Introduction

The genus *Salmonella* is named after the American veterinarian Daniel E. Salmon, who first isolated *Bacterium cholerae-suis* (*Salmonella enterica* serotype Choleraesuis abbreviated to *S. enterica* serotype Choleraesuis or *S. Choleraesuis*) from a pig (Salmon and Smith, 1885). The members of this genus are gram-negative facultative-anaerobic and peritrichously flagellated rods. *Salmonella* serotypes are distinguished from members of other genera of the *Enterobacteriaceae* by a combination of biochemical reactions, including the production of hydrogen sulfide, the use of tetrathionate as a terminal electron acceptor and others. The genus *Salmonella* contains 2579 serotypes, which can be distinguished serologically based on antigenic properties of their lipopolysaccharide (LPS) sugar repeat units (O-antigens) and their structural protein subunits of flagella (H-antigens) (Grimont and Weill, 2007). Members of the genus *Salmonella* can be divided into two species, termed *Salmonella enterica* (Le Minor and Popoff, 1987), which contains 2557 serotypes, and *Salmonella bongori* (Reeves et al., 1989), which comprises the remaining 22 serotypes. All *Salmonella* serotypes are considered potential pathogens of humans and/or animals (Kelterborn, 1967).

In a first approximation, diseases caused by *Salmonella* serotypes can be divided into two broad categories, those that remain localized to the intestinal tract (self-limiting gastroenteritis) and those that are associated with systemic dissemination of the bacteria. Localized and systemic infections are associated with different *Salmonella* serotypes. One goal of this overview is to illustrate that in immunocompetent hosts, these two different syndromes are a result of distinct virulence strategies being employed by the respective pathogens. Research on *Salmonella* pathogenesis suggests that these two distinct virulence strategies reflect differences in how the respective pathogens ensure their transmission to a new susceptible host. After a brief overview of the two major disease manifestations associated with *Salmonella* serotypes, this chapter will discuss the virulence factors responsible for host–pathogen interactions observed in both settings.

Clinical Manifestations

Gastroenteritis

The vast majority of *Salmonella* serotypes are capable of causing gastroenteritis in humans,
an infection that remains localized to the terminal ileum, colon and mesenteric lymph nodes (Zhang et al., 2003). Bacterial invasion of these tissues leads to the development of inflammatory infiltrates that are dominated by neutrophils (McGovern and Slavutin, 1979; Murphy and Gorbach, 1982). Neutrophil influx in the terminal ileum and colon is accompanied by necrosis of the upper mucosa and the presence of neutrophils in the faeces (Harris et al., 1972). The host inflammatory response is largely responsible for the characteristic symptoms produced during infection, which include diarrhoea, nausea, vomiting, intestinal cramping and fever. Importantly, host responses are elicited rapidly after ingestion of the organism, as indicated by an average incubation period of less than 1 day (Glynn and Palmer, 1992).

Localized infections that are similar to human gastroenteritis are caused by Salmonella serotypes in other large animal species, e.g. cattle and horses (Baker, 1970; Reynolds et al., 1986). Salmonella serotypes associated with gastroenteritis transmit through the faecal–oral route, either directly or via contaminated food or water. This mode of transmission ensures circulation of these pathogens within their respective animal reservoirs, from where they can become introduced into the human food supply. While a large number of Salmonella serotypes can cause gastroenteritis, a select few serotypes account for the majority of cases in any given animal reservoir. In the case of humans, S. enterica serotypes Typhimurium and Enteritidis (S. Typhimurium and S. Enteritidis) are associated most frequently with this diarrheal disease (Rabsch et al., 2001).

**Systemic disease**

*Typhoid fever*

A small number of Salmonella serotypes represent specialists that are associated with systemic infections in immunocompetent hosts. Perhaps the most infamous of these diseases is typhoid fever, which, in the absence of treatment, leads to a fatality rate of approximately 10%. Typhoid fever is caused by the strictly human-adapted S. enterica serotype Typhi (Raffatellu et al., 2008b). After ingestion, the organism invades the mucosa of the small intestine, followed by its dissemination throughout the body. However, the initial spread of the pathogen does not evoke overt host responses, as indicated by the fact that typhoid fever has an average incubation period of 2 weeks (Olsen et al., 2003).

Pathological changes in the intestine are characterized by a slow development of inflammatory infiltrates that are dominated by mononuclear cells (macrophages and dendritic cells) while neutrophils are scarce (Sprinz et al., 1966; Mukawi, 1978; Kraus et al., 1999; Nguyen et al., 2004). The organism spreads to internal organs, most frequently the bone marrow, the liver and the spleen where it is found in histiocytic granulomas, known as typhoid nodules (Nasrallah and Nassar, 1978). Spread to the gall bladder or urinary bladder can lead to chronic carriage, which is important for human-to-human spread of the disease. Symptoms of typhoid fever are non-specific, commonly including fever and a slowed heart rate (bradycardia). Splenomegaly, hepatomegaly, or rose spots are encountered less frequently (Nasrallah and Nassar, 1978). Unlike gastroenteritis, typhoid fever is not considered a diarrhoeal disease, because this symptom develops late, after the onset of fever, in only a fraction (approximately one-third) of typhoid fever patients, while the remaining individuals remain either diarrhoea free or become constipated.

*Salmonella* Paratyphi A, and less frequently S. Paratyphi B and Paratyphi C, are associated with paratyphoid fever, a disease that is milder in its course but otherwise indistinguishable from typhoid fever. Together with S. Typhi, these pathogens are commonly referred to as typhoidal Salmonella serotypes.

*Bacteraemia in humans*

IMMUNOCOMPETENT INDIVIDUALS. Two non-typhoidal Salmonella serotypes can cause a systemic infection in immunocompetent humans that can be distinguished clinically from typhoid fever and has been termed
bacteraemia (Saphra and Wassermann, 1954). These pathogens are S. Choleraesuis and S. Dublin (Saphra and Wassermann, 1954; Fang and Fierer, 1991). Bacteraemia in immunocompetent individuals is not a diarrhoeal disease and may manifest in the absence of enteric pathology. Similar to typhoid fever, only about one-third of bacteraemia patients develop diarrhoea (Saphra and Wassermann, 1954; Cherubin et al., 1969; Fang and Fierer, 1991).

**IMMUNOCOMPROMISED INDIVIDUALS.** In immunocompromised humans, bacteraemia is a common complication of infections with non-typhoidal Salmonella serotypes that are normally associated with gastroenteritis in immunocompetent individuals (Gordon, 2008). The organisms most frequently associated with bacteraemia in immunocompromised individuals are S. Typhimurium and Enteritidis. Bacteraemia in immunocompromised individuals commonly develops in the absence of symptoms of gastroenteritis (Green and Cheesbrough, 1993; Brown and Eykyn, 2000). Knowledge about the specific immune defects that render individuals susceptible to bacteraemia with non-typhoidal Salmonella serotypes is instructive, as this information points to mucosal barrier functions that successfully prevent bacterial dissemination during gastroenteritis. Furthermore, Salmonella serotypes, such as S. Typhi, which are associated with systemic disease in immunocompetent hosts, are expected to possess specific virulence factors that enable them to overcome the mucosal barrier functions, which prevent bacterial dissemination during gastroenteritis.

Immune defects that increase the risk of developing bacteraemia include an advanced infection with human immunodeficiency virus (HIV) (Gordon et al., 2001, 2002), severe paediatric malaria (Graham et al., 2001; Walsh et al., 2000), anaemia (Okuonghae et al., 1993), neutropenia (Noriega et al., 1994; Tumbarello et al., 1995), interleukin (IL)-12/IL-23 deficiency (MacLennan et al., 2004) and chronic granulomatous disease (Winkelstein et al., 2000). Severe anaemia and advanced HIV infections are associated with a reduced microbiocidal activity of neutrophils (Supan et al., 1990; Coffey et al., 1998; George et al., 1998), while chronic granulomatous disease decreases the microbiocidal activity of phagocytes in general (Tauber et al., 1983). Mutations in the shared p40 subunit of IL-12 and IL-23 increase the susceptibility to bacteraemia by an interferon (IFN)-γ-independent mechanism (MacLennan et al., 2004), which could be related to the role of IL-23 in recruiting neutrophils to the intestinal mucosa during S. Typhimurium infection (Godinez et al., 2009). Reduced neutrophil recruitment due to depletion in the intestinal mucosa of CD4+ cells, specifically Th17 cells, has also been postulated to increase the risk of bacteraemia during advanced HIV disease (Raffatellu et al., 2008a). Collectively, these clinical data point to an important role of neutrophils in preventing bacterial dissemination beyond the mesenteric lymph node during gastroenteritis.

**Systemic infections in other host species**

A small number of Salmonella serotypes are associated with systemic infections in immunocompetent animals. As is the case for the human-restricted serotype Typhi, these pathogens are considered host adapted and generally have a host range that is narrow compared to that of serotypes associated with gastroenteritis. For example, isolates of S. Gallinarum can be further subdivided into the biotypes Gallinarum and Pullorum, which cause systemic infections in poultry known as fowl typhoid and pullorum disease, respectively (Shivaprasad, 2000). Salmonella Choleraesuis and S. Dublin cause systemic disease in pigs and cattle, respectively (Turk et al., 1992; Visser et al., 1992). Systemic dissemination of S. Abortusequi and S. Abortusovis in horses and sheep, respectively (Perrin et al., 1950; Jack, 1968). Finally, a subset of S. Typhimurium strains can be distinguished by phage typing from strains associated with gastroenteritis in humans and are associated with systemic disease in pigeons (Rabsch et al., 2002).

While the genetic basis for adaptation to different host species is poorly understood,
the virulence strategies used by host-adapted *Salmonella* serotypes to spread systemically are likely to be variations of a common theme that involves an ability to overcome mucosal barrier functions and disseminate throughout the body. These virulence attributes are absent from *Salmonella* serotypes associated with gastroenteritis.

**Virulence Factors Associated with Gastroenteritis**

**Invasion**

*The invasion-associated type III secretion system (T3SS-1)*  

Although gastroenteritis differs in important aspects from the systemic infections discussed above, *Salmonella* serotypes associated with either of these two different disease manifestations share a number of virulence strategies. Most importantly, all *Salmonella* serotypes enter the intestinal mucosa by actively invading the epithelial lining. This event occurs very early during host–pathogen interaction and is observed within 10 to 15 min after infection of ligated ileal loops (Frost *et al.*, 1997; Santos *et al.*, 2002). The use of tissue culture models to study invasion of epithelial cells led to the identified two virulence factors critical for this process, flagella-mediated motility (Liu *et al.*, 1988) and the type III secretion system (T3SS-1) (Galán and Curtiss III, 1989) encoded by genes located on *Salmonella* pathogenicity island 1 (SPI1) (Mills *et al.*, 1995) (Fig. 5.1).

T3SS-1-mediated invasion of polarized epithelial cells requires the function of the non-fimbrial adhesin SiiE, which is encoded by SPI4 (Gerlach *et al.*, 2008). Expression of the T3SS-1 apparatus and the SiiE adhesin is coordinately regulated through HilA, the master regulator of invasion genes encoded on SPI1 (Lee *et al.*, 1992; Ahmer *et al.*, 1999; De Keersmaecker *et al.*, 2005; Gerlach *et al.*, 2007; Thijs *et al.*, 2007; Main-Hester *et al.*, 2008). The main function of the SPI1-encoded T3SS-1 apparatus is to translocate proteins, termed effectors, into the host cell cytosol. Up to 15 effector proteins are known to be translocated by the T3SS-1 into host cells (Ibarra and Steele-Mortimer, 2009). These effector proteins are encoded by genes located either within SPI1 (e.g. *sipA*, *sipB*, *sipC*, *sipD* and *sptP*), on SPI5 (e.g. *sopB* (*sigD*)), on pathogenicity islets (e.g. *slrP*) or on bacteriophages (e.g. *sopE*).

A subset of these effector proteins, including *SipA*, *SipC* (*SspC*), *SopA*, *SopB* (*SigD*), *SopD*, *SopE* and *SopE2* cooperate to induce actin rearrangements that promote bacterial entry into epithelial cells (Hardt *et al.*, 1998a; Hong and Miller, 1998; Hayward and Koronakis, 1999; Zhou *et al.*, 1999; Ahmer *et al.*, 1999; Friebel *et al.*, 2001; Jepson *et al.*, 2001; McGhie *et al.*, 2001; Raffatellu *et al.*, 2005b). In vivo, invasion of the intestinal epithelium is accompanied by a rapid induction of inflammatory responses (Santos *et al.*, 2009). Inactivation of the T3SS-1 ameliorates or abrogates pathological changes at early time points after *S. Typhimurium* infection of calves (Watson *et al.*, 1998; Tsolis *et al.*, 1999a; Zhang *et al.*, 2002a, b) or streptomycin pre-treated mice (Hapfelmeier *et al.*, 2004). Only effector proteins contributing to epithelial invasion *in vitro* have been shown to contribute to the elicitation of inflammatory responses *in vivo* (Zhang *et al.*, 2002a, b; Hapfelmeier *et al.*, 2004).

Many of the genes involved in T3SS-1-mediated invasion are highly conserved among the genus *Salmonella* but are absent from the genomes of close relatives, such as *Escherichia coli*. This phylogenetic distribution is observed for SPI1 (Li *et al.*, 1995), SPI4 (Vernikos *et al.*, 2007), SPI5 (Mirolid *et al.*, 2001), *sopE2* and *sopD* (Mirolid *et al.*, 2001). The phylogenetic distribution of these genes explains why the ability to invade epithelial cells is a shared virulence strategy among *Salmonella* serotypes that distinguishes these pathogens from the closely related *E. coli* (Bäumler, 1997). Some T3SS-1 effector proteins are not conserved among *Salmonella* serotypes and may have accessory functions. For example, the *sopE* gene is encoded by a lysogenic bacteriophage (Hardt *et al.*, 1998b) that is only present in a small group of *S. Typhimurium* strains (Mirolid *et al.*, 1999). Phage-mediated horizontal transfer of the
**Fig. 5.1.** Schematic representation of host–pathogen interactions that unfold during gastroenteritis. For explanation see text.

sopE gene may enable the pathogen to fine-tune the level of T3SS-1-dependent intestinal inflammation it elicits (Zhang et al., 2002a). As discussed below, intestinal inflammation promotes transmission of *Salmonella* serotypes during gastroenteritis (Lawley et al., 2008). By fine-tuning the level of intestinal inflammation, phage-mediated horizontal transfer of effector genes might optimize transmission, thereby giving rise to epidemic phage types. Consistent with this idea, phage-mediated horizontal transfer of the sopE gene gave rise
to S. Typhimurium strains that were associated with epidemics in cattle (Mirold et al., 1999).

**Motility**

A majority of studies has focused on eliciting the mechanisms by which the T3SS-1 contributes to bacterial invasion. However, a second factor, flagella, is of considerable importance during this process. The contribution of flagella to invasion might be largely indirect, by increasing bacterial contact with host cells. None the less, flagella-mediated motility contributes markedly to the efficiency of epithelial invasion by *Salmonella* serotypes in vitro (Khoramian-Falsafi et al., 1990; Jones et al., 1992; Van Asten et al., 2000; Winter et al., 2009a, b). Inactivation of flagella biosynthesis genes markedly reduces intestinal inflammation at early time points after *S. Typhimurium* infection of calves (Schmitt et al., 2001; Winter et al., 2009a) or in a murine colitis model (Stecher et al., 2004). While the structural protein subunits of flagella (flagellin) can serve as a pathogen-associated molecular pattern (PAMP) that activates innate pathways of inflammation, motility per se contributes significantly to the ability of flagellated *S. Typhimurium* to trigger inflammation in vivo (Winter et al., 2009a).

Expression of flagella is controlled in a hierarchical fashion by the master regulator FlhDC (Chiodini and Sundberg, 1981; Macnab, 2004). FlhDC activates transcription of genes encoding the alternative sigma factor FliA, the anti-sigma factor FlgM, the regulator FliZ, as well as genes required for hook-basal body formation. Secretion of FlgM through the completed hook-basal body frees FliA to induce expression of flic, encoding phase 1 flagellin (H1-antigen) (Hughes et al., 1993). FliZ controls expression of the T3SS-1 by activating hilD, a gene encoding a positive regulator of hilA expression in *S. Typhimurium* (Lucas et al., 2000; Ellermeier et al., 2005; Kage et al., 2008). Thus FlhDC regulation ensures that two virulence factors critical for invasion of the epithelial lining, flagella and T3SS-1, are coordinately expressed during infection.

**Survival in tissue**

After penetrating the epithelial lining, *Salmonella* serotypes become exposed to host defences encountered in the interstitial fluid, including complement. The complement system can be activated through various initiating events. However, all pathways rely on the fact that complement component 3 (C3) senses conserved microbial structures, such as the LPS of Gram-negative bacteria, and thus may serve as a pattern recognition event (Gasque, 2004; Winter et al., 2010a).

This results in the deposition of a C3 cleavage product (C3b) on the bacterial surface, a process known as C3 fixation. The mechanism of C3 fixation involves a reactive thioester group in C3b, which forms esters with free hydroxyl groups of LPS sugar moieties (Sahu et al., 1994). C3 fixation has at least two consequences important for *Salmonella* infection.

One consequence of C3 fixation is the initiation of complement-mediated killing by triggering the formation of a membrane attack complex composed of the proteins C5b, C6, C7, C8 and C9 (Müller-Eberhard, 1986). *Salmonella* serotypes associated with gastroenteritis can withstand this killing mechanism, a property known as serum resistance. A key virulence factor in mediating serum resistance is the synthesis of long O-antigen chains in LPS, which is controlled by the *wzz*ST and *wzz*FEP genes (Bravo et al., 2008; Holzer et al., 2009) (Fig. 5.1). Long O-antigen chains reduce neither C3 fixation nor consumption of C5b, C6, C7, C8 and C9 (Joiner et al., 1982a). Instead, long O-antigen chains confer serum resistance because the membrane attack complex forms at a great distance to the cell surface and fails to insert into the bacterial outer membrane (Joiner et al., 1982b). In addition, *S. Typhimurium* possesses some accessory components that can further reduce the likelihood that the membrane attack complex reaches its target, the outer membrane. One of these accessory factors is Rck (Heffernan et al., 1992a, b), an outer membrane protein encoded on the virulence plasmid, which binds complement.
regulatory protein factor H (fH), a protein preventing complement deposition on host cells (Ho et al., 2010). However, in smooth strains with long O-antigen chains, Rck does not appear necessary. Another accessory factor is the outer membrane protease PgtE, which can cleave C3b, C4b and C5 when deposited close to the outer membrane, but a contribution of PgtE to serum resistance is only observed in strains lacking long O-antigen chains (Ramu et al., 2007).

A second consequence of C3 fixation is the opsonization of bacteria, which promotes phagocytosis through complement receptor (CR) 3 (Fig. 5.1). Salmonella serotypes associated with gastroenteritis are serum resistant, but this virulence trait does not reduce C3 fixation (Joiner et al., 1982a), presumably because deposition of C3b in the outer regions of the O-antigen remains unaffected by serum resistance mechanisms. An efficient C3 fixation may be one of the reasons why Salmonella serotypes associated with gastroenteritis remain susceptible to neutrophils, which can readily phagocytose these pathogens through CR3 (Joiner et al., 1989; Troelstra et al., 1999). While phagocytosis by neutrophils results in killing, Salmonella serotypes can survive when they become internalized by macrophages through CR3-dependent phagocytosis (Santos and Bäumler, 2004).

Macrophage survival

Salmonella Typhimurium is efficiently taken up by phagocytes in the intestinal mucosa and is observed microscopically in mononuclear cells (dendritic cells or macrophages) and neutrophils (Frost et al., 1997; Santos et al., 2002). A major bactericidal mechanism of phagocytosis is the release of reactive oxygen intermediates (ROI) through the respiratory burst, followed later during infection by production of reactive nitrogen intermediates (RNI), a consequence of host cells producing high levels of inducible nitric oxide synthase (iNOS) (Vazquez-Torres and Fang, 2001). A certain level of intrinsic resistance to these killing mechanisms is required for survival in tissue. For example, the sodCl and sodCII genes of S. Typhimurium encode periplasmic superoxide dismutases, which scavenge superoxide radicals (O_2•⁻) and peroxynitrite (ONOO•). Both genes are required for full mouse virulence (De Groote et al., 1997; Fang et al., 1999). A mutation in metL, an enzyme involved in homocysteine biosynthesis, results in hypersusceptibility to nitric oxide (NO) and is required for mouse virulence (De Groote et al., 1996).

Macrophages are the preferred intracellular niche for persistence of Salmonella serotypes in tissue (Santos and Bäumler, 2004). A key virulence factor required for survival in macrophages is the type III secretion system encoded by SPI2 (T3SS-2) (Ochman et al., 1996). Genes on SPI2 that encode the T3SS-2 apparatus are present in all members of the species S. enterica, but are absent from S. bongori or E. coli (Ochman and Groisman, 1996; Hensel et al., 1997). T3SS-2-mediated survival in macrophages is thus a shared virulence strategy among S. enterica serotypes and is found in pathogens associated with gastroenteritis as well as in serotypes associated with invasive disease (Bäumler, 1997) (Fig. 5.1). T3SS-2-mediated macrophage survival enhances the severity of intestinal inflammation and pathology in bovine and murine models of gastroenteritis (Tsolis et al., 1999a; Coburn et al., 2005).

The T3SS-2 translocates at least 16 effector proteins into the host cell cytosol, including SpiC, SseF, SseG, SlrP, SspH1, SspH2, SifA, SifB, SseI, SseJ, PipB, PipB2, SseK1, SseK2, GogB and SopD2 (Abrahams and Hensel, 2006). Although the molecular functions are known for some of these effector proteins, it remains unclear in most cases how they contribute to T3SS-2-mediated macrophage survival. One purpose of the T3SS-2 seems to be altering the properties of the Salmonella-containing vacuole (SCV) by manipulating vesicular trafficking events (Uchiya et al., 1999; Beuzon et al., 2000; Vazquez-Torres et al., 2000). For example, the T3SS-2 has been implicated in inhibiting phagolysosomal fusion of the SCV (Uchiya et al., 1999), although this finding was later called into question (Drecktrah et al., 2007). The T3SS-2 enables S. Typhimurium to evade the respiratory burst in macrophages (Vazquez-Torres et al., 2000) by interfering with the assembly of the NADPH oxidase complex in the
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As a result, S. Typhimurium prevents the production of superoxide radicals ($O_2^-$) in the SCV. While this process protects the pathogen in the SCV from oxidative damage, it does not reduce the overall superoxide production by macrophages (Vazquez-Torres et al., 2000).

Some evidence suggests that the $spv$RABCD operon is also involved in the interaction of Salmonella serotypes with macrophages (Libby et al., 2000). The $spv$B gene encodes an ADP-ribosyl transferase (Otto et al., 2000), which is translocated into the host cell cytosol (Gotoh et al., 2003) where it ADP-ribosylates actin (Tezcan-Merdol et al., 2005). The $spv$ operon is required for full mouse virulence of S. Typhimurium (Gulig and Curtiss, 1987; Gulig et al., 1992). Furthermore, inactivation of the $spv$ operon reduces the number of bacteria recovered from the ileal mucosa of calves infected with S. Typhimurium (Tsolis et al., 1999b).

The $spv$ operon is located on virulence plasmids present in a small number of S. enterica subsp. enterica serotypes (Woodward et al., 1989; Gulig, 1990; Rotger and Casadesus, 1999) or on the chromosome of S. enterica subsp. arizonae serotypes (Libby et al., 2002). This limited phylogenetic distribution suggests that the $spv$ operon is not part of a virulence strategy shared by all Salmonella serotypes associated with gastroenteritis.

Interaction with neutrophils

As discussed above, the actions of SopCI, SodCII and T3SS-2 confer some level of resistance to killing by ROI and contribute to macrophage survival of S. Typhimurium (De Groote et al., 1997; Fang et al., 1999; Vazquez-Torres et al., 2000). However, patients with phagocyte oxidase deficiencies such as chronic granulomatous disease are defective in limiting systemic bacterial dissemination (Winkelstein et al., 2000), which suggests that ROI-dependent killing mechanisms still constitute an effective barrier against the spread of S. Typhimurium. This ROI-dependent barrier function is likely mediated by neutrophils. Neutrophil depletion markedly increases the susceptibility to systemic dissemination of S. Typhimurium in humans (Noriega et al., 1994; Tumarello et al., 1995; Fierer, 2001) and leads to the appearance of extracellular bacteria in tissue of mice (Conlan, 1996; Vassiloyanakopoulos et al., 1998; Cheminay et al., 2004). The extracellular stage observed in neutropenic mice might represent bacteria transiting from a deceased host cell to a new macrophage. Consistent with this idea, macrophage cell death, termed pyroptosis, is a known consequence of S. Typhimurium interaction with macrophages (Cookson and Brennan, 2001; Bergsbaken et al., 2009) and the resulting release of bacteria leads to efficient clearance by ROI-dependent killing mechanisms of neutrophils (Miao et al., 2010). These data suggest that the need to transit between host cells renders S. Typhimurium susceptible to neutrophil attack (Fig. 5.1).

C3 fixation results in efficient uptake of S. Typhimurium by neutrophils through CR3 (Joiner et al., 1989), a process that is associated with the generation of a respiratory burst (Ross, 1986). A greater magnitude of the respiratory burst in neutrophils compared to resting macrophages may help explain why neutrophils have the capacity to prevent bacterial dissemination to internal organs. Macrophage survival is likely an important strategy to, at least temporarily, shelter S. Typhimurium from neutrophil killing mechanisms (Vassiloyanakopoulos et al., 1998).

Intestinal colonization

Transmission

Gastroenteritis is a self-limiting infection with symptoms subsiding within less than 10 days. Invasion and colonization of intestinal tissue appears to be a dead end for Salmonella serotypes associated with gastroenteritis, because the pathogen is eventually cleared from intestinal tissues when adaptive immune responses develop (Mastroeni, 2002). However, the acute inflammatory response triggered by T3SS-1-mediated invasion and T3SS-2-mediated macrophage survival promotes the ability of Salmonella
serotypes to colonize the lumen and increases their relative abundance in intestinal contents (Stecher et al., 2007; Barman et al., 2008). This luminal outgrowth of Salmonella serotypes during gastroenteritis is important for their transmission by the faecal–oral route (Lawley et al., 2008). This luminal outgrowth of Salmonella serotypes during gastroenteritis is important for their transmission by the faecal–oral route (Lawley et al., 2008).

Inactivation of T3SS-1 and T3SS-2 renders S. Typhimurium unable to trigger acute intestinal inflammation, prevents its outgrowth of the microbiota (Stecher et al., 2007; Barman et al., 2008) and blocks transmission in a mouse model (Lawley et al., 2008). IL-10-deficient mice develop severe intestinal inflammation and support outgrowth of a T3SS-1/T3SS-2-deficient S. Typhimurium mutant (Stecher et al., 2007). Thus, outgrowth of Salmonella serotypes in the lumen is driven by intestinal inflammation and the actions of T3SS-1 and T3SS-2 are required for inducing inflammation, but not subsequently for enhancing growth of the pathogen in the inflamed gut.

The picture emerging from these studies is that T3SS-1 and T3SS-2, the main virulence factors important for gastroenteritis, generate a luminal environment that tips the balance in the competition between Salmonella serotypes and the microbiota in favour of the pathogen. Furthermore, the overall pathogenic strategy of Salmonella serotypes associated with gastroenteritis appears to be an induction and subsequent exploitation of the host inflammatory response to enhance their transmission to a new host (Stecher and Hardt, 2008; Santos et al., 2009; Winter et al., 2010a).

Resistance to antimicrobials

One of the changes in the luminal environment during inflammation is the epithelial release of antimicrobials, including defensins and lipocalin-2 (Raffatellu et al., 2007, 2009) (Fig. 5.1). Defensins are peptides exhibiting a membrane-targeted antimicrobial activity against a wide range of bacteria (Ouellette, 2006). PmrAB, a two-component regulatory system, controls expression of defensin resistance genes, which are involved in modifying LPS to reduce its negative charge by adding phosphoethanolamine or 4-amino-4-deoxy-L-arabinose moieties to phosphate groups in lipid A or the LPS core region (Gunn, 2008). Inactivation of pmrAB attenuates S. Typhimurium by the oral route of inoculation, but not when mice are infected by the peritoneal route (Gunn et al., 2000). These data suggest that defensin resistance is primarily a requirement for survival in the intestine.

Lipocalin-2 is an antimicrobial protein whose secretion into the intestinal lumen is induced during inflammation (Raffatellu et al., 2009). Lipocalin-2 prevents bacterial iron acquisition by binding enterobactin (Goetz et al., 2002; Flo et al., 2004; Berger et al., 2006), a siderophore produced by Salmonella serotypes and many other Enterobacteriaceae (Pollack and Neilands, 1970; Pollack et al., 1970). Salmonella enterica serotypes can synthesize a glycosylated derivative of enterobactin, termed salmochelin (Hantke et al., 2003). Salmochelin is not bound by lipocalin-2 and its production therefore confers lipocalin-2 resistance (Fischbach et al., 2006). The biosynthesis and uptake of salmochelin are encoded by the iroBCDEN gene cluster, which is present in all members of the species S. enterica, but absent from S. bongori and commensal E. coli isolates (Bäumler et al., 1996, 1997, 1998). The presence of the iroBCDEN genes in S. Typhimurium confers a growth advantage in the intestinal lumen during inflammation, when large amounts of lipocalin-2 are released into the gut (Raffatellu et al., 2009). However, the iroBCDEN genes confer no luminal growth advantage in the absence of intestinal inflammation or in lipocalin-2 deficient mice.

Outgrowth in the inflamed intestine

The above examples illustrate that Salmonella serotypes possess antimicrobial resistance mechanisms that contribute to intestinal colonization during gastroenteritis. A marked increase in the epithelial release of antimicrobials during inflammation might benefit Salmonella serotypes during their competition with commensal microbes that lack adequate resistance mechanisms. However, to a large part, the ability of Salmonella serotypes to outgrow the microbiota revolves around competition for nutrients.
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Gastroenteritis is a diarrhoeal disease during which intestinal contents are removed from the intestinal lumen by flushing (Santos et al., 2009). In this environment, microbes depend increasingly on nutrients present in the mucous layer for growth. Consistent with this idea, *S. Typhimurium* requires motility and chemotaxis towards mucous carbohydrates to increase its abundance in the intestinal lumen (Stecher et al., 2008). The pathogen can colonize this niche using fimbrial adhesins that bind carbohydrate moieties present in the mucous layer (Chessa et al., 2009). However, to outgrow the microbiota, the pathogen utilizes nutrients generated as a consequence of the host inflammatory response.

In the anaerobic environment of the gut, microbes rely on fermentation to produce energy for growth. Fermentation by the microbiota is accompanied by production of large quantities of hydrogen sulfide (H₂S), a highly cytotoxic compound. To protect itself from the toxic effects of hydrogen sulfide, host enterocytes produce enzymes that oxidize this compound to thiosulfate (S₂O₃²⁻) (Levitt et al., 1999; Furne et al., 2001).

During gastroenteritis, neutrophils transmigrate into the intestinal lumen in large numbers, giving rise to an abundance of faecal leukocytes, which characterizes inflammatory diarrhoea (Harris et al., 1972). The ROI produced during the respiratory burst of luminal neutrophils oxidize thiosulfate (S₂O₃²⁻) into tetrathionate (S₄O₆²⁻) (Winter et al., 2010b) (Fig. 5.1). The ttrBCA ttrRS gene cluster enables *Salmonella* serotypes to use tetrathionate as a terminal electron acceptor (Hensel et al., 1999). Through this mechanism, inflammation provides a respiratory electron acceptor in the gut, enabling the pathogen to outgrow the microbiota in the lumen, thereby enhancing its transmission to the next host by faecal shedding of the organism (Fig. 5.1).

**Virulence Factors Associated with Systemic Disease**

**Evasion of mucosal barrier functions and systemic dissemination**

*Invasion and entry into tissue*

Although *Salmonella* serotypes are associated with systemic infections in a number of animal reservoirs, typhoid fever is the disease that has been studied most extensively and will be discussed in greater detail throughout this section.

T3SS-1 and T3SS-2, two major virulence factors important for gastroenteritis, are also key players during the pathogenesis of systemic infections in immunocompetent hosts (Fig. 5.2). However, while infection with *S. Typhimurium* causes symptoms of gastroenteritis within less than 1 day after infection (Glynn and Palmer, 1992), invasion by *S. Typhi* does not elicit overt intestinal host responses during the first 2 weeks after ingestion (Olsen et al., 2003). Virulence factors that...
enable S. Typhi to prevent the generation of host responses early after infection are encoded by the viaB locus, a DNA region located on SPI7 (Parkhill et al., 2001). The viaB locus is absent from Salmonella serotypes associated with gastroenteritis and is only found in two other pathogens in the genus, S. Dublin and S. Paratyphi C, both of which are associated with systemic infections in immunocompetent hosts (Raffatellu et al., 2006).

The viaB locus encodes genes for the positive regulation (tviA), the biosynthesis (tviBCDE) and the export (vexABCDE) of the virulence (Vi) capsular polysaccharide (Virlogeux et al., 1995). TviA is thought to form hetero-dimers with the response regulator RcsB, thereby increasing its affinity for promoter regions (Virlogeux et al., 1996). The RcsCDB phosphorelay system represses flhDC (Cano et al., 2002), encoding a positive regulator of flagella and T3SS-1 in Salmonella serotypes (Frye et al., 2006). Expression of tviA is controlled by the two-component system EnvZ/OmpR, which responds to changes in osmolarity. As a result, tviA is repressed at an osmolarity encountered in the intestinal lumen (approximately 300 mM) but expression is induced under osmotic conditions encountered in tissue (150 mM). In the
absence of tviA (e.g. in S. Typhimurium), RcsB represses flhDC transcription in an osmolarity-independent fashion. By sensing the availability of TviA, RcsB incorporates a new regulatory signal, osmolarity, into the RcsCDB regulon of S. Typhi (Winter et al., 2009b).

The presence of a new regulatory protein, TviA, in S. Typhi has important consequences for host–pathogen interaction. Since TviA is not expressed under high osmolarity, S. Typhi remains invasive, motile and non-capsulated while it resides in the intestinal lumen. However, TviA expression is induced at tissue osmolarity, resulting in production of the Vi capsule and a rapid repression of T3SS-1 and flagella gene expression when the pathogen transits through the intestinal epithelium (Tran et al., 2010; Winter et al., 2010c). One of the functions of the intestinal epithelium is to serve as a sentinel for microbial translocation from the gut lumen by expressing Toll-like receptor (TLR) 5 on its basolateral surface (Gewirtz et al., 2001). TLR5 is a pattern recognition receptor of the innate immune system whose stimulation by its cognate PAMP, flagellin, initiates inflammatory responses leading to the recruitment of neutrophils. By repressing flagellin expression during the transition through the intestinal epithelium, TviA enables S. Typhi to evade this sentinel function and to increase its dissemination to internal organs (Winter et al., 2010c) (Fig. 5.2).

**Evasion of C3 fixation and opsonophagocytosis**

A second consequence of TviA-mediated gene regulation is that the Vi capsule is expressed during the transition of S. Typhi through the intestinal epithelium (Tran et al., 2010), which ensures that the pathogen is Vi-capsulated when it encounters complement. Vi-capsulated S. Typhi isolates deposit less C3b on their surface than non-capsulated isolates (Looney and Steigbigel, 1986), which can arise during laboratory passage by a spontaneous loss of SPI7 (Bueno et al., 2004; Nair et al., 2004). These data suggest that the Vi capsule is a virulence factor that prevents C3 fixation (Fig. 5.2). The Vi capsule is a homopolymer of \((1\rightarrow4)-2\text{-acetamido-3-O-acetyl-2-deoxy-\alpha-d-galacturonic acid}\) (Heyns and Kiessling, 1967), a structure that does not contain free hydroxyl groups available for complement deposition. The lack in the Vi polysaccharide of free hydroxyl groups may explain why expression of this surface structure inhibits C3 fixation. An inhibition of C3 fixation is a property that distinguishes S. Typhi from Salmonella serotypes associated with gastroenteritis and helps explain differences in the disease manifestations between localized and systemic infections.

Vi-capsulated S. Typhi isolates are internalized more efficiently by phagocytes than non-capsulated isolates (Looney and Steigbigel, 1986), presumably because inhibition of C3 fixation prevents phagocytosis through CR3. Furthermore, inhibition of CR3-mediated phagocytosis by Vi-capsulated S. Typhi isolates prevents the generation of a respiratory burst in neutrophils (Miller et al., 1972; Kossack et al., 1981; Looney and Steigbigel, 1986). These properties likely contribute to the ability of S. Typhi to overcome a neutrophil barrier and spread beyond the mesenteric lymph node. Salmonella Dublin, which causes an invasive infection in cattle and can express the Vi capsular antigen (Hashimoto and Khan, 1997), exits from the mesenteric lymph node into the efferent lymphatics extracellularly (Pullinger et al., 2007). This extracellular phase may render the pathogen susceptible to neutrophil attack and may therefore necessitate the use of antiphagocytic mechanisms, such as expression of the Vi capsular antigen, to ensure successful dissemination from the lymph node into the bloodstream. Such an extracellular phase during the initial dissemination of S. Typhi would explain why antibody responses elicited by vaccination with purified Vi capsular polysaccharide are protective in humans, because a defect in CR3-mediated clearance can now be compensated for by Fc receptor-mediated phagocytosis (Robbins and Robbins, 1984; Tacket et al., 1986; Klugman et al., 1987).

**Anti-inflammatory properties of the Vi capsule**

The Vi capsule attenuates inflammatory responses generated by TLR4, a pattern
recognition receptor, which recognizes the lipid A moiety of LPS (Hirose et al., 1997; Raffatellu et al., 2005a; Wilson et al., 2008) (Fig. 5.2). Furthermore, expression of the Vi capsule reduces intestinal inflammation and neutrophil recruitment in bovine and murine models of gastroenteritis (Raffatellu et al., 2007; Haneda et al., 2009). These anti-inflammatory properties of the Vi capsule might be a consequence of its ability to impair C3 fixation. An important role for complement in triggering LPS-induced inflammatory responses has surfaced in a number of previous studies. While TLR4 is necessary for triggering inflammatory responses to intraperitoneally administered LPS in mice, complement is crucial for fully inducing these responses in vivo (Li et al., 2005; Perlik et al., 2005; Zhang et al., 2007). Similarly, while LPS-induced gene expression in macrophages is TLR4-dependent, TLR4 is not sufficient and requires CR3 to facilitate signalling in vitro (Perera et al., 2001; Vogel et al., 2001; Noubir et al., 2004). During the interaction of S. Typhimurium with phagocytes, TLR4-dependent responses are attenuated when phagocytosis is blocked using anti-CR3 antibodies. In contrast, a TLR4-blocking peptide attenuates TLR4-dependent responses without reducing uptake of S. Typhimurium (van Bruggen et al., 2007). These data point to a sequential interaction of bacteria with CR3 and TLR4, which might explain why opsonophagocytosis is necessary for triggering TLR4-dependent responses.

In summary, evasion of C3 fixation by the Vi capsule helps to explain a number of clinical differences between typhoid fever and gastroenteritis (Raffatellu et al., 2006; Tsolis et al., 2008). First, the Vi capsule attenuates intestinal inflammatory responses triggered by bacterial invasion, which likely contributes to the long incubation period of typhoid fever and the scarcity of neutrophils in intestinal infiltrates. Second, the Vi capsule prevents phagocytosis and ROI-mediated killing by neutrophils, which likely promotes systemic dissemination during typhoid fever. However, the viaB locus is absent from a number of Salmonella serotypes associated with systemic diseases and the mechanisms by which these pathogens overcome mucosal barrier functions remain to be discovered.

Chronic carriage

The ability to disseminate throughout the host and take residence in internal organs provides access to new potential routes of transmission. The utilization of such new routes of transmission is a common trait among Salmonella serotypes associated with systemic infections and this property distinguishes these pathogens from Salmonella serotypes associated with gastroenteritis. For example, the most important route of transmission in fowl typhoid and pullorum disease is transovarian infection resulting in vertical spread via the egg and to the chick or poult (Shivaprasad, 2000). In cattle, long-term S. Dublin carrier animals contribute to transmission of the pathogen within infected herds by periodically shedding bacteria through faeces or milk (Nielsen et al., 2004).

The human-restricted serotype Typhi can establish chronic carriage in the gall bladder or urinary bladder of a fraction of patients and the respective individuals, termed ‘typhoid Marys’, are important reservoirs for transmission within the human population. The initial colonization of the urinary bladder during typhoid fever is associated with the passing of turbid urine, indicative of bacteria being present at concentrations above $10^8$ bacteria ml$^{-1}$ (Horton-Smith, 1900). This disease manifestation develops in the absence of symptoms of cystitis, which illustrates the stealthy design of S. Typhi. Colonization of the urinary bladder has become rare since the advent of antibiotic therapy. As a result, chronic gall bladder carriage is currently the main route of transmission through chronic carriers.

The estimated age of the S. Typhi lineage is approximately 50,000 years (Kidgell et al., 2002), suggesting that the pathogen evolved when the human population existed in the form of small groups of hunters and gatherers. Transmission through ‘typhoid Marys’ was likely essential for maintaining the infection within groups of some 100 to 200 individuals that existed during this time and enabled the pathogen to become strictly human adapted (Kingsley and Bäumler, 2000).
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The mechanisms important for establishing a chronic carrier status are largely unknown. The formation of gallstones increases the risk of developing chronic gall bladder carriage after recovering from typhoid fever. Colonization of gallstones involves biofilm formation of *S. Typhi* (Crawford *et al.*, 2010). An O-antigen capsule encoded by the *yihU-yshA* and *yihV-yihW* genes (Snyder *et al.*, 2006), which is distinct from the Vi capsular polysaccharide, is required for biofilm formation on gallstones (Crawford *et al.*, 2008), and thus may contribute to transmission success (Fig. 5.2).

**Pseudogene formation during host adaptation**

The genome of *S. Typhi* contains more than 200 pseudogenes, compared to only 39 present in *S. Typhimurium* (McClelland *et al.*, 2001; Parkhill *et al.*, 2001). Pseudogene formation in the *S. Typhi* lineage may reflect, at least in part, a loss of genes no longer required because of changes in the mode of transmission. For example, genes that promote intestinal colonization, which is important for transmission during gastroenteritis, include fimbrial operons, many of which contain pseudogenes in *S. Typhi* (Townsend *et al.*, 2001). Furthermore, one pseudogene found in *S. Typhi* strain CT18 is *ttrS*, encoding a regulator of tetrathionate respiration genes (Parkhill *et al.*, 2001). An elevated number of pseudogenes is also detected in the genomes of host-adapted *S. Gallinarum* and *S. Choleraesuis* and correlates with a host range that is more restricted than that of *Salmonella* serotypes associated with gastroenteritis (Chiu *et al.*, 2005; Thomson *et al.*, 2008).

In summary, the principal pathogenic strategy of *Salmonella* serotypes associated with systemic disease in immunocompetent hosts builds on an approach common to host-pathogen interactions of all *S. enterica* serotypes. This common approach includes a T3SS-1-mediated invasion of the intestinal epithelium followed by a T3SS-2-mediated macrophage survival. However, *Salmonella* serotypes associated with systemic disease have acquired additional virulence mechanisms that enable them to overcome mucosal barrier functions and spread systemically throughout the body. Systemic dissemination results in colonization of internal organs, which is essential for their transmission (Fig. 5.2).

While this overall virulence strategy is shared by all *Salmonella* serotypes associated with systemic disease in immunocompetent hosts, each pathogen has evolved different approaches to transmit from its respective reservoirs in internal organs to the next host. These include horizontal transmission from reservoirs in the gall bladder or urinary bladder, vertical transmission from reservoirs in the udder or the ovaries, or horizontal transmission from reservoirs in the placenta through abortion.

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**References**


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