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Review

Salmonella spp. survival strategies within the host gastrointestinal tract

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Human salmonellosis infections are usually acquired via the food chain as a result of the ability of *Salmonella* serovars to colonize and persist within the gastrointestinal tract of their hosts. In addition, after food ingestion and in order to cause foodborne disease in humans, *Salmonella* must be able to resist several deleterious stress conditions which are part of the host defence against infections. This review gives an overview of the main defensive mechanisms involved in the *Salmonella* response to the extreme acid conditions of the stomach, and the elevated concentrations of bile salts, osmolytes and commensal bacterial metabolites, and the low oxygen tension conditions of the mammalian and avian gastrointestinal tracts.

Introduction

Non-typhoidal human salmonellosis is characterized by the acute onset of fever, abdominal pain, nausea, diarrhoea, and sometimes vomiting (Hohmann, 2001). The disease is usually self-limiting, lasting a few days. However, in some cases, more serious complications follow, especially in immunocompromised people, pregnant women, the elderly and children.

In 2009, salmonellosis was second only to campylobacteriosis in terms of reported zoonotic disease in humans in the European Union, with 108 614 confirmed cases. The two most commonly reported *Salmonella* serovars were *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) and *S. enterica* serovar Typhimurium (*S.* Typhimurium), representing 52.3 and 23.3 % of all reported serovars (EFSA, 2011). However, this number represents only a portion of the true number of illness cases, due to underreporting (EFSA, 2008). The most common reservoir of *Salmonella* is the intestinal tract of a wide range of domestic and wild animals, which can result in a variety of foodstuffs acting as vehicles of infection. Human *S.* Enteritidis cases are most commonly associated with the consumption of contaminated eggs and poultry meat, while *S.* Typhimurium cases are mostly associated with contaminated pig, poultry and bovine meat (EFSA, 2011).

In order to cause foodborne disease in humans, *Salmonella* must colonize the gastrointestinal tract of the reservoir host, survive in the food chain and cross the species gap to colonize the human. After food ingestion, *Salmonella* encounters several (sub)lethal environmental conditions which are part of the host defence against infections. Prior to invasion, *Salmonella* must survive extreme acid conditions within the stomach, and elevated concentrations of bile salts, osmolytes and commensal bacterial metabolites, and low oxygen tension conditions within the intestine. Consequently, *Salmonella* has evolved adaptive networks to cope with the challenges of a changing environment. The aim of this review is to compile the numerous research studies carried out in recent decades in order to elucidate the primary cellular defence systems employed by *Salmonella* spp. to protect themselves against the stress conditions prevailing in these host environments. If not specifically stated, the information given refers to experiments performed with the two most frequent serovars responsible for foodborne infections, *S.* Typhimurium and *S.* Enteritidis.

Survival strategies in the stomach – resistance to low pH

The acid environment of the stomach can be considered one of the host's first lines of defence against ingested bacteria. The importance of human gastric fluid as an antibacterial barrier against enteric pathogens is well-documented (Smith, 2003). Humans produce approximately 1–2 l of gastric fluid per day, with a pH as low as 1.5 and HCl levels of 150–160 mEq l^{-1} (Johnson, 2001; Smith, 2003). Furthermore, several organic acids can be present in the stomach, including lactic, acetic, propionic and butyric acids, which can also contribute to the antimicrobial effect

(Mikkelsen *et al.*, 2004). Thus, a number of studies have associated hypochlorhydria (inability to produce stomach acid) with an increased risk of infection (Howden & Hunt, 1987; Nwokolo *et al.*, 1994), and it has been shown that H^+/K^+-ATPase subunit-deficient mice, unable to produce gastric HCl (stomach pH ~7.0) are more susceptible to colonization by *Salmonella* than wild-type BALB/cCrSlc mice (Tennant *et al.*, 2008).

Generally, the infective dose (ID) of enteric pathogens is related to a number of factors, including their ability to cope with acid. Although in clinical trials the ID for non-typhoid *Salmonella* has been estimated to be at least 10^6 bacteria, various reports have indicated that when cells are ingested with a food source, *Salmonella* can have a much lower ID ($\leqslant$100 cells) (Waterman & Small, 1998; Gawande & Bhagwat, 2002). It is reasonable to assume that the temporary increase in pH following consumption of food may help *Salmonella* to survive the harsh stomach conditions (Rychlik & Barrow, 2005). In addition, it has been suggested that certain solid food sources, especially those rich in fat or with a high protein content, protect *Salmonella* against stomach acidity (Waterman & Small, 1998; Álvarez-Ordóñez *et al.*, 2009a), and feed composition has been shown to influence the gastric pH and the stomach organic acid content (Mikkelsen *et al.*, 2004). With regard to liquid foods, such as water and juices, their short emptying time from the stomach could also facilitate *Salmonella* survival (Smith, 2003).

Despite being neutrophilic bacteria, *Salmonella* may be able to survive in the hostile environment of the stomach through the induction of the so-called acid tolerance response (ATR), which can be defined as the capacity to undergo an adaptive response to moderately acidic pH that enhances the subsequent survival under conditions of lethal pH, such as those prevalent in the stomach (Foster, 2000; Audia *et al.*, 2001; Álvarez-Ordóñez *et al.*, 2011). There is evidence to suggest that this process increases invasive capacity and virulence potential *in vivo* (Gahan & Hill, 1999). This section will deal with the response of *Salmonella* to low-pH environments. A schematic overview of the main systems of the *Salmonella* response to acid stress is shown in Fig. 1.

pH homeostatic systems

It is well known that Gram-negative bacteria try to keep intracellular pH relatively constant at pH 7.6–7.8, even as extracellular pH dramatically changes. The intracellular pH is maintained by pumps that extrude protons from the cytoplasm in low-pH environments (Foster, 2000). In addition, it has become clear that inducible lysine decarboxylase and arginine decarboxylase systems (Figs 1a and 2a) play an important role in the maintenance of intracellular pH in *Salmonella*.

A number of studies have demonstrated a role for lysine in pH homeostasis in *Salmonella* (Park *et al.*, 1996; de Jonge *et al.*, 2003; Morita *et al.*, 2006; Lee *et al.*, 2007a, 2008; Álvarez-Ordóñez *et al.*, 2010b). The lysine decarboxylase system is composed of a transcriptional regulator (CadC) of the *cadBA* operon, a lysine decarboxylase enzyme (CadA), and a lysine–cadaverine antiporter (CadB). Under conditions of low external pH in the presence of lysine, CadC activates the transcription of the *cadBA* operon. Following induction, the enzyme CadA converts intracellular lysine to cadaverine with the consumption of a proton, thus increasing the intracellular pH. Subsequently, cadaverine is exchanged for extracellular lysine via the CadB antiporter. It is important to note that studies using *in vivo* expression technologies have shown that *cadC* and *cadB* are induced during infection of BALB/c mice (Heithoff *et al.*, 1997) and macrophages (Eriksson *et al.*, 2003). These findings together with the report of Lee *et al.* (2007a), who have described the role of CadC as a global regulator linked to the OmpR–EnvZ regulatory system that controls the expression of $\geqslant$36 proteins during acid exposure in *S.* Typhimurium, suggest the importance of the lysine decarboxylase system for *Salmonella* in transiting the stomach.

Kieboom & Abee (2006) confirmed the existence of an active arginine decarboxylase system in *S.* Typhimurium, composed of an arginine decarboxylase (AdiA), which converts arginine into agmatine in the cytoplasm with the consumption of a proton, an arginine/agmatine antiporter (AdiC), which expels agmatine from the cell in exchange for external arginine, and a transcriptional activator (AdiY). Those authors showed an enhanced relative expression level for *adiA* and *adiY* under acid conditions, and a reduced acid resistance for *S.* Typhimurium cells disrupted in *adiA*, *adiC* and *adiY*. In their experiments, the oxygen level was critical, with only anaerobically grown cells being able to display the arginine-dependent acid-resistant phenotype. However, recent studies by Álvarez-Ordóñez *et al.* (2010b) have also demonstrated the presence of an active acid-inducible arginine decarboxylase system under aerobic conditions. These latter authors compared the acid tolerance of acid-adapted and non-acid-adapted cells at pH 2.5 in a minimal medium (MM) and MM supplemented with arginine, and they observed that whereas the inclusion of this amino acid in the challenge medium did not modify the acid resistance of non-acid-adapted cells, it significantly increased the acid tolerance of acid-adapted cells. In addition, they determined the relative expression levels of the genes encoding the main components of the arginine decarboxylase system (i.e. *adiA*, *adiY*) by quantitative PCR, and they found that these genes were significantly upregulated after bacterial growth under acid conditions. Therefore, they concluded that the arginine decarboxylase system is present in *S.* Typhimurium, but is only active under acid growth conditions.

Synthesis of acid shock proteins (ASPs)

During the ATR, several groups of ASPs are induced in order to prevent or repair the macromolecular damage

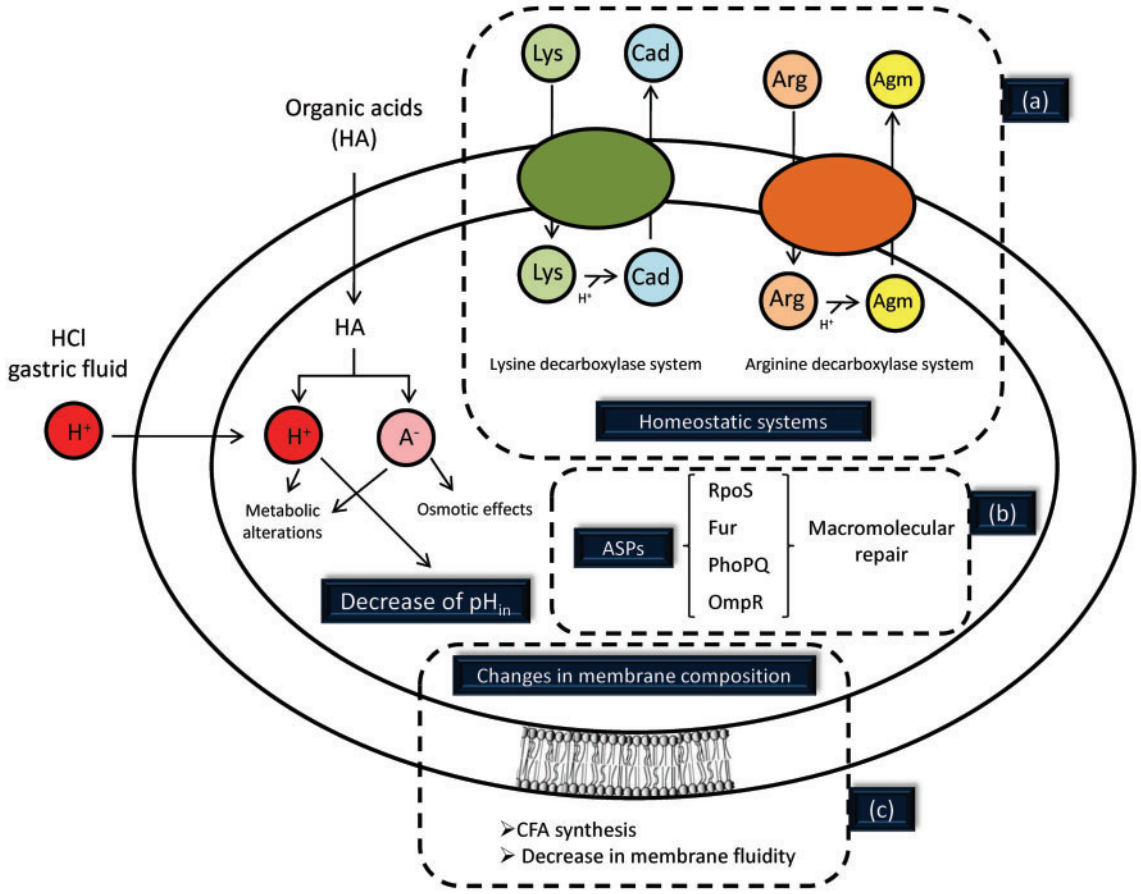

Fig. 1. Schematic view of a *Salmonella* spp. cell containing all the known components of inducible acid-tolerance and acid-resistance systems. A low internal pH (pH_{in}) induces the expression of amino acid decarboxylase systems (a), the synthesis of ASPs (b), and modifications to membrane FA composition (c). Cad, cadaverine; Agm, agmatine.

caused by acid stress (Audia *et al.*, 2001). An extensive research effort has been made in recent decades to identify and characterize these stress proteins in *S.* Typhimurium, and several regulatory genes controlling the expression of different subsets of ASPs have been described, including RpoS, Fur, PhoP/PhoQ and OmpR. Most of the identified ASPs are involved in cellular regulation, molecular chaperoning, energy metabolism, transcription, translation, synthesis of fimbriae, regulation of the cellular envelopes, colonization and virulence (Foster, 2000; Audia *et al.*, 2001).

The alternative sigma factor RpoS is important for *Salmonella* survival in stationary phase, as well as under various stress conditions (reviewed by Hengge-Aronis, 2002). RpoS has been shown to be involved in the acid inducible exponential-phase ATR of *S.* Typhimurium, controlling the expression of at least 10 ASPs (Foster, 2000; Tu *et al.*, 2006). In addition, RpoS is responsible for the non-acid-inducible stationary-phase ATR, expressed upon entry into stationary phase as part of the general stress response (Audia *et al.*, 2001). Various studies have shown *rpoS* null mutants to display increased susceptibility to acid pH and attenuated virulence in BALB/c mice after the peroral route of infection (Fang *et al.*, 1992; Lee *et al.*, 1995; Coynault *et al.*, 1996; Bearson *et al.*, 2006; Domínguez-Bernal *et al.*, 2008; Karasova *et al.*, 2009). Although many genes with known functions in stress responses have been identified within the *rpoS* regulon of *S.* Typhimurium (Ibanez-Ruiz *et al.*, 2000), further work is required in order to determine the nature of the full regulon and to elucidate the environmental factors that influence its expression within specific microenvironments in the host.

The Fur protein is usually linked to the regulation of bacterial iron metabolism. In addition, it has been shown to control a subset of ASPs in an iron-independent manner, which contribute to the *S.* Typhimurium exponential-phase ATR, and confer protection mainly against organic acid stress (Hall & Foster, 1996; Foster, 2000). However, little is known about the identity of the acid-induced Fur-regulated genes. Moreover, several studies have shown that disruption of Fur in *Salmonella* attenuates its virulence

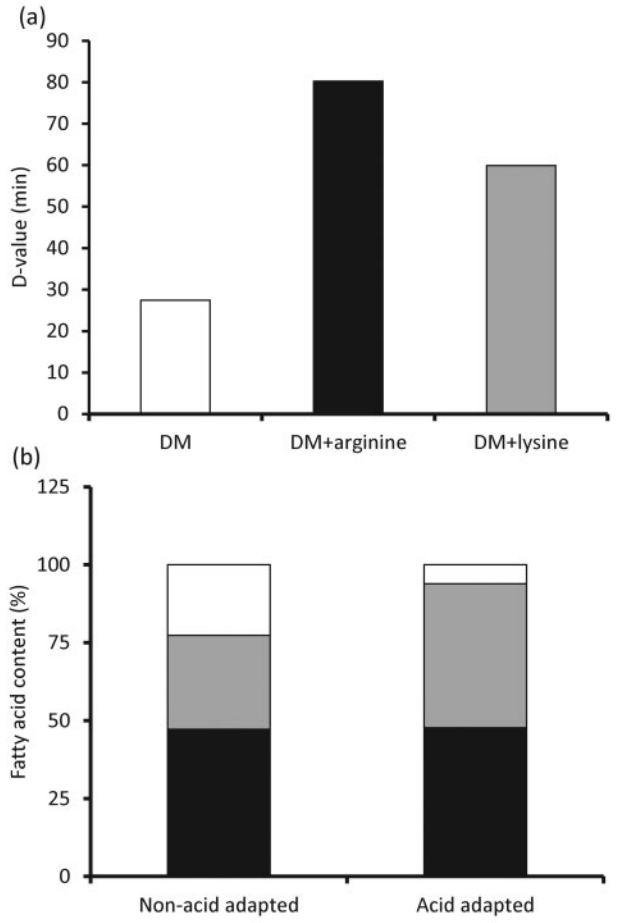

Fig. 2. (a) Inactivation of acid-adapted *S.* Typhimurium at pH 2.5 for 3 h in a defined medium (DM) unsupplemented (white bar) or supplemented with 0.01 % (w/v) arginine (black bar) or 0.01 % (w/v) lysine (grey bar). Adapted from Álvarez-Ordóñez *et al.* (2010b). (b) Membrane content of *S.* Typhimurium non-acid-adapted and acid-adapted cells with respect to saturated FAs (black), cyclopropane fatty acids (grey) and UFAs (white). Adapted from Álvarez-Ordóñez *et al.* (2008).

(Wilmes-Riesenberg *et al.*, 1996; Curtiss *et al.*, 2009; Karasova *et al.*, 2009), and a new role for Fur as a regulator of the expression of the *Salmonella* pathogenicity island 1 (SPI-1) type III secretion system has been described (Ellermeier & Slauch, 2008), suggesting a role for this regulator in pathogenicity.

The *Salmonella* PhoP/PhoQ two-component system modulates a large regulon that controls expression of ~3 % of the genome (Groisman, 2001; Charles *et al.*, 2009). The main signal regulating the two-component system is the concentration of Mg^{2+}. The sensor protein PhoQ responds to low Mg^{2+} concentrations by promoting phosphorylation of the response regulator PhoP, which binds to its target promoters to stimulate transcription of PhoP-activated genes (García Véscovi *et al.*, 1996; Shin & Groisman, 2005). In addition, several studies have shown that acid pH also regulates the transcription of certain PhoPQ-regulated genes, mainly conferring protection in exponential phase against inorganic acid stress (Foster, 2000). Thus, Bearson *et al.* (1998) demonstrated the induction of PhoP *in vitro* by acid pH in the presence of high concentrations of Mg^{2+}, and Prost *et al.* (2005) showed that PhoQ is directly activated by pH 5.5. It is also worth mentioning that Alpuche Aranda *et al.* (1992) demonstrated PhoP-activated gene transcription within acidified macrophage phagosomes, and Martin-Orozco *et al.* (2006) indicated that vacuolar acidification itself suffices to induce PhoP in the *Salmonella*-containing vacuole. Finally, Merighi *et al.* (2005) demonstrated the induction of PhoP *in vivo* inside macrophages and in the gastrointestinal tract of BALB/c mice, and several studies have shown *Salmonella* strains harbouring null alleles of the *phoP* or *phoQ* gene to be highly attenuated for virulence (Miller *et al.*, 1989; Lee *et al.*, 2007b; Domínguez-Bernal *et al.*, 2008; Karasova *et al.*, 2009).

The OmpR protein reacts to a variety of environmental factors, with osmolarity being the most actively studied (Bremer & Krämer, 2000). Acid shock induces OmpR by means of its phosphorylation from the phosphate donor acetyl phosphate. OmpR, in its phosphorylated state, triggers the expression of various genes involved in the acid-inducible stationary-phase ATR (Bang *et al.*, 2000, 2002; Zhao & Houry, 2010). However, further studies are necessary to clarify how it is induced and which OmpR-dependent genes are involved in this adaptive response. Interestingly, several studies have connected OmpR with *Salmonella* virulence, mainly through the regulation of both SPI-1- and SPI-2-encoded genes (Lee *et al.*, 2000; Kim & Falkow, 2004; Fass & Groisman, 2009; Karasova *et al.*, 2009).

Modifications of the membrane composition

It is generally accepted that bacteria change their membrane composition in response to the environmental signals found in their surroundings, with the purpose of maintaining a degree of membrane fluidity compatible with life (Beney & Gervais, 2001). The composition of membrane fatty acids (FAs) is responsible for membrane fluidity, and a number of studies have suggested a relationship between membrane fluidity and stress tolerance for *Salmonella* (de Jonge *et al.*, 2003; Álvarez-Ordóñez *et al.*, 2008, 2009b, 2010a, c). Experiments performed by Álvarez-Ordóñez *et al.* (2008) showed that when *S.* Typhimurium cells are exposed to acid pH, a membrane adaptation, characterized by a decrease in the unsaturated FA (UFA) to saturated FA ratio and in membrane fluidity, occurs. An increase in the membrane content of cyclic FAs (CFAs) was also observed (Fig. 2b). Qualitatively similar changes in membrane composition and fluidity were subsequently described for *Salmonella enterica* serovar Senftenberg (Álvarez-Ordóñez *et al.*, 2009b) and *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis)

(Alonso-Hernando *et al.*, 2010). Therefore, it seems clear that during *Salmonella* acid adaptation a significant proportion of the UFAs are converted to their cyclic derivatives (CFAs). The CFAs are formed by a CFA synthase (encoded by the *cfa* gene) through the addition of a methylene group from *S*-adenosyl L-methionine to the *cis* double bond of the UFA moiety of the phospholipid (Grogan & Cronan, 1997). Kim *et al.* (2005) showed CFA to be important for *S.* Typhimurium acid tolerance, since they described *cfa*-defective mutants that were sensitive to low pH, a phenotype which could be partially restored by the introduction of a functional *cfa* gene. Interestingly, those authors described an increased CFA synthase expression at early stationary phase linked to RpoS. However, the contribution of CFAs to bacterial membrane properties is not yet fully understood. A prominent theme among the various hypotheses put forward is that CFA formation changes the fluidity or other physical properties of bacterial membranes in a biologically relevant way (Dufourc *et al.*, 1984; Grogan & Cronan, 1997). In contrast, other studies have hypothesized that the primary function of CFA formation is to change membrane chemical properties. CFAs appear to be considerably less reactive than the corresponding UFAs towards certain forms of oxidation (Grogan & Cronan, 1997), which suggests that CFAs exert their protective effect through the reduction of cellular oxidative damage. Finally, the cyclization of membrane FAs has also been proposed as a means of controlling the penetration of undesirable molecules from the cellular surroundings (Chang & Cronan, 1999). In any event, there is every indication that CFA synthesis and the regulation of membrane fluidity are important for *Salmonella* survival in the stomach, and the report by Haque *et al.* (1996) that *Helicobacter* spp. isolates identified as gastric colonizers tend to produce larger amounts of CFA than intestinal colonizers, suggests that CFA formation provides bacteria with an evolutionary advantage for survival in extreme acid niches.

Survival strategies in the gut

Salmonella response to bile and colonization of the gall bladder

Bile, the major constituent of which is bile salts, is synthesized in the liver from cholesterol, is stored and concentrated in the gallbladder inter-digestively, and is released after food intake into the duodenum, where it plays an essential role in the digestion of lipids. The ability to act as detergents also confers potent antimicrobial properties on bile salts. Bile primarily exerts its effects on bacterial cell membranes, but can also have numerous other effects including induction of secondary structure formation in RNA, induction of DNA damage and misfolding or denaturation of proteins (reviewed by Gunn, 2000; Begley *et al.*, 2005; Merritt & Donaldson, 2009).

Physiology of bile tolerance. *Salmonella* is considered to be inherently bile tolerant. Indeed, bile salts are used in agars for selective enrichment, e.g. *Salmonella–Shigella* agar, MacConkey agar, bile aesculin agar and violet red bile agar. The MICs of ox bile for stationary-phase cells of several *S.* Typhimurium and *Salmonella enterica* serovar Typhi (S. Typhi) strains have been determined to be 18 and 12 %, respectively, and minimal bactericidal concentrations (MBCs) were >60 % for *S.* Typhimurium and 18 % for *S.* Typhi (van Velkinburgh & Gunn, 1999).

Salmonella can adapt to bile, as exposure to sublethal levels can result in increased survival when challenged with otherwise lethal levels. Experiments by van Velkinburgh & Gunn (1999) suggest that this phenotype is both growth phase- and bile concentration-dependent. Pre-treatment of exponential-phase cells of *S.* Typhimurium with 15 % bile resulted in increased survival when challenged with 24 % bile. Exposing stationary-phase cells to the same concentration of bile did not increase their resistance when subsequently challenged with 24 % bile (van Velkinburgh & Gunn, 1999). It was also noted that exposure of exponential-phase cells to low levels of bile (1–3 %) did not affect resistance.

Genetics of bile tolerance. Many of the genetic loci that contribute to *Salmonella* bile tolerance have been uncovered and, given the numerous effects that bile can have on bacterial cells, it is unsurprising that a variety of genes have been shown to be involved (Table 1). Similar to other bacteria, these include genes that encode efflux pumps, two-component signal transduction systems, transcriptional regulators and proteins involved in DNA repair or the maintenance of membrane integrity (reviewed by Begley *et al.*, 2005). The lipopolysaccharide (LPS) has been shown to play a major role in *Salmonella* bile resistance and loss of the O-antigen, which creates a 'rough' colony phenotype, results in increased bile sensitivity (Lacroix *et al.*, 1996, Prouty *et al.*, 2002a). Mutations in the *wecD* and *wecA* genes, involved in the biosynthesis and assembly of enterobacterial common antigen (ECA), render *S.* Typhimurium more sensitive to the bile salt deoxycholate (Ramos-Morales *et al.*, 2003). The *S.* Typhimurium AcrAB pump is also required for bile resistance (Lacroix *et al.*, 1996, Nikaido *et al.*, 1998, Prouty *et al.*, 2004b). Mutations in *tol* genes have been shown to affect bile resistance; three of the 15 *S.* Typhimurium bile-sensitive transposon mutants isolated by Prouty *et al.* (2002a) were located in the *tolQRA* region.

Loci regulated by the two-component system PhoPQ are also required for bile resistance in *S.* Typhimurium. Mutants lacking PhoPQ are killed at significantly lower bile concentrations, while strains with constitutively active PhoPQ are able to survive prolonged incubation with bile at concentrations of >60 % (van Velkinburgh & Gunn, 1999). Reporter gene fusions indicate that the PhoPQ regulon does not sense and respond to bile. This suggests that the PhoPQ regulon and the bile regulon contain overlapping genes. Prouty *et al.* (2004b) revealed that the antibiotic-resistance operon *marRAB* contributes to bile tolerance. This study also implies that the ability of *S.*

Table 1. Genetic loci that contribute to *Salmonella* spp. bile tolerance

Gene or protein	Function of encoded protein	Link to bile tolerance	Reference(s)
phoPQ	Two-component system	*phoPQ* mutant is sensitive to bile	van Velkinburgh & Gunn (1999); Langridge *et al.* (2009)
marRAB	Regulatory genes	*marRAB* are upregulated by bile, *mar* mutants are sensitive to bile	Prouty *et al.* (2004b)
acrAB	Efflux pump	*acrAB* mutant is sensitive to bile, *acrAB* are upregulated by bile	Lacroix *et al.* (1996); Prouty *et al.* (2004b); Langridge *et al.* (2009)
tolQRA, *tolC*	Efflux pump function	*tol* mutants are sensitive to bile	Prouty *et al.* (2002a); Langridge *et al.* (2009)
dam	DNA adenine methylase	*dam* mutants are sensitive to bile	Heithoff *et al.* (2001); López-Garrido *et al.* (2010); Prieto *et al.* (2004); Langridge *et al.* (2009)
wecD, *wecA*	Biosynthesis and assembly of enterobacterial common antigen	*wecA* and *wecD* mutants are sensitive to bile	Prouty *et al.* (2002a)
xthA and *nfo*	Exonuclease and endonuclease, respectively, involved in DNA repair	Mutant lacking both *xthA* and *nfo* is sensitive to bile	Prieto *et al.* (2006)
recA, *B*, *C*, *D*, *J*	Repair and maintenance of DNA	*rec* mutants are sensitive to bile	Prieto *et al.* (2006)
dinB	DNA repair	*dinB* mutant is bile-sensitive	Prieto *et al.* (2006)
seqA	GATC-binding protein	*seqA* mutant is bile-sensitive	Prieto *et al.* (2007); Langridge *et al.* (2009)
hupA	DNA-binding protein	*hupA* mutant is bile-sensitive	Langridge *et al.* (2009)
mrcA, *mrcB*	Penicillin-binding proteins 1a and 1b	*mrcA*, *mrcB* mutants are bile-sensitive	Langridge *et al.* (2009)
sanA	Uncharacterized membrane protein	*sanA* mutant is bile-sensitive	Langridge *et al.* (2009)
sbcB	Exonuclease, involved in DNA repair	*sbcB* is upregulated by bile	Prieto *et al.* (2006)
YciF	Unknown function	YciF expression increases in the presence of bile	Prouty *et al.* (2004a)
STM4242	Unknown function	STM4242 expression increases in the presence of bile	Prouty *et al.* (2004a)

Typhimurium to adapt to sublethal levels of bile may involve some *mar*-dependent pathways.

Exposure of *Salmonella* to bile salts induces the SOS response, indicating DNA damage (Prieto *et al.*, 2006). Bile increases the frequency of point mutations and chromosomal rearrangements (Prieto *et al.*, 2004), and induces the expression of genes belonging to the OxyR and SoxRS regulons (*dps*, *katG*, *nfo*, *fumC*), suggesting that bile salts may cause oxidative damage. Rec mutants (*recA*, *recB*, *recC* and *recD*) are extremely sensitive to bile, providing evidence that bile-induced damage may impair DNA replication. *S.* Typhimurium DNA adenine methylase (DAM) mutants also exhibit enhanced sensitivity to bile salts (Heithoff *et al.*, 2001). This enzyme may be important in repairing DNA damage induced by exposure to bile. Disruption of the inner-membrane protein DamX causes severe sensitivity to bile (López-Garrido *et al.*, 2010).

2D gel electrophoresis analyses by van Velkinburgh & Gunn (1999) demonstrated that numerous proteins are affected both positively and negatively by bile and deoxycholate in *S.* Typhimurium and *S.* Typhi. Bile and deoxycholate resulted in ~15 and 14 easily observable changes, respectively, in *S.* Typhimurium. Fewer alterations were observed in *S.* Typhi (two for bile, six for deoxycholate). Minimal overlap was observed in the proteins affected for each serovar, suggesting that the regulatory factors or the targeted genes differ. A later study by the same authors analysed five of the protein spots that were altered in *S.* Typhimurium and identified them as PagC, OmpD and FljB (all repressed by bile), and YciF and a hypothetical membrane protein, STM4242 (both activated by bile) (Prouty *et al.*, 2004a). DNA microarrays have been employed to examine the global effect of bile on transcription in *S.* Typhimurium. It was observed that 230 genes (101 activated, 129 repressed) were more than threefold affected when exponential-phase cells were exposed to 3 % bile (Prouty *et al.*, 2004a).

Langridge *et al.* (2009) generated an estimated 1.1 million *S.* Typhi transposon mutants and compared the growth of this mutant pool in the presence or absence of bile (10 % ox bile). A total of 169 genes were identified as being required for bile tolerance, including several that have been previously implicated in bile tolerance, such as the *waa* genes, *acrAB*, *tolC*, *seqA*, *dam*, *phoP* and *phoQ*. Genes which had not been implicated in bile tolerance prior to this study included *hupA*, *mrcA*, *mrcB* and *sanA*. Thirty hypothetical genes important for bile tolerance were also identified.

Antunes *et al.* (2011) used a metabolomics approach to study the changes elicited in the chemical composition of murine bile by *Salmonella*. The authors observed that the concentrations of multiple glycerophospholipids, such as phosphatidylcholine, phosphatidylethanolamine, lysopho-

sphatidylcholine and lysophosphatidylethanolamine, were significantly decreased upon *Salmonella* growth in bile *in vitro*. Examination of bile from mice infected with *Salmonella* demonstrated a similar reduction in glycerophospholipids in *vivo*. These findings strongly suggest that glycerophospholipids are used as substrates by *Salmonella* during growth in bile.

Relationship between bile and pathogenesis. As a large variety of proteins contribute to bile tolerance it is likely that some of these will, either directly or indirectly, contribute to *in vivo* survival and colonization of the intestinal tract by *Salmonella*. For example, AcrB, Dam, PhoPQ and Wec mutants have reduced virulence in BALB/c mice (Miller *et al.*, 1989; Lacroix *et al.*, 1996; van Velkinburgh & Gunn, 1999; Ramos-Morales *et al.*, 2003; Prieto *et al.*, 2004). It is also becoming increasingly evident that bile plays a role in *Salmonella* pathogenesis by acting as a signal molecule, and the bacterium can modulate gene expression in response to bile to induce genes that assist survival and repress others. Prouty & Gunn (2000) demonstrated that *S.* Typhimurium grown in the presence of bile is able to invade epithelial cells at only 4 % of the level of cells grown in the absence of bile. Transcription of invasion gene regulators (*sirC* and *invF*) was shown to be repressed in the presence of bile, resulting in decreased transcription of SPI-1 genes (Prouty & Gunn, 2000; Prouty *et al.*, 2004a). The authors hypothesize that *Salmonella* may use bile as an environmental signal to repress its invasive capacity in the intestinal lumen, where bile concentrations are high, and invasion may then be initiated after transiting the mucus layer. In addition, microarray experiments have revealed reduced expression of flagellar biosynthesis genes, including *flhC*, *flgC* and *fliC*, in the presence of bile, a finding that was corroborated phenotypically by motility assays (Prouty *et al.*, 2004a). As motility is energetically costly, bile-mediated repression of motility may be energetically favourable in the intestinal environment.

Survival in the gallbladder. Menendez *et al.* (2009) used a mouse model of acute typhoid fever to demonstrate that *Salmonella* could replicate extracellularly in the gallbladder lumen and could also infect and replicate intracellularly within gallbladder epithelial cells. *S.* Typhi has been shown to colonize the human gallbladder and persist in an asymptomatic carrier state that is often associated with the presence of gallstones. Residing in this location may allow the bacterium to escape the host immune system and antibiotics, and to be released in bile back into the intestine to reinfect the same host or be shed in the faeces. There is a strong correlation between gallbladder abnormalities, particularly gallstones, and asymptomatic *Salmonella* carrier state development (Lai *et al.*, 1992). It is therefore important to elucidate the mechanisms by which *Salmonella* survives in the gallbladder.

Prouty *et al.* (2002b) demonstrated that *S.* Typhimurium, *S.* Enteritidis and *S.* Typhi could form biofilms on human gallstones *in vitro*. Efficient biofilm formation on gallstones was dependent upon the presence of bile, as a biofilm did not form on gallstones within 14 days in culture medium alone (Prouty *et al.*, 2002b). As cholesterol is the primary constituent of human cholesterol gallstones, cholesterol-coated Eppendorf tubes have been employed to mimic human gallstones *in vitro*, and *Salmonella* has been shown to form biofilms on these tubes (Crawford *et al.*, 2008). Screening of a random transposon bank for mutants that were impaired in biofilm formation on cholesterol-coated Eppendorf tubes led to the identification of 49 mutants with this phenotype (Crawford *et al.*, 2010a). The results revealed that genes involved in flagellum biosynthesis and structure primarily mediated attachment to cholesterol (Crawford *et al.*, 2010a).

A recent study by Crawford *et al.* (2010b) provides direct evidence that gallstone biofilms occur in mice and humans and that they play a significant role in *Salmonella* gallbladder colonization and carriage. *Salmonella*-resistant (Nramp1$^{+/+}$) mice were fed a lithogenic diet to induce gallstones. Mice were subsequently infected with *Salmonella* and it was observed that the number of bacteria in the gallbladder epithelium and gallbladder bile was significantly higher than that observed in *Salmonella*-infected mice that did not harbour gallstones (Crawford *et al.*, 2010b). In addition, mice harbouring gallstones shed a three-log higher number of *Salmonella* in their faeces. Crawford *et al.* (2010b) subsequently demonstrated the presence of *S.* Typhi in 4.9 % of the patients enrolled for surgical gallbladder removal in a hospital in Mexico City. *Salmonella* biofilms could be visualized on gallstones. The authors of this study suggest that gallstone biofilms may represent a novel therapeutic target against the spread of typhoid fever.

Salmonella response to other gut-related stresses

Osmotic shock. The lumen of the gastrointestinal tract is a region of relatively high salt concentration (equivalent to 0.3 M NaCl). Bacteria react to environments of elevated osmolarity by means of a biphasic response, which involves the stimulation of potassium uptake (and its counter-ion glutamate) followed by a dramatic increase in the cytoplasmic concentration (by synthesis and/or uptake) of the so-called compatible solutes (reviewed by Sleator & Hill, 2002).

With regard to *Salmonella*, an inducible high-affinity system (Kdp) and two low-affinity systems (Trk, Kup) have been shown to be important for potassium uptake (Frymier *et al.*, 1997; Balaji *et al.*, 2005; Su *et al.*, 2009). On the other hand, glycine-betaine, carnitine, ectoine, proline and trehalose have emerged as the principal compatible solutes (Csonka & Epstein, 1996; Bremer & Krämer, 2000; Howells *et al.*, 2002; Sleator & Hill, 2002; García-Estepa *et al.*, 2006). The genetic loci responsible for the synthesis or uptake of compatible solutes and potassium are highlighted in Fig. 3 and Table 2, in addition to other osmoregulated genes that do not directly contribute to potassium or compatible solute accumulation.

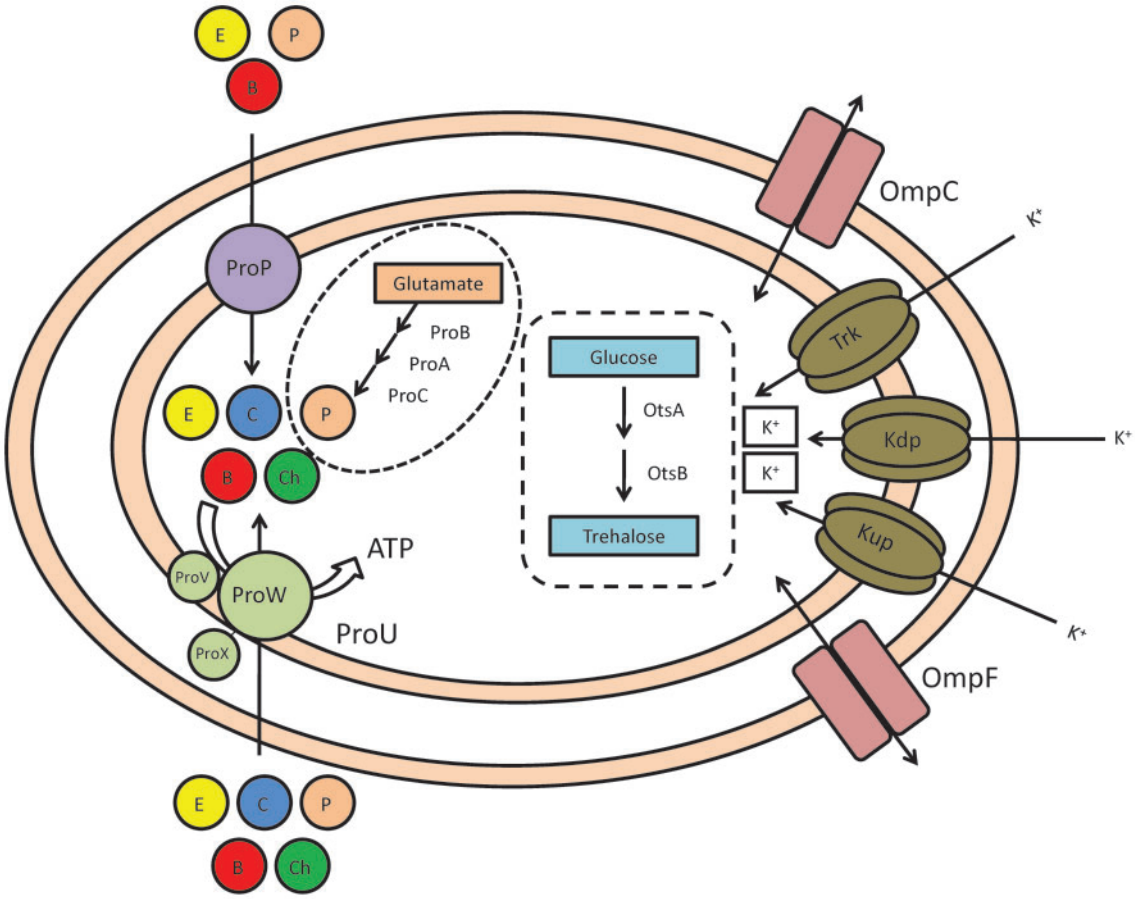

Fig. 3. Schematic view of the systems for uptake and/or synthesis of potassium (K^+), betaine (B), proline (P), choline (Ch), ectoine (E), carnitine (C) and trehalose in *Salmonella* spp.

Intestinal anaerobiosis. During passage through the gastrointestinal tract the oxygen availability decreases, and as a result, in the large intestine the environment is predominantly anaerobic. Two major regulatory circuits, dependent on Fnr and ArcAB, are responsible for the regulation of the cellular metabolic activity in anaerobic environments.

Fnr, also named OxrA in *Salmonella*, is a cytoplasmic oxygen sensor that can bind promoter sequences, and interacts with the RpoA subunit of RNA polymerase to increase the efficiency of transcription of a variety of genes required for anerobic metabolism, while it represses many of the genes encoding enzymes involved in aerobic electron transport, oxidative phosphorylation and some tricarboxylic cycle enzymes (Unden *et al.*, 1995; Wei & Miller, 1999; Perrenoud & Sauer, 2005; Weber *et al.*, 2005).

ArcAB is a two-component signal transduction system induced under microaerobic and anaerobic conditions that suppresses the expression of genes encoding enzymes of the tricarboxylic cycle, with a consequent decrease in the production of harmful oxygen radicals and conservation of endogenous energy sources (Sevcík *et al.*, 2001). Therefore, it regulates the defence against reactive oxygen and nitrogen intermediates (Lu *et al.*, 2002).

It is important to note that several authors have described a role for *fnr* and *arcAB* in *Salmonella* pathogenesis and virulence (see Table 2) and that disruption of the tricarboxylic cycle has been recently shown to increase the ability of *S.* Typhimurium to survive within murine macrophages, which suggests a link between anaerobic metabolism and pathogenicity (Bowden *et al.*, 2010).

Commensal micro-organisms. In recent decades it has become clear that the interaction of *Salmonella* with the gastrointestinal microflora is an important factor determining the outcome of the infection process. It is evident that the presence of a numerically dominant gut microflora results in a competition with *Salmonella* for the adhesion receptors in the gut epithelium and for the uptake of the available nutrients. This leads to the induction of a starvation stress response (Spector, 1998; Rychlik & Barrow, 2005). In addition, gastrointestinal commensal bacteria are known to produce metabolites with anti-*Salmonella* activity, such as bacteriocins and short chain

Table 2. Genetic loci that contribute to *Salmonella* tolerance to different gut-related stresses (osmotic shock, intestinal anaerobiosis, commensal bacteria metabolites and antimicrobial peptides)

Gene	Function of encoded protein	Link to stress tolerance	Reference(s)
Osmotic shock			
kdp	High-affinity K^+ transport system	Osmotic shock induces the expression of *kdp*	Frymier *et al.* (1997); Balaji *et al.* (2005)
trk	Low-affinity K^+ transport system	Trk modulates *Salmonella* virulence	Su *et al.* (2009)
proP	Permease involved in compatible solute uptake	Osmotic shock induces the expression of *proP*	Cairney *et al.* (1985); Balaji *et al.* (2005)
proU	Permease involved in compatible solute uptake	Osmotic shock induces the expression of *proU*	Balaji *et al.* (2005)
otsA	Trehalose-6-phosphate synthase (involved in synthesis of trehalose)	Osmotic shock induces the expression of *otsA*; *otsA* mutants grow poorly under high-osmolarity conditions	Howells *et al.* (2002)
otsB	Trehalose-6-phosphate phosphatase (involved in synthesis of trehalose)	Osmotic shock induces the expression of *otsB*	Balaji *et al.* (2005)
proA	γ-Glutamyl phosphate reductase (involved in synthesis of proline)	*proA* confers osmotolerance	Mahan & Csonka (1983)
proB	γ-Glutamyl kinase (involved in synthesis of proline)	*proB* confers osmotolerance	Mahan & Csonka (1983)
ompC	Outer-membrane channel porin	Osmotic shock induces the expression of *ompC*; *ompC* mutants are attenuated *in vivo* for BALB/c mice	Chatfield *et al.* (1991); Balaji *et al.* (2005)
ompF	Outer-membrane channel porin	*ompF* mutants are attenuated *in vivo* for BALB/c mice	Chatfield *et al.* (1991)
rpoS	Alternative sigma factor	Osmotic shock induces the expression of *rpoS*; *rpoS* mutants survive poorly under high-osmolarity conditions	Balaji *et al.* (2005); McMeechan *et al.* (2007)
rpoE	Alternative sigma factor	*rpoE* mutants survive poorly under high-osmolarity conditions	McMeechan *et al.* (2007)
Intestinal anaerobiosis			
fnr	Cytoplasmic oxygen sensor	*fnr* regulates the expression of genes involved in flagellar biosynthesis, motility and chemotaxis, and activates the transcription of many SPI-1 genes and genes important for *Salmonella* pathogenesis and invasiveness; *fnr* mutants are attenuated in macrophages and mice	Rollenhagen & Bumann (2006); Fink *et al.* (2007); Zbell *et al.* (2007); Ammendola *et al.* (2008)
arcAB	Two-component signal transduction system	*arcA* controls resistance to reactive nitrogen and oxygen intermediates; *arcA* regulates *hyb* and *hyd*, important for virulence	Lu *et al.* (2002); Zbell *et al.* (2007)
Commensal bacterial metabolites			
sdiA	AHL receptor	*sdiA* regulates the *rck* operon ('for resistance to complement killing'); *sdiA* becomes active during the transit of *Salmonella* through the gastrointestinal tract of turtles	Ahmer *et al.* (1998); Smith *et al.* (2008)
luxS	AI-2 synthase	*luxS* mutants are attenuated in the expression of the virulence genes of SPI-1. *luxS* regulates genes involved in bacterial metabolism, transcription, transport, biofilm formation and motility	Choi *et al.* (2007); Jesudhasan *et al.* (2010)
Antimicrobial peptides			
rpoE	Alternative sigma factor	*rpoE* mutant is more susceptible to antimicrobial peptides than the parent strain	Crouch *et al.* (2005)

fatty acids (SCFAs) (Nava *et al.*, 2005). Finally, pathogenic and commensal bacteria are able to interact by the phenomenon of quorum sensing, responsible for the development of intraspecies, interspecies and inter-Kingdom communication networks.

Quorum sensing is a widespread phenomenon used by bacteria as a communication mechanism in order to make 'collective decisions' based on the synthesis and secretion of small chemical signal molecules which at high concentrations modulate gene expression and the behaviour of the whole population (Ahmer, 2004; Miller *et al.*, 2004; Walters & Sperandio, 2006; Boyen *et al.*, 2009). In *Salmonella*, the main signal molecules are *N*-acylhomoserine lactone (AI-1) and 2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF, AI-2). *Salmonella* lacks the genetic machinery needed to synthesize AI-1, but is able to sense the AI-1 produced by other gastrointestinal bacteria by means of SdiA, which regulates the expression of virulence genes with different functions, ranging from resistance towards complement killing to the expression of fimbriae (Boyen *et al.*, 2009; Dyszel *et al.*, 2010). On the other hand, the *luxS* gene is responsible for *Salmonella* AI-2 synthesis (Table 2).

Some reports have also described the ability of *Salmonella* to sense and respond to neurotransmitters and hormones produced by the host, such as epinephrine and norepinephrine (Walters & Sperandio, 2006; Bearson & Bearson, 2008; Bearson *et al.*, 2008).

Antimicrobial peptides. *Salmonella* must be able to tolerate antimicrobial peptides encountered in the host small intestinal lumen, such as defensins. Evidence provided by Salzman *et al.* (2003) supports the hypothesis that mucosal defensins play an important role in mammalian host defence against *Salmonella* infection. Those authors demonstrated that transgenic mice expressing a human intestinal defensin (HD-5) were more resistant to oral challenge with *S.* Typhimurium than control mice. Crouch *et al.* (2005) demonstrated that the alternative sigma factor σ^E is required for *Salmonella* resistance to killing by the bactericidal/permeability-increasing protein (BPI)-derived peptide P2 and the murine α-defensin cryptdin-4 (Crp4). Their experiments with immunocompromised gp91$phox^{-/-}$ mice that lack a functional NADPH phagocyte oxidase suggest that σ^E plays an important role in resistance to non-oxidative mucosal host defences such as antimicrobial peptides (Crouch *et al.*, 2005). Further research is necessary to investigate other molecular mechanisms employed by *Salmonella* to tolerate host-produced antimicrobial peptides in the intestine. It is possible that the systems that have been shown to be important for the tolerance of antimicrobials in the macrophage phagosome, such as PhoPQ, PmrAB and RcsCDB (Bader *et al.*, 2003; Farris *et al.*, 2010), might be involved. *Salmonella* may also have to tolerate antimicrobial peptides produced by gut bacteria, i.e. bacteriocins. The systems used by *Salmonella* to sense and respond to bacteriocins have not yet been investigated.

Conclusions

Salmonella must be able to cope with stresses encountered in its various ecological niches as well as in the gastrointestinal tract of its hosts in order to persist and cause disease. Following food consumption, pathogenic bacteria are exposed to the extreme stomach pH, and later to bile salts, high osmolarity and low oxygen tension in the intestine. Furthermore, the competition for nutrients with gastrointestinal commensal bacteria and the production by the latter of bacteriocins and metabolites with antimicrobial activity also represent a significant challenge. Therefore, in addition to the 'true' virulence factors (e.g. invasins and toxins), other factors involved in environmental stress management (more correctly regarded as 'niche factors') may also be considered as directly influencing the outcome of the infection.

The recent availability of numerous genome sequences and the development of novel molecular methodologies have led to an increasing number of genomics-driven transcriptomics and proteomics studies, from which the functions of different genes and macromolecules have been predicted. Among the genes described as important for the gastrointestinal phase of salmonellosis, some act as global regulators of the *Salmonella* stress response, since they are induced by a wide spectrum of stress conditions and control the expression of numerous genes that mediate the adaptation to suboptimal environments and govern virulence. This suggests that *Salmonella* utilizes common adaptive stress pathways in response to a diverse range of environmental conditions, and indicates the existence of a coordinated response to the great variety of insults that *Salmonella* overcomes on its way through the gastrointestinal tract. Furthermore, the regulatory connections between some of these stress-induced genes and genes located in SPI-1 and -2 highlight the importance of mounting an orchestrated and functional stress response for bacterial invasiveness and survival within host cells.

The recent advances in the understanding of the survival strategies of *Salmonella* in the host may lead to the design of more ambitious strategies for the prevention of human and animal salmonellosis. During the last few years the search for biomarkers for bacterial resistance and virulence has emerged as an indispensable tool for the design of rational therapeutic and diagnostic strategies. This article describes several gene candidates which could be considered as biomarkers, or as therapeutic targets, or alternatively for the development of live attenuated vaccines. The next few decades will be an exciting phase in the battle to control salmonellosis.

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