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Salmonella enterica Serovar Typhimurium Skills To Succeed in the Host: Virulence and Regulation

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SUMMARY

Salmonella enterica serovar Typhimurium is a primary enteric pathogen infecting both humans and animals. Infection begins with the ingestion of contaminated food or water so that salmonellae reach the intestinal epithelium and trigger gastrointestinal disease. In some patients the infection spreads upon invasion of the intestinal epithelium, internalization within phagocytes, and subsequent dissemination. In that case, antimicrobial therapy, based on fluoroquinolones and expanded-spectrum cephalosporins as the current drugs of choice, is indicated. To accomplish the pathogenic process, the *Salmonella* chromosome comprises several virulence mechanisms. The most important virulence genes are those located within the so-called *Salmonella* pathogenicity islands (SPIs). Thus far, five SPIs have been reported to have a major contribution to pathogenesis. Nonetheless, further virulence traits, such as the pSLT virulence plasmid, adhesins, flagella, and biofilm-related proteins, also contribute to success within the host. Several regulatory mechanisms which synchronize all these elements in order to guarantee bacterial survival have been described. These mechanisms govern the transitions from the different pathogenic stages and drive the pathogen to achieve maximal efficiency inside the host. This review focuses primarily on the virulence armamentarium of this pathogen and the extremely complicated regulatory network controlling its success.

INTRODUCTION

Gastrointestinal diseases of infectious origin usually arise upon ingestion of contaminated foods or water and can have a wide number of etiological agents, known as enteric pathogens. Among them, the genus *Salmonella* is of particular clinical relevance in both developed and developing countries, where this pathogen is one of the most common causes of food-borne illness and is a major cause of diarrheal diseases, respectively (1–5). According to the information published by the CDC (http://www.cdc.gov/salmonella/general/index.html), approximately 40,000 cases of salmonellosis are reported each year in the United States alone, despite the real number supposedly being 30-fold greater or more due to the absence of diagnosis or reporting of many milder cases. This illness is detected predominantly in young children, the elderly, and immunocompromised patients, leading to the death of 400 persons each year due to acute salmonellosis in the United States. Moreover, food-borne pathogens usually emerge in outbreaks and may affect a significant number of patients. Several outbreaks attributed to different *Salmonella* serovars are reported each year, highlighting the frequency of *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis among the most common causal agents (1, 5).

The pathogenesis triggered by *S.* Typhimurium has been extensively studied over the last few years. Knowledge about the

virulence mechanisms of this pathogen is increasing and has led to a comprehensive study of the five *Salmonella* pathogenicity islands (SPIs) reported thus far which most significantly contribute to host cell interactions (6, 7). Additional virulence determinants, such as those encoded within the pSLT virulence plasmid, adhesins, flagella, and biofilm-related proteins, are also under study and have been reported to contribute to several stages of the disease (8–13). This huge armamentarium of virulence factors is under the control of an extremely complicated regulatory network, which coordinates and synchronizes all the elements involved. This regulation is important not only from the point of view of guaranteeing the expression of individual virulence elements but also to confer a cross talk between all of these determinants to ensure the appropriate response of the bacteria in which all the stages are subsequently activated following a temporal hierarchy (14–16).

The aim of this review is to provide an overview of the main virulence elements and their sequential contribution to the pathogenesis of *S.* Typhimurium as well as to understand the regulatory network behind the control and coordination of its armamentarium. The regulators that are involved in the regulation of several elements and are responsible for cross talk are emphasized in this review. For better understanding, the pathogenetic process is also reported following an introduction to the pathogen, which describes the clinical aspects of the disease as well as the most appropriate antimicrobial therapy and resistance patterns.

THE GENUS *SALMONELLA*

The discovery of the genus *Salmonella* goes back to 1885 when Daniel Elmer Salmon, an American veterinary pathologist, and Theobald Smith, his assistant, had been searching for the cause of common hog cholera. Smith isolated a new species of bacteria, formerly called *S. cholerae-suis*, from ill pigs and proposed it as the casual agent. Nonetheless, despite Smith being the actual discoverer, Salmon claimed credit for the discovery, and the organism was subsequently named after him. Later research, however, revealed that this organism rarely causes enteric symptoms in pigs and was therefore not the agent they were seeking (which was eventually shown to be a virus) (17).

The genus *Salmonella*, which is closely related to the genus *Escherichia*, is composed of Gram-negative, non-spore-forming, rod-shaped bacteria belonging to the *Enterobacteriaceae* family. These microorganisms range in diameter from around 0.7 to 1.5 μm, with a length of 2 to 5 μm. They are facultative anaerobes and show predominantly peritrichous motility. This genus refers to primary intracellular pathogens leading to different clinical manifestations in the development of infection in humans (18, 19).

Classification

The World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France, defines and updates the classification of this genus based on the Kauffmann-White scheme (20). Accordingly, the genus *Salmonella* consists of two species, *S. enterica* and *S. bongori*. In turn, *S. enterica* can be divided into six subspecies: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and *S. enterica* subsp. *indica* (VI). *S. bongori* (V) was initially considered to be another subspecies but it has now been classified separately from the rest of the *S. enterica* lineages as a distinct species. *S. bongori* as well as subspecies II, IIIa, IIIb, IV, and VI are rarely isolated from clinical specimens but rather are found principally in cold-blooded vertebrates and in the environment. Therefore, almost all *Salmonella* organisms that cause disease in humans and domestic animals belong to *S. enterica* subspecies *enterica* (I) (20–22).

Alternatively, *S. enterica* strains can also be classified on the basis of the O (lipopolysaccharide [LPS]) surface antigen into 67 serogroups and into 2,557 serotypes or serovars when strains are differentiated by both their O and H (flagellar) antigens. Among them, 1,531 serovars are recognized to belong to subspecies I. Before this taxonomy was established, serovar names were wrongly treated as species and hence were italicized. Nowadays, according to the current classification, the familiar names given to serovars, such as *S. enterica* serovar Typhimurium, Enteritidis, or Choleraesuis, are maintained and not replaced by their antigenic formulas. Nonetheless, the nomenclature should be *S. enterica* followed by the serovar (e.g., *S. enterica* serovar Typhimurium) (20).

Clinical Identification

Identification in the clinical laboratory is performed by the growth of stool samples on different solid media. Plates are examined after 24 h of growth at 37°C based on the macroscopic characteristics. MacConkey agar plates are generally used in all laboratories and represent a low-selectivity medium in which *Salmonella* colonies are colorless due to the lack of lactose fermentation. However, other solid selective media, such as *Salmonella-Shigella* (SS) agar, xylose-lysine-deoxycholate (XLD) agar, and Hektoen enteric (HE) agar plates, are used for more specific isolation and identification. Hydrogen sulfide production, a metabolic trait characteristic of this genus, is shown by colonies with black centers in these three types of selective media.

In addition, several specific biochemical properties corroborate the identification of this enteric pathogen. These properties include the production of gas and hydrogen sulfide on Kligler's iron agar (KIA) and triple sugar iron (TSI) agar as well as dextrose fermentation leading to yellow coloration. Both media are used to determine the ability to ferment glucose and/or lactose, although the TSI medium also detects sucrose fermentation. *Salmonella* can ferment glucose but not lactose or sucrose. Lack of lysine decarboxylase production is also characteristic of the genus *Salmonella*. Moreover, further identification of the serovar involved is obtained with the use of specific antisera. There are 7 polyvalent O antiserum mixtures available in the market, and among these, OMA and OMB are used in the clinical setting to detect approximately 98% of the *Salmonella* strains isolated from humans and warm-blooded animals (including serogroups A, B, C, D, E, F, G, H, and L).

At present, an increasing number of clinical laboratories are replacing the former biochemical characterization of *Salmonella* by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry analysis because it is a simple, rapid, inexpensive method for routine identification. Unfortunately, the identification of *Salmonella* clinical isolates with MALDI-TOF analysis cannot reach the serovar level (23, 24).

Clinical Relevance

Two major clinical syndromes caused by *Salmonella* infection in humans are enteric or typhoid fever and colitis/diarrheal disease.

Enteric fever is a systemic invasive illness caused by the exclusively human pathogens *S. enterica* serovar Typhi and *S. enterica* serovar Paratyphi A and B. Clinical manifestations include fever, headache, abdominal pain, and transient diarrhea or constipation, and infection can produce fatal respiratory, hepatic, spleen, and/or neurological damage. Without treatment, the mortality is 10 to 20%, decreasing to <1% among patients treated with the appropriate antibiotics (25, 26).

In contrast, there are many nontyphoidal *Salmonella* (NTS) strains that cause diarrheal disease in humans and can, in addition, infect a wide range of animal hosts (25, 27). According to data obtained from the World Health Organization, *S.* Enteritidis and *S.* Typhimurium are the two serovars most commonly isolated in clinical practice. In all regions except North America and Oceania, *S.* Enteritidis is more prevalent than *S.* Typhimurium. Nonetheless, these two serovars rank in opposite order in these two regions, globally accounting for 65% and 12% of all isolates, respectively, in 2002. In contrast to these results, *S.* Typhimurium was the most commonly reported serotype among nonhuman isolates in 2002, accounting for 17% of isolates (28).

In an immunocompetent host, NTS serovars cause self-limiting diarrhea that has an untreated case fatality rate of approximately 0.1% in developed countries. Risk factors for NTS diarrheal disease include age, alteration of the endogenous bowel flora (e.g., as a result of previous antimicrobial therapy or surgery), achlorhydria, atrophic gastritis or previous gastric surgery, and diabetes, and of particular importance is the dramatically more severe and invasive presentation in immunocompromised adults, particularly in the context of HIV (27, 29).

Enteric infection with *Salmonella* cannot be reliably clinically distinguished from that caused by other enteric bacterial pathogens. Patients typically present an acute onset of fever, cramping, abdominal pain, diarrhea with or without blood associated with inflammation of the large bowel, and very often nausea and vomiting as well; there is a wide spectrum of severity of illness (19). Disease usually occurs after the ingestion of greater than 50,000 bacteria in contaminated food or water and after an incubation period of approximately 6 to 72 h, which depends on host susceptibility and inoculum (30). Approximately 5% of individuals with gastrointestinal illness caused by NTS develop bacteremia, a serious and potentially fatal problem. Bacteremia is more likely to occur in young children, immunologically compromised patients, and patients with comorbid medical conditions (e.g., HIV, malaria, or malnutrition). These hosts are also more likely to develop focal infection, including meningitis, septic arthritis, osteomyelitis, cholangitis, and pneumonia. A feared complication of *Salmonella* bacteremia in adults is the development of infectious endarteritis, especially that which involves the abdominal aorta (19, 29, 31).

On the other hand, the mortality rate due to NTS is as high as 24% in developing countries, where *Salmonella* infections contribute to childhood diarrhea morbidity and mortality and are a common cause of hospital admission among children, being among the most frequent etiological agents causing bacteremia (>20% of cases). This high impact is the consequence of the marked intensity of the symptoms observed in children with enterocolitic infection, such as increased inflammatory severity, bloody diarrhea, and increased duration of infection and risk of complication, which is particularly important when malnutrition is also concomitant (19, 31–33).

Another particular aspect of the clinical impact of *Salmonella* infections is the so-called carrier state. This condition, which is valid for NTS infections in both humans and livestock and for typhoid fever, corresponds to a persistent colonization of the gut, established durably upon the initial infection (over 10 weeks postinfection). Biofilm production is often among the virulence traits supporting such chronic persistence. These carriers are characterized by a symptom-free condition and can act as reservoirs and hence contribute to the propagation of the disease, which is particularly important in the case of food workers. Unfortunately, there is a scarcity of results concerning the immune response and the efficacy of antimicrobial treatment in these silent infections that could be used to develop suitable prophylactic and therapeutic modalities (34–36).

Antimicrobial Treatment and Resistance

For gut-limited NTS infections, treatment of fluid and electrolyte imbalances by oral or intravenous rehydration is necessary when fluid loss is substantial. In this type of disease, the symptoms usually last between 5 and 7 days and resolve spontaneously. Antimicrobial therapy is indicated only for patients who are severely ill, when positive signs of invasive disease have been detected, and for patients with risk factors, such as those mentioned above, for extraintestinal spread of infection. However, there is controversy about the efficacy of antibiotics in decreasing either the duration of illness or the severity of symptoms. Children under 1 year of age should also be treated to prevent invasion. Usually 3 to 7 days of treatment is reasonable (19, 29). Antibiotics may also be useful when rapid interruption of fecal shedding is needed to control outbreaks of salmonellosis in institutions (37).

Efficient therapies include treatment with fluoroquinolones, trimethoprim-sulfamethoxazole (TMP-SMZ), ampicillin, or expanded-spectrum cephalosporins (e.g., ceftriaxone or cefixime). However, the increasing rates of antibiotic resistance among *S.* Typhimurium isolates have led to less use of TMP-SMZ and ampicillin, since resistance to these antimicrobial compounds is common. Even worse, resistance to multiple antimicrobial agents (multidrug resistance [MDR]) can be particularly high among *S.* Typhimurium isolates (>55%) (38, 39). Spread of this MDR phenotype is supported by dissemination of dominant resistant clones, such as definitive phage type DT104, which carries several chromosomally located genes conferring the ACSSuT resistance type (resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline) (40). On the other hand, dissemination of strains carrying hybrid plasmids (see below) is a potential problem. These strains, which are resistant to ampicillin, chloramphenicol, streptomycin-spectinomycin, sulfonamides, and tetracycline, have already been detected in Spain and the United Kingdom, and indirect evidence has suggested their presence in other European countries (41–43).

As a result, there has been an increasing use of expanded-spectrum cephalosporins and quinolones when susceptibilities are unknown (44). Unfortunately, in line with these therapeutic strategies, an increasing rate of resistance has been observed, not only to nalidixic acid, a phenotype which usually correlates with decreased susceptibility to ciprofloxacin and appears to be a predictor of clinical "fluoroquinolone hyporesponsiveness," but also to expanded-spectrum cephalosporins, which are also widely used in the clinical setting, especially among children, for whom these agents are the current drugs of choice (29, 45–47). Particularly, the

phenotype of reduced susceptibility to ciprofloxacin (MIC ≥ 0.125 μg/ml) has been associated with a delayed response or clinical failure following treatment with these antimicrobial agents (46, 48). As a result, the Clinical and Laboratory Standards Institute (CLSI) has adapted the breakpoints for quinolones, which have been currently proposed to be ≤0.06 mg/liter for susceptibility, 0.12 to 1.0 mg/liter for the intermediate phenotype, and ≥2 mg/liter for resistant bacteria (49). Recently, however, a relationship between acquisition of high levels of fluoroquinolone resistance and decreased cell invasion ability has been reported, and this may explain why, in general terms, a high prevalence of fluoroquinolone-resistant *S. enterica* strains remains rare among clinical isolates. These resistant bacteria show an impaired growth rate which may appear as a consequence of the acquisition of fluoroquinolone resistance and compromise the expression of the invasion genes (50–52). In view of these results, azithromycin and aztreonam are alternative agents that may be useful for patients with multiple allergies or for organisms with unusual resistance patterns (29).

Treatment of bacteremia can usually be successfully completed within 10 to 14 days of therapy. However, treatment of life-threatening bacteremia complications now includes both an expanded-spectrum cephalosporin and a fluoroquinolone until the susceptibilities of the antimicrobial agents are known. If endocarditis or infectious arteritis is eventually reported, surgery should be undertaken as soon as possible for the best chance of achieving a cure (29).

PATHOGENESIS MODEL

Salmonella Typhimurium infection begins with the ingestion of organisms in contaminated food or water. The first obstacle to overcome within the host is the acidic pH of the stomach. To protect itself against severe acid shock, *S.* Typhimurium activates the acid tolerance response (ATR), which provides an inducible pH-homeostatic function to maintain the intracellular pH at values higher than those of the extracellular environment (53). After entering the small bowel, salmonellae must reach and traverse the intestinal mucus layer before encountering and adhering to intestinal epithelial cells. In mice, salmonellae appear to preferentially adhere to and enter the M cells of the Peyer's patches (PPs) in the intestinal epithelium, although invasion of normally nonphagocytic enterocytes can also occur (54, 55). Shortly after adhesion, the invasion process appears as a consequence of engaged host cell signaling pathways leading to profound cytoskeletal rearrangements (56, 57). These internal modifications disrupt the normal epithelial brush border and induce the subsequent formation of membrane ruffles that engulf adherent bacteria in large vesicles called *Salmonella*-containing vacuoles (SCVs) (58–60), the only intracellular compartment in which *Salmonella* cells survive and replicate (58, 59). Simultaneously, induction of a secretory response in the intestinal epithelium initiates recruitment and transmigration of phagocytes from the submucosal space into the intestinal lumen. This process is associated with the production of several proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-8 (IL-8) (25, 61). Lastly, the apical epithelial brush border reconstitutes (62) (Fig. 1).

SCVs are initially integrated within the early endocytic pathway. However, they need to be later uncoupled to bypass delivery of lysosomal enzymes. This action depends on *Salmonella*-directed changes in host endocytic trafficking and function to eventually avoid fusion with secondary lysosomes (63, 64). During SCV maturation, *Salmonella* induces *de novo* formation of an F-actin meshwork around bacterial vacuoles, a process which is termed vacuole-associated actin polymerization (VAP) and is important for maintenance of the integrity of the vacuole membrane (65). SCVs then migrate to a perinuclear position, in close proximity to the Golgi apparatus, presumably to facilitate interception of endocytic and exocytic transport vesicles to obtain nutrients and/or membrane fragments. This event appears to be essential for bacterial replication (66, 67). In addition, it has been observed that intracellular *Salmonella* can induce the formation of long filamentous membrane structures called *Salmonella*-induced filaments (SIFs) (68, 69). SIFs are tubular aggregates along a scaffold of microtubules and originate from the SCVs and extend throughout the cell. Although the biological role of the induction of SIFs is not completely understood, it has been postulated that this process may lead to an increased availability of nutrients that may otherwise be limited within the SCV (70).

Thereafter, a fraction of these SCVs transcytose to the basolateral membrane. Once across the intestinal epithelium, salmonellae are engulfed by phagocytes. Three types of phagocytes are reported to interact with these invading bacteria: (i) neutrophils, (ii) inflammatory monocytes which differentiate into macrophages, and (iii) dendritic cells, another type of monocytes which function as antigen-presenting cells. The first two types of cells are both recruited from blood in response to the inflammatory signals (71–75). In general terms, since most of the bacterial cells have breached the epithelium through the M cells, they directly reach the PPs and then the mesenteric lymph nodes (MLNs) via the intestinal lymph, most likely being transported by dendritic cells (76). Nonetheless, dendritic cells have also been reported to directly take up bacteria from the intestinal lumen by opening the tight junctions and sending dendrites to the lumen (77). Experiments performed by Rydstrom and Wick indicate that inflammatory monocytes (macrophages) are those phagocytes which accumulate predominantly in PPs and MLNs, followed by neutrophils (78). Salmonellae are then phagocytosed and internalized again within SCVs, triggering a response similar to that reported inside epithelial and M cells to ensure bacterial survival and replication (25, 78, 79). Migration of these infected phagocytes, predominantly macrophages, facilitates systemic dissemination of the bacteria via the bloodstream to several additional tissues, such as the spleen and liver, where this pathogen preferentially replicates (25, 79). Alternatively, direct blood access of *Salmonella*-infected phagocytes from the basolateral side of the intestine has also been suggested to contribute to systemic dissemination. This hypothesis is supported by the finding of infected phagocytes in the blood within minutes after oral infection and is attributed to a manipulation of the motility of the infected cells (80) (Fig. 1).

VIRULENCE FACTORS AND STRATEGIES

In order to overcome the pathogenic process described above, *S.* Typhimurium possesses many virulence strategies employed to interact with the above-mentioned host defense mechanisms. The majority of the genes encoding the most important virulence factors are located within highly conserved *Salmonella* pathogenicity islands (SPIs), whereas others are found on a virulence plasmid (pSLT) or in the chromosome. Thus far, a total of five SPIs (SPI-1 to SPI-5) have been identified as being clearly involved in *S.* Typhimurium virulence, together with further virulence components such as the pSLT plasmid-carried *spv* operon, several types

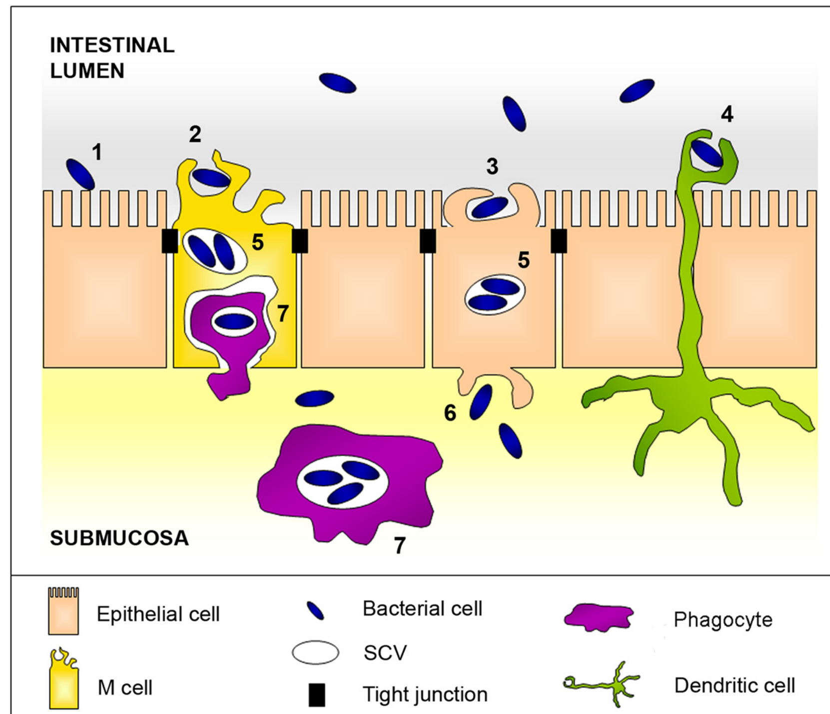

FIG 1 Pathogenesis model of *Salmonella enterica* serovar Typhimurium. 1, *Salmonella* cells attach to the intestinal epithelium by means of adhesins, such as those encoded within SPI-3 and SPI-4. 2 and 3, Invasion of bacteria follows, and engulfment is mediated by virulence factors encoded within SPI-1 and SPI-5. 4, Alternatively, bacterial cells can also be directly taken up by dendritic cells from the submucosa. 5, Once inside the cytoplasm, *Salmonella* is localized within the SCV, where it replicates. Factors encoded within SPI-2 and the pSLT plasmid are essential for survival. 6, The SCVs transcytose to the basolateral membrane and release the internal cells to the submucosa. 7, Bacteria are internalized within phagocytes and located again within an SCV, where SPI-3, in addition to SPI-2 and the pSLT plasmid, play an important role. Lastly, these infected phagocytes can disseminate through the lymph and the bloodstream. (Modified from reference 347 with permission from the BMJ Publishing Group.)

of adhesins, flagella, and the essential components for biofilm formation (6, 19, 81). In this review, the most relevant proteins contributing to pathogenesis of *S.* Typhimurium are considered and described below to provide a sequential overview of the steps that this pathogen overcomes inside the host (Table 1). First, an initial description of each block of virulence determinants is presented.

Virulence Determinants

SPIs. SPI-1 encodes several effector proteins which mostly trigger invasion of epithelial cells by mediating actin cytoskeletal rearrangements and hence internalization of the bacteria. These effectors are translocated into the host cell by means of a type III secretion system (T3SS), termed T3SS-1, also encoded within SPI-1 (Fig. 2). The *prg*/*org* and *inv*/*spa* operons encode the needle complex *per se*, whereas the *sic*/*sip* operons encode the effector proteins and the translocon (SipBCD), a pore-forming structure that embeds in the host cell membrane and delivers these effectors to the host cytosol. Other injected effectors, however, have been reported to be encoded elsewhere on the chromosome (82–84). In addition, several chaperones are also encoded within SPI-1. Through specific binding to their targets (secreted or effector proteins), these chaperones protect SPI-1-related proteins from degradation, prevent premature interactions, and/or mediate their recognition by T3SS-1 (Table 1).

SPI-2 is divided into two segments. The smaller portion contains the *ttrRSBCA* operon, which is involved in tetrathionate reduction, and seven open reading frames (ORFs) of unknown function. Initial results suggested that these genes do not significantly contribute to systemic infections in mice (85). Conversely, recent evidence has attributed a growth advantage over the microbiota to the expression of these genes (86). The larger portion of this island was initially characterized to be of key importance for the ability of *Salmonella* to survive and replicate inside host cells (epithelial cells and macrophages) within the SCV (87, 88). The SPI-2-related events are triggered by the action of effector proteins injected into the host cytoplasm by means of its own T3SS, T3SS-2 which also encodes its proper translocon machinery (SseBCD). In general terms, SPI-2 harbors four types of genes which are important for virulence: *ssa*, the genes encoding the T3SS-2 apparatus; *ssr*, encoding the regulators; *ssc*, encoding the chaperones; and *sse*, encoding the effectors (Fig. 2) (Table 1) (6, 89).

The remaining three SPIs have not been studied in as much detail as SPI-1 and SPI-2, and therefore, less information is available regarding their function. Unlike the two other SPIs, only four ORFs within SPI-3 encoding proteins with a known function have been studied (Fig. 2). This island encodes proteins with no obvious functional relationship to each other, since it is involved in both initial attachment and long-term persistence (MisL) as well as survival during systemic dissemination (MgtCB). MarT is a regulator also encoded within SPI-3, the function of which will be defined below (90–92). SPI-4 contains only six ORFs, arranged in a single operon termed *siiABCDEF*, and plays a role during the initial interaction with the intestinal epithelium and possibly con-

TABLE 1 Function, targets and chromosomal localization of the major proteins and virulence determinants contributing to *Salmonella* Typhimurium pathogenesis

Virulence determinant	Localization	Known target(s)[a]	Function(s)	Reference(s)
Flagella	Chromosome		Approach to the intestinal epithelium	9
			Efficient access to intestinal nutrients, outgrowth in the intestine	148
		TLR5	Induction of proinflammatory response, inhibition of apoptosis in epithelial cells	142, 143, 192
		IPAF	Early macrophage pyroptosis	197, 198
Type I fimbriae	Chromosome	Laminin	Adhesion to epithelial cells	116, 117
			Biofilm formation	206
Curli fimbriae	Chromosome	Fibronectin	Adhesion to epithelial cells	119, 120
		TLR2	Induction of proinflammatory response	110
			Biofilm formation	118
Pef fimbriae	pSLT plasmid	Le^x blood group antigen	Adhesion to crypt epithelial cells	122
			Induction of proinflammatory response	107
			Biofilm formation	12
Lpf fimbriae	Chromosome		Biofilm formation	12
Std fimbriae	Chromosome	α(1-2)Fucose receptors	Adhesion to epithelial cells	109, 123
AvrA	SPI-1	JNK	Inhibition of apoptosis in epithelial cells, inhibition of macrophage pyroptosis	190, 191
BapA	Chromosome		Adhesion to epithelial cells, biofilm formation	11
DsbA	Chromosome		Full activation of T3SS-1	127
		SsaC*	Full activation of T3SS-2	154
IacP	SPI-1	SigD*, SopD*, SopA*	Posttranslational modification	141
InvB	SPI-1	SipA*, SopE*, SopE2*, SopA*	Chaperone	339, 340, 341
MisL	SPI-3	Fibronectin	Adhesion to epithelial cells	92
MgtCB	SPI-3		Intramacrophage survival	90
PipA	SPI-5		Development of systemic infection	99
PipB	SPI-5		Accumulation in lipid rafts, development of systemic infection[b]	69
PipB2	Chromosome	Kinesin	Kinesin accumulation in the SCV, inhibition of SCV perinuclear migration	166, 170
SicA	SPI-1	SipB*, SipC*	Chaperone	342
SicP	SPI-1	SptP*	Chaperone	343
SigD	SPI-5		Chloride secretion, induction of proinflammatory response	133, 134
		RhoG	Actin cytoskeletal rearrangements, invasion of epithelial cells	98, 134
			Inhibition of vesicular trafficking, SCV formation and size	156
		Akt	Inhibition of apoptosis in epithelial cells	97
SigE	SPI-5	SigD*	Chaperone	96
SiiE	SPI-4		Adhesion to epithelial cells	94
SifA	Chromosome	SKIP	Decrease of kinesin accumulation in the SCV, modulation of vesicular trafficking, SCV perinuclear migration, SCV membrane integrity	159, 160, 166
SipA	SPI-1	Actin	Stabilization and localization of actin filaments during invasion, stabilization of VAP, correct localization of SifA and PipB2, SCV perinuclear migration and morphology	135, 136, 161
SipB	SPI-1		Adhesion to epithelial cells	126
			Early macrophage pyroptosis	195, 196
			Macrophage autophagy	203
SipC	SPI-1		Adhesion to epithelial cells	126
SipD	SPI-5		Adhesion to epithelial cells	126
SlrP	Chromosome	Trx, ERdj3	Apoptosis of epithelial cells	185, 186
SopE	Chromosome	Cdc42, Rac-1	Actin cytoskeletal rearrangements, invasion of epithelial cells, induction of proinflammatory response	61, 98, 129
			Nitrate respiration, outgrowth in the intestine	150
SopE2	Chromosome	Cdc42, Rac-1	Actin cytoskeletal rearrangements, invasion of epithelial cells, induction of proinflammatory response	131, 132
SopD	Chromosome		Epithelial cell invasion in cooperation with SigD	138
			Replication inside macrophages	179, 180
SopA	Chromosome		Induction of proinflammatory response	140
SptP	SPI-1	Cdc42, Rac-1	Disruption of the actin cytoskeleton by antagonizing SopE, SopE2, and SigD	145

(Continued on following page)

TABLE 1 (Continued)

Virulence determinant	Localization	Known target(s)[a]	Function(s)	Reference(s)
SpvB	pSLT plasmid	Actin	Inhibition of actin polymerization, inhibition of VAP and SIF formation, apoptosis of epithelial cells, delayed macrophage pyroptosis	13, 164, 165, 200, 201
SpvC	pSLT plasmid	ERK	Inhibition of inflammation	8, 146
SsaB	SPI-2	Hook3	Disruption of Golgi apparatus and lysosomes, inhibition of SCV-lysosome fusion	152, 155
SsaE	SPI-2	SseB*	Chaperone	338
SscA	SPI-2	SseC*	Chaperone	344
SscB	SPI-2	SseF*	Chaperone	345
SseA	SPI-2	SseB*, SseD*	Chaperone	346
SseF	SPI-2		SCV perinuclear migration	66, 67, 177, 178
			Microtubule bundling, SIF formation	177, 178
SseG	SPI-2		SCV perinuclear migration	66, 67, 177, 178
			Microtubule bundling, SIF formation	177, 178
SseI	Chromosome	Filamin	Remodeling of VAP	13
		TRIP6	Stimulation of macrophage motility, acceleration of the systemic spread	80
SseJ	Chromosome	RhoA	SIF formation	158, 175, 176
SseL	Chromosome		Delayed macrophage pyroptosis	202
SspH2	Chromosome	Filamin, profilin	Remodeling of VAP	13
ttr genes	SPI-2		Tetrathionate respiration, outgrowth in the intestine	86

[a] Targets marked with an asterisk refer to bacterial proteins. This is particularly the case for all chaperones, DsbA, and IacP.
[b] This function has been suggested according to the regulation pattern. However, there is no clear information about its role.

tributes to long-term persistence (Fig. 2) (93, 94). Finally, SPI-5 is involved in accomplishing several pathogenic processes during infection (95). The *sigDE* operon encodes SigD (SopB), a multifaceted effector involved in several steps of pathogenesis, and SigE (PipC), its presumed chaperone (Fig. 2) (Table 1) (96–98). Other genes, e.g., *pipB* and *pipA*, translocated through T3SS-2 are presumed to contribute to systemic infection in mice (99). However, more information is required in order to specifically understand the roles of the proteins encoded within these SPIs.

pSLT plasmid. Among the high number of *Salmonella* serovars, only a few harbor a serovar-specific virulence plasmid. Strains belonging to clinically important serovars, e.g., *S.* Enteritidis, *S.* Typhimurium, *S.* Choleraesuis, and *S.* Dublin, are usually positive for this trait. This specificity can be exemplified by plasmid size, ranging from 50 to 95 kb depending on the serovar. In the particular case of *S.* Typhimurium, the plasmid is approximately 95 kb and has been termed pSLT. Nonetheless, they all share a highly conserved 8-kb region of five genes, the *spvRABCD* locus, which can restore virulence to plasmid-cured strains in a mouse model (6, 100, 101). The first gene, *spvR*, encodes a regulator which will be defined in the next section. SpvB and SpvC are the only effector proteins with known functions: SpvB is a cytotoxic protein whose role is related to the intracellular stage of the disease, whereas SpvC is important primarily during the proinflammatory response of the host (8, 13). In contrast, SpvA, found exclusively in the outer membrane, and SpvD, primarily exported outside the cell, play roles in *Salmonella* virulence that are yet to be elucidated (101).

Alternatively, unusual virulence plasmids have been detected to additionally harbor antimicrobial resistance genes, and their size is significantly greater (102). In the case of *S.* Typhimurium, such hybrid plasmids (e.g., pUO-StRV2) are 140 kb in size and originate from pSLT through acquisition of a complex resistance island. Although these plasmids do not preserve all the genes located in pSLT, the *spv* operon is still detected (41).

Adhesins. Sequencing of the *S.* Typhimurium LT2 genome revealed the existence of 13 predicted fimbrial loci (103). Type I fimbriae and curli fimbriae are the only two operons which can be expressed *in vitro* under standard laboratory conditions, whereas the remaining 11 operons appear to be poorly expressed (104). In order to solve the question of whether such operons are expressed *in vivo*, the same authors studied the roles of 11 major fimbrial subunits (FimA, CsgA, LpfA, PefA, StdA, BcfA, StbA, SthA, StcA, StiA, and StfA) as antigens during infections in mice. The results showed the seroconversion of the animals to positivity in all cases, despite most animals seroconverting to only a subset of these fimbrial antigens. These findings suggest that all these structures are expressed *in vivo* (105). Several studies performed *in vitro* and *in vivo* have reported that fimbriae are involved in several pathogenic processes: adhesion to specific epithelial cells (e.g., type I fimbriae, curli fimbriae, Pef, Lpf, and Std) (106–109), intestinal fluid accumulation (e.g., curli fimbriae and Pef) (107, 110), intestinal persistence in mice (e.g., Lpf, Bcf, Stb, Stc, Std, and Sth) (111), and biofilm formation (e.g., curli fimbriae) (112). However, individual inactivations of these operons trigger a moderate alteration in mouse virulence, whereas a combination of such mutations significantly increases their lethal effects, suggesting that their contribution can be masked by the plurality and functional compensation effects of these determinants (10).

Flagella and chemotaxis. Flagella are surface appendages of *S.* Typhimurium that are required not only for motility and chemotaxis but also for several other processes in pathogenesis. The synthesis and function of the flagellar and chemotaxis system requires the expression of more than 50 genes which are divided among at least 17 operons (*flh*, *flg*, *fli*, *flj*, *mot*, *che*, *tar*, *tsr*, and *aer*) that constitute the large and coordinately regulated flagellar regulon (113).

Approach and Attachment to the Intestinal Epithelium

Once *Salmonella* has reached the intestinal lumen, the pathogen needs to establish initial contact with the epithelium to interact

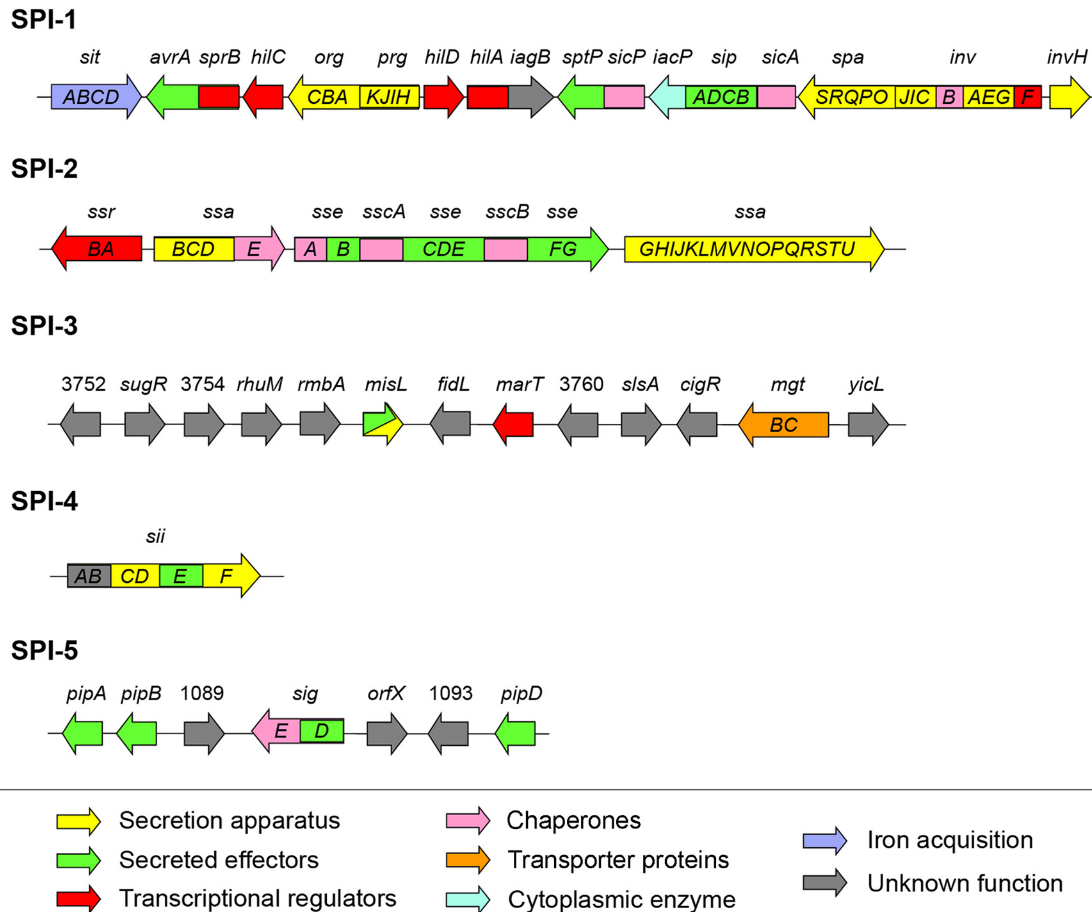

FIG 2 Schematic representation of the genes carried within the five SPIs and their putative functions.

with the target cells. This process is initially enhanced by motility and chemotaxis and is then driven by several virulence determinants, such as several types of fimbriae and adhesins, as well as the T3SS-1 translocon *per se*.

Approach. Motility is a facilitating prerequisite for *Salmonella* cells to increase the chance of encountering the intestinal epithelium and hence be able to establish adhesion to and invasion of these mammalian cells (114). Therefore, *Salmonella* strains lacking functional flagella or chemotaxis display a reduced capacity to approach the intestinal monolayer during the early phase of infection (9).

Attachment. Intimate attachment between bacteria and the eukaryotic cells is an indispensable prerequisite for the translocating activity of T3SS-1 (115). Close contact with host cells can then be established through several virulence determinants. Despite several fimbrial operons reportedly being carried within the *S.* Typhimurium genome, no information is available about the binding specificity of their products. Only those structural units with available information about their interaction with host cells (type I fimbriae, curli fimbriae, Pef fimbriae, and Std fimbriae) are considered in this review. Type 1 fimbriae of *Salmonella* are encoded by the *fim* genes, which are arranged in a single cluster which is composed mainly of the six-gene operon *fimAICDHF*, encoding structural subunits, and three regulatory genes, *fimZ*, *fimY*, and *fimW* (103). The resulting fimbrial structure binds the extracellular matrix glycoprotein laminin through its oligomannoside chains and mediates adhesion to a broad range of eukaryotic cells (116, 117). The genes encoding production of curli fimbriae (also termed tafi, for thin aggregative fimbria) are organized into two adjacent, divergently transcribed operons, *csgBAC* and *csgDEFG* (118). Curli fibers participate in several bacterial processes; however, a contribution to *Salmonella* adhesion and invasion of eukaryotic cells by binding to the extracellular matrix protein fibronectin was the initial phenotype attributed to these genes (119, 120). In contrast to the other fimbrial operons, the *pef* genes are located on the pSLT virulence plasmid of *S.* Typhimurium (121). On overexpression of these genes in an *Escherichia coli fim* mutant, Pef fimbriae specifically bind to the trisaccharide Galβ1-4(Fucα1-3)GlcNAc, also known as the Lewis X (Le^x) blood group antigen. In the human intestine, Le^x is expressed mainly by crypt epithelial cells, which remain intact once the inflammatory reaction has been initiated (in contrast to the usual cell targets of *Salmonella*). These results raise the possibility that the pathogen may bind to human crypt epithelium at later stages of infection (122). Absence of the *std* operon has been shown to cause a competitive disadvantage during long-term persistence in the ceca of mice (111). Moreover, upon turning on the expression of this operon *in vitro*, Std fimbriae contribute to intestinal colonization by mediating attachment to human colonic epithelial cell lines by binding to ter-

minal α(1-2)fucose receptors expressed in the cecal mucosa (109, 123).

Other proteins with adhesive properties, such as large adhesins (SiiE and BapA) or autotransporter proteins (e.g., MisL), have also been reported to take part in the adhesion process. The *misL* gene, located within SPI-3, encodes an autotransporter protein (91). Autotransporters are related to the type V secretion pathway, which transports proteins across the outer membranes of Gram-negative bacteria. In these systems the secreted substrate and the transport functions are in the same protein (124). The outer membrane protein MisL has been reported to bind to fibronectin, an extracellular matrix component, in *in vitro* experiments and hence to promote colonization of intestinal epithelial cells (92). The SiiC, SiiD, and SiiF proteins, encoded within SPI-4, reportedly form a type 1 secretion system (T1SS) showing homology to the TolC-like outer membrane protein, the membrane fusion protein, and the transport ATPase, respectively (93, 125). SiiE is a giant nonfimbrial adhesin exported by this T1SS and mediates contact-dependent adhesion to epithelial cells, whereas SiiA and SiiB are not secreted but represent inner membrane proteins whose function has yet to be determined. These two proteins, however, are not required for the secretion of SiiE, and mutations within the respective genes do not seem to affect the expression of SiiE or other SPI-4 gene products (94). Similarly, the large cell surface protein BapA is also secreted through a T1SS (BapBCD) encoded downstream from the *bapA* gene. Despite the attribution of its main role as being contribution to biofilm formation, the absence of this protein is also related to lower colonization of the intestinal epithelium. Thus, analogously to the function of SiiE, BapA might be involved in mediating adhesion and colonization of the host mucosa (11).

Additionally, recent experiments have provided evidence that the T3SS-1 translocon members, SipB, SipC, and SipD, and presumably the assembly of the translocon, are essential for close association with cultured mammalian cells. First, SipD is exposed on the bacterial surface prior to contact with target host cells, and it may be localized at the tip of the needle complex. This potential position could then mediate intimate attachment. Next, upon contact with host cells, SipB and SipC may also become extracellularly exposed to act in concert in promoting this interaction (126).

Invasion and Engulfment by Epithelial Cells and Induction of Inflammation

Following attachment, salmonellae cells need to fully activate the exporting machinery so that a feedback-regulated expression of effector proteins can be initiated. Thereafter, effectors are translocated through T3SS-1 to engage the host signaling pathways. This action triggers cytoskeletal rearrangements, which are essential for membrane ruffling and bacterial engulfment, and a proinflammatory response, eventually leading to the induction of colitis. This process is dependent primarily on SPI-1, although other, unrelated proteins also participate in the response.

T3SS-1 activation. Ellermeier and Slauch have stated that T3SS-1 is not yet fully functional at this step unless DsbA, an effector translocated into the host cytosol, accomplishes its function (127). DsbA is a disulfide oxidoreductase involved in the formation of periplasmic disulfide bonds which eventually contribute to the proper folding and assembly of specific proteins (128). In relation to *Salmonella* virulence, this protein is required for translocation and secretion of effectors via T3SS-1, and it has been hypothesized that it contributes to the correct assembly or proper functioning of this system. Moreover, these results indicate that production of effectors is dependent on a fully functional T3SS-1 (127).

Cytoskeletal remodeling and inflammation. Initiation of cytoskeletal remodeling and induction of a proinflammatory response are attributed mainly to the effectors SopE, SopE2, and SigD (encoded within SPI-5), which cooperate in a functionally redundant fashion. SopE functions as a guanidine exchange factor (GEF) that activates Cdc42 and Rac-1 by stimulating GDP/GTP nucleotide exchange (129). These two small Rho GTPases are components of the host cell signaling pathways involved in the actin cytoskeletal rearrangements and in the stimulation of nuclear responses, such as rapid reprogramming of host gene expression through the transcriptional factor NF-κB. Eventually, this signaling cascade triggers induction of proinflammatory cytokines such as IL-8 and TNF-α, eliciting mucosal inflammation (61, 98). However, not all *Salmonella* strains carry the *sopE* gene (130), raising the question of whether and how such strains engage the host cellular actin polymerization machinery. Covering the absence of *sopE*, SopE2, another GEF protein highly homologous to SopE, has been detected in all *S.* Typhimurium strains, also triggering a similar phenotype (131, 132).

SigD was initially reported in *S.* Dublin to promote fluid secretion, phagocyte accumulation, and inflammatory responses in the infected ileum (133). Further studies performed with the same serovar reported that this protein has inositol phosphoinositide phosphatase activity which causes derangement of the phosphatidylinositol signaling pathway, which indirectly leads to increased chloride secretion and eventually diarrhea (134). SigD has also been shown to activate a GTPase of the Rho family, the RhoG protein, in *S.* Typhimurium. This effector, however, mediates its activation through an indirect effect on an endogenous exchange factor as a result of its phosphatase activity. Likewise, the RhoG GTPase is involved in the stimulation of cell actin cytoskeletal modifications, thereby implying a role in invasion (98).

However, the actin rearrangements induced need to be localized so that host cells efficiently engulf the invading bacteria. Thereafter, the actin binding protein SipA (SPI-1 effector) stabilizes actin filaments by inhibiting their depolymerization at early stages of infection (135, 136). SipA is thought to increase the net accumulation of actin filaments at the point of bacterium-host cell contact, since it promotes outward extension of the membrane ruffles that result from the activation of Rho GTPases. Moreover, SipA contributes to bacterial localization in clusters in the invasion area, thereby facilitating bacterial uptake (136).

Three additional effectors, SopD, SopA, and IacP, also contribute to enteropathogenesis. On one hand, SopD cooperatively acts with SigD in the induction of enteritis by promoting fluid secretion and inflammatory responses in bovine ligated ileal loops (137). SopD recruitment to the site of invasion is SigD dependent and contributes to host cell membrane internalization during invasion. Thus, SopD presumably increases inflammation and fluid secretion during gastroenteritis by directly promoting *Salmonella* invasion (138). On the other hand, SopA, first described in *S.* Dublin, is an ubiquitin ligase mimicking the mammalian HECT E3 protein (139, 140). On expression of a catalytically incompetent SopA mutant, *Salmonella*-induced neutrophil transepithelial migration is reduced, suggesting that SopA ubiquitinates bacteri-

al/host proteins that are involved in intestinal inflammation (140). Finally, the cytoplasmic enzyme IacP was initially identified as a putative acyl carrier protein (ACP) by sequence similarity (135). However, recent experiments have shown that it is not involved in the biosynthesis of essential lipids as expected, but instead it presumably accounts for the posttranslational modification of SigD, SopD, and SopA, a process necessary for the secretion and translocation of these effectors (141).

In addition to these T3SS-1 effectors, there are other virulence determinants not related to the SPIs which also contribute to elicit inflammation. These bacterial structures, such as flagella and curli fimbriae, are identified as pathogen-associated molecular patterns (PAMPs) that stimulate innate pathways of inflammation (IL-8 production and neutrophil influx) upon recognition by their cognate Toll-like receptor (TLR) (110). Flagellin, the monomer which forms the filaments in the bacterial flagellum, interacts with TLR5, leading to activation of NF-κB and IL-8 secretion. These signaling responses promote fluid secretion and leukocyte influx, eventually triggering colitis. Since TLR5 is a cell surface receptor expressed exclusively on the basolateral membrane of the intestinal epithelia, these findings suggest that this interaction may be a sensor of pathogens that invade or translocate flagellin through the intestinal mucosa (142, 143).

Adhesins are reported to contribute not only to mediating adhesion but also, for some of them, to fluid accumulation. The initial experiments reporting the adhesive properties of Pef fimbriae also showed that mutant bacteria lacking a functional *pef* operon triggered diminished fluid accumulation in infant mice. To demonstrate the specificity of Pef in causing this phenotype, the authors tested an *S.* Typhimurium *fim* mutant and observed no change in fluid accumulation (107). Further experiments have extensively analyzed the contributions of 11 fimbrial operons (*fim*, *csg*, *pef*, *lpf*, *bcf*, *stb*, *stc*, *std*, *stf*, *sth*, and *sti*). The results have shown that only the absence of curli fimbriae reduces fluid accumulation in a statistically significant way. Inactivation of the *csgBA* genes causes a reduction in the severity of neutrophil infiltration in *in vitro* tests. On studying the molecular mechanism, the authors reported that curli fimbriae induce IL-8 production in human macrophage-like cells through interaction with TLR2 (110). Nonetheless, it is worth mentioning that Pef fimbriae, despite not contributing in a statistically significant manner, showed the lowest response after that of curli fimbriae. Thus, these results do not necessarily contradict the presumed contribution of the *pef* operon to this phenotype (110).

Downregulation of inflammation. The internalization process and proinflammatory response eventually lead to epithelial damage (144), allowing essential nutrients to become available for *Salmonella*. Unfortunately, overactivation of these signaling pathways will result in significant alterations of the host cell homeostasis that may be detrimental to the ability of the bacteria to survive, replicate, and disseminate inside the host. Consequently, *Salmonella* has evolved mechanisms to downregulate these inflammatory responses by delivering antagonic and hence anti-inflammatory effectors (e.g., SptP and SpvC). The SPI-1-encoded effector SptP functions as a GTPase-activating protein (GAP) by stimulating the intrinsic GTPase activity of Rac-1 and Cdc42. This activity inactivates these enzymes and disrupts the actin cytoskeleton, thereby antagonizing the responses induced by SopE and presumably by SopE2 and SigD. It has been suggested that *Salmonella* might deliver SopE and SptP into the host cell either sequentially or in different amounts in order to stimulate the appropriate responses (145).

SpvC is a phosphothreonine lyase that has been reported to remove phosphate groups and hence inactivate extracellular signal-regulated kinase (ERK), a mitogen-activated protein kinase (MAPK) signaling pathway required for *Salmonella*-induced inflammation. This plasmid-encoded effector can be delivered into the host cell cytoplasm by both T3SS-1 and T3SS-2 (8, 146). Initially, SpvC was reported to be required for systemic infection in mice (100). Recent experimental evidence indicates that mice infected with *spvC* mutant bacteria show pronounced colitis compared to those infected with wild-type bacteria. Particularly, the enzymatic activity of SpvC reduces expression of proinflammatory cytokines (IL-8 and TNF-α) and diminishes inflammation and neutrophil infiltration at infection sites during early stages of infection (8, 146). Moreover, the absence of this gene triggers attenuation in the mouse model of systemic infection upon intraperitoneal inoculation, since lower numbers of bacteria are recovered in the spleen. These results are in agreement with the ability of T3SS-2 to also export this effector and suggest that both SPI-1 and SPI-2 contribute to attenuation of inflammatory responses during *S.* Typhimurium infection at different sites in the host (8, 146).

Outgrowth of *S.* Typhimurium against Commensal Bacteria in the Inflamed Gut

A high density of commensal microbiota inhabits the intestine and protects against infection. However, enteropathogenic bacteria can successfully compete with the microbiota and overcome colonization by means of specific virulence strategies. The results reported indicate that inflammation can shift the balance between the protective microbiota and the pathogen in favor of the pathogen. The mechanisms involved guarantee an important boost in *S.* Typhimurium growth, which may explain why a very low oral dose of infecting *Salmonella* is required to establish successful infection.

Nutrient access. Stecher et al. have reported that the inflammatory host response induced by *S.* Typhimurium changes the composition of the microbiota and suppresses its growth, thereby offering *Salmonella* a growth advantage (147). Particularly, the absence of flagella or chemotactic movement attenuates disease and reduces the fitness of salmonellae in the inflamed gut (9). Since mucosal inflammation provides a localized source of high-energy nutrients, motility and chemotaxis allow *Salmonella* to efficiently access these nutrients and accumulate in proximal areas, resulting in faster replication and a fitness benefit over the microbiota (148).

Tetrathionate respiration. Colonic bacteria produce large quantities of H_2S, a highly toxic compound which is converted to thiosulfate by the cecal mucosa as a protective response (149). However, during *Salmonella*-induced gut inflammation processes, reactive oxygen species (ROS) are released by the neutrophils recruited. These compounds react with thiosulfate to form a new respiratory electron acceptor, tetrathionate, which, in the presence of the *ttr* genes located in SPI-2, can be utilized as an electron acceptor. This advantageous ability is particularly important under the anaerobic growth conditions encountered in the intestinal mucus layer, since it confers the opportunity to outgrow the fermenting commensal competitors (86).

Nitrate respiration. On the other hand, SopE, which largely

contributes to intestinal inflammation as noted above, also induces the expression of nitric oxide synthase, a mammalian enzyme that generates nitric oxide and is produced mainly by activated macrophages (150). This compound reacts with ROS to eventually lead to nitrate. Nitrate is then preferentially used as an anaerobic electron acceptor for *Salmonella*, even in comparison with tetrathionate (presumably because of its higher standard redox potential) (151). The growth benefit conferred by tetrathionate respiration is diminished in the presence of nitrate, presumably because SopE markedly reduces the expression of *ttrA* in luminal *S.* Typhimurium. Thus, the increased nitrate production by macrophages promotes the luminal abundance of those *S.* Typhimurium strains carrying the *sopE* gene by boosting growth through nitrate respiration (150).

Intracellular Survival in Epithelial Cells and Macrophages

Once engulfment has been completed, bacterial cells are localized within the SCVs inside the eukaryotic cytoplasm. The next events triggered by the pathogen are focused primarily on the biogenesis and maintenance of the SCV by preventing the delivery of antimicrobial host factors (e.g., free-radical-generating complexes) by modifying the organization of the host cell cytoskeleton and impairing vesicular transport (70, 89, 152). Preserving the SCV membrane integrity undoubtedly plays a role in permitting *Salmonella* replication inside its intracellular niche (65). These procedures are driven mainly by the transporting action of the T3SS-2 and its translocon machinery, the SseBCD complex (153). Thus, the required effectors, encoded both inside and outside SPI-2, are translocated to interact with the host cell cytoskeleton and promote success in the intracellular environment. Similar mechanisms have been reported to occur inside epithelial cells immediately following intestinal invasion and once bacteria has been internalized by macrophages during the systemic spread of the infection.

T3SS-2 activation. Similar to the findings previously reported regarding DsbA and the complete activation of T3SS-1, this disulfide oxidoreductase is also crucial and equally required for the proper function of T3SS-2 (127). In this case, however, Miki et al. have identified a DsbA substrate, since the absence of the two cysteine residues present in SsaC, a component of T3SS-2, triggers a loss of SPI-2 function *in vitro* and *in vivo* (154).

SCV maturation and trafficking. Internalization of bacteria inside the SCV is followed by processes of SCV maturation and trafficking. At this stage, two effectors, SigD and SsaB (SpiC), have been reported to interact with this vesicular trafficking to escape from the normal degradation pathway, which ends upon fusion with lysosomes. The SsaB protein, which is a component of the T3SS-2 apparatus, also functions as an effector *per se* (152). Once delivered to the cytosol, SsaB inactivates the mammalian protein Hook3, a component of the endocytic compartment which links microtubules with organelles, leading to disruption of Golgi apparatus and lysosomes and thereby inhibiting intracellular trafficking by blocking the fusion of the SCVs with lysosomes (152, 155). According to the SigD-mediated ability to modulate phosphoinositide metabolism and because phosphoinositides are important in vesicular trafficking, SigD is also thought to impair the vesicular trafficking pathway by arresting the progression of the SCV, a process which results in enlarged vesicles. These spacious phagosomes may provide a favorable environment where *Salmonella* can reside and build its replicative niche. Thus, SigD is important in forming and determining the size of the SCVs to allow bacterial replication (156).

Moreover, SifA, a major SPI-2 virulence protein that is localized in the SCV membrane (157), has also been reported to interact with the endocytic pathway. The SifA C-terminal domain contains the WxxxE G-protein mimic signature motif (158, 159). On one hand, *in vitro* experiments show that SifA binds to the inactive and GDP-bound form of RhoA, despite no direct GEF activity having been demonstrated (158). In contrast, simultaneous results published by Jackson et al. revealed that the WxxxE motif is critical for the ability of SifA to bind to SKIP, a mammalian protein located in SCVs. The SifA-SKIP interaction is reported to then compete in binding with Rab9 (159). Rabs are small GTPases involved in the regulation of endocytic trafficking, and this type of G-protein antagonism contributes in driving the SCV progression and maintenance along the endocytic pathway (159, 160).

VAP formation. Several hours after bacterial uptake, *Salmonella* induces VAP formation in close association with the SCV. This actin assembly process is required for maintenance and stability of the SCV membrane, since treatment with actin-depolymerizing agents releases bacteria into the host cell cytoplasm and abolishes their replication (65). Recent experiments have revealed that not only the classical T3SS-2-dependent effectors (e.g., SspH2, SseI, and SpvB) but also other bacterial factors initially classified as SPI-1-related effectors (e.g., SipA) are involved in this process. Thus, the T3SS-1 effector and actin binding protein SipA, which has been reported to persist after bacterial internalization, is exposed on the SCV and stabilizes the actin filaments induced during VAP (161).

Additionally, SspH2 and SseI have also been suggested to contribute to or remodel VAP formation. SspH2, a protein containing leucine-rich repeats (162), colocalizes with the actin filaments induced during VAP (13). By means of yeast two-hybrid assays, SspH2 has been shown to interact with two mammalian proteins: filamin, a protein which cross-links actin fibers in areas of active polymerization, and profilin, a molecule that enhances actin polymerization through direct interaction. Moreover, *in vitro* experiments show that SspH2 inhibits actin polymerization (13). Similarly, SseI strongly interacts with filamin (13). Thus, these two effector proteins have been deduced to interact with the actin cytoskeleton to direct localization and organization of the actin filaments around the SCV. However, since strains with mutations in *sspH2* and *sseI* retain the ability to form VAP, these experiments are not conclusive for the essential role of SspH2 or SseI in VAP formation. Accordingly, the authors have suggested that these two proteins have a subtle effect on the actin cytoskeleton and hypothesize that this lack of phenotype may be explained by functional redundancy among effectors (13).

Conversely, the plasmid-encoded protein SpvB, expressed in cultured macrophages and epithelial cells (163), acts as an ADP-ribosylating toxin that uses actin as a substrate. This cytotoxic activity prevents actin polymerization, thereby leading to loss of the actin cytoskeleton (164, 165). Moreover, cells infected with an *spvB* mutant strain do show an effect on VAP formation; particularly, a significant increase in this phenotype is clearly detected (13).

SCV migration and SIF formation. As the SCV matures and is surrounded by actin polymerization events, it migrates toward a perinuclear position depending on the balanced activity of two microtubule proteins, kinesin and dynein, which are, respectively,

the major plus-end-directed and minus-end-directed motors. Evidence suggests that these two motor proteins transport cargo inside the host cell, i.e., kinesin toward the cell periphery and dynein to the nucleus (166, 167). Once SCVs are correctly located, bacteria start replicating and initiate SIF formation. Several effectors, such as SifA, SipA, SseJ, SseF, and SseG, enhance the successful establishment of this bacterial intracellular niche by promoting SIF formation and maintenance of the integrity of the SCV membrane. Moreover, these factors counteract the opposite action of effectors such as PipB2 and SpvB. Among these effectors, SifA localizes in SIFs in addition to the SCV membrane (157). Deletion of this gene leads to diverse phenotypes, including redistribution of SCVs from a juxtanuclear position to the cell periphery (166) and loss of vacuole integrity (168). Moreover, its absence also leads to replication defects in cultured cells (168) and marked attenuation of virulence in mice (169), presumably as a consequence of the first two phenotypes. The N-terminal domain of SifA also binds to the mammalian protein SKIP. This interaction antagonizes and hence reduces kinesin accumulation on the SCV, which is initially promoted by the action of PipB2 upon its binding to the kinesin light chain (166, 170). The precise balance between SifA and PipB2 is influenced by SipA, which stabilizes SifA through its actin modification effects. Otherwise, the absence of SipA leads to aberrant kinesin recruitment and hence aberrant SCV positioning and morphological defects (161).

SseJ, whose amino acid sequence similarity and further *in vitro* studies indicate it to be an acyltransferase/lipase (171, 172), localizes in the phagosome membrane during infection (173). However, there has been controversy in the literature regarding its role. Initially, SseJ was suggested to negatively modulate SIF formation because the absence of this protein led to increased production of these structures (171, 174). In contrast, recent results have shown an increase in SIF-like structures when SseJ is coexpressed with either SifA or activated RhoA in comparison with the activity of SifA alone (158). Moreover, SseJ recruits active RhoA to the SCV, and this interaction stimulates the lipase activity of SseJ, resulting in the esterification of cholesterol in the host cell membrane. These changes eventually alter the cholesterol membrane composition, a condition that presumably contributes to membrane tubulation and hence SIF formation (175, 176). The next two effectors, SseF and SseG, share significant amino acid similarity and have been reported to interact functionally and physically with each other (66). These proteins localize in the SCV membranes and SIFs in addition to the Golgi network. Moreover, both proteins are required for the aggregation of endosomal compartments along microtubules leading to the formation of massive bundles of microtubules (177, 178). As a result, SseF and SseG contribute to SCV migration to the perinuclear region in close proximity to the Golgi network, where they facilitate surrounding of the SCVs by membranes of this compartment and induce microtubule bundling that can then serve as a scaffold for SIF formation (66, 67). In contrast, but in agreement with the above-mentioned cytotoxic effect of the SpvB protein, its actin-depolymerizing activity negatively modulates SIF formation in the same way that it reduces VAP formation (13).

Non-SPI-2-related effectors. Additionally, there are several other effectors, such as SopD, MgtCB, PipB, and PipA, whose function has been associated with intracellular replication and the systemic stage of disease. Nonetheless, more details are needed in order to better comprehend their interaction with the host cells and hence the specific role they play in virulence. SopD is reported to be an SPI-1 effector by promoting *Salmonella* invasion (138). However, its expression has been found to be maintained at later stages of infection when other SPI-1 effectors are not expressed, suggesting that this effector may also play a role in systemic infection (179). It has been hypothesized that SopD may also be translocated through T3SS-2. Accordingly, deletion of the *sopD* gene leads to impaired bacterial replication in mouse macrophages, despite no effect being detected in human epithelial cells (180).

The *mgtC* and *mgtB* genes constitute the SPI-3-encoded *mgtCB* operon, which is required for growth and replication within macrophages. Nonetheless, while inactivation of the *mgtC* gene triggers a macrophage survival defect, absence of *mgtB*, encoding an Mg^{2+} transport protein, leads to only a milder phenotype, which could be due to an indirect effect on *mgtC* expression. Blanc-Potard and Groisman have reported the ability of this operon to allow growth in Mg^{2+}-limiting environments, such as that thought to exist inside the phagosome, presuming this scarcity to be a host defense mechanism since Mg^{2+} is an important biochemical cofactor (90). Further experiments, however, have revealed that the ability of MgtC to promote growth in low-Mg^{2+} medium is not sufficient to promote intramacrophage replication and that its role is not linked to Mg^{2+} adaptation, indicating that the phagosome does not necessarily constitute a low-Mg^{2+} environment (181).

PipB, encoded within SPI-5, was initially reported to contribute to bovine enteropathogenesis in *S.* Dublin (95). Later, it was also reported to localize in the SCVs and SIFs once expressed under SPI-2-inducing conditions and translocated by T3SS-2. Nonetheless, this protein is not required for either the formation or maintenance of either of these two intracellular structures or for intracellular replication in phagocytic cells (99). Further results have shown that PipB concentrates in intracellular lipid rafts that are present on the membranes of the SCVs and SIFs, facilitating its interaction with host cell signaling pathways, even though no clear and direct role has yet been attributed to this effector (69). In contrast, PipA, which is located downstream from *pipB* (both genes are transcribed as an operon), contributes to the development of systemic disease in mice (99).

Programmed Cell Death and Systemic Dissemination

Internalization of the infecting *Salmonella* within the SCV is followed by systemic spread through other target organs, such as the spleen and liver. Several bacterial strategies have been reported to contribute to this systemic stage of the disease. On one hand, effectors of both T3SS-1 and T3SS-2 have been reported to trigger a cytotoxic effect by inducing programmed host cell death through different mechanisms. These programs are believed to initially be a host defense mechanism to clear the infection; however, they can also facilitate systemic dissemination of the pathogen on the basis that *Salmonella* has developed specific tools to survive inside the macrophages attracted during the inflammatory response associated with some of these cell death programs. On the other hand, *Salmonella* cells have been reported to influence the motility of macrophages.

Apoptosis of epithelial cells. On one hand, *Salmonella* induces epithelial cell death featuring the characteristic morphological changes of apoptosis: maintenance of an intact plasma membrane to prevent release of inflammatory intracellular contents. Membrane-bound apoptotic bodies can be taken up by phagocytes or

neighboring cells, allowing for degradation of cellular components in a generally noninflammatory process. This event is induced at relatively late stages after prolonged exposure, at least 12 h after *in vitro* infection (182). This program is independent of caspase-1 activation and instead involves caspase-3 as well as T3SS-2 (182). The main effector reported to induce epithelial cell death is SpvB. The cytotoxic effects reported as a result of the actin depolymerization activity of this plasmid-encoded protein are thought to eventually induce apoptosis of cultured epithelial cells (183), although the exact mechanism still remains to be elucidated.

More recently, SlrP, another *Salmonella* effector which can be translocated through both T3SS-1 and T3SS-2 (172), has been reported to contribute to epithelial cell death. This protein was initially characterized as a leucine-rich repeat protein involved in *in vivo* colonization of mice (although not of calves) (184). However, recent studies have shown that SlrP promotes cell death by two complementary interactions, with Trx and with ERdj3. First, SlrP has been characterized as an E3 ubiquitin ligase which interacts with mammalian thioredoxin-1 (Trx), a multifunctional protein involved in stimulating cell growth and inhibiting apoptosis. Stable expression of SlrP in HeLa cells results in a significant decrease of Trx activity and in an increase of cell death (185). Moreover, SlrP has also been reported to interact with ERdj3, a member of the Hsp40/DnaJ family of chaperones, in a manner independent of the SlrP-ubiquitin ligase activity. This interaction is thought to promote accumulation of unfolded proteins, a process that, under chronic activating conditions, can eventually induce apoptosis (186). Thus, since SlrP expression in HeLa cells makes them more prone to death (185), SlrP may promote cell death by two complementary interactions, with Trx and with ERdj3.

Nonetheless, according to the existing delay in the induction of apoptosis, several bacterial components seem to counteract early epithelial cell death (e.g., SigD, AvrA, and flagellin). The SPI-5-encoded effector SigD phosphorylates and hence activates Akt, which is a proto-oncogene product involved in the regulation of cell proliferation and survival (187). A *sigD* deletion mutant fails to activate Akt, resulting in increased levels of apoptosis. Thus, through its phosphatase activity, SigD acts as an antiapoptotic effector (97). In addition, AvrA, another T3SS-1 effector (188), is rapidly phosphorylated by the ERK pathway shortly after translocation and in this way remains within the cell for an extended period of time (189). Thus, this effector possesses acetyltransferase activity toward specific MAPKKs and potently inhibits Jun N-terminal protein kinase (JNK), a key regulator of many cellular events, including programmed cell death (190). Accordingly, on inactivation of the *avrA* gene, mutant bacteria induce higher levels of caspase-3-dependent apoptosis (190, 191). Lastly, the interaction between flagellin and the TLR5 has been described above to activate NF-κB and contribute to the initial proinflammatory response. Nonetheless, further experiments have shown that aflagellate mutants increase enterocyte apoptosis and disease severity due to enhanced activation of caspase-3, an action observed to be independently related to the lack of motility in the mutant strain. These observations are consistent with the inability of these bacteria to activate NF-κB, which is widely accepted to play an antiapoptotic role. Thus, the pathway activated upon flagellin recognition also counteracts apoptosis at later stages postinfection (192).

Macrophage pyroptosis. On the other hand, *Salmonella* can cause macrophage death in several different ways (193). This effect is thought to enable bacterial systemic dissemination throughout infected organs by incoming uninfected macrophages engulfing either infected dying cells or bacteria released by these cells into the extracellular space (194). This cytoxicity can appear very rapidly after phagocytosis (requiring T3SS-1) or can be induced several hours later (essentially requiring T3SS-2). In general terms, this process is termed pyroptosis and is dependent on the inflammasome, a multiprotein complex that mediates activation of caspase-1, which in turn leads to proteolytic activation of IL-1β and IL-18. These two multifunctional cytokines play central roles in acute and chronic inflammation and potently stimulate recruitment of immune cells, thereby contributing to the inflammatory outcome predicted for this programmed cell death (182). Early pyroptosis is mediated by SipB, which, in addition to being a member of the T3SS-1 translocon system, can also directly engage targets inside macrophages by binding to caspase-1 and hence acting as a cytotoxic effector (195, 196). Moreover, in addition to activating TLR5 in the basolateral membrane, flagellin can also be recognized by the cytosolic mammalian molecule IPAF, a protease that transmits a proinflammatory signal to activate the inflammasome, eventually triggering rapid macrophage pyroptosis (197, 198). In order to reach the appropriate localization for this interaction, flagellin has been reported to penetrate inside the macrophage cytoplasm by injection through T3SS-1 (199).

In contrast, delayed pyroptosis is mediated through the action of several effectors. First, the SpvB protein has also been reported to trigger late cell death in macrophages (200). Browne et al. have shown that at 18 to 24 h after infection, actin is depolymerized by the SpvB protein, and cell detachment and pyroptosis follow (201). Second, SseL displays deubiquitinating activity on ubiquitin-modified proteins which accumulate in infected macrophages. The authors have observed that SseL activity decreases the accumulating amounts and have hypothesized that this action might interfere with a signaling pathway to promote macrophage killing, possibly via caspase-1 activation (202). Third, SipB contributes to this cell death, despite its activity being independent of caspase-1 activation. In contrast, in mice deficient in caspase-1, SipB has been reported to mediate the formation of unusual multimembrane structures resembling autophagosomes. These structures require the localization of SipB to membrane mitochondria and contain mitochondrial and endoplasmic reticulum markers. These results suggest that SipB induces autophagy of macrophages, another type of programmed cell death (203).

Similar to the antagonistic effect reported for AvrA regarding apoptosis of epithelial cells, this effector can also inhibit pyroptosis in macrophages. Despite induction of a more severe inflammatory response and higher levels of leukocyte recruitment, on inactivation of the *avrA* gene, mutant *Salmonella* cannot establish the typical intracellular niche within macrophages. Moreover, loss of intracellular carriage as well as an increased microbial parenchymal burden in systemic lymphoid tissues in the later stages of systemic infection is observed. Thus, AvrA dampens proinflammatory responses and represses host cell death during multiple stages of the infectious process (190, 191).

Influence on macrophage motility. The initial information regarding the function of the SseI effector suggested that it participates in VAP remodeling (13). However, further studies have reported new evidence. SseI has been shown to interact with the host protein TRIP6, an adaptor protein that binds components of the

Rac signaling pathway, which is critical for cell motility, and the NF-κB inflammatory pathways. Particularly, the SseI-TRIP6 interaction is thought to promote macrophage motility *in vitro* and accelerate the systemic spread of infection away from the lumen of the intestine in mice (80).

Biofilm Production and Chronic Infections

The ability to form biofilm is also an important factor in the virulence of *Salmonella* and has been shown to promote the survival of bacteria when they are exposed to limited nutrient availability, heat, acidic pH, low temperatures, and antimicrobials (204). In this pathogen, two extracellular matrix components play an important role in biofilm formation: the exopolysaccharide cellulose and curli fimbriae (112). Cellulose biosynthesis depends on proteins encoded within two constitutively expressed operons, *bcsABZC* and *bcsEFG* (205). The information for production, biosynthesis, and assembly of curli fimbriae is encoded within the two above-mentioned operons *csgBAC* and *csgDEFG* (118). More recently, a third component, the large cell surface BapA protein, has also been shown to be required for biofilm formation, since absence of the *bapA* gene leads to loss of the capacity to produce a biofilm. Nonetheless, overproduction of curli fimbriae and not cellulose has been reported to compensate for the biofilm deficiency observed in this mutant strain (11). Furthermore, according to additional experiments, adhesion mediated by type 1 fimbriae, Lpf, and Pef also contributes to biofilm formation in *in vitro* tissue culture epithelial cells and in *in vivo* models (12, 206).

Persistent colonization of the gut triggers the so-called carrier state. This particular situation, mentioned above, has been more extensively studied in the case of *S.* Typhi. Presumably, these chronic carriers play a more relevant role in the transmission of typhoid, since this pathogen is restricted to humans whereas NTS serovars can also infect animals, which can then act as reservoirs. Similar to the pathogenesis described for *S.* Typhimurium, *S.* Typhi also needs to invade and cross the intestinal epithelium to cause systemic dissemination. Once the pathogen has reached the liver, bacteria can then be shed into the gallbladder, where they can cause active (cholecystitis) or chronic (carrier state) infection. The latter condition is often asymptomatic and frequently associated with gallbladder abnormalities, such as gallstones (207).

It has been found in *in vitro* experiments that both *S.* Typhi and *S.* Typhimurium form biofilm on the surfaces of gallstones, a presumed protective advantage against high concentrations of bile and antibiotics (208). Nonetheless, little knowledge is available for *S.* Typhimurium infections in humans and their carriage in the gallbladder. The information available suggests that biofilm production may rather represent an advantageous trait to promote survival outside the host. Accordingly, through *in vivo* imaging experiments, White et al. reported an absence of expression of curli genes during infection but activation once *S.* Typhimurium has passed out of the mice into the feces. These results suggest that biofilm formation may aid long-term survival by offering mechanisms of resistance to the extreme conditions encountered outside the host and hence mediate transmission between hosts (209, 210). Thus, further research is needed in order to provide new insight into the contribution of biofilm formation to the pathogenesis of *S.* Typhimurium and elucidate whether this NTS pathogen equally forms biofilm inside the host.

REGULATION

Gene regulation plays an extremely important role in the efficacy of the pathogenesis of *Salmonella* in order to coordinate, at the correct time and location, all the virulence traits. This regulation is under a temporal hierarchy in which virulence elements need to be progressively expressed. Since the SPIs carry the genes playing the most important role in invasion and survival, their regulation is crucial, particularly in the case of SPI-1. A large number of regulators affecting virulence have been described to date, and this section highlights the most important regulators controlling the SPIs and those synchronizing expression of several virulence traits. These regulatory proteins are listed in Table 2.

General Regulatory Traits

SPIs. The extremely complex regulation of SPI-1 genes depends on the balance between the interactions of the regulators encoded both inside and outside the island (Fig. 3). These regulators detect the best environmental options to allow invasion, and hence upon the expression of the SPI-1 genes, *Salmonella* initiates the pathogenic process. SPI-1 is maximally transcribed during *in vivo* invasion of epithelial cells, whereas only those conditions resembling the host intestinal lumen, i.e., low oxygen and high osmolarity, activate these genes during exponential growth in *in vitro* assays (211, 212). SPI-2 genes have been shown to be rapidly induced after entry into macrophages or epithelial cells and are continually expressed throughout infection. Thus, the *in vitro* SPI-2-inducing conditions are those mimicking the intracellular environment, consisting of low osmolarity, low calcium concentration, and acidification when bacteria are in the stationary phase of growth (213). Only four ORFs within SPI-3 have been well characterized, and these encode proteins with functions not related to each other. Regulation of these genes does not follow a unique pattern. Interestingly, no gene with a proposed regulatory function has been identified within the SPI-4 locus. The peak in expression of the *sii* operon is detected at the late exponential phase and a rapid drop on entering the stationary phase has been reported, similarly to the SPI-1 expression pattern (214). SPI-5 encodes proteins whose function and secretion are related to those of either SPI-1 (T3SS-1) or SPI-2 (T3SS-2). Therefore, the regulation of these genes obviously follows differential expression patterns which correlate with these two SPIs, respectively.

pSLT plasmid. Activation of the *spv* genes *in vitro* is exhibited as bacteria enter the stationary phase of growth, whereas *in vivo spv* expression appears to be dependent on the intracellular environment (163).

Adhesins. The most important and characteristic member among adhesins is the type I fimbria, which is encoded by the *fim* genes. Their expression, which is maximal in the stationary phase, is first controlled by genes within the same loci as well as by a few external regulators encoded elsewhere on the bacterial chromosome (215–217).

Flagella and chemotaxis. According to the hierarchical transcription observed, the flagellar genes are divided into early, middle, and late, and the corresponding promoters are referred to as class 1, class 2, or class 3, respectively. There is only one class 1 promoter; it transcribes the two early genes of the *flhDC* master operon, which encode the two key transcriptional activators for the expression of the subsequent genes (113). Genes under control of class 1 (or early) promoters are expressed before genes belonging to class 2 (or middle) and even more than those belonging to

TABLE 2 Influence of the regulatory proteins on the virulence determinants reported

	Effect[a] on virulence determinant:								
Regulator	SPI-1	SPI-2	SPI-3[c]	SPI-4	SPI-5[c]	pSLT	Type I fimbriae	Flagella and motility	Biofilm
HilA	A[a]	R		A	A			R	
InvF	A				A				
HilD	A	A		A*					
HilC	A			A*					
RtsA	A								
HilE	R			R*					
SsrA-SsrB		A			A				
PhoQ-PhoP	R/A[b]	A	A	R*				R	
BarA-SirA	A			A*	A*		A	R*	
RcsCDB	R	A		R	R/A	A	A	R	A
QseC-QseB	A	A	A		A			A	
EnvZ-OmpR	A	A							A
PhoR-PhoB	R								
H-NS	R	R	R	R		R		A	A
Hha	R	R							
YdgT		R							
IHF	A	A	A	A	A	A		A	A
Fis	A	A	R	A	A			A	
HU	A	A						A	
MarT			A						
SpvR						A			
FimWYZ	R						A	R	
FlhDC								A	
FliZ	A						R		
CsgD									A
RtsB	R*						A*	R	
Lrp	R	R							
Lon	R								
DnaK	A							A	
Fur	A								
Mlc	A								
RNase E	R								
FadD	A								
Pag	R								
SlyA		A							

[a] A, activation; R, repression. An asterisk indicates that the regulatory effects have not been proven to act directly on a particular trait. According to the information currently available, the effect rather is indirect and mediated by another regulator.
[b] Two effects have been reported, depending on the genes within the same virulence determinant. The first effect is the most important.
[c] SPI-3 and SPI-5 harbor genes whose function is related to either SPI-1 or SPI-2. The information regarding which particular effector is influenced is indicated in the text.

class 3 (or late). Expression of flagellar genes occurs at the early exponential growth phase, since they play the most initial role in pathogenesis, allowing *Salmonella* cells to reach the intestinal epithelium for a proper interaction (15).

Biofilm. Several environmental conditions have an impact on biofilm production. For example, biofilm formation has been reported to be maximal at the stationary phase and under reduced nutrient availability, aerobic conditions, low osmolarity, and a low temperature (28°C) (218).

Key Regulators Controlling SPI Expression

HilA. HilA is a transcriptional activator belonging to the OmpR/ToxR family, which is encoded within SPI-1 (219) and can be negatively autoregulated (220). This regulator plays the central role in invasion, not only because all the regulatory systems and environmental signals affect its expression (83, 212, 221) but also because a deletion of *hilA* has been shown to be phenotypically equivalent to a deletion of the entire SPI-1 locus (222). HilA activates all the operons encoding the functional T3SS-1: the *prg*/*org* and *inv*/*spa* operons are activated by a direct binding of HilA to their promoters, whereas the *sic*/*sip* operons are induced mainly via the activation of InvF (212, 219, 223). More recent findings, however, have revealed that HilA can also activate several effectors secreted through T3SS-1 (224).

HilA transcriptional activity also activates expression of the *sii* operon (SPI-4) as well as the *sigD* gene (SPI-5). This regulator is necessary to induce *siiA* transcription, SiiE secretion, and bacterial adhesion (214, 225) by direct binding of HilA to the *siiA* promoter (224). However, little or no effect is seen on increasing *siiE* expression in the absence of SPI-1, suggesting that this protein acts in coordination with another member of SPI-1 (225). Similarly, HilA directly interacts with the promoter of the *sigD* gene (T3SS-1 effector) to coordinate its expression under SPI-1-inducing conditions (99, 224), despite previous reports showing that HilA could not activate SigD in the absence of InvF (226).

In contrast, under invasion-inducing conditions, HilA surprisingly represses expression of the SPI-2 genes (i.e., *ssaH* and *sseL*)

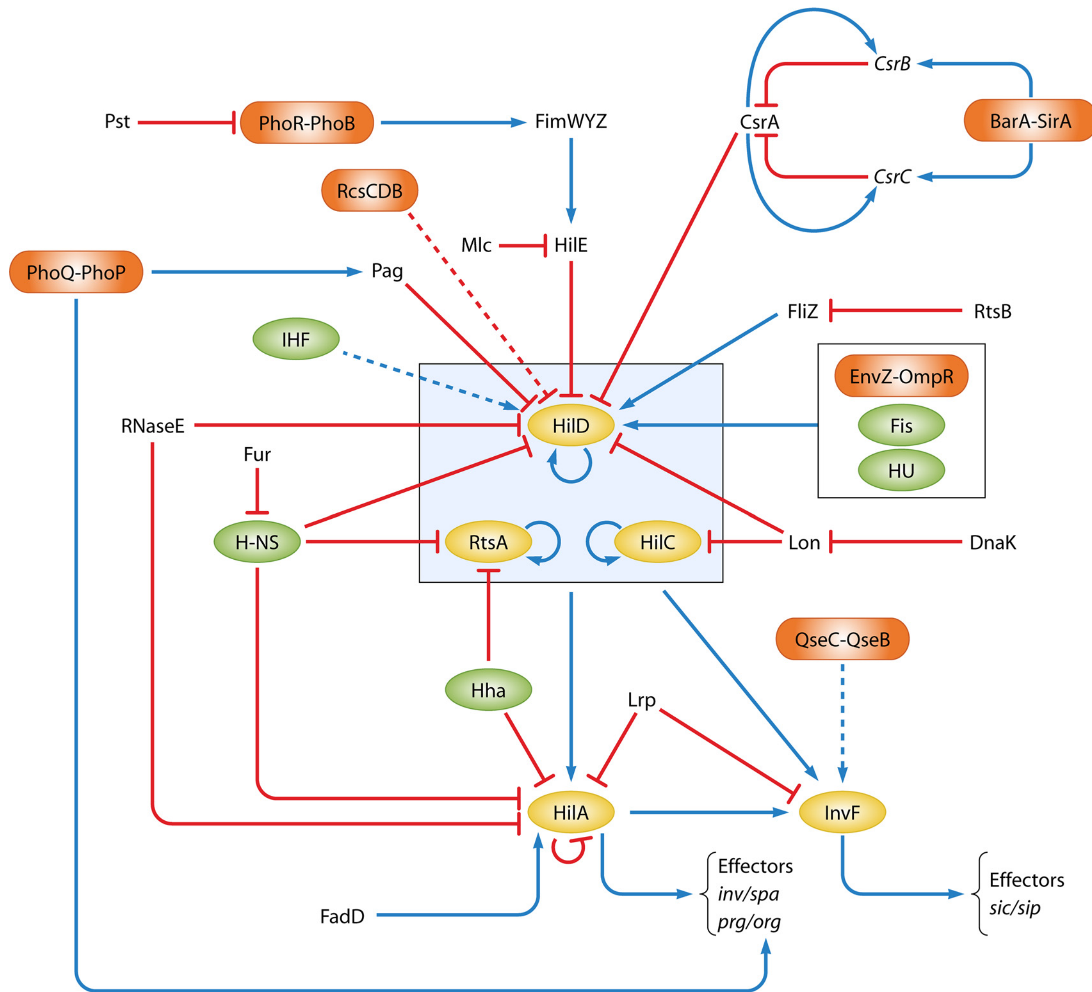

FIG 3 SPI-1 regulatory network. Blue arrows indicate activation or autoactivation, whereas red blunt-end arrows indicate repression or autorepression. Discontinuous arrows suggest the putative regulatory target proposed in this model. Regulators in yellow are those encoded within SPI-1 (with the exception of RtsA) that play a critical role in the regulation of the invasion phenotype. Green refers to NAPs, whereas light orange is used for 2CRSs. The positive regulatory interactions between HilD, HilC, and RtsA have been omitted to avoid complicating the figure. The putative direct activation of HilA by SirA has not been included in this model due to lack of corroborative data. IHF activation is deduced to be mediated through HilD as for Fis and HU according to the information provided in the text. The PhoQ-PhoP repressive effect on the *prg* genes is proposed to occur through repression of HilA by means of posttranscriptional repression of HilD, since no direct effect has been reported in these SPI-1 genes. According to this model, all the 2CRSs exert their effect through HilD, with the exception of QseC-QseB, which is currently known to affect only InvF. For this reason, we have used a discontinuous arrow despite the possibility that it may also be acting at the level of HilD. Moreover, most of the regulatory signals are integrated at the level of HilD, mainly by posttranscriptional modulation, which heads the hierarchy of the SPI-1-encoded regulators.

and decreases motility through reported direct binding to the *ssaH* promoter and to the *flhD* upstream region (a key regulator for flagellar gene expression; see below) (224). Corroborative results have shown that in the absence of SPI-1, flagellar genes are expressed longer (15). Nonetheless, controversial results have been reported supporting the contention that HilA does not affect expression of an *flhD* transcriptional fusion, at least during growth in motility agar (227).

InvF. The AraC family member InvF is also encoded within SPI-1 (by the first gene of the *inv* operon) (228) and can be activated in a HilA-dependent or -independent manner. Particularly, InvF acts together with the chaperone SicA to activate the expression of the downstream virulence genes (229), which are mainly the effectors encoded within SPI-1 in the *sic*/*sip* operons and elsewhere on the chromosome (e.g., *sptP*) (223). These effectors also include the *sigDE* operon, and it is suggested that InvF directly activates them since the absence of this regulator compromises *sigDE* expression even in the presence of *hilA* (223). Thus, HilA and InvF cooperatively regulate the expression of invasion genes through different, albeit similar, sets of target genes.

HilD, HilC, and RtsA. Expression of HilA is controlled by the combined action of three AraC-like transcriptional activators: HilC and HilD, both encoded within SPI-1 (230), and RtsA, encoded within an independent island (231). Each activator can bind to the *hilA* promoter and activate its expression and can also significantly induce its own expression as well as activate the other regulators (222, 232). Nonetheless, the positive effect of HilC and RtsA on HilD transcriptional activation is suggested to play a minor role (83). As a consequence, in the model proposed by Ellermeier and Slauch, HilD, the most important activator of HilA *in*

vitro, is at the top of the hierarchy and activates the transcription of HilC and RtsA. Then, the combined action of all three activators can amplify the signal and act as a switch for HilA transcription (83). Alternatively, all three activators can activate InvF expression in a *hilA*-independent manner, supposedly by direct interaction with an alternative *invF* promoter (231, 233). Moreover, the effectors SlrP and DsbA are also activated by RtsA alone or by these three regulators (127, 231). Accordingly, they seem to reinforce expression of the SPI-1 genes by different routes.

In addition, HilD can also induce expression of the SPI-2 genes by direct binding to the *ssrAB* promoter (the local regulatory system carried within SPI-2; see below). This effect, observed only at the late stationary phase, is thought to counteract the reported H-NS repression (14). Lastly, individual inactivation of the *hilC* and *hilD* genes has been shown to strongly reduce expression of *siiE* (225), despite this effect probably being mediated through HilA.

HilE. The most important negative regulator of HilA expression is HilE. It is encoded outside SPI-1, by a gene located in a zone in the chromosome which has many characteristics of a pathogenicity island (234). Overexpression of HilE superrepresses *hilA* transcription, whereas disruption of this locus leads to increased expression of *hilA* together with increased invasion under low-oxygen conditions (232, 234, 235). Since HilE interacts with HilD at the protein level, this posttranscriptional interaction is deduced to be the mechanism which negatively controls *hilA* transcription (234). Accordingly, mutations in *hilE* are reported to enhance *siiE* expression (225), presumably through its effect on HilA transcription.

SsrA-SsrB. The most important and essential regulatory system required for SPI-2 gene expression is the two-component regulatory system (2CRS) SsrA-SsrB, encoded in a single locus located within SPI-2 (for a definition of 2CRS see below). SsrA is the membrane-located sensor kinase, whereas SsrB is the transcriptional regulator (87, 236). This system is also necessary to express T3SS-2 effectors carried outside SPI-2, such as the *sifA* and *sifB* genes (213). Interestingly, two promoters have been characterized within this locus, one upstream of each gene, leading to different regulation and uncoupled production. There is evidence suggesting that SsrB can be autoregulated and can activate SsrA expression, albeit to a lesser extent (237). Moreover, the SsrB requirement in the absence of H-NS (see below) is substantially reduced, suggesting a dual role for SsrB in SPI-2 induction: activation of transcription and countering H-NS-mediated repression (238). Moreover, the *pipB* gene (SPI-5), which is expressed under SPI-2-inducing conditions, has an expression profile comparable to that of the SPI-2-borne gene *sseB*. The proof of coregulation of these genes is the finding that *pipB* expression is dramatically reduced in the absence of *ssrB* and that PipB is exported via the T3SS-2 (99).

MarT. The last of the four best known ORFs within SPI-3 encodes the regulator MarT. It is a ToxR-like regulatory protein which has only a local effect by inducing expression of the MisL adhesin due to direct binding to its promoter (239).

pSLT Local Regulator

SpvR. *spvR* is the first gene of the *spv* region and encodes a transcriptional regulator which shares homology to members of the LysR family of transcriptional activators (240). This region includes two transcriptional units. On one hand, it can autoregulate itself while, on the other hand, SpvR has been shown to positively modulate the *spvABC* promoter and separately activate this operon by means of *lacZ* transcriptional fusion (241, 242). Thus, it is considered the local activator of the *spv* locus.

Type I Fimbria Local Regulators

FimWYZ. The three genes encoding FimWYZ constitute the main activation complex of type I fimbriae: FimZ belongs to the response regulator family of proteins showing DNA binding ability (215), FimY cooperatively acts with FimZ to activate the fimbrial genes (216), and FimW exerts a negative influence on the expression of the *fim* genes (243). In addition, FimZ negatively influences expression of HilA as well as the flagellar genes and motility. In the former situation, FimZ directly binds to and hence activates the *hilE* promoter. As a result, the absence of *fimZ* leads to increased *hilA* transcription, whereas *fimYZ* overexpression triggers the opposite effect (244). The latter regulatory effect causes an absence of motility as a consequence of repression of the flagellar *flhDC* operon (see below) (245).

Flagellum Local Regulators

FlhDC. The *flhDC* master operon represents a crucial regulatory point at which a number of global regulatory signals, including many environmental cues and growth phase, influence the decision as to whether to synthesize flagella (113). Its regulatory role is focused on inducing expression of the class 2 flagellar genes (113), including *fliA*, which is an alternative sigma factor required for transcription of the class 3 genes (246), and *fliZ*, an activator of both class 2 and 3 genes (whether it exerts a direct effect on expression of class 3 genes or whether this phenomenon is the consequence of *fliA* activation remains undetermined) (217).

An association of the expression of FliZ with invasion genes and type I fimbriae has also been established. FliZ overproduction increases *hilA* transcription in a HilD-dependent manner, suggesting that FliZ activates *hilA* by posttranscriptionally controlling HilD (247). In contrast, this regulator represses expression of type I fimbriae, since *fliZ* deletion leads to increased expression of *fimA*, whereas its overexpression concurs with repression of FimZ and eventually the *fim* genes. The regulatory link is not completely elucidated and can imply either posttranscriptional regulation of FimZ or prevention of FimZ binding to the *fimA* promoter and consequent activation (15, 217).

Biofilm Key Regulator

CsgD. CsgD is a LuxR family member, encoded within the curli *csgDEFG* operon, which has been described as the master regulator of biofilm production since it plays a key role in synchronizing expression of several determinants involved in this process. CsgD increases curli fimbriae and *bapA* expression and posttranscriptionally activates cellulose biosynthesis. Particularly, CsgD activates AdrA transcription, leading to increased levels of the signaling molecule cyclic di-GMP (c-di-GMP), which in turn mediates posttranscriptional activation of cellulose biosynthesis (11). CsgD also controls expression of the *rdar* morphotype (for red, dry and rough), being clearly dependent on the expression of curli fimbriae and cellulose (248, 249).

Two-Component Regulatory Systems

Two-component regulatory systems are used by microorganisms to sense and respond to changes in the environment. In general terms, these systems consist of a membrane-bound histidine ki-

nase that senses a specific environmental stimulus and a corresponding response regulator that mediates the cellular response, mostly through differential expression of target genes. The sensor protein autophosphorylates at a conserved histidine (His) residue, located in the transmitter domain (H1), in response to an environmental cue. The phosphoryl group is then transferred to a conserved aspartate (Asp) residue located in the receiver domain of the corresponding response regulator. These regulators also contain an effector domain, which is often a DNA binding moiety activated upon phosphorylation to eventually alter gene transcription (250, 251).

PhoQ-PhoP. Low extracellular cation concentrations, such as those detected within the SCV, and low pH have been reported to activate the sensor kinase PhoQ, which, in turn, activates the regulator PhoP (252). Regarding expression of SPI-1 genes, PhoP has been reported to repress the *prg* genes (for PhoP-repressed genes) and *hilA* (212, 253, 254), whereas on the other hand, PhoP activates transcription of the *pag* genes (for PhoP-activated genes), which are required for bacterial survival within macrophages (255, 256). Moreover, on deletion of the *pag* gene, located within *pagK* and *pagM*, increased *hilA* expression is observed only in the presence of HilD (232). Thus, it might be deduced that once inside the SCV, PhoP activates the *pag* genes, which in turn reduce *hilA* transcription through HilD and eventually trigger repression of the *prg* genes.

The PhoP repressive effect can be extended to the *sii* and flagellar genes. In the absence of a functional PhoP protein, enhanced expression of the *siiA* and *siiE* genes is observed (214, 225), whereas in the background of *phoP*(Con) (constitutive for PhoP), a strong reduction in the *siiA* expression has been reported (214). However, there is no evidence of a direct effect on the *sii* promoter and this effect rather may mirror the PhoP-mediated repression of HilA, leading to the consequent lack of activation of the SPI-4 genes. Moreover, pH acidity has been reported to progressively diminish cell motility until pH 3, when no motility is detected. Accordingly, corroborative results show that overexpression of this 2CRS decreases motility on swarm plates and that PhoP represses transcription of the *fliC* gene (257).

Conversely, since PhoP is activated within macrophages, it has been characterized as a positive regulator of the genes necessary for survival at this stage of disease (SPI-2, *orgBC*, and *mgtCB*). First, PhoP seems to be essential for SPI-2 expression. Experiments have shown that PhoP controls SsrA posttranscriptionally and directly activates the *ssrB* gene by binding to its promoter (258). Nonetheless, in the absence of *phoP* inside macrophages, there is still expression of SPI-2 genes, implying that PhoP is dispensable under these circumstances (259, 260). These results suggest that PhoQ-PhoP activation may be necessary to promote SPI-2 expression only under particular conditions (e.g., in the preexisting environment before intramacrophage localization, as a preadaptation process) (261). Alternatively, more recent experiments have shown that PhoP directly activates the *orgBC* operon, which is located within SPI-1 and indirectly activated by HilA during invasion. Thus, the *org* genes are thought to be expressed during and after *Salmonella* entry and hence play an additional role after host cell internalization (262). Moreover, transcriptional fusions have revealed that the SPI-3 operon *mgtCB* is positively regulated by PhoP (263).

BarA-SirA. The sensor kinase BarA and its cognate regulator SirA are involved in carbohydrate metabolism, motility, biofilm formation, and invasion (227, 264–267). According to the current regulatory model, SirA activates the expression of two small RNA molecules, *csrB* and *csrC*, which inhibit the production of CsrA, an RNA binding protein. CsrA is a posttranscriptional regulatory protein that alters mRNA stability of target mRNAs and, in turn, induces the production of both *csrB* and *csrC* (268, 269). Since SirA activates expression of a *hilA*::*lacZ* transcriptional fusion only in the presence of HilD (222), CsrA has been hypothesized to bind to the *hilD* mRNA and impair its translation. Thus, SirA activates both *csrB* and *csrC*, which prevent CsrA action and preserve HilD activity. Nonetheless, both *csrA* loss and overexpression are detrimental for the cell, suggesting that CsrA must be under tight control to allow optimal invasion (83, 268, 269). Alternatively, gel shift experiments have shown that SirA can directly bind to the *hilA* and *hilC* promoters *in vitro* (227), although there is controversy in concluding that this interaction leads to direct activation of *hilA in vivo* (83).

Lack of a functional SirA protein leads to reduced expression of *siiA* and *siiE* as well as diminished secretion of the latter gene in correlation with reduced bacterial adhesion to *in vitro* cultured cells (214, 225). *sigD* has also been identified among the positively SirA-regulated genes. However, it seems likely that SirA may activate SigD and the SPI-4 genes through HilA activation (270). The possible presence of a direct interaction of SirA with their respective promoters remains to be demonstrated.

Opposite effects have been reported regarding type I fimbriae and flagella. SirA activates expression of the *fim* genes through two different pathways: (i) by direct binding of SirA to the *fimA* operon and (ii) by activation of the Csr system, leading to increased expression of the mRNA transcripts *csrB* and *csrC*, which in turn eventually activate *fim* expression (266). However, transcriptional fusions to several flagellar genes (early, middle, and late) significantly increase transcription in a *sirA* mutant only when bacteria are chemotaxing and growing in motility agar. Accordingly, it has been inferred that SirA represses flagellar genes, despite the *sirA* mutant being nearly identical to the wild type in terms of swarm size (271). On the other hand, CsrA positively influences motility in *Salmonella*, since a *csrA* mutant exhibits diminished transcription of the *flg* and *fli* operons as well as genes involved in motility and chemotaxis, in addition to being aflagellate and nonmotile (272). According to these data, no clear conclusion about the regulatory cascade can be deduced, since this effect may be a direct consequence of the SirA-mediated repression of CsrA and consequent activation of HilA, which in turn could repress the *flhDC* genes.

RcsC-RcsD-RcsB. Despite the Rcs system being included in the category of 2CRSs in this review, it should rather be referred to as a phosphorelay system since more than two proteins are required to transfer the phosphoryl group to the receiver domain of the response regulator. While RcsB is the response regulator, RcsC is a hybrid histidine kinase and RcsD (YojN) is an essential intermediate phosphotransmitter. This system plays a role in the maintenance of cell wall integrity, cell division, stationary-phase sigma factor activity, biofilm development, motility, and virulence. Several accessory proteins have been reported to influence this system: RcsA is required for RcsB activation of certain genes of the regulon, whereas RcsF (outer membrane protein) and IgaA (inner membrane repressor) act upstream of RcsC by sensing extracellular signals. Activation occurs upon growth on a solid surface, os-

motic shock, desiccation, or growth at a low temperature (20°C) in particular media (273).

The Rcs system affects many virulence determinants, and appropriate levels of Rcs activation are crucial to trigger a positive or negative effect, depending on the target genes. By means of microarray analysis and transcriptional fusions, several SPI-1 genes (e.g., *hilA*, *hilC*, *hilD*, *invF*, *invH*, *prgH*, and effectors encoded outside SPI-1), the *siiE* gene (SPI-4), and the *sigDE* genes (SPI-5) are highly repressed by this system. These results have been deduced not only upon *rcsB* inactivation but also upon Rcs overproduction as a consequence of an *igaA* missense mutation (274, 275). More details have been reported in the case of the flagellar genes. Standard activity or overactivation of this system triggers loss of motility in correlation with repression of the *flhDC* genes (274, 276, 277). Indeed, RcsB regulates flagellar gene expression both negatively and positively. The former effect occurs solely at the initiation of transcription of the master operon and operates through direct binding to the *flhDC* promoter. Positive regulation, however, is mediated by direct activation of the *fliPQR* middle genes and is antagonized by RcsA (274). Nonetheless, the global effect on motility is repression of the flagellar genes.

In contrast, the microarray analyses performed by Wang et al. (274) have revealed dual regulation for SPI-2 (including the *ssrAB* locus), the *pipB* gene (SPI-5), the plasmid-borne genes *spvABC*, and, surprisingly, several *fim* genes. Absence of the *rcsB* gene causes repression, suggesting that RcsB acts as an activator, whereas high activation of the Rcs system reduces their expression. Nonetheless, the fold expression values reported for the *spv* genes are lower than those observed for the other virulence determinants (274). Similarly, analysis of a strain overproducing the Rcs system shows that the *bapA* gene is activated by this phosphorelay system (275). Corroborative results have been provided by other research groups. On one hand, the absence of a functional *rcsC* causes attenuation in mouse virulence at late stages of infection (278). On the other hand, constitutive expression of this phosphorelay system also results in avirulence in mice upon intraperitoneal administration (276). Thus, under standard growth conditions, the Rcs system is an activator of all these genes, although repression is seen upon high-level production of the Rcs proteins.

QseC-QseB. The QseC-QseB 2CRS, in which QseC is the sensor kinase and QseB is the response regulator, is regulated by quorum sensing (279). The initial role attributed to this 2CRS in *E. coli* was, in fact, regulation of flagella and motility by transcriptional activation of the *flhDC* operon (279). In *Salmonella* QseC also enhances motility by significantly increasing flagellar gene expression (e.g., the regulators *flhC* and *fliA*) (280). More recently, QseC has been shown to play a global function in *Salmonella* virulence, since it activates several genes located in different SPIs. Deletion of the *qseC* gene has been studied in *in vitro* and *in vivo* experiments, and the results have revealed decreased invasion ability, a marked reduction in intramacrophage survival, and attenuated systemic infection in mice. These phenotypes correlate with reduced expression of the SPI-1-borne genes *invF* and *sipA*, the SPI-2-related gene *sifA*, the SPI-3-located gene *mgtB*, and the *sigD* gene, which is carried within the SPI-5 (281). Unfortunately, no effect on *hilA* transcription has been studied in order to clarify how it activates SPI-1 (in general, all 2CRSs are reported to affect HilA through modifications in HilD). Thus, QseC is an activator of all of these genes, although the exact or direct mechanism underlying this regulation has not been completely elucidated.

EnvZ-OmpR. In the EnvZ-OmpR 2CRS, EnvZ is the sensor protein and OmpR is the cognate transcriptional regulator, and it is activated in response to extracellular osmolarity (282, 283). Deletion of the *envZ* gene leads to decreased expression of a *hilC*::*lacZ* transcriptional fusion, whereas no effect on the *hilD* promoter is detected (284). Nonetheless, this OmpR-mediated regulation is reported to act through HilD, supposedly by posttranscriptional activation (222). A functional OmpR protein also activates SPI-2 gene expression. An intracellular location inside macrophages determines the need of *Salmonella* for this 2CRS to efficiently transcribe the *ssrAB* genes immediately after entry into macrophages. This effect is mediated by direct binding to the promoters of these two local regulators (237, 260).

Regarding biofilm formation, up to 6 different binding sites for the OmpR regulatory protein have been described in the *csgD* promoter. D1 is the first, where OmpR binds with higher affinity and triggers a positive effect on *csgD* transcription. The second binding site, D2, has been located upstream of D1. There, the binding affinity of OmpR is reportedly lower than that for D1 and is associated with a repressive effect. The remaining 4 binding sites play roles of limited importance and are located in a region whose length suggests that all of these sites must be simultaneously occupied (285). Thus, in general terms, OmpR is deduced to promote biofilm formation by enhancing CsgD expression.

PhoR-PhoB. PhoR is the sensor kinase that activates the transcriptional regulator PhoB under conditions of low inorganic phosphate (P_i) extracellular concentration. The Pst system, encoded in the *pstSCAB-phoU* operon, is a high-affinity P_i uptake system which is required for negative control of PhoR-PhoB (286). This 2CRS, in turn, induces expression of the *fimYZ* genes, which activate HilE to eventually repress HilA (16). Lucas et al. have shown that a mutation within the *pstS* gene leads to repressed *hilA* expression, whereas the absence of a functional Pst system activates this 2CRS (287). Thus, phosphorylated PhoB triggers the repression of *hilA* through the appropriate action of the intermediary regulators.

Nucleoid-Associated Proteins

Bacterial nucleoid-associated proteins (NAPs) represent a class of regulatory proteins whose number is gradually increasing. They possess DNA binding activity and an ability to alter the topology of the DNA molecule by bending, wrapping, or bridging it. Consequently, the levels of DNA supercoiling are modified, and hence expression of many genes can be influenced either positively or negatively. Thus, NAPs contribute to both nucleoid structure and gene regulation, and they may perform both roles simultaneously (288).

H-NS. H-NS usually acts as a repressor of bacterial gene transcription and plays a critical role in regulating gene expression and determining the topology of the DNA (289, 290). Initial studies supported *hilA* repression under noninducing conditions (291). More recently, H-NS has been shown to repress the expression of HilA, HilD, HilC, and RtsA under noninducing conditions. However, direct binding to these promoters has been reported only for the *hilA* and *rtsA* genes. In the absence of H-NS, the *hilA* promoter appears to be highly active, even in the absence of positive regulators. Indeed, under inducing conditions, HilD and HilC, and

probably RtsA, counteract the strong HilA and RtsA depression triggered by H-NS (292, 293).

These repressive effects can also be extended to several SPI-2 genes (e.g., *ssaB*, *ssaG*, *ssaM*, and *sseA*), the SPI-3 *misL* gene, the *siiE* gene (SPI-4), and the plasmid-borne genes *spvR* and *spvB* (225, 238, 239, 294). Repression mediated by direct binding to their promoters has been reported for the first two classes of genes (238, 239). These silencing effects indicate that positive regulators are required to overcome repression under the appropriate inducing conditions. Accordingly, since the MarT homolog CadC has been proven to counteract H-NS and since HilA is no longer required for *siiE* expression in the absence of this NAP, MarT binding to the *misL* promoter and HilA activation of the SPI-4 genes are thought to antagonize H-NS repression (225, 239).

Surprisingly, a positive effect has also been associated with the regulatory influence of H-NS. This activation is exerted on the *flhD* flagellar master regulator and the *csgD* regulator of curli fimbriae and biofilm. These results are reinforced by the fact that on mutation of the *hns* gene, diminished swarming ability and decreased expression of the *rdar* morphotype are observed (285, 295). Nonetheless, further activators are suggested to be necessary for full activation of the flagellar genes (295).

Hha. The Hha regulator also contributes to gene regulation despite playing a less relevant role than H-NS. Initially, it was found to repress *hilA* transcription by direct binding to its promoter (296). Further results have supported new evidence that it could also repress *rtsA* and *hilC* transcription, despite direct binding being demonstrated only for the *rtsA* promoter. Similar to the findings for H-NS regarding regulation of *hilA* and *rtsA*, HilD and HilC, and possibly RtsA, counteract the Hha-mediated silencing of these two genes. Moreover, H-NS and Hha have been proven to synergistically contribute to such repression under noninducing conditions (292, 293).

Additionally, Hha is the major repressor in silencing SPI-2 genes before bacteria are located in the intracellular environment. A potential synergistic interaction between Hha and YdgT, an Hha homolog protein (see below), has been proposed to explain the higher attenuation of an *hha ydgT* double mutant than of individual *hha* and *ydgT* mutants (297, 298).

YdgT. The YdgT NAP is a negative regulator of SPI-2 genes, since it is transcriptionally repressed during early intracellular infection, when SPI-2 is activated. Deletion of this gene leads to a surprising biphasic phenotype: enhanced early survival in macrophages followed by an attenuated intracellular phenotype. Early increased SPI-2 expression in the *ydgT* mutant can initially be advantageous, since wild-type bacteria are still adapting to the intracellular environment. Nonetheless, this protein is necessary for full virulence during systemic colonization, since moderate YdtG expression in macrophages together with reduced SPI-2 expression is observed later in infection. These findings suggest that tight regulation of this protein is necessary to accommodate the intracellular growth rate to guarantee bacterial persistence (298).

IHF. Integration host factor (IHF) plays an important role in DNA bending and compactation and in the transcriptional regulation of many genes. It is a heterodimeric protein composed of two highly homologous subunits (IHFα and IHFβ) (299) whose intracellular concentration is growth phase dependent (300). After inactivating both subunits, Mangan et al. (301) used microarrays to show that IHF can activate all five SPIs, motility, and the *spv* genes depending on the growth phase. SPI-1 genes, the *siiBCD* genes (SPI-4), *sigD*, and the flagellar and chemotaxis genes are significantly downregulated at 1 and 4 h of growth in the absence of IHF. Moreover, reduced motility is also observed. These findings suggest that IHF activates these invasion-related genes during exponential growth, despite no uniform role and less clear results being reported for the stationary phase (301). Concerning SPI-1 regulation, since similar activating effects are seen for HilA, HilD, and HilC, it is possible that IHF activates SPI-1 genes at the level of HilD, which can then influence expression of the downstream regulators.

In contrast, but according to the maximal IHF expression levels detected in the late logarithmic phase, SPI-2 genes, the *mgtCB* operon, *pipB*, and the *spvABC* genes are particularly activated after 6 h of growth (when favorable conditions for SPI-2 induction and intracellular survival have been described). Variable results are observed at 1 and 4 h of growth. Thus, IHF is essential for activating these genes at the stationary phase, when they are thought to contribute to the intracellular stage of the disease (301). Additional results have revealed that IHF binds to the *spvR* upstream region and that upon its inactivation, *spvR* and *spvB* transcriptional levels are reduced (302). Lastly, the absence of a functional IHF protein leads to a reduced *rdar* morphotype, and consistently diminished CsgD expression is detected. Moreover, mobility shift assays have revealed that IHF directly binds to the *csgD* promoter, thereby explaining its positive effect as an activator (285).

Fis. Fis is a global regulatory NAP involved in the processes of replication, recombination, and transcription. It modulates the topology of the DNA in a growth-phase-dependent manner (303). On deletion of the *fis* gene, diminished *hilA* transcription, invasion, and *hilA*-independent *invF* expression (304) are observed, as well as a marked reduction in the *hilD* mRNA levels (291). Thus, Fis, which is maximally expressed at 1 h of growth, is an activator of invasion genes that works primarily by affecting HilD transcription (291). Activation can also be extended to the SPI-2, SPI-4, and SPI-5 genes, since in the absence of this global regulator, there is diminished expression of several SPI-2 genes (particularly at 1 h of growth), as well as the *siiABCDEF* and *sigDE* operons and the *pipB* gene (particularly at 4 h of growth) (305). Further proteomic analyses have corroborated that the SPI-2 genes are positively influenced by Fis, and Fis has been shown to directly bind to the *ssrA* and *ssaG* promoters with the use of DNA shift mobility assays (305, 306). In contrast, in a *fis* mutant increased transcription of the *mgtCB* operon after 1 and 4 h of growth (being more important at the onset of the exponential phase) suggests a repressive effect. Differential and less significant results have been reported for the *marT* and *misL* genes. Thus, despite Fis not having a consistent regulatory effect on all SPI-3 genes, a repressive action on the Mgt proteins can be assumed (305).

Concerning the flagellar genes, inactivation of the *fis* gene has been associated with diminished expression of the flagellar and motility genes (highlighting a strong effect on expression of *rtsB*, a regulator of flagellar genes [see below]) at 4 h of growth by means of transcriptomic analysis (305, 306). Transcriptional fusions to several early, middle, and late genes have corroborated these results, as have motility tests, which show impaired motility in a *fis* mutant. Moreover, gel retardation assays show that Fis directly binds, at least, to the *flhDC* promoter (305). Altogether, these results suggest a positive effect of Fis on flagellar gene expression.

HU. The most abundant NAP in *Enterobacteriaceae* is the heterotypic dimer HU. The subunits HU-2 (or α) and HU-1 (or β)

are encoded within the *hupA* and *hupB* genes, respectively, and lead to the formation of three different dimers ($\alpha\beta$, α_2, and β_2). The genes belonging to the HU regulon are involved in anaerobiosis, acid stress, high osmolarity, and SOS induction (307, 308). Regarding virulence, HU is deduced to activate SPI-1 and SPI-2 genes. By means of microarray analysis, the *hupA hupB* double mutant, despite showing diminished fitness, has been observed to trigger diminished expression of *hilA*, *hilC*, and *hilD* as well as other SPI-1 structural genes (at 1, 4, and 6 h of growth). In addition, this mutant also shows a marked and significant reduction in epithelial cell invasion. Nonetheless, since the *hup* mutations do not trigger impaired *hilA* transcription in the absence of HilD or affect *hilD* transcription, it has been deduced that HU transcriptionally activates HilA expression by posttranscriptional modulation of HilD (291, 309). Similarly, several SPI-2 genes, including the 2CRS *ssrAB* genes, are downregulated in the HU double mutant after 4 h of growth (309).

Activation of flagellar genes and motility has also been deduced from analysis of the *hupA hupB* double mutant. In this particular situation, expression of flagellar genes is reduced at all time points, whereas motility genes are downregulated only at 1 and 6 h of growth, while at 4 h of growth there is no significant change in expression. Confirmatory results have been obtained from motility assays on soft agar plates, which show significantly decreased motility (309).

Other Regulators

RtsB. The regulator RtsB is encoded within the same islet where RtsA is encoded. This regulator has been reported to repress expression of the flagellar genes by binding to the *flhDC* promoter region and hence decreasing the expression of the entire flagellar regulon (231). Particularly, RtsB represses the dynamics of the flagellar genes, since flagellar genes in the *rtsB* mutant are expressed longer whereas constitutive *rtsB* expression completely inhibits flagellum synthesis (15). Moreover, RtsB also influences expression of SPI-1 genes and type I fimbriae. Upon constitutive expression of RtsB, the SPI-1 genes are repressed, whereas in the absence of this regulator, no significant change can be detected. However, RtsB has been reported not to directly act on the SPI-1 genes, and this effect mirrors that observed in the absence of FliZ (15, 231). Alternatively, constitutive expression of RtsB accelerates induction of type I fimbriae, despite being only weakly delayed in the *rtsB* mutant. A direct effect on the *fim* genes has been ruled out, since RtsB has no effect in the absence of FliZ (15). Thus, these two indirect effects are assumed to be mediated by FliZ; RtsB represses FliZ, which has been shown to activate SPI-1 expression but repress type I fimbriae.

Lrp. The Lrp global regulator of metabolism is involved in several bacterial processes. Its regulon includes genes responsible for amino acid, carbon, and energy metabolism, pilus synthesis, macromolecular biosynthesis, the stress response, etc (310, 311). A substantial fraction of these operons are also influenced by leucine, which antagonizes or potentiates the repressing or activating effects of Lrp, whereas the remaining operons are not influenced (311). Recently, the Lrp effect on virulence has been tested in *Salmonella*. Lrp constitutive expression dramatically attenuates virulence, leading to defects in invasion, cytotoxicity, and colonization, whereas *lrp* deletion enhances these activities. Particularly, Lrp represses transcription of the *hilA*, *invF* (SPI-1), *ssrA* (SPI-2), and *spvA* (pSLT plasmid) genes by direct binding to a consensus DNA motif (302, 312). The effects seen on *hilA* and *ssrA* expression are independent of the presence of leucine (312). In contrast, *invF* repression does require leucine, whereas expression of the *spvABCD* genes, which is prevented by Lrp protection of the transcriptional start site of the *spvA* gene, is antagonized by leucine (302, 312).

Lon and DnaK. Heat shock proteins (HSPs) constitute a cellular system for folding, repair, and degradation of proteins. Major HSPs are proteases (e.g., Lon) and molecular chaperones (e.g., DnaK and DnaJ) activated during a heat shock response in which the shutoff phase is partially mediated by DnaK (313). These two HSPs influence the expression of SPI-1 and contribute to survival during the intracellular stage of disease. DnaK alone, however, has been reported to fully activate the expression of the flagellar genes through modulation of the native protein complex composed of FlhD and FlhC (314).

On one hand, concerning SPI-1 regulation, a *lon* mutant shows a dramatic enhancement in *hilA* and *invF* transcription and increased secretion of SipA, SipC, and SipD (315, 316). Moreover, in the absence of Lon, HilC and HilD accumulate intracellularly (317). Conversely, the DnaK/DnaJ chaperone machinery has been shown to be necessary for invasion of epithelial cells (318). Accordingly, it has been suggested that SPI-1 gene expression is controlled by a feedback regulatory loop. First, Lon is induced to control turnover of HilD and HilC and hence limit the expression of SPI-1 genes, which is of particular relevance after invasion of epithelial cells. Second, DnaK negatively modulates *lon* expression thereby promoting the invasion phenotype (317, 319).

On the other hand, Lon positively influences virulence at this stage. Disruption of *lon* leads to an inefficient proliferation within the spleen and absence of lethal systemic disease in mice. Moreover, these mutants cannot survive or proliferate within macrophages (320). Similarly, the DnaK/DnaJ chaperone machinery has been shown to be necessary for survival within macrophages (318).

Fur. Fur is the primary iron-regulatory protein in *Salmonella* and *E. coli*. When bound to a divalent cation (mainly Fe^{2+}), this regulator binds to DNA sites to directly repress downstream genes (321). Ellermeier and Slauch initially reported that Fur activates *hilA* transcription in a HilD-dependent manner, and since Fur is thought to be capable of acting only as a repressor, it should repress a repressor that controls HilD (322). More recently, Troxell et al. have given new insight into this by showing that Fur binds to the promoter of the *hns* gene (323). Accordingly, Fur represses H-NS, and thereby HilD is no longer repressed.

Mlc. The global regulator Mlc is involved in carbohydrate metabolism and in the regulation of sugar utilization (324). Lim et al. have reported that an *mlc* mutant shows decreased expression of *hilD*, *hilA*, and *invF* and that Mlc can directly bind to the *hilE* promoter. Thus, Mlc acts as an activator of *hilA* transcription by repressing *hilE* expression (325).

RNase E. RNase E or endoribonuclease E is encoded by the *ams* gene and plays a general role in RNA decay, being involved in cleavage of A/U-rich single-strand RNA regions (326). An *ams* mutant has been reported to be much more invasive than the parent strain under high- and low-oxygen conditions. Therefore, since the message for HilA has a long 5′ untranslated region which is A/U rich, it has been hypothesized that RNase E degrades *hilA* mRNA to repress invasion (235). Nonetheless, further results indicate that increased *hilA* expression is detected only in the pres-

ence of HilD, suggesting that *ams* may also exert a posttranscriptional effect on HilD (232).

FadD. The *fadD* gene encodes acyl coenzyme A (CoA) synthetase, an enzyme required for uptake and degradation of long-chain fatty acids (LCFA) (327). Loss of FadD has been reported to repress *hilA* transcription, and therefore, fatty acid derivatives may act as intracellular signals to regulate *hilA* expression (287). Nonetheless, the mechanism by which regulation occurs remains unknown, and there is still controversy about the role of fatty acids in virulence (328–331).

SlyA. The SlyA regulator is required for virulence and survival within macrophages (332). The intracellular behavior of an *slyA* mutant in infected cells is consistent with inefficient SPI-2 expression (including effectors encoded outside the island, such as by *sifA* and *sifB*) (333). In addition, SlyA stimulates the transcriptional activity of an *ssrA*::*lacZ* transcriptional fusion (334) and directly binds to the *ssrA* promoter (335). Therefore, it has been deduced that SlyA activates SPI-2 gene expression in an SsrA-dependent manner (333).

CROSS TALK

An obvious consequence of proteins sharing the same functional properties is that they usually share regulatory pathways. It has been stated that there is cross talk between these pathogenicity elements, since different global and specific regulators influence the expression of several SPIs, thereby maximizing the efficiency of *Salmonella*. As a result, SPI-1 is the key point at which many regulatory inputs are processed at the level of HilA to evaluate whether it is appropriate to proceed with the invasion process (e.g., most of the 2CRS regulatory systems interact with HilD posttranscriptionally, which then activates HilA; in turn, HilA transcription is under the control of other regulators, such as several NAPs).

On one hand, regulation of all the SPIs is cross talked through several regulators, exerting a positive (Fig. 4A) or negative (Fig. 4B) action. Among these regulators are three which are encoded within SPI-1 itself (HilA, HilD, and InvF) and guarantee their internal synchronization. Additionally, there are six 2CRSs (EnvZ-OmpR, BarA-SirA, PhoQ-PhoP, QseC-QseB, RcsCDB, and SsrA-SsrB) which also take part in this regulatory event. Nonetheless, whether or not they exert a direct effect on all the SPIs they regulate is something that remains to be completely elucidated: (i) mutations within *hilD*, *hilC*, and *hilE* influence expression of the SPI-4 genes, despite a direct effect not having been reported, possibly as a direct consequence of their influence on HilA (214); (ii) the BarA-SirA system influences expression of SPI-4 and SPI-5, although this action might be similarly mediated through HilA (evidence show that HilA is required in the case of SPI-5) (270); (iii) the RcsCDB system influences a large number of virulence elements (274), making it therefore reasonable to think that intermediate regulatory proteins are required, such as HilD (as seen for most of the other 2CRSs), which eventually has an effect on four SPIs by its own action or via HilA; and (iv) likewise, the repressive effect stated for PhoQ-PhoP on the SPI-4 genes (214) may also be the result of its known repression of HilA and consequent absence of activation of the *sii* genes. Alternatively, five NAPs (H-NS, Fis, HU, IHF, and Hha) have also been reported to contribute to this purpose. They exert either a positive or negative effect on a particular operon within the SPIs, depending on the growth phase and on the type of genes within the same island.

This information reveals a crucial role for the extracellular signals sensed by the 2CRSs (e.g., PhoP is activated inside macrophages) and growth phase. Growth phase determines the expression levels of several regulators (e.g., Fis is maximally expressed at 1 h of growth, and IHF peaks during the transition into the stationary phase [301, 305]) and influences its regulatory action (e.g., HilD induces SPI-2 genes during the late stationary phase [14]). As a result, transcriptional transition from the invasion process *per se* to the intracellular survival stage depends on the relative concentrations of all the regulators involved. There are, among these regulators, not only important activators leading to gene induction or counterrepression at the proper step but repressors shutting off the virulence traits which are expressed later in pathogenesis or those no longer required (e.g., SPI-2 genes are repressed by Hha and YdgT before encountering the intracellular environment [297]).

On the other hand, cross talk is also important for synchronizing expression of invasion (particularly SPI-1), flagella, type I fimbriae, the pSLT plasmid, and biofilm formation. Several global regulatory proteins, including a subset of the regulators involved in the cross talk between SPIs, take part in regulating these virulence traits. These regulators embrace five 2CRSs (EnvZ-OmpR, BarA-SirA, PhoQ-PhoP, QseC-QseB, and RcsCDB), four NAPs (H-NS, Fis, IHF, and HU) and several regulators (i.e., RtsA, RtsB, CsgD, Lrp, and DnaK) (Fig. 5).

Following a temporal order, flagellar and motility genes are expressed at the early exponential growth phase according to their initial role in pathogenesis. At this stage, the FliZ flagellar activator has been reported to simultaneously repress the fimbrial genes but activate SPI-1 through HilD (15, 287). Next, when *Salmonella* enters the late exponential growth phase, these primarily invasion genes are maximally transcribed, and further results suggest that HilA may act as a repressor of flagellar genes which are no longer required (224). Moreover, since the RtsA and RtsB proteins are encoded within the same operon, they coordinate induction of invasion and repression of motility in the small intestine, respectively (231). Later, upon extracellular entry in the stationary phase, virulence gene transcription is focused on fimbrial genes, and among these genes, those for the regulators FimWYZ have also been reported to repress SPI-1 (via HilE) as well as the flagellar genes and motility, since they are no longer useful. It has been speculated that fimbrial adhesion is also important for persistence of those bacterial cells which have not breached the intestinal epithelium (15, 245). Thus, FimZ, FliZ, and HilA mediate coordination between motility, invasion, and adhesion as sequential and, sometimes, opposite steps (Fig. 5).

Alternatively, the *spv* genes also show an expression pattern integrated within this regulatory network in relationship to the role that these genes play in pathogenesis. IHF and the RcsCDB system coordinately regulate this plasmid-borne operon together with SPI-2 and the *pipB* gene, as seen by the strong activation at the stationary phase and the dual regulation according to the degrees of activation reported for these regulators (274, 301).

Lastly, biofilm production is also connected with the expression of other virulence traits. Despite the scarcity of reports concerning this cross talk, the key regulator of biofilm, CsgD, is of marked importance, since it has been attributed a global role in virulence. First, it increases the c-di-GMP levels, which in turn inhibits invasion of epithelial cells (336). Second, studies performed in *E. coli* have shown that CsgD represses flagellar genes (337). Thus, CsgD promotes the transition from the invading and

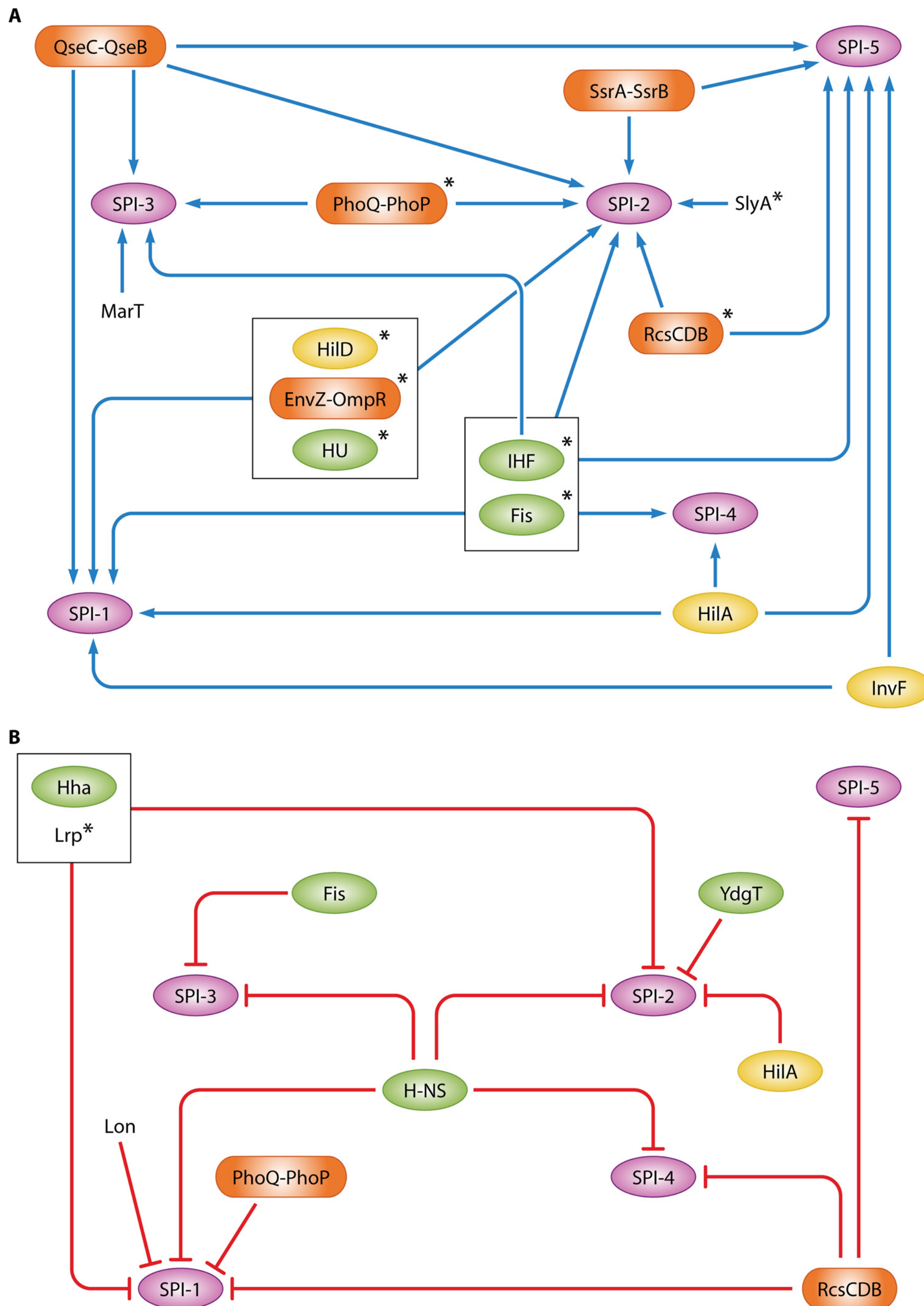

FIG 4 Cross talk between all the SPIs. The regulatory effects which may be explained through an indirect pathway have not been represented to make comprehension easier (i.e., the effects that HilD, HilC, HilE, PhoQ-PhoP, and SirA-BarA exert on either SPI-4 or SPI-5). According to the evidence reported in the text, the regulators marked with an asterisk are those proposed to act in this model on the SPI-2 genes via SsrA-SsrB. The roles reported for Lon and DnaK regarding systemic virulence have not been included in this model, since there is no specific information about their target genes. (A) Regulators positively influencing expression of the SPI genes. (B) Regulators leading to a repressor effect.

motile bacteria characteristic of acute infections toward the settled noninfective biofilm status associated with chronic infection.

In summary, as observed from the results presented and as an obvious consequence of this cross talk, the genes playing a cooperative role, such as those involved in the first stage of infection or those required for survival within macrophages, have a similar expression profile, thereby maximizing the efficiency of the pathogenic process.

CONCLUDING REMARKS

Salmonella Typhimurium is an excellent intracellular pathogen whose abilities to colonize and succeed within the host are ex-

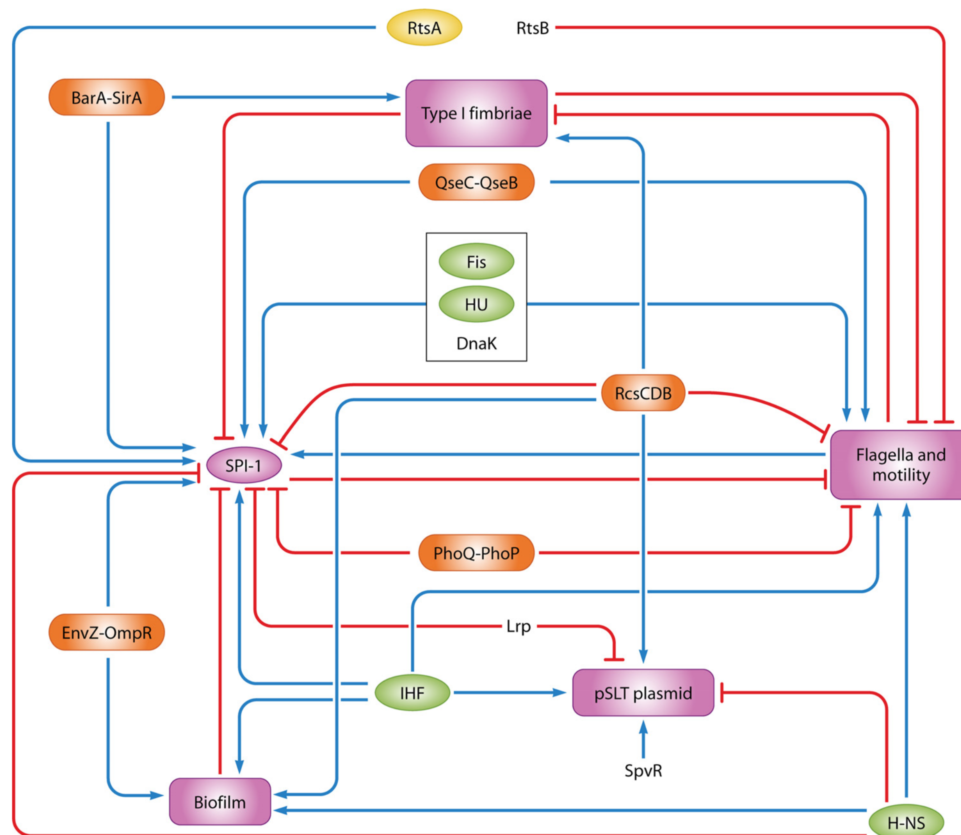

FIG 5 Cross talk between the virulence elements. The reported effects of SirA and CsrA on flagellar genes are not considered in this scheme, since no direct evidence of independent HilA activity have been presented. The regulatory effects that flagellar genes exert on SPI-1 and type I fimbriae are mediated by FliZ, whereas SPI-1 repression of flagellar genes is dependent on HilA activity. The influence of biofilm on SPI-1 expression is mediated by CsgD.

tremely versatile. Its genome includes several virulence systems, including genes required for motility and chemotaxis, adhesion, invasion, and replication and survival within host cells, as well as biofilm formation, which cover the whole pathogenic process from the intestinal stage to systemic dissemination. As a result, *Salmonella* has evolved to a complex state of interactions with the human body in which a large number of effectors trigger specific actions on the host signaling pathways. These inputs lead to sometimes redundant mechanisms which must be perfectly balanced to ensure the intracellular changes that allow internalization and survival of the pathogen. Moreover, coordination in the incredibly large set of bacterial virulence properties plays a critical role, since the effectors can, at times, show antonistic functions: activation of signaling pathways can be followed by repression mediated by other effectors to counteract the dangerous responses of the host which may end in bacterial clearance. As a consequence, this bacterial armamentarium is perfectly synchronized following a temporal hierarchy in which extensive cross talk facilitates expression of the appropriate virulence properties at the correct times and locations. Specific and global regulators organize this incredible orchestra and mirror the complicated interactions between the invading *Salmonella* and the host in the attempt to overcome the infection.

According to the above-mentioned information regarding the biological role and regulation of the known SPIs, SPI-1 is required primarily for the first stage of disease, as is SPI-4, which reportedly complements SPI-1 in adhesion and in the inflammatory response processes (214, 225, 270). In contrast, SPI-2 is required primarily for the growth and survival of bacteria within the host cells during the systemic phase of disease (87). Nonetheless, recent findings suggest that the boundaries between the functions of these two SPIs are not sharply defined. The facts that some SPI-1 effectors are detected hours or days after infection and that some effectors can be secreted by both T3SS-1 and T3SS-2 support this point of view. Strikingly, SPI-3 and SPI-5 play a dual role in pathogenesis, since the two islands encode proteins involved in both invasion and intracellular survival. This dual role is particularly evident in the case of SPI-5, since it encodes effectors secreted via T3SS-1 and T3SS-2 (95, 96, 239, 338). Additional ORFs are located on each of these two islands, although their function has not been completely elucidated, making understanding of their overall role and transcription difficult.

In terms of regulation, the extremely complex regulatory network coordinates the expression of the genes involved in central metabolism, cell wall integrity and division, response to extracellular stimuli, quorum sensing, and global gene regulation to ensure that only when all conditions are favorable does *Salmonella* fully activate its virulence machinery, thereby contributing to its success as a pathogen. Moreover, several of these regulators play a master role in the timing of virulence, since they drive the transition from the exponential growth to the stationary phase (e.g., Fis and IHF) as well as from the extracellular to the intracellular en-

vironment (e.g., PhoQ-PhoP). Therefore, such cross talk mainly controls gene expression dynamics, i.e., transitions between different phases of gene expression.

Thus far, in spite of the large number of regulators reported to influence this regulatory cascade, not all of these regulators exert a clear and well understood role in regulation. Further studies are needed to specifically determine the real contribution of some of these regulators, their activation under particular stimuli, and the molecular pathways involved in the regulation *per se*. Likewise, more specific reports focused on host-pathogen interactions are required to fully understand the cooperative role of the bacterial effectors which have been already characterized and of those whose function still remains undetermined.

Thus, despite most infections being restricted to the intestinal area and not meriting antimicrobial therapy, new insight into regulation and virulence may help to develop new antibacterial strategies for the situations in which *Salmonella* cells traverse the intestinal epithelium, leading to a systemic spread, and the normal antimicrobial prescriptions fail due to the emergence and dissemination of mechanisms of resistance to several current drugs.

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