SPI2 genes and other genes encoding effectors specifically secreted through T3SS2 (Xu and Hensel, 2010). (ii) The ancestral PhoQ/PhoP regulatory system is a master two-component system that regulates more than 100 genes (Tran *et al.*, 2016; Zwir *et al.*, 2005) in response to environmental signals including low Mg^{2+}, acidic pH, and cationic antimicrobial peptides (Bader *et al.*, 2005; Chamnongpol *et al.*, 2003; Prost *et al.*, 2007). Since PhoP regulates expression of *ssrA* and *ssrB* (Bijlsma and Groisman, 2005), it also regulates expression of genes in the SsrB regulon. (iii) Finally, the Rcs phosphorelay system has been shown to play an important role in virulence in mice, in particular during systemic infections (Detweiler *et al.*, 2003; Domínguez-Bernal *et al.*, 2004; Erickson and Detweiler, 2006; García-Calderón *et al.*, 2005). Repression of P*srfJ* by RcsB correlates with previous reports suggesting that high level of activation of the system negatively regulates expression of SPI1 and SPI2 genes (Wang *et al.*, 2007), Thus, this pattern of regulation indicates that P*srfJ* functions as a typical promoter of a T3SS2-related gene that responds specifically to intravacuolar signals. Consistent with this, transcription form this promoter was induced in LPM at pH 5.8, a medium that mimics intracellular conditions (**Figure R.1.2**). More importantly it was also induced in bacteria phagocytized by macrophages (**Figure R.1.7**) which are conditions known to induce the expression of the T3SS2.

Regulation by IolR and *myo*-inositol was more puzzling. Nonetheless, we were able to show that these act through a different, distal promoter, P*iolE*. The transcription start site corresponding to this promoter is an adenine located 99 bases upstream of *iolE* (**Figure**

R.1.6) and 2130 bases upstream of *srfJ*. Two lines of evidence support the existence of an operon encompassing *iolE*, *iolG1* and *srfJ*: (i) RT-PCR carried out with appropriate oligonucleotide pairs on an RNA sample obtained from an *iolR*-mutant strain yielded products of the expected size (**Figure R.1.5**) if the three genes are transcriptionally linked. (ii) P*iolE* can drive expression of the reporter *lux* operon in response to an *iolR* mutation (**Figure R.1.4**) or *myo*-inositol supplementation (not shown) from a proximal (pSB377-P*iolE*) and a distal position (pSB377-P*iolE*-P*srfJ*).

In contrast to P*srfJ*, P*iolE* does not respond to intravacuolar signals but to *myo*-inositol. This carbohydrate, produced by most of the plants, is important for plant growth and development (Loewus and Loewus, 1983): oxidation of *myo*-inositol is an important pathway in cell wall polysaccharide biogenesis (Loewus, 2006; Loewus and Murthy, 2000); inositol and derived molecules are involved in stress-related responses (Loewus and Murthy, 2000); and *myo*-inositol is used as a precursor of inositol-containing signalling molecules including phosphatidylinositol and phosphoinositides (Gillaspy, 2011). The presence of this carbohydrate in plant extracts explains expression of *srfJ* from P*iolE* in LM and TM (**Figure R.1.10** and **Figure R.1.11**). The observation that this expression is detected 24 h but not 8 h after the inoculation of the medium is in agreement with a previously reported extended lag phase during the growth of *Salmonella* in the presence of *myo*-inositol as the sole carbon source (Kröger and Fuchs, 2009). The authors of this report exclude catabolite repression as an explanation but suggest that the *iol* genes in *Salmonella* could be under a tight repression or under the action of an additional unknown regulatory factor. Interestingly, our results suggest that expression of *srfJ* from the *myo*-inositol responsive promoter P*iolE* could be important during the plant colonization by *Salmonella* (**Figure R.1.12**). This could also explain the chromosomal location of *srfJ* inside the *myo*-inositol island from an evolutionary point of view.

Several reports suggest the important role of *Salmonella* T3SS during plant colonization. *Salmonella* lacking T3SS1 and T3SS2, *prgH*, and *ssaV* mutants, respectively, showed reduced proliferation in syringe-infiltrated leaves of *Arabidopsis thaliana* (Schikora *et al*., 2011). Symptoms caused by these mutants were more pronounced in comparison with the wt strain, indicating that T3SSs are involved in suppressing the hypersensitive response (HR), an induced, localized cell death, which limits the spread of pathogens. Furthermore, transcriptome analyses showed that a *prgH* mutant induced stronger defense gene expression than wt bacteria in *Arabidopsis* seedlings (García *et al*., 2014; Schikora *et al*., 2011). Similarly, experiments in *Nicotiana tabacum* have shown that the T3SS1 is

essential for the active suppression of defense mechanisms by *Salmonella* (Shirron and Yaron, 2011). Interestingly, the response was different in other plants or even in different organs of the same plants: mutants lacking T3SS1 (*sipB* or *spaS*) colonized *Medicago sativa* roots, *A. thaliana* roots, and *Triticum aestivum* roots and seedlings in significantly greater numbers than the wt strain 14028 (Iniguez *et al*., 2005). Because the *sipB* mutation did not enhance colonization in a *nrp1 Arabidopsis* mutant, which is defective in both salicylic acid (SA)-dependent and SA-independent defense responses (Ton *et al*., 2002), the authors concluded that T3SS1 is involved in the induction of both kinds of plant responses. In contrast, another study concluded that T3SS1 and T3SS2 were not involved in suppression of plant defenses in *Nicotiana bethamiana* leaves (Meng *et al*., 2013). These discrepant results indicate that the exploration of a variety of experimental conditions and host models will be necessary to ascertain the role of *Salmonella* T3SSs and particular effectors in plants. Interestingly, although *Salmonella*-mediated delivery of effector proteins into plant cells have not been shown yet (García and Hirt, 2014), effectors SseF and SspH2 were able to trigger cell death through resistance-gene-mediated signalling in *N. benthamiana* when heterologously delivered using *A. tumefaciens* or *Xanthomonas campestris* (Bhavsar *et al*., 2013; Üstün *et al*., 2012).

An important aspect of this work was the analysis of the contribution of SrfJ to the survival of *Salmonella* inside animal and plant hosts. Our results suggest that the expression of this effector at the appropriate time is a relevant factor in the interaction of *Salmonella* with mice macrophages and with lettuce and tomato leaves: (i) The results obtained with the *iolR* mutant and the *iolR srfJ* double mutant indicate that the ectopic production of SrfJ caused by the absence of the IolR repressor decreases survival/proliferation of *Salmonella* inside RAW264.7 macrophages (**Figure R.1.9**). (ii) The lack of production of SrfJ in plants caused by a deletion of the coding sequence of *srfJ* or a deletion of the distal promoter P*iolE* leads to an improved survival of *Salmonella* in plants (**Figure R.1.13**). This result is in line with the reduced defense gene activation displayed by the *srfJ* mutant compared to the wt (**Figure R.1.14**) and suggests that SrfJ could act in this system as an avirulent protein (Mansfield, 2009). These proteins are effectors of plant pathogens that, in the course of plant-pathogen co-evolution, have been recognized by plant receptors to activate defense responses. As such, SrfJ could have a virulent role in other sensitive plant strains or species.

Additional experiments will be needed to ascertain if SrfJ expressed in *Salmonella* during plant colonization can be secreted through T3SS2. In this context, it should be noted that

in our experimental model P*srfJ* was not active during plant colonization with *Salmonella*. This result suggests that, under these conditions, the SsrB regulon, including, SPI2, would not be expressed and therefore, SrfJ, although expressed from P*iolE*, would not be secreted through T3SS2. Nevertheless, the phenotype of the *ssaV* mutant noted above (Schikora *et al*., 2011) argues for the expression of this system at some point of the colonization of plants. Our previous results showed that translocation into macrophages was specifically T3SS2-dependent (Cordero-Alba *et al*., 2012), and here we have shown that P*srfJ* is the only promoter that drives the expression of *srfJ* inside these cells (**Figure R.1.7** and **Figure R.1.8**). However, secretion of SrfJ through T3SS1 is another interesting possibility that could be explored in different cell types or hosts. Several effectors can be secreted through both systems. For example, for PipB2, considered as a T3SS2 effector, we have previously shown the possibility of translocation through T3SS1 into a variety of cell types (Baisón-Olmo *et al*., 2012). Specificity of secretion is achieved, at least for some effectors, simply by coexpression between the particular T3SS and its effectors. An example is SseK1: when expressed from a constitutive promoter it can be secreted by T3SS1 at earlier time points p.i. than when expressed from its own promoter (Baisón-Olmo *et al*., 2015). Coexpression of T3SS1 and *srfJ* from P*iolE* may take place in plants due to the presence of *myo*-inositol, making it possible the delivery of the effector through this way. A recent report suggests that *S.* Typhimurium is unable to translocate T3SS effectors into cells of beet roots or pepper leaves (Chalupowicz *et al*., 2018). However, there is the possibility of translocation in other plant systems. Alternatively, some effectors could be secreted and exert their action in the apoplast rather than inside plant cells.

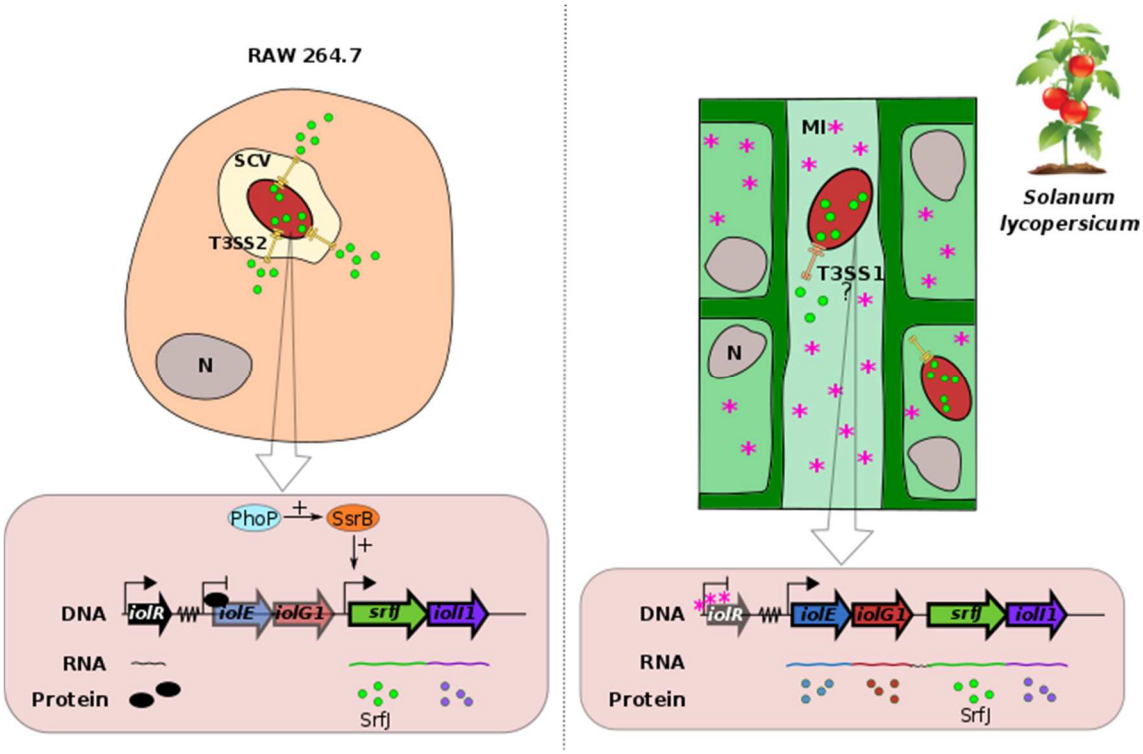


Figure D.1. Dual expression of the *Salmonella* effector SrfJ in mammalian cells and plants. SCV: *Salmonella* containing vacuole; MI: *myo*-inositol; N: nucleus.

The function of SrfJ inside the host is presently unknown. Its amino acid sequence and its structure (Kim *et al.*, 2009) suggest that it may have glucosylceramidase activity. This enzymatic activity catalyzes hydrolysis of glucosylceramide into glucose and ceramide, the simplest member of the family of sphingolipids. These lipids not only represent essential structural elements of membranes, but several members of this family, including ceramide, are also secondary messenger molecules that regulate intra- and intercellular processes (Ilan, 2016). Ceramides can affect cellular proliferation, differentiation, cell death, insulin resistance, oxidative stress, and inflammation (Kogot-Levin and Saada, 2014; Kuzmenko and Klimentyeva, 2016; Pandey *et al.*, 2007; Saddoughi and Ogretmen, 2013). Glycosphingolipids are membrane components that can affect numerous cellular events including homeostasis, adhesion, growth, motility, apoptosis, proliferation, stress, and inflammatory responses (Ilan, 2016). Interestingly, glucosylceramide is the only glycosphingolipid that plants, fungi, and animals have in common (Warnecke and Heinz, 2003). Glucosylceramide is important in animals for the activation of antigen-presenting cells, induction of Th1 and Th7 responses, and neutrophil recruitment (Pandey *et al.*, 2012). There is less information about the functions of glucosylceramide in plants, but it has been suggested to be necessary for normal Golgi-mediated protein trafficking (Melser

et al., 2010, 2011). A more recent report has shown that null mutants for glucosylceramide synthase failed to develop beyond the seedling stage and that glucosylceramide is critical for cell-type differentiation and organogenesis (Msanne *et al.*, 2015). Future research should focus on the relevance and consequences of the putative effect of SrfJ on this important lipid target both in animal and plant cells.

The putative glucosylceramidase activity of SrfJ may be responsible for different effects in the host cell during infection. As a way to get some insight into the possible targets of this effector, we explored the transcriptome changes induced by *S.* Typhimurium in the mammalian host cell, and specifically the role of SrfJ in these changes. Two different mammalian cell lines were used, (i) HeLa (ECAC no. 93021013), human epithelial cell line; and (ii) RAW264.7 (ECACC no.91062702), murine macrophage cell line.

First, we analysed the changes induced by wt *Salmonella* infections in both cell types using *Clariom*^{TM} *S Assay* microarrays from Affymetrix. Infected HeLa cells showed showed 121 differentially expressed genes with a fold change > 2 and $p < 0.05$ compared to non-infected cells. A much higher number of genes (1991) was obtained for infected RAW264.7 macrophages, so, for these cells, we decided to focus on 185 differentially expressed genes with a fold change of > 8. Although only 7 specific genes were differentially expressed in both cell types (*TNFAIP3, IRF1, SOD2, IER3, ZFP36, JUNB, IL6*), a GO enrichment analysis revealed that biological processes related to innate immune response, adaptive immune response, cell adhesion and cell proliferation were significantly enriched in the sets of up-regulated genes in response to *Salmonella* infection in both cellular models (**Tables R.2.2**, **R.2.4** and **R.2.5**). Innate immune response is the first barrier against pathogen invasion. Its activation in infected HeLa cells is observed by the enrichment of signaling pathways in response to LPS, like TLR (*Toll-like receptor)* signaling pathway, and ERK1/ERK2 cascades (Akira and Takeda, 2004; Dommett *et al.*, 2005). This activation leads to the production and secretion of different cytokines, which trigger the inflammatory process. Cytokines play several roles, like chemoattractants of other components of the immune system such as T-helpers cells (Th), which indicate the involvement of the adaptive immune response (Kunkel and Butcher, 2002; Schluns and Lefrançois, 2003). There is a wide activation of the innate immune response in infected macrophages, with routes such as fever and the secretion of several cytokines (Arango Duque and Descoteaux, 2014), including interleukins (IL-1, IL-6, IL-2, IL-10) (Beuscher *et al.*, 1990; Hu *et al.*, 2013; Matsui, 1999; Nguyen *et al.*, 2013) and chemokines (Chandrasekar *et al.*, 2013), which ultimately promote the attraction of

neutrophils (Kunkel and Butcher, 2002). An enrichment of the production of IFN-γ and IFN-β as well as of TNF (Robinson, 2018; Robinson *et a*l., 2012) is also observed. This is consistent with the activation of the macrophage response against *Salmonella*. Normally, resident infected macrophages recognize cytosolic flagellin via the NLRC4 inflammasome complex to activate caspase-1 and induce pro-inflammatory cytokines IL-1β and IL-18 (Pham and McSorley, 2015), so our results fits with previous descriptions. Other defensive processes that are activated during *Salmonella* infection, are the production of reactive oxygen species and nitric oxide (NO). These are microbicidal molecules that destroy the microbes after phagocytosis (van der Heijden *et al*., 2015). These responses are probably the result of the activation of ERK1/ERK2, NF-κB, TLRs and JAK-STAT pathways that has been previously described in macrophages after *Salmonella* infection (Carey *et al*., 2012; Li and Cherayil, 2003; Procyk *et al*., 1999; Ramos-Marquès *et al*., 2017). Adaptive immune response processes are also enriched among the genes up-regulated in infected macrophages. These processes include the recruitment of T-helpers and myeloid leukocytes (Kunkel and Butcher, 2002; von Andrian and Mackay, 2000).

It is also interesting to note the enrichment of negative regulation pathways of apoptosis among the up-regulated genes in both cells types, which suggests that during *Salmonella* infection these cells are prevented from dying so that bacteria retain their niche (Wu *et al*., 2012; Ruan *et al*., 2016). Regulation of cell-cell and cell-substrate adhesion were also enriched processes. As mentioned in the **Introduction**, several T3SS1 effectors are involved in destabilizing tight junctions (Boyle *et al*., 2006). This is also consistent with previous reports showing that *Salmonella* perturb tight junction integrity and suggesting that, during infection by these and other bacterial pathogens, gap junctions favor the cell-to-cell diffusion of the bacterial peptidoglycan that would then activate IL-8 production in bystander, non-infected cells (Bonazzi and Cossart, 2011). Finally, regulation of cell proliferation and cell cycle are additional terms over-represented in the set of genes up-regulated in HeLa and RAW264.7.

Among the genes down-regulated in *Salmonella* infected macrophages it is specially striking the presence of a large set of genes encoding variants of histones H1, H2A, H2B and H3. Human and murine cells contain multiple copies of core histone genes. Most of them are clustered in two loci, *HIST1*, with almost 80% of histone genes, and *HIST2* (Mei *et al*., 2017). All the histone genes found in this study were located in cluster *HIST1*. It has been described that methylation of H3K4, H3K9, H3K36, H3K79, and H4K20 is

needed during inflammatory response in murine RAW264.7 cells upon LPS stimulation. They play a critical role in the regulation of LPS-induced expression and release of IL-6 and TNF-α (Zhao *et al*., 2018). Furthermore, several histones are phosphorylated (H2B1F, H4, and H15), in late stages of *Salmonella* infection (Imami *et al*., 2013). But, little is known about down-regulation of histone expression in *Salmonella* infected cells. Interestingly, a previous transcriptomic study of bone marrow-derived macrophages infected with uropathogenic *E. coli* showed down-regulation of the genes encoding histones H1, H2, and H4 (Mavromatis *et al*., 2015). Since the expression of these histone genes is coupled to DNA-replication (Marzluff *et al*., 2002), the down-regulation observed could be a consequence of growth-inhibitory effects of bacterial infections on macrophages.

Once we analysed the general processes altered in the host cells upon *Salmonella* infection, we focused on the specific effects of SrfJ. Two approaches were used: (i) comparison of the effects of wt *Salmonella* and a *srfJ* null mutant on RAW264.7 macrophages, and (ii) analysis of the effects of transfection of SrfJ in HeLa cells.

The first approach revealed that 22 genes were down-regulated and 11 genes were up-regulated during *Salmonella* infection in a SrfJ-dependent manner (fold change>2, $p<0.05$). A GO enrichment analysis detected some biological processes that were overrepresented in the set of down-regulated genes (**Table R.2.9**). These processes were related to immune response, cell communication, and cell death. Especially interesting was the down-regulation of inflammatory processes through the down-regulation of specific cytokines and chemokines (*IL1A, IL1B, CCL5, CCRL2*). These results suggest that SrfJ, through a still unknown mechanism of action, is preventing the expression of these cytokines. SrfJ may counteract the global activation of the inflammatory responses that produces *Salmonella* infection in macrophages, controlling an overreacted response, protecting bacteria from cell death and promoting its survival and replication in the SCV. This effect has already been described for other T3SS2 effectors that reverse the changes caused by earlier secreted effectors. For instance, SptP and SspH1 participate in downregulating IL-8; SspH1 localizes to the nucleus and inhibits NF-κB-dependent gene expression. This observation has lead to the compelling hypothesis that suppressing proinflammatory responses is critical to intracellular survival and *Salmonella* pathogenesis (Haraga and Miller, 2003; Hardt *et al*., 1998; Srikanth *et al*., 2011).

The second approach is a more direct way to test the effect of SrfJ on host cells, without the potentially confounding effects of the simultaneous presence of other bacterial factors. Given the technical difficulties associated to transfection of macrophages, these experiments were carried out in HeLa cells. Twenty-four differentially expressed genes (fold change> 2, $p<0.05$) were found, 19 of them down-regulated. In **Table R.2.11** are represented the biological processes enriched among the GO terms associated to the differentially expressed genes. The enriched pathways are related to defense response against biotic stimuli. These results support our data obtained during macrophage infections, suggesting that SrfJ down-regulates genes related with host defense against pathogens and immune response, preventing an explosive activation of the inflammatory response. Analysing specific common genes differentially expressed in response to SrfJ in macrophage infections and HeLa transfections, we can observe that *CCL5* is very down-regulated. *CCL5* encodes a chemotactic cytokine that attracts T-cells, eosinophils, basophiles and, hence, plays and important active role recruiting leukocytes to inflammated tissues (Marques *et al.*, 2013). Further experiments are needed to validate these results, including qRT-PCR analysis of some representative genes.

In addition to the transcriptomic analysis, we used a complementary approach to try to understand through which intracellular transduction signaling pathways the effector SrfJ operates. We used a phospho-kinase array to determine the effect of SrfJ on the phosphorylation levels of 43 protein kinases and 2 related proteins. Changes were observed in the phosphorylation pattern of some proteins from RAW264.7 cells infected with wt *S.* Typhimurium compared to those that were not infected: p38α, CREB, c-Jun proteins are less phosphorylated during the infectious process; whereas WNK1, Akt, and STAT3 are more phosphorylated.

Interestingly, the presence of SrfJ was important to determine the degree of phosphorylation of WNK1 during *Salmonella* infection. This kinase is more phosphorylated during infection with the *srfJ* mutant than during infection with wt bacteria. The with-no-lysine (K) (WNK) protein kinase family is an evolutionarily conserved, atypical group of serine/threonine kinases with the conserved ATP-binding lysine shifted to a different position within the kinase domain (Veríssimo and Jordan, 2001; Xu *et al.*, 2000). WNK1 is a complex 32-exon gene with multiple tissue-specific isoforms. In total, 9 WNK1 exons are alternatively spliced, some expressed in a tissue-specific manner (Vidal-Petiot *et al.*, 2012). WNK1 and WNK4 were identified in 2001 as the genes responsible for the autosomal dominant hereditary hypertensive disease

pseudohypoaldosteronism type II (PHAII) (Wilson *et al.*, 2001). The best-characterized function of WNKs is their binding and activation of downstream target kinases, oxidative stress responsive 1 (OSR1) and SPS/STE20-related proline-alanine–rich kinase (SPAK) (Moriguchi *et al.*, 2005; Ramer *et al.*, 1993; Vitari *et al.*, 2005). Once activated, OSR1 and SPAK phosphorylate and regulate downstream cation-chloride cotransporters of the SLC12 family (Dowd and Forbush, 2003; Piechotta *et al.*, 2002; Vitari *et al.*, 2006). This WNK–SPAK/OSR1 pathway enables cells to adjust intracellular ions and cell volume in response to ion imbalances and osmotic stress (Gagnon *et al.*, 2006). In addition, WNKs have been linked to the regulation of cell proliferation (Moniz *et al.*, 2007; Tu *et al.*, 2011), cell death (Veríssimo *et al.*, 2006), cell migration (Dbouk *et al.*, 2014; Mäusbacher *et al.*, 2010; Moniz *et al.*, 2013; Zhu *et al.*, 2014), endocytosis (Cai *et al.*, 2006; Cha and Huang, 2010; Cheng and Huang, 2011; He *et al.*, 2007), angiogenesis (Dbouk *et al.*, 2014; Xie *et al.*, 2009), and autophagy (Gallolu Kankanamalage *et al.*, 2016). They also impact multiple signal transduction pathways, including the ERK1/2 and ERK5 MAP kinase pathways (Anselmo *et al.*, 2006; Xu *et al.*, 2004). Interestingly, the phosphorylation state of WNK1 in macrophages has alredy been shown to be dependent on T3SS2 effectors (Imami *et al.*, 2013). However, the specific effectors involved were not investigated and the regulated phosphorylation site was not clearly determined. Our results suggest that SrfJ contributes to decrease the level of WNK1 phosphorylated on Thr60. This residue can be phosphorylated by Akt (Jiang *et al.*, 2005; Vitari *et al.*, 2004) but this phosphorylation does not alter activity or localization of WNK1. A recent study has shown that phosphorylation of WNK1 by Akt3 in adipocytes leads to WNK1 polyubiquitination and degradation via proteasome pathway (Ding *et al.*, 2017). Our experiments show an increase in Akt phosphorylation in RAW264.7 macrophages infected with wt *Salmonella*. This increase, which is slightly reduced in cells infected with the *srfJ* mutant, could lead to activation of Akt, phosphorylation on Thr60 and degradation of WNK1. This hypothesis could be tested by measuring not only the levels of Thr60-phosphorylated but also the total levels of WNK1 in infected macrophages.

Our proteomic assay also revealed a very significant increase in the total level of HSP60 when RAW264.7 cells were infected with a *srfJ* mutant of *S.* Typhimurium. Heat shock proteins (HSPs) are ubiquitous and evolutionarily conserved proteins induced by cell stress. HSP60, in particular, is a typical mitochondrial molecular chaperone that is known to assist nascent polypeptides to reach a native conformation. It is also found in extramitochondrial compartments including cytosol, outer mitochondrial surface, cell

surface, intracellular vesicles, nucleus, extracellular space and in blood circulation (Meng *et al.*, 2018). HSP60 elicits potent proinflammatory response in cells of the innate immune system and serves as a danger signal of stressed or damaged cells (Nakamura and Minegishi, 2013). HSP60 is an innate signal for macrophages and dendritic cells that are innate nodes in the immune system network. In this cells, TLR4 signaling is activated in response to HSP60. It is clear that HSP60 on its own can activate innate immune receptors. Furthermore, NF-κB is involved in the regulation of transcription of genes encoding HSP, while members of HSP family are modulators of NF-κB activation, which occurs as a result of bacterial infections and leads to the development of inflammation (Struzik *et al.*, 2015). HSP60 protein levels have also been found as being regulated at the post-transcriptional level, for example, HSP60 can exert pro-apoptotic function, which could be regulated by DNA-PK activity (Um *et al.*, 2003). Results in **Figure R.2.6** suggest that SrfJ prevents an increase in the level of HSP60 induced by *Salmonella* infection. This could be important to avoid immune detection of the pathogen by the host.
An applied aspect of this thesis was the design of a *Salmonella* live vaccine based on the T3SS. Previous studies have shown that T3SS-mediated translocation can be used for efficient delivery of heterologous antigens in fusion with effector proteins to the cytosol of antigen-presenting cells (APC) (Hegazy *et al.*, 2012; Panthel *et al.*, 2008; Rüssmann, 2003; Xiong *et al.*, 2011). Effector SseJ showed its usefulness as a carrier of model antigens in live vaccine design (Hegazy *et al.*, 2012). Therefore, in **Chapter 3** we have tested the possibility of using SseJ and other effectors studied in our laboratory, SrfJ, SlrP, SteA and SseK1, as carriers in the design of a vaccine against *P. aeruginosa*, a clinically relevant Gram-negative opportunistic bacterial pathogen. Antigens used were the outer membrane proteins OprF and OprI (Baumann *et al.*, 2004; Hassan *et al.*, 2015; Gilleland *et al.*, 1988; Lee *et al.*, 2000b; Matthews-Greer and Gilleland, 1987; von Specht *et al.*, 1996), and the *P. aeruginosa* V-antigen (PcrV), a component of the virulence-related T3SS of these bacteria (Le Moigne *et al.*, 2015; Taylor *et al.*, 2015).
This study shows that the T3SSs of *Salmonella* can be used as vectors for efficient delivery of *P. aeruginosa* antigens and elicitation of protective immunity against this opportunistic pathogen. Several constructs were prepared in a low-copy-number plasmid and, among them, the expression of a fusion between the *Salmonella* effector SseJ and the *Pseudomonas* antigen PcrV under the control of P*sseA* yielded good results. Since P*sseA* responds to intravacuolar signals and SseJ is a specific substrate of the T3SS2, the fusion protein SseJ-PcrV is expected to be expressed inside the vacuole of the antigen-

presenting cell and translocated from the vacuole into the host cell cytosol. Previous findings suggested that translocation of fusion proteins results in MHC class-I and MHC class II-restricted presentation and that a fusion of the model antigen listeriolysin to SseJ induced T cell responses after vaccination in mice (Hegazy *et al.*, 2012). Here, we show that mice immunized with *Salmonella* expressing a fusion of SseJ to PcrV, a relevant *P. aeruginosa* antigen, produced high levels of specific anti-PcrV IgG and were able to survive an otherwise lethal infection with *Pseudomonas*. The protective effect of this vaccination was confirmed because immunized mice displayed lower bacterial load in systemic organs, and lower serum levels of pro-inflammatory cytokines.

Surprisingly, a similar construct with the OprF/I antigen was unable to elicit the production of specific antibodies or to provide protection against Pseudomonas. The reasons for the superior immunogenicity of PcrV compared to OprF/I in our system are unclear. The recombinant hybrid protein OprF(190-342)/OprI(21-83) was shown to be immunogenic and protective in previous studies (Arnold *et al.*, 2004; Baumann *et al.*, 2004; Bumann *et al.*, 2010; Göcke *et al.*, 2003; Knapp *et al.*, 1999; Mansouri *et al.*, 1999, 2003; Rello *et al.*, 2017; Saha *et al.*, 2006; von Specht et al., 1996b, 1995; Zhang *et al.*, 2015), and a vaccine based on this hybrid protein, IC43 (Rello *et al.*, 2017; Westritschnig *et al.*, 2014), was evaluated in a phase III clinical trial (NTC01563263). The N-terminal fusion to SseJ, the C-terminal fusion to the FLAG epitope, or the way translocated proteins are processed, could be factors that influence the response to a specific antigen. We were able to detect expression and translocation to macrophages of 5 different combinations of promoter and effector in fusion with OprF/I. All of them were tested in mice but none of them elicited specific antibody production.

PcrV is an essential component of the T3SS. This system and its effectors (ExoS, ExoT, ExoU and ExoY) are the major virulence determinant of *P. aeruginosa* (Galle *et al.*, 2012). ExoS and ExoT are toxins that prevent the production of reactive oxygen species burst in neutrophils (Vareechon *et al.*, 2017). ExoT also promotes death of host cells (Wood *et al.*, 2015b, 2015a). ExoU is a highly cytotoxic phospholipase (Sato and Frank, 2004). ExoY is an adenylate cyclase that causes actin cytoskeleton disorganization, cell necrosis and alteration of endothelial cell barrier integrity following lung injury (Stevens *et al.*, 2014). Thus, the T3SS is an appealing target for new therapies, especially against acute infections, and a number of pharmacological inhibitors have been discovered (Anantharajah *et al.*, 2016). In particular, PcrV, as part of the translocon, the most accessible part of the T3SS, has been selected for targeting by antibodies and some of

them have been developed for clinical use (Baer *et al.*, 2009; DiGiandomenico *et al.*, 2014; François *et al.*, 2012; Warrener *et al.*, 2014). However, few vaccines have been described based on this antigen, presumably because of solubility problems during the purification of the protein (Yang *et al.*, 2017). The methodology employed here, circumvents these problems, since the antigen is produced by *Salmonella* and directly translocated through the T3SS2 into the antigen-presenting cells. Among the combinations of promoters and effectors tested, only one yielded a fusion protein, SseJ-PcrV with a detectable level of translocation into RAW264.7 macrophages. This construct was very efficient in immunization experiments and showed a high protective potential in a mouse model of *P. aeruginosa* infection. These results are in agreement with a previous study that showed that fusion proteins based on SseJ elicited potent immune responses against model antigens (ovalbumin and listeriolysin), although protection assays were not carried out in that work (Hegazy *et al.*, 2012). The use of and in vivo inducible promoter, like P*sseA*, instead of a constitutive promoter, is advantageous to enhance stable expression and immunogenicity of foreign antigens expressed by *Salmonella* (Dunstan *et al.*, 2003). The pWSK29 vector carries the pSC101 replicon that produces 6 to 8 copies per cell (Wang and Kushner, 1991) and is sufficiently stable for in vivo applications (Hegazy *et al.*, 2012).

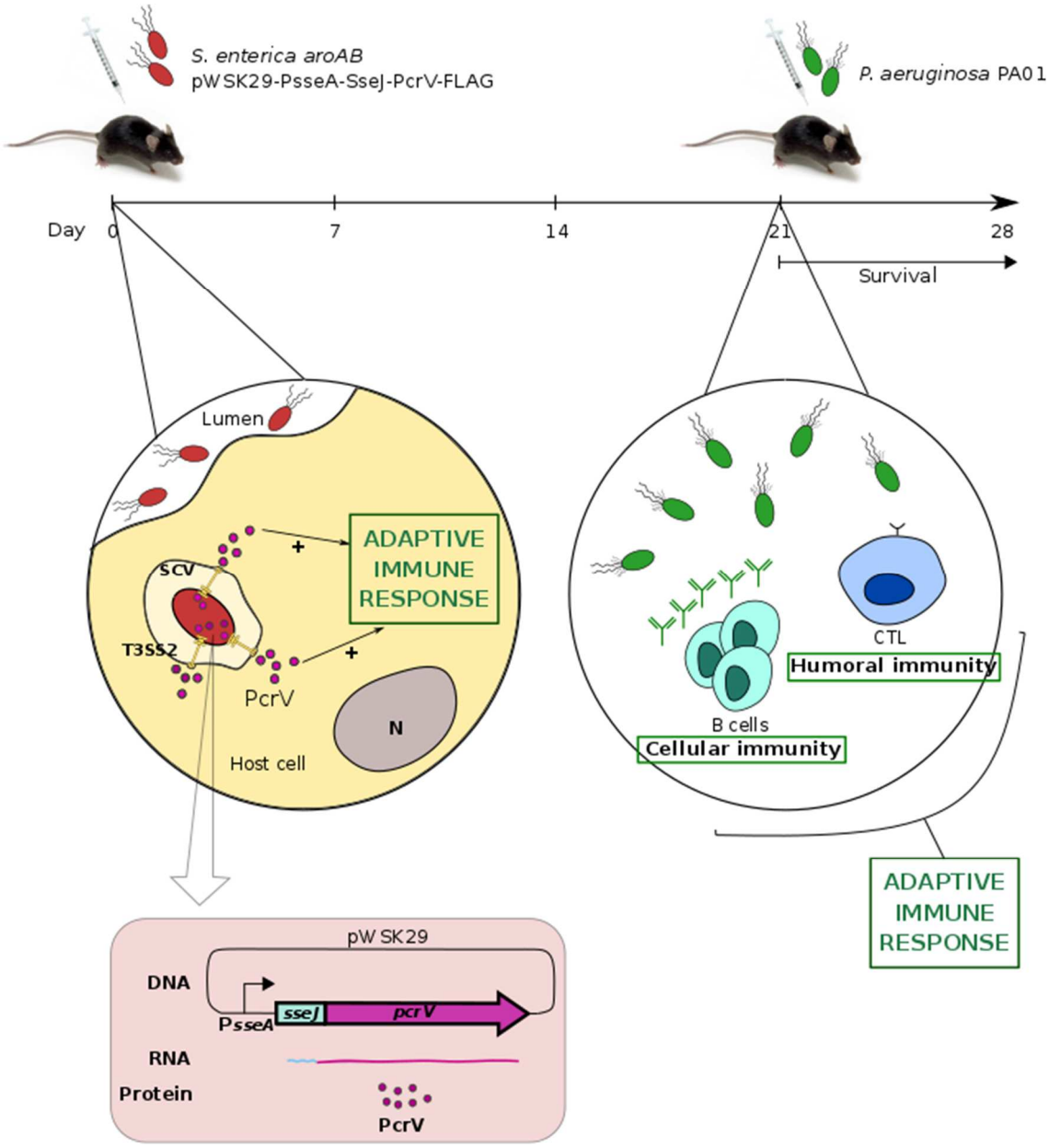


Figure D.2. Immunization against *P. aeruginosa* using *S. enterica aroAB* as a live vaccine. SCV: *Salmonella*-containing vacuole; CTL: Cytolytic T Lymphocyte.

Although this is an important step towards the development of an effective vaccine for the prevention of infections with *P. aeruginosa*, additional aspects could be optimized in future experiments. (i) Several other effectors could be tested as carriers for the PcrV antigen, taking into account that the fusion with the highest level of expression in vitro is not necessarily the most immunogenic in vivo (Hegazy *et al.*, 2012). (ii) Since *Salmonella* is also a pathogen, strains used to develop a live vaccine should be attenuated. However, they should also be able to reach, multiply and persist temporarily in lymphoid organs to stimulate protective immune responses. Thus, a balance between attenuation and immunogenicity is essential for the success of a live vaccine. The *aro* mutants of *S.*

Typhimurium used here are auxotrophic for aromatic amino acids and several essential vitamins. These mutants are safe and immunogenic in the mouse model (Hoiseth and Stocker, 1981), where this serovar can reach systemic organs, and have previously been used as carriers of heterologous antigens (Bachtiar *et al.*, 2003; Coloe *et al.*, 1995; Dougan *et al.*, 1987). Since *S.* Typhimurium causes only self-limited intestinal disease in immunocompetent humans and does not reach systemic sites in this host, the human-restricted *S.* Typhi is also being tested as a live vector for humans (Galen *et al.*, 2009). A double *aro* mutant of *S.* Typhi is highly immunogenic but additional attenuation could be desirable in humans (Dilts *et al.*, 2000; Tacket *et al.*, 1992), therefore, other means of enhancing vaccine efficacy and safety have been proposed, including a combination of regulated delayed attenuation mutations (Wang *et al.*, 2013a). (iii) Another important aspect to be considered for a future development of this vaccine is the route of immunization. In addition to the intraperitoneal route used here, mice can also be inoculated with *Salmonella* via the intravenous, subcutaneous or oral routes. The latter more closely resembles the natural route of infection in humans but requires the highest number of bacteria (Simon *et al.*, 2011). Additional routes of mucosal administration, including intranasal and rectal, have also been successfully explored (Spreng *et al.*, 2006). In conclusion, the last part of the thesis shows that the SPI2 related T3SS of *Salmonella* is an appropriate vehicle to deliver the *Pseudomonas* antigen PcrV and to generate a protective live vaccine against this human pathogen.

CONCLUSIONS

1. The expression of the *Salmonella* gene *srfJ* is driven by two promoters: a proximal promoter, P*srfJ,* and a distal promoter, P*iolE.*

2. P*srfJ* is induced in macrophages in response to intravacuolar signals and is regulated by the phosphorelay systems PhoQ/PhoP, SsrA/SsrB, and Rcs.

3. P*iolE* is induced in plants in response to *myo*-inositol and is negatively regulated by IolR.

4. Sixteen genes are significantly down-regulated and 12 genes are significantly up-regulated in response to the presence of SrfJ in human epithelial HeLa cells and murine RAW264.7 macrophages.

5. SrfJ contributes to dephosphorylation of WNK1 and prevents induction of HSP60 in RAW264.7 macrophages.

6. A live *Salmonell*a vaccine delivering the *Pseudomonas* antigen PcrV through the type III secretion system protects against *P. aeruginosa*.

CONCLUSIONES

1. La expresión del gen *srfJ* de *Salmonella* está dirigida por dos promotores: un promotor proximal, P*srfJ*, y un promotor distal, P*iolE*.

2. P*srfJ* se induce en macrófagos en respuesta a las señales intravacuolares y está regulado por los sistemas de regulación PhoQ/PhoP, SsrA/SsrB y Rcs.

3. P*iolE* se induce en plantas en respuesta a *mio*-inositol y está regulado negativamente por IolR.

4. Dieciséis genes están significativamente menos expresados y 12 significativamente más expresados debido a la presencia de SrfJ en células epiteliales humanas HeLa y en macrófagos de ratón RAW264.7.

5. SrfJ contribuye a la desfosforilación de WNK1 y evita la inducción de HSP60 en macrófagos RAW264.7.

6. Una vacuna viva de *Salmonella* que transloca el antígeno de *Pseudomonas* PcrV a través de un sistema de secreción de tipo III protege frente a *P. aeruginosa*.

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