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Salmonella Pathogenicity Islands in host specificity, host pathogen-interactions and antibiotics resistance of *Salmonella enterica*

Funktionen von Salmonella *Pathogenitätsinseln bei der Wirtspezifität, Erreger-Wirts-Interaktion und Antibiotika-Resistenz von* Salmonella enterica

Roman G. Gerlach, Michael Hensel

Summary

Salmonella enterica is a pathogen highly successful in causing a variety of gastrointestinal and systemic diseases in animals and humans. While some serovars of *S. enterica* are able to infect a broad range of host organisms, other serovars are highly restricted to specific host species. The colonization of hosts by *S. enterica* depends on the function of a large number of virulence determinants. The molecular analyses of virulence genes demonstrated that most of these loci are clustered within *Salmonella* Pathogenicity Islands (SPI). SPI1 and SPI2 each encode type III secretion systems (T3SS) that confer main virulence traits of *S. enterica*, i.e. invasion, enteropathogenesis and intracellular survival and proliferation. Further SPI encode factors that contribute to intracellular survival, different types of adhesins, or effector proteins of the SPI1-T3SS or SPI2-T3SS. The availability of genome sequences of several serovars of *S. enterica* also revealed serovar-specific SPI. In this review, the main characteristics of the currently known SPI are summarized with focus on their roles in various animal hosts and putative functions in human infections.

Keywords: Bacterial protein secretion, Pathogenicity island, Gram-negative pathogen, invasion, intracellular pathogen, PAI, SPI, *Salmonella*

Zusammenfassung

Salmonella enterica ist ein Erreger, der äußerst erfolgreich gastrointestinale und systemische Erkrankungen bei Menschen und Tieren auslöst. Während einige Serovare von *S. enterica* in der Lage sind verschiedenste Wirtsorganismen zu infizieren, sind andere Serovare hochgradig wirtsadaptiert. Für die Pathogenese von Salmonellen-Infektionen ist die Funktion einer großen Zahl von Virulenzfaktoren erforderlich. Die molekulargenetische Analyse dieser Faktoren zeigte, dass die Mehrheit der Virulenzgene in *Salmonella* Pathogenitätsinseln (SPI) lokalisiert sind. Sowohl SPI1 und SPI2 kodieren für Typ-III-Sekretionssysteme (T3SS), die eine entscheidende Rolle während der Pathogenese spielen, wie etwa der Invasion von Wirtszellen, die Enteropathogenität, und der Fähigkeit zu intrazellulärem Überleben und Vermehrung. Weitere SPI kodieren für Faktoren, die zum intrazellulären Überleben beitragen, unterschiedliche Typen von Adhäsinen, oder für Effektorproteine der SPI1-T3SS oder SPI2-T3SS. Die momentan verfügbaren Genomsequenzen verschiedener Serovare von *S. enterica* lassen die Anwesenheit von Serovar-spezifischen SPI erkennen, deren Funktionen noch weitgehend unbekannt sind. Mit besonderem Blick auf die Rolle in verschiedenen Tiermodellen und der möglichen Bedeutung für Infektionen des Menschen sollen in diesem Übersichtsartikel die wichtigsten Eigenschaften der zurzeit bekannten SPI dargestellt werden.

Schlüsselwörter: Bakterielle Proteinsekretion, Pathogenitätsinsel, Gram-negative Erreger, Invasion, intrazelluläres Pathogen, PAI, SPI, *Salmonella*

TABLE 1: *Characteristics of Salmonella Pathogenicity Islands.*

Designation (alternative)	Size* in kb	base composition % G+C (range)	insertion point	distribution	variability (stability)	virulence functions
SPI1	39.8	47	*flhA-mutS*	*Salmonella* spp.	conserved	T3SS, iron uptake
SPI2	39.7	44.6	tRNA *valV*	*S. enterica*	conserved	T3SS, tetrathionate reductase
SPI3	17.3	47.3 (39.8–49.3)	tRNA *selC*	*Salmonella* spp.	variable	Mg^{2+} uptake, MisL adhesin
SPI4	23.4	44.8	(tRNA-like)	*Salmonella* spp.	conserved	T1SS, adhesin
SPI5	7.6	43.6	tRNA *serT*	*Salmonella* spp.	variable	T3SS effectors SopB, PipB
SPI6 (SCI)	59	51.5	tRNA *aspV*	subsp. I, parts in IIIB, IV, VII	conserved in subsp. I	Saf fimbriae
SPI7 (MPI)	133	49.7 (44–53)	tRNA *pheU*	subsp. I serovars	instable	Vi antigen, pilus assembly, SopE
SPI8	6.8	38.1	tRNA *pheV*	sv. Typhi	?	unknown
SPI9	16.3	56.7	prophage	subsp. I serovars	?	T1SS, adhesin BapA
SPI10	32.8	46.6	tRNA *leuX*	subsp. I serovars	variable	Sef fimbriae
SPI11	14	41.3	prophage	*S.* Choleraesuis	?	unknown
SPI12	6.3	49.9	tRNA *pro*	*S.* Choleraesuis	?	unknown
SPI13	n.d.	48.1	tRNA *pheV*	*S.* Gallinarum *S.* Typhimurium	?	unknown
SPI14	n.d.**	41.0	–	*S.* Gallinarum, *S.* Typhimurium	?	unknown
SPI15	6.3	n.d.	tRNA *gly*	*S.* Typhi	?	unknown
SPI16	4.5	n.d.	tRNA *arg*	*S.* Typhi, and others	?	serotype conversion
SPI17	5.1	n.d.	tRNA *arg*	*S.* Typhi, and others	?	serotype conversion
SGI1	43	48.4	*thdF–yidY*	subsp. I serovars	variable	5 antibiotic resistance genes
CS54	23.2	57	*xseA-yfgK*	subsp. I serovars	?	adhesion

* Size of the PAI as calculated for S. Typhi or the serovars indicates; ** n.d., not determined.

Introduction

Salmonella spp. are Gram-negative bacteria and members of the family enterobacteriaceae. The genus comprises the two species *Salmonella enterica* and *Salmonella bongori*, the latter being considered as the phylogenetical older species (Boyd et al., 1996). *Salmonella enterica* is classified into seven subspecies: I, II, IIIa, IIIb, IV, VI and VII. Further characterization was done by typing of their O (somatic), K (capsular) and H (flagellar) antigens, with more than 2,500 serovars being identified.

A remarkable feature of *Salmonella* is the ability to cause a broad spectrum of diseases in infected humans and animals, ranging from localized intestinal inflammation and gastroenteritis to typhoid fever as a life-threatening systemic infection. Persistent infections known as carrier state are a major problem in public health. There is also variation in the host adaptation among certain serovars being highly adapted to human hosts such as *S. enterica* serovar Typhi and Paratyphi, others causing predominantly diseases in farm animal species such as *S.* Gallinarum in fowl or *S.* Choleraesuis in swine, and others showing broad host ranges such as *S.* Enteritidis or *S.* Typhimurium.

In contrast to many other bacterial pathogens, classical exotoxins do not play an important role in *Salmonella* pathogenesis. Rather, most of the virulence factors of *Salmonella* identified so far are linked to protein translocation and secretion systems, either being parts of secretion systems itself or transported proteins.

Pathogenicity Islands (PAI) are large chromosomal regions that are present in pathogenic bacteria and confer virulence properties. The first indications for the role of PAI came from the genetic analyses of unstable virulence traits of uropathogenic *Escherichia coli* (Hacker et al., 1983)

Since then, PAI have been discovered in a large number of bacterial pathogens and the concept of the acquisition of new virulence functions by integration of PAI into the chromosomes is now a paradigm for the evolution of bacterial virulence. Briefly, PAI can be characterized by (i) their large size (several kb to more than 100 kb), (ii) a base composition different to the core genome (e.g. lower percentage of bases G+C within the PAI sequence), (iii) association with 'mobile DNA elements' such as integrases, insertion sequence (IS) elements, direct repeats, bacteriophage genomes, etc., (iv) genetic instability resulting in loss of the PAI, (v) frequent association with genes encoding tRNA, (vi) presence of one or more virulence genes. It should be noted that only few PAI have all of these characteristics. It has also been observed that the acquisition and chromosomal integration of large fragments of DNA is not restricted to virulence traits but can also be observed for metabolic functions. The resulting loci have been termed 'fitness islands' or 'genomic islands' (Dobrindt et al., 2004).

More detailed descriptions of the genetics of PAI and the role of PAI in pathogenesis of bacterial infections can be found in recent reviews (Dobrindt et al., 2004; Gal-Mor and Finlay, 2006; Hacker and Kaper, 2000; Schmidt and Hensel, 2004).

Salmonella virulence factors and Pathogenicity Islands

The molecular basis of virulence of *Salmonella enterica* has been approached by various screens for attenuated mutants and resulted in the identification of many single genes that contribute to key virulence traits such as invasion or intracellular replication. The first indication that many of these genes are clustered in loci that represent PAI came from the molecular characterization of invasion genes (see below). PAI in *Salmonella* spp. are commonly termed '*Salmonella* Pathogenicity Island' or SPI. It is now obvious that *S. enterica* harbors a surprisingly large number of SPI and that these loci are the key elements of *Salmonella* virulence. In the following parts, we will describe the main features of the SPI with focus on their contribution to pathogenesis in human and animal hosts. Important features of currently known SPI are summarized in Table 1.

SPI1

Salmonella spp. is able to invade non-phagocytic host cells. Several genes required for the host cell invasion phenotype of *S. enterica* serovar Typhimurium were initially identified by screening of mutant banks (Galan and Curtiss, 1989). Later work by the group of C.A. Lee revealed that all of the host cell invasion genes identified so far were clustered within a region at centisome 63 of the *Salmonella* chromosome (Mills et al., 1995). The identification of a second cluster of genes required for ability of *Salmonella* to proliferate in various organs of an infected host (systemic virulence) prompted researchers to name the invasion locus *Salmonella* Pathogenicity Island 1 (SPI1) and, accordingly, the newly identified locus SPI2 (Shea et al., 1996). The designation 'SPI' has been extended to most PAI subsequently identified in *Salmonella* spp. SPI1 is about 40 kb in size and encodes a type III secretion system (T3SS), type III-translocated effectors and their chaperones, regulatory components as well as the Sit iron uptake system.

T3SS are complex, supramolecular assemblies which span the inner membrane, the periplasmic space, the outer membrane of the bacteria, the extracellular space and a membrane of a eukaryotic host cell. T3SS have been isolated in species of several Gram-negative bacteria (e.g. *Salmonella, Yersinia, Shigella, E. coli, Pseudomonas*) and are consisting of at least 20 different subunits which enable these bacteria to translocate specific substrates (or 'effectors') directly into the host cell cytoplasm in order to exert a broad range of virulence functions (Ghosh, 2004). Because of their shape and their ability to translocate proteins in a cell contact-dependent manner T3SS are also referred to as 'injectisomes' or 'molecular needles' (reviewed in Cornelis, 2006).

The SPI1-encoded T3SS enables *Salmonella* to actively invade non-phagocytic cells and plays an important role in *Salmonella*-induced inflammatory responses (Fig. 1) (reviewed in Schlumberger and Hardt, 2006). The coordinated action of a whole set of translocated effectors leads to temporal reorganization of the host cell actin cytoskeleton and induces uptake of the bacteria by means of macropinocytosis. The effectors SopE, SopE2 and SopB act on small GTPases of the Rho-family, thereby manipulating signaling pathways. While SopE and SopE2 function as G-nucleotide exchange factors (GEFs) on Cdc42 and Rac1 (Friebel et al., 2001), SopB is a phosphatidylinositol phosphatase which activates Rho-GTPases indirectly by generating several potential second messengers (Zhou et al., 2001). A set of two other effectors, namely SipA and SipC, were shown to act directly on actin and mediate polymerization and bundling (McGhie et al., 2001). Another effector called SptP was shown to be a GTPase activating protein (GAP) acting on Rac1 and Cdc42, thus being able to reverse the cytoskeletal rearrangements induced by SopE/E2 and SopB (Fu and Galan, 1999).

Rather diverse effects on host cells are elicited by SopB, which is encoded by a gene in SPI5 (see below). Together with the effector molecules SopA and SopD its function is linked to diarrheal symptoms in a bovine infection model. In detail, SopB activates chloride channels in the membrane of epithelial cells, finally leading to the secretion of chloride and loss of fluid into the intestinal lumen (Norris et al., 1998). Furthermore, SopB was shown to be essential for the early steps of the maturation of the organelle harboring the internalized bacteria: the *Salmonella*-containing vacuole (SCV). Enzymatic activity of SopE depletes PtdIns(4,5)P2 from the invaginating membrane (Terebiznik et al., 2002) and maintains high levels of PtdIns(3)P in the SCV membrane at later stages (Hernandez et al., 2004).

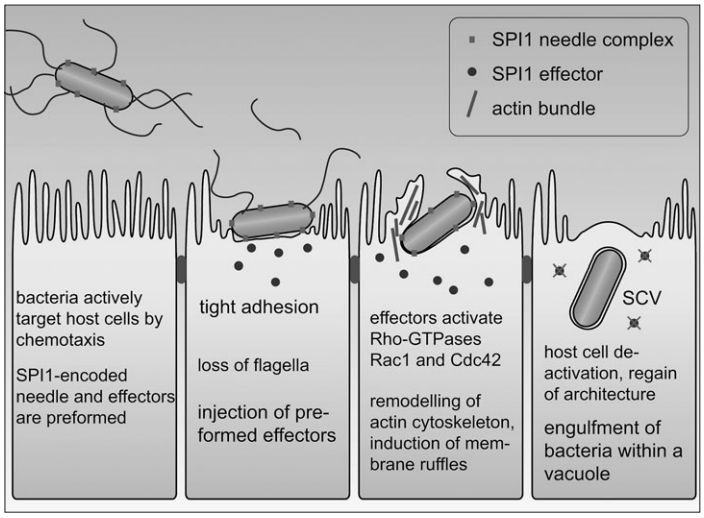

FIGURE 1: *SPI1-mediated invasion of Salmonella into non-phagocytic cells. Upon contact to eukaryotic host cells, the SPI1-encoded T3SS inject a cocktail of different effectors into the host-cell cytoplasm. Effector-mediated activation of small Rho-GTPases leads to massive rearrangements of the cytoskeleton with subsequent bacterial uptake. After uptake of the bacteria by a process termed macropinocytosis, Salmonella remains in a vacuole and the host cells regains a normal architecture. Further details are given in the text.*

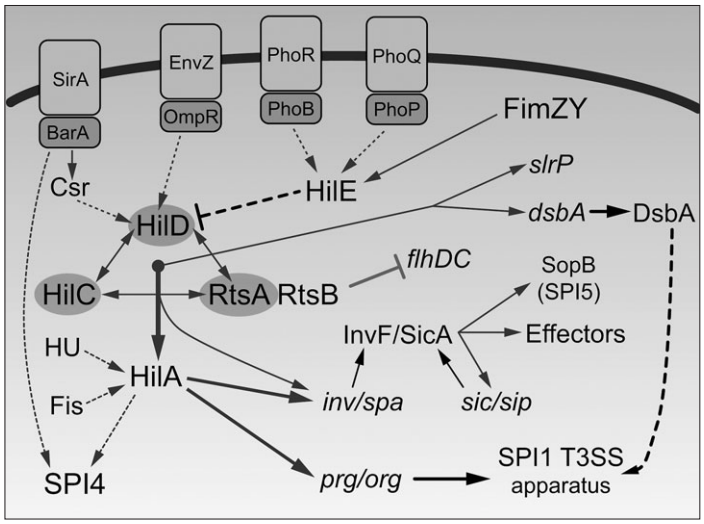

FIGURE 2: *The SPI1 regulon. The key regulatory factor of SPI1 expression is HilA. HilA-activity is mainly regulated on the transcriptional level and depends on a complex network of transcription factors and two-component regulatory systems. Arrows indicate activation of gene expression. Factors which promote their own expression (positive feedback loop) are highlighted by bold arrows. Repression is noted as lines with blunt ends. Solid lines represent direct transcriptional regulation. Thin dashed lines represent regulation that is not known to be direct or indirect and bold dashed lines represent post-translational effects. See text for details (adapted from Ellermeier et al., 2005).*

The SPI1 regulon

The central transcription factor driving SPI1-expression is the SPI1-encoded HilA, a member of the OmpR/ToxR family of transcriptional regulators (Ahmer et al., 1999). An overview of the complex regulatory mechanisms involved in SPI1 expression is shown in Fig. 2. Expression of the SPI1 regulon is controlled in response to a specific combination of environmental signals that presumably act as cues for the appropriate anatomic location of the bacteria. Environmental signals known to regulate SPI1-expression are oxygen, osmolarity, pH, antimicrobial peptides and presumably several other unknown signals (Bader et al., 2003; Bajaj et al., 1996). These signals are sensed by a set of two-component regulatory systems: BarA/SirA (Ahmer et al., 1999), OmpR/EnvZ (Lucas and Lee, 2001), PhoBR (Lucas et al., 2000) and PhoPQ (Pegues et al., 1995). The phosphorylated cognate response regulators can promote the expression of either HilD or HilE, thereby stimulating or repressing SPI1-expression. PhoB and PhoP as well as FimZY (Baxter and Jones, 2005) can activate *hilE* expression. It was shown that HilE and HilD interact with each other in a bacterial two-hybrid screen and that HilE can negatively influence *hilA* expression (Baxter et al., 2003). HilD together with HilC and RtsA constitute a feed-forward loop, where each factor can promote the expression of itself, *rtsA, hilC* and *hilA*, thus integrating and greatly enhancing the signal (Ellermeier et al., 2005). In addition, the nucleoid proteins HU (Schechter et al., 2003) and Fis (Wilson et al., 2001) are required for *hilA* expression.

The transcription factor HilA activates the *prg/org* and *inv/spa* operons within SPI1 by binding to *cis*-elements present in the respective promoters (Lostroh and Lee, 2001). This activation leads to the production of InvF, a member of the AraC family of transcriptional regulators (Kaniga et al., 1994). InvF, together with the chaperone SicA induces expression of a set of genes encoded within SPI1 and on various loci elsewhere in the chromosome (e.g. *sopB* on SPI5, Darwin and Miller, 2000).

RtsA/HilD/HilC can activate the expression of *dsbA*, a periplasmic disulphide isomerase which is required for the function of the SPI1-encoded T3SS (Ellermeier and Slauch, 2004). The three regulatory proteins can also act independently of HilA in activating the *invF* operon (Ellermeier and Slauch, 2003). The genes *rtsA* and *rtsB* constitute one operon, where RtsB negatively regulates *flhDC* and hence the flagellar regulon (Ellermeier and Slauch, 2003).

Role of SPI1 in livestock species

A role of SPI1 in the intestinal colonization of pigs has been reported (6-week-old farm-reared Landrace/Large White cross male piglets, Boyen et al., 2006b). This study also indicated that the colonization of tonsils, another organ that can harbor *Salmonella* in infected pigs, could occur independent from the function of SPI1. A large scale signature-tagged mutagenesis (STM) screen performed with S. Typhimurium in chicken and calves revealed the involvement of SPI1 genes in colonization of the calf (twenty-eight-day-old Friesian bull calves) intestine, but indicated the lack of a role of SPI1 genes in the chicken model (1-day-old light Sussex chicks, Morgan et al., 2004). Similar observation has been reported before using host-adapted *S.* Gallinarum in the chicken model (three-week-old specific-pathogen-free Rhode Island Red chickens, Jones et al., 2001). In contrast, a previous study reported the attenuation of *S.* Typhimurium and *S.* enteritidis SPI1 mutant strains in a chicken infection model (1-day-old White Leghorn chicks, Porter and Curtiss, 1997). Shah et al. (2005) applied a STM screen to *S.* Gallinarum in a chicken infection model (1-day-old White Leghorn chicks) and identified attenuated mutants defective in SPI1 genes. As a reason for these obviously disparate results, the different susceptibility of chicken strains to *Salmonella* infection has to be considered.

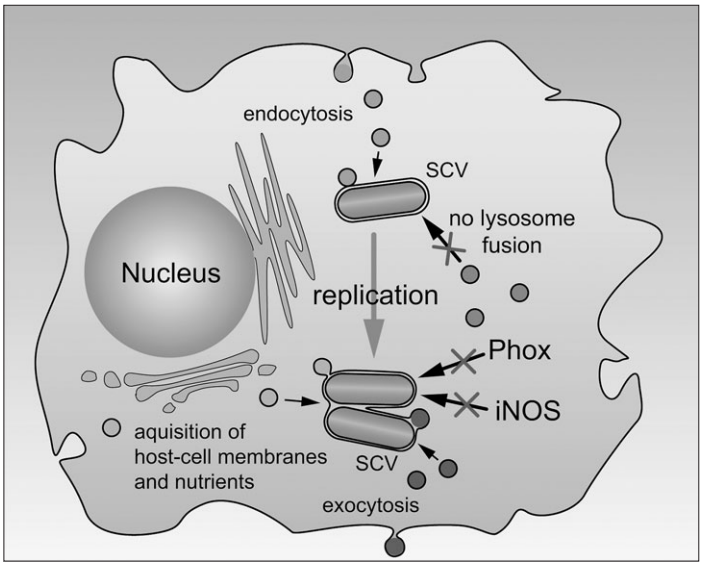

FIGURE 3: *SPI2 function is important for intracellular survival of S. Typhimurium. Intracellular Salmonella employ the SPI2-encoded T3SS to translocate effector molecules over the SCV-membrane into the host cell cytoplasm. Functions of SPI2 effectors ensure intracellular survival and replication mainly by influencing the vesicular trafficking. This includes the acquisition of nutrients and membranes as well the evasion of certain host responses directed against intracellular bacteria.*

SPI2

Genes within SPI2 were initially identified by STM screening of a mutant bank for clones with reduced capacity to survive and replicate in the murine model of systemic *Salmonella* infections (Hensel et al., 1995; Shea et al., 1996). Later, a portion of SPI2 was also identified as a *Salmonella*-specific chromosomal region (Ochman et al., 1996). A second T3SS is encoded by SPI2 which is expressed during intracellular life of *Salmonella*. SPI2-activity is required to establish and maintain the SCV as an intracellular niche in which *Salmonella* can survive and replicate (for a model, see Fig. 3). Most of the SPI2-phenotypes characterized so far are linked to the manipulation of host-cell vesicle trafficking, thus ensuring nutrient supply and evading bactericidal activities. It has been shown that *Salmonella* can prevent fusion of the SCV with vesicles containing phagocyte oxidase (Phox, Vazquez-Torres et al., 2000) as well inducible nitric oxide synthase (iNOS, Chakravortty et al., 2002). To date, 17 effectors are known to be translocated over the SCV membrane into the host-cell cytoplasm, most of them encoded outside the SPI2-locus (reviewed in Kuhle and

Hensel, 2004). Only 3 effectors are encoded within SPI2: SpiC, SseF and SseG. SpiC is an effector which was demonstrated to block fusion of the SCV with lysosomes (Uchiya et al., 1999). Recently, a SseFG-dependent redirection of exocytic transport processes could be shown (Kuhle et al., 2006). Furthermore, SseF plays an important role in maintaining a juxtanuclear position of the SCV in HeLa cells (Abrahams et al., 2006). In HeLa cells, tubular, membranous extensions from the SCV can be observed, termed '*Salmonella*-induced filaments' or SIF, (Garcia-del Portillo et al., 1993). This phenotype depends on SifA, an effector shown to be required to maintain the integrity of the phagosomal membrane of the SCV during intracellular proliferation (Stein et al., 1996). SifA mutants loose their SCV membrane in epithelial cells and macrophages, a process presumably driven by the action of SseJ (Ruiz-Albert et al., 2002). An enzymatic activity as an acyltransferase could be assigned to SseJ (Ohlson et al., 2005). The molecular mechanisms by which SPI2 effectors can manipulate intracellular trafficking are subjects of ongoing research. There is experimental evidence available showing the influence of effectors on motor proteins of the cytoskeleton (reviewed in Abrahams and Hensel, 2006). Furthermore, direct interactions with the actin cytoskeleton (Miao et al., 2003) as well as with the microtubule network (Kuhle et al., 2004) were demonstrated.

Like the SPI1, SPI2 harbors additional genes encoding proteins involved in metabolism (Hensel et al., 1999). Of importance for virulence functions could be the tetrathionate reductase system since it might be beneficial for *Salmonella* to colonize certain anaerobic habitats.

The SPI2 regulon

Similar to SPI1, the expression of SPI2 genes is controlled by a local, and modulated by global regulatory system. The SPI2-encoded SsrAB system is a typical two-component system and essential for the expression of the SPI2 regulon in intracellular bacteria as well as under *in vitro* conditions mimicking the intravacuolar environment of the SCV. Main global regulatory systems that affect the expression levels of SPI2 genes are the EnvZ/OmpR (Garmendia et al., 2003) and PhoPQ (Bijlsma and Groisman, 2005) two-component systems, SlyA (Navarre et al., 2005) and Fis (Lim et al., 2006) (see Fig. 4 for a model).

Role of SPI2 in other animal hosts

Although SPI2 has been identified in a murine model of typhoid fever, there is evidence that this virulence locus is also important for *Salmonella* virulence in infection models with other animals. Tsolis et al. (1999) reported that SPI2 was not required for diarrheal disease in calves (3 to 4 week-old milk-fed male Friesian-Holstein calves). However, a subsequent study (twenty-eight-day-old Friesian calves, Bispham et al., 2001) reported that SPI2 mutant strains of S. Dublin were attenuated in both the induction of diarrheal symptoms and systemic disease in a calf model of infection. Using *S.* Gallinarum (three-week-old specific-pathogen-free Rhode Island Red chickens, Jones et al., 2001) and *S.* Pullorum (1-day-old Brown egg layer chicks, Wigley et al., 2002) infection models, a role of SPI2 for the systemic pathogenesis of *Salmonella* in chicken was observed. Mutant strains in SPI2 genes were less able to proliferate in host organs. Data on the role of SPI2 for *Salmonella* pathogenesis in porcine infection models are limited. A recent STM screen with *S.* Choleraesuis in a porcine model (5- to 8-week-old white crossbred piglets) identified highly attenuated mutant strains in SPI2 genes encoding the T3SS (Ku et al., 2005). These mutant strains were successfully used as attenuated live vaccines.

SPI3

The SPI3 locus is inserted at the *selC* tRNA gene locus. The best characterized virulence factor in SPI3 is the *mgtCB* operon. This operon was shown to be important for intra-macrophage survival, virulence in mice and growth in low Mg^{2+} media (Blanc-Potard and Groisman, 1997). *mgtB* encodes for a high affinity magnesium uptake system whose expression might be an adaptation to the nutrient-poor environment of the SCV (Snavely et al., 1991). Whether MgtC is involved in magnesium uptake is still unclear (Blanc-Potard et al., 1999). Recently, MgtC was shown to activate Na^+-K^+-ATPases, thus it might be involved in regulating the membrane potential (Gunzel et al., 2006).

SPI3 also encodes MisL, a classical autotransported protein that is not homologous to family members of the trimeric autotransporter adhesins (Dorsey et al., 2005) and shows significant similarity to the autotransported AIDA-1 adhesin of enteropathogenic *E. coli* (EPEC). Proteins secreted by the autotransporter pathway (also referred to as type V secretion system, T5SS) are of modular composition: An N-terminal signal sequence targets the protein to the general secretion pathway at the inner membrane, the passenger domain harbors the specific effector function and the C-terminal translocation unit forms, once inserted into the outer membrane, a β-barrel secondary structure which allows secretion of the passenger domain. Autotransporters are synthesized as pre-pro-proteins, after cleavage of the signal-peptide the pro-protein is released into the periplasm. The

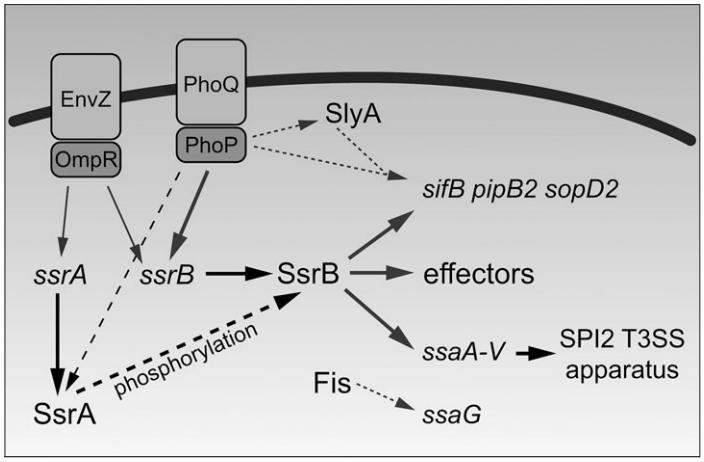

FIGURE 4: *Model of SPI2 regulation. The key regulatory factor of SPI2 expression is SsrB. SsrB is the response regulator of the SsrAB two-component signal transduction system. It is regulated on the transcriptional level by PhoP and OmpR. SsrB is phosphorylated by SsrA in response to yet unknown environmental signals. Solid line arrows indicate activation of gene expression and direct transcriptional regulation. Thin dashed lines represent regulation that is not known to be direct or indirect. Bold dashed lines represent post-translational effects.*

passenger domain is, depending on the particular autotransporter, cleaved off its translocation unit after passing the outer membrane, and in some T5SS, the passenger domain is released into the extracellular milieu.

MisL was shown to function as an adhesin (Dorsey et al., 2005). Furthermore, Dorsey and colleagues demonstrated that MisL is required for long-term intestinal persistence of *Salmonella* in mice, as shown before for the ShdA adhesin (Kingsley et al., 2002). In *S.* Typhimurium, another monomeric autotransporter, ShdA, was shown to have a function in adhesion and virulence. The surface-located protein, encoded on the CS54 PAI, is induced *in vivo* within the murine caecum and its expression might play a role in the long-term colonization of mice. GST-fusion proteins of the passenger domain of ShdA were shown to bind fibronectin *in vitro* (Kingsley et al., 2002). ShdA has no role in longterm fecal shedding of *Salmonella* by pigs (6-week-old landrace piglets, Boyen et al., 2006a)

SPI4

Salmonella Pathogenicity island 4 (SPI4) was first identified as a *S.* Typhimurium-specific genomic fragment by subtractive hybridization to genomic DNA of *E. coli* K-12 (Wong et al., 1998). The highly repetitive nature of SPI4 sequences led to a wrong annotation with 18 open reading frames (ORFs). Final sequencing of the genome of S. Typhimurium strain LT2 revealed that the locus only contains the six ORF STM4257 - 4262 (McClelland et al., 2001). Recently, SPI4 genes were renamed *siiA-F* for '*Salmonella* intestinal infection' due to their role in virulence (see below). Three of the six putative proteins encoded within SPI4 show a significant sequence similarity to the components of a type I secretion system.

Type one secretion systems (T1SS) or ABC (ATP-binding cassette) transporters are heterotrimeric complexes consisting of an inner membrane ABC exporter, a membrane fusion protein (MFP) and a pore-forming, outer membrane protein (OMP). T1SS allow the secretion of a wide range of substrates (proteinaceus and non-proteinaceus) from the cytoplasm into the extracellular space in a single step, without a stable periplasmic intermediate. Most protein substrates described so far possess a C-terminal signal sequence which is characterized by loosely conserved secondary structures (Stanley et al., 1991) and is not cleaved off during secretion. This implies that cotranslational secretion is not possible (reviewed in Delepelaire, 2004). The mechanism of secretion by T1SS was studied in great detail at the α-hemolysin (HlyA) secretion found in some uropathogenic *E. coli* (UPEC, Thanabalu et al., 1998).

One SPI4 gene, *siiE*, is of highly repetitive nature and encodes for a protein with a predicted mass of 600 kDa. The predicted protein SiiE shows a limited homology to the AdaH adhesin of *Burkholderia cenocepacia* (Urban et al., 2005). The first two genes *siiA* and *siiB* encode predicted proteins of 210 and 462 amino acids, the latter showing a weak similarity to methyl-accepting chemotaxis proteins of the MotA family.

SPI4 seems to be highly conserved among different *Salmonella* serovars (Edwards et al., 2002; Soto et al., 2006). Using Southern blot analyses we found the locus being absent only in some members of the *Salmonella* subspecies IIIa and IIIb (our unpublished observations).

SPI4 regulation

Although the molecular mechanism by which SPI4-encoded proteins contribute to *Salmonella* virulence has not been shown the regulation of these genes was examined in several unbiased approaches. A study of De Keersmaecker et al. (2005) suggested a role for SPI4 in intra-macrophage survival as shown for SPI2. Using gene-arrays, an upregulation of STM4260/4261/4262 (*siiDEF*) was observed after a shift of bacterial cultures to minimal media with a pH of 5. This study is contradictory to the data published by several other groups and could not be strengthened by functional tests.

A screen of random *lacZY* fusions revealed 5 fusions within *siiE* regulated in a SirA- and HilA-dependent manner (Ahmer et al., 1999). The authors suggested SirA as a global regulator of genes mediating enteropathogenesis (Ahmer et al., 1999). A similar approach of random *lacZY*-fusions was used to identify genes regulated depending on RtsA/B. A RtsA/B-dependent fusion within *siiE* (or *icgA* for invasion coregulated gene) was identified (Ellermeier and Slauch, 2003). The activity of this transcriptional fusion within the *siiE* gene was shown to depend on HilA but not on InvF. This supports a model where SPI4 genes are co-coordinately upregulated together with SPI1 genes during invasion. Gene-profiling of *Salmonella* growing within J774-A1 macrophages revealed significant down-regulation of SPI4-genes, as observed for SPI1 genes (Eriksson et al., 2003).

Role of SPI4 in animal models of Salmonella infection

A STM screen with *S.* Typhimurium performed in bovine and chicken models of salmonellosis revealed SPI4 as an important factor required for optimal colonization of cattle (twenty-eight-day-old Friesian bull calves, Morgan et al., 2004). These findings led to the renaming of ORFs STM4257 - 4262 to *siiA-F* (*Salmonella* intestinal infection). The molecular mechanism underlying the phenotype described by Morgan et al. (2004) remained unclear. Due to sequence similarities to the SPI9-encoded BapA protein, SiiE was tested in an in vitro system elucidating its role for *Salmonella* biofilm formation. Although it was suggested SiiE being a member of the growing Bap-family of repetitive proteins, there was no defect in biofilm formation found in a SiiE-deficient strain (Latasa et al., 2005). Our group has recently been able to demonstrate the function of SiiE as a non-fimbrial adhesin for the binding of *S.* Typhimurium to polarized epithelial cells and a contribution of the SPI4 to enteropathogenesis in murine colitis model (Gerlach et al., 2007). Interestingly, in the highly human-adapted *Salmonella* serovars sequenced so far, for example *S.* Typhi CT18 (Parkhill et al., 2001), a nonsense mutation in the *siiE* homolog resulted in two ORFs that may encode non-functional fragments of SiiE.

SPI5

The SPI5 locus was identified using *sopB*-specific probes to screen a cosmid library and subsequent sequencing (Wood et al., 1998). SopB itself was previously shown to be translocated into HeLa cells by *S.* Dublin in a SPI1-dependent manner (Galyov et al., 1997). *In vivo* studies revealed a significant attenuation of SPI5-deficient *Salmonella* in enteropathogenicity in a cattle infection model. In contrast, if assessed in a mouse model of sys-

temic infection, SPI5 mutants showed only a minor virulence defect (Wood et al., 1998). Another effector encoded on this PAI, PipB, is translocated depending on the SPI2-encoded T3SS and localizes to SIF within HeLa cells. PipB was neither needed for intracellular survival nor for systemic virulence in mice (Knodler et al., 2002). The mosaic structure of SPI5 is an example for regulatory and functional crosstalk between pathogenicity islands.

SPI6

The SPI6 locus has also been termed '*Salmonella* centisome 7 genomic island' or SCI by Folkesson et al. (2002). In S. Typhimurium, SPI6 is 47 kb in size and contains the *saf* gene cluster encoding a fimbrial adhesin. A microarray analysis indicated the conservation of SPI6 among serovars of *S. enterica* subspecies I serovars.

It is worthwhile to mention that a very large redundancy of fimbrial and non-fimbrial adhesins is found in *S. enterica*. The complete genome sequence of *S.* Typhimurium revealed the presence of 13 operons with homology to fimbrial gene sequences. There is evidence for the expression of at least 11 of them in vivo as shown by sero-conversion of *Salmonella*-resistant CBA mice (Humphries et al., 2005). The presence of a whole set of putative fimbriae together with phase variation demonstrated for some of the fimbrial operons (*fim, lpf* and *pef*), might be an adaptation which enables *Salmonella* to colonize a broad range of hosts and to evade immune responses. In addition to these fimbrial adhesins, at least 4 non-fimbrial adhesins are present, all encoded by SPI (SPI3, SPI4, SPI9 and CS54).

SPI7 and SPI8

The SPI7 and SPI8 loci are restricted to *S.* Typhi and a few other serovars. SPI7 is also referred to as 'major pathogenicity island' and represents the largest SPI with a size of 134 kb. The best characterized virulence factor is the Vi antigen biosynthesis gene cluster. The Vi antigen is the constituent of the exopolysaccharide capsule of serovars Typhi, Paratyphi C and Dublin. The locus is instable and loss of the capsule can be observed among *S.* Typhi isolates. SPI7 also encodes a type IV fimbrial adhesin. SPI8 is a small PAI of 6.8 kb and appears to be specific for *S.* Typhi. The function of SPI8 genes has not been reported so far.

SPI9

Like SPI4, the SPI9 locus harbors ORFs predicted to encode a large repetitive protein (STM 2689) and the components of a T1SS (STM2690-2692). A recent study showed a function of the pathogenicity island for biofilm formation (Latasa et al., 2005). Latasa and co-workers renamed the ORFs to *bapA-D* (biofilm-associated protein) because of sequence homologies of BapA to members of the BAP-family of repetitive proteins. Bap was recently described in *Staphylococcus aureus* as a cell wall-associated protein able to strongly promote biofilm-formation (Cucarella et al., 2001). The published genome sequence of *S.* Typhimurium LT2 shows a frame shift in the STM2689 sequence which was not present in the strains sequenced by Latasa et al., leading to a putative protein of 386 kDa. Deletion of BapA caused a loss of capacity to form a biofilm and overexpression reinforced biofilm pellicle formation by *Salmonella* (Latasa et al., 2005).

SPI10

SPI10 has been defined as an insertion at the tRNA *leuX* gene. This locus appears to be hypervariable and a point of insertion for various different DNA fragments (Bishop et al., 2005). In *S.* Enteritidis the *sef* and *pef* gene clusters encoding fimbrial adhesins were detected. In *S.* Typhi and *S.* Paratyphi A this locus contains a bacteriophage genome and *sef* and *pef* gene clusters with various pseudogenes. In contrast, *S.* Typhimurium has an insertion of an element with entirely different gene content. These observations and the comparison to related bacterial species lead to the suggestion that the *leuX* locus represents a hot spot for the insertion of various mobile genetic elements (Bishop et al., 2005).

SPI11 and SPI12

The genome sequence of *S. enterica* serovar Choleraesuis revealed the presence of two new putative SPI, termed SPI11 and SPI12 as well as two genomic islands conferring metabolic functions (Chiu et al., 2005). Both SPI show characteristics of PAI such as association with bacteriophage genomes and tRNA genes and a low G+C content of 41.32 % was observed for SPI11. The role of SPI11 and SPI12 in *Salmonella* virulence has not been demonstrated experimentally and the putative virulence genes in these loci await further characterization.

SPI13 and SPI14

A STM screen was performed to identify virulence genes in the avian-adapted *S.* Gallinarum, the causative agent of fowl typhoid (Shah et al., 2005). Attenuated mutant strains were identified in an infection model in 1-day-old White Leghorn chicks. This screen identified many previously known virulence genes in SPI1, SPI2 and SPI10. Interestingly, two new large loci were identified that have characteristic features of PAI. These loci were termed SPI13 and SPI14. SPI13 is adjacent to the tRNA *pheV* gene and comprises 18 ORFs. SPI14 consists of 6 ORFs, is not associated with a tRNA gene, but shows a G+C content of 41 % in contrast to the about 52 % for the *Salmonella* core genome. Both SPI13 and SPI14 are absent in *S.* Typhi and *S.* Paratyphi A but present in *S.* Typhimurium and *S.* Enteritidis, indicating a possible role of the loci in host specificity. The role of genes in SPI13 and SPI14 for the fowl typhoid has not been analyzed in detail and awaits further molecular characterization.

SPI15, SPI16, and SPI17

Using a bioinformatics approach, Vernikos and Parkhill (2006) identified three additional loci in the genome

sequence of *S.* Typhi. The regions were termed SPI15, SPI16 and SPI17 and all show association with tRNA genes. SPI16 and SPI17 harbor genes that are involved in LPS modification. SPI15 is only found in *S.* Typhi isolate CT18, while SPI16 and SPI17 are present in *S.* Typhi and most other *S. enterica* genome sequences.

SGI1

The emergence of antibiotic resistant strains is a common phenomenon of pathogenic bacteria and is also observed in *S. enterica*. Many antibiotic resistant *Salmonella* isolates harbor resistance plasmids of variable size and composition of resistance genes. However, the molecular investigation of the epidemic strain S. Typhimurium DT104 indicated that a multidrug resistance phenotype is conferred by a PAI termed '*Salmonella* genomic island 1' or SGI1 (for recent review, see Mulvey et al., 2006). SGI1 was first identified in the multidrug-resistant epidemic strain *S.* Typhimurium DT104 but is also present in other strains. The SGI1 confers resistance to the antibiotics ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline but variant SGI1 with other compositions of resistance genes have been identified. The SGI1 locus is flanked by direct repeats and contains genes encoding integrase and excisionase, suggesting the instability and mobility of this SPI. The excision and integration of SGI1 has been demonstrated experimentally (Doublet et al., 2005). There are controversial observations on the presence of virulence factors in addition to the resistance factors on SGI1 (Mulvey et al., 2006).

Further putative SPI and genomic islands

Based on the common features of SPI, i.e. association with tRNA genes and different base composition, a systematic screen of tRNA loci for *Salmonella*-specific insertions was performed that indicated the presence of additional putative PAI or genomic islands (Hansen-Wester and Hensel, 2002). There is also a large number of smaller *Salmonella*-specific loci that only contain one or few virulence genes. These loci were termed 'Pathogenicity Islets' and it is likely that an acquisition by horizontal gene transfer occurred similar to those of the larger PAI.

Conclusions and Outlook

PAI are a common phenomenon in pathogenic bacteria and many important virulence determinants are encoded by PAI. The characterization of individual virulence genes and genome sequences revealed a remarkably large number of PAI in *S. enterica* serovars. The availability of several genome sequences of *S. enterica* serovars with broad or narrow host ranges allowed the characterization of the content of SPI and may allow first clues how the provision with SPI might correlate with host adaptation. However, in addition to the presence and composition of the SPI loci it will be important to investigate the functionality of the SPI-encoded virulence factors. There are several indications that SPI are present in host-adapted *Salmonella* serovars but harbor pseudogenes and thus will not confer the specific virulence function associated with the SPI.

For a few SPI, such as SPI1 and SPI2, a detailed understanding of the molecular functions and roles in virulence is available. However, even for these intensively studied SPI remarkable differences in the functions in pathogenesis in various animal models were observed. For most of the SPI, the understanding of the molecular function is still elusive. For SPI unique to *S.* Typhi and other serovars highly restricted to human, suitable infection models are a limitation. Other SPI are likely to have host species-related functions and it will require future careful investigation in non-murine animal models to understand their role in pathogenesis.

We expect that the further molecular analysis of SPI will allow the improvement of prevention and treatment of *Salmonella* infection, most importantly by the identification of novel mutant strains as live vaccines for use in human and veterinary medicine. In addition, such studies should unravel the evolutionary mechanisms that lead to development of variants of a bacterial species that have different host specificities and cause different degrees of disease severity. These investigations should have impact on the overall understanding of bacterial pathogenesis.

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