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Salmonella stress management and its relevance to behaviour during intestinal colonisation and infection

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Abstract

The enteric pathogen *Salmonella enterica* is exposed to a number of stressful environments during its life cycle within and outside its various hosts. During intestinal colonisation *Salmonella* is successively exposed to acid pH in the stomach, to the detergent-like activity of bile, to decreasing oxygen supply, to the presence of multiple metabolites produced by the normal gut microflora and finally it is exposed to cationic antimicrobial peptides present on the surface of epithelial cells. There are four major regulators controlling relevant stress responses in *Salmonella*, namely RpoS, PhoPQ, Fur and OmpR/EnvZ. Except for Fur, inactivation of genes encoding the other stress regulators results in attenuated virulence and such mutants can therefore be considered as vaccine candidates. In contrast, a decrease in oxygen supply monitored by Fnr and ArcAB, or oxidative stress controlled by OxyR and SoxRS is not regarded as a stress associated with host colonisation since inactivation of either of these systems does not result in reductions in colonisation. The role of quorum-sensing through *luxS* and *sdiA* is also considered as a regulator of virulence and colonisation.
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Keywords: *Salmonella*; Acid resistance; Bile; Cationic antimicrobial peptide; Heat shock; Virulence; Quorum sensing; Short chain fatty acid

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1. Introduction

An enteric bacterial pathogen such as *Salmonella* is exposed to a number of stressful environments during its life cycle and the ways in which it responds to different and multiple stresses are correspondingly complex. Stress occurs when the bacterial cell experiences sudden changes in its environment. Under laboratory conditions *Salmonella* may experience stress naturally when nutrients or electron acceptors become limited and *Salmonella* enters the stationary phase of growth. Outside the laboratory, in the organism's real life, this may happen whenever *Salmonella* enters a host from the environment but also when *Salmonella* leaves the host into the environment. Genes and proteins identified as being central to the mechanism employed by the bacterium to cope with stress, particularly those involved in first contact with the host, may act as potential targets for immune intervention. The major stress factors and the genes and proteins required for control of stress management by *Salmonella* are the subject of this review. Naturally, different serotypes (or even different strains within a given serotype) may respond differently to various stresses. Therefore, if not specifically stated, information given in this review was obtained by experiments with the two most frequent serovars Typhimurium and Enteritidis and may not necessarily be representative of other serotypes.

2. Stresses encountered by *Salmonella* on entering the host – resistance to low pH

When *Salmonella* enters a host, it first senses an increase in temperature followed by a dramatic change in pH. In the stomach, the pH suddenly drops to values which may approach 1–2, although locally the pH can be higher as a result of the buffering capacity of feed. From the point of view of acid stress adaptation, animal hosts may be divided into those with simple or more complex stomach systems, exemplified for the purposes of this review by mice, pigs and humans, and on the other hand gallinaceous birds. In non-ruminant mammals, *Salmonella* and other bacteria pass immediately to the stomach on ingestion. In gallinaceous birds including the chicken, *Salmonella* first reaches the crop where the pH is between 4 and 5, as a result of bacterial lactic acid fermentation, a pH which enables *Salmonella* to adapt to much higher ΔH^{+} and thereby resist the antibacterial effects of the stomach.

Salmonella is relatively resistant to low pH when in the stationary phase of growth [1]. When growing exponentially, however, it is less acid resistant and can survive exposure only to moderately low pH values of between 4 and 5. However, in both the cases, *Salmonella* can become more acid resistant after a short period of adaptation at moderate pH. This phenomenon is called the acid tolerance response (ATR) [1–4]. When this occurs in exponentially growing cells, there are two distinct steps to adaptation. Transient adaptation is achieved after 20 min exposure to moderate pH with a second level of sustained adaptation requiring ≈60 min of exposure.

The proteins and genes induced by *Salmonella* in response to low pH can be identified by a number of techniques, including the generation of random promoter fusions and selection of the promoters of genes induced specifically under low pH. This approach has allowed the identification of genes coding for proteins related to cell–surface structure and maintenance (*aas*, *pbpA* and *cld*), stress response (*dps* and *rna*) and generalised efflux pump *mar* and *emr* [5]. However, although individual effector proteins are important for acid survival, regulatory proteins are equally important. *Salmonella* harbours several regulons which enable it to adapt to acidification, especially those controlled by RpoS, Fur, PhoPQ, and OmpR/EnvZ. Two of them, PhoPQ and OmpR/envZ, are two-component signal transduction systems while the other two genes/proteins operate apparently individually in the bacterial cytoplasm. *rpoS* and *fur* are essential for response to low pH induced by organic acids in log phase while the *phoPQ* system is tuned to response to inorganic low pH stress [6]. *ompR*/*envZ* is necessary for acid resistance in stationary phase cells [7,8]. An additional gene, *oxrG* is also involved in regulation of the low pH response although almost nothing is known about its function [9].

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2.1. RpoS and the acid stress response

RpoS is one of the sigma subunits of RNA polymerase. It was first described in *Escherichia coli* and subsequently in *Salmonella* where, in addition to its association with the starvation stress response and virulence it was found to be associated with acid response [10]. RpoS binds the core RNA polymerase primarily under stress conditions and controls the expression of a specific subset of genes which increases resistance to a variety of stresses. In vitro RpoS is expressed in stationary phase during nutrient limitations [11] or in a low pH environment [12–14]. RpoS is known to be expressed also in vivo in the eucaryotic intracellular environment [13]. Genes known to belong to the RpoS regulon in *Salmonella* include *spv* [15–17], *ots* [18], *katE* [13], *poxB* and *ogt* [19], or *narZYWV* [20]. There are also more than 10 other open reading frames (ORFs) of unknown function regulated by RpoS in stationary phase of growth [19] and 7 other loci regulated after exposure to low pH [14]. As a consequence, *rpoS* mutants are defective of prolonged survival in nutrient-depleted media or survival in low pH environment. Genes regulated by RpoS in *E. coli* are better described [21] and it can be expected that majority of genes regulated by RpoS in *E. coli* will be regulated in the same manner in *Salmonella*.

The *rpoS* regulon in *Salmonella* in stationary phase is responsible for stress tolerance including resistance to pH. RpoS is also involved in the log phase *Salmonella* acid tolerance response. To observe the log phase *rpoS*-dependent acid resistance, the adaptation period must last for at least 60 min. During such a period of adaptation (upto 120 min), a subset of about 50 proteins is induced [22], seven of which are RpoS-dependent [14]. Shorter moderate acid adaptation also increases total acid resistance of *Salmonella* but this process is *rpoS*-independent and requires functional Fur protein (see below). During the adaptation period, the intracellular level of RpoS increases. Because RpoS competes for the core RNA polymerase with the other sigma subunits [23], when more RpoS is available in the cytoplasm, more RNA polymerase interacts with it resulting in greater induction of the RpoS regulon.

Consistent with the central role of *rpoS* in the stress response in *Salmonella* is its complex regulation. Total *rpoS* expression is controlled at all levels starting from transcription [24–26], regulation of mRNA stability, translational efficiency, and regulation of RpoS proteolysis. Stability and efficiency of translation of *rpoS* mRNA is controlled by small regulatory RNAs such as DsrA or RprA [27–29]. Interestingly, the DsrA RNA in conjunction with Hfq can positively regulate RpoS translation but suppress H-NS expression by stabilisation of *rpoS* mRNA but increasing turnover of *hns* mRNA [27]. In log phase acid-shocked *Salmonella*, the level of RpoS can increase by increased translation through a mechanism independent of DsrA and RprA. The 566 nt untranslated 5′ end region (UTR) of the *rpoS* mRNA controls acid shock-induced translation. Except for the initial 51 nucleotides of the mRNA, the remaining part of the untranslated region is essential for acid induced increases in translation, probably due to the formation of competing stem loop structures of UTR of *rpoS* mRNA in response to acid shock [30]. Thus, on passage through the stomach transcription of the whole set of *rpoS*-regulated genes is likely to be initiated, with associated increases in resistance to a number of other stresses. The pH of the small intestine is between 6 and 7, due to the presence of organic acids including short chain fatty acids (SCFA) produced by the normal microflora, mainly lactic acid bacteria and buffered by bicarbonate ions. These acids can be toxic to *Salmonella*; however, acid tolerance, induced in the stomach, also protects *Salmonella* against their action [31]. As a corollary, contact with increased concentrations of SCFA also results into increased acid resistance in *Salmonella* [32] which may, thus, maintain a degree of acid resistance prior to entry of *Salmonella* into host cells and the phagolysosome where pH values are around 5. Interestingly, SCFA also induce expression of the *spv* genes [33], known to be necessary for *Salmonella* intracellular survival. *Salmonella* can therefore utilise changes in pH to monitor the environment and modulate the infection process.

RpoS levels can also increase by decreased protein degradation. This is dependent on the ClpXP protease complex and MviA (RssB) [12,34,35]. RssB is a response regulator in which the phosphorylated form exhibits a high affinity for RpoS and makes it available to proteolytic degradation by ClpXP [36]. Mutants with inactivated *mviA* (*rssB*) or *clpP* thus accumulate higher levels of RpoS even in non-stressed cells and are generally more acid resistant [12].

rpoS null mutants are attenuated for mice both after per oral and intraperitoneal routes of infection [37]. Nickerson and Curtiss found that RpoS-regulated genes are essential for colonisation of gut-associated lymphoid tissue despite the fact that the *rpoS* mutant colonised the gut as efficiently as did the wild-type strain [38]. This would indicate that *rpoS* mutants are attenuated mainly due to the regulation of *spv* genes during the systemic phase of disease and not due to decreased acid resistance during passage through stomach. Consistent with this are observations showing that *ClpP* or *MviA* mutants, with increased RpoS levels and increased acid resistance, are also of attenuated virulence for mice [12,39]. In chickens the situation seems to be different from mice. We did not find a difference in virulence of the wild-type strain and *rpoS* mutants of *S. typhimurium* and *S. enteritidis* for chickens after oral infection [40]. Another experiment showed that of three wild-type strains with

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a naturally defective *rpoS* gene, only two were attenuated for chickens while one of them was fully virulent [41]. Whether acid pre-adaptation of *Salmonella* in the crop of birds, which is missing in mammals, plays any role in these apparently contradictory results in mice and chicken remains unclear.

2.2. Fur and the acid stress response

The Fur protein is usually linked to the regulation of bacterial iron metabolism. Upon complexing with Fe^{2+}, Fur recognises a specific DNA sequence, the *fur* box and binds to it. The consensus *fur* box is 19 bp long GATAATGATAATCATTATC sequence and it is most frequently found between the −35 and −10 sequences of sigma 70 promoters. When Fe^{2+}–Fur is bound to the *fur* box, gene transcription is prevented. If iron becomes limiting, Fe^{2+} dissociates from the complex making Fur unable to bind and thus allowing the transcription of normally repressed genes. Because the Fur regulon includes genes for iron uptake and transport, such regulation results in increased expression of genes for the efficient iron uptake only when iron is a growth-limiting factor [42]. Despite the fact that Fur is primarily a negative regulator, it can also act as a positive regulator since at least nine proteins require the presence of both Fur and iron for their expression and six proteins require a functional Fur free of iron for their expression [43]. However, in such cases it is not clear whether Fur directly regulates such genes or whether this may be caused by the indirect action of Fur as a negative regulator of a second negative regulator.

Surprisingly, it was found that *fur* mutants show reduced adaptation to acidification [2,43]. Proteins regulated by Fur in response to iron starvation or low pH form two distinct clusters with the exception of seven proteins which are influenced by both these factors. Like RpoS, Fur is also involved in the acid tolerance response of log phase cells [14,43], and predominantly responds to organic acid stress [6]. Unlike RpoS, Fur is essential for rapid but transient induction of a set of proteins at ~pH 5 which allows *Salmonella* to survive subsequent challenge at pH 3. This set of proteins is induced 20–40 min post-exposure to pH 5 but it disappears after 60 min exposure, during which time RpoS-controlled systems of ATR are induced [14,22].

Why Fur integrates the iron and pH responses is not known. A possible explanation for this observation is that in the acid environment, Fe^{3+} is more easily available and consequently, intracellular iron concentration increases. However, experimental increases or decreases of iron at neutral pH did not result in increased acid resistance in either *Salmonella* or *Helicobacter pylori* [44,45], and pH-regulated genes do not respond to iron availability [9]. Finally, Hall and Foster showed that the iron and acid regulation of Fur can be separated genetically. Mutation H90R of the Fur protein sequence resulted in the mutant showing a deregulated iron response but still being capable of an acid tolerance response. Histidine 90 of the Fur primary structure is therefore indispensable for iron-dependent regulation but is not necessary for pH-dependent regulation [45].

None of this, however, explains why evolution selected for Fur sensing both iron concentration and pH. Although iron is the fifth most important ion for the living cell, its excess can be toxic. The primary reason for this is Fenton's reaction during which Fe^{2+} is responsible for generation of toxic hydroxyl radicals from hydrogen peroxide which can damage cellular structures [46]. Because exposure to low pH also leads to oxidative stress, it is thus possible that the unifying action of Fur lies in its protection against oxidative damage. This may be supported by observations in *E. coli* where levels of *fes* or *ydiE* mRNA, genes controlled by Fe–Fur, were found to be the same in *fur* mutants as in the wild-type strain when treated with H_2O_2 [47]. If oxidative stress is caused by excess of iron, this could lead to the cell attempting to limit iron uptake. Recently, Fur has been associated also with the response and resistance to nitrosative stress [47,48]. Furthermore, a number of Fur protein molecules per bacterial cell (5000–10,000) is much more than that of typical transcriptional regulators [49]. It is therefore possible that the function of Fur is slightly different from more usually understood regulatory proteins. Little is known about identity of acid-induced Fur-regulated genes [9]. As a result of this, it would be interesting to compare the promoter sequences of known Fur-regulated genes responsive to iron restriction, acid pH and nitrosative stress and analyse the presence or absence of *fur* boxes in them. And the most surprisingly, despite the in vitro results showing clearly a role for Fur in different stress responses, a *fur* mutant of *S. typhimurium* SL1344 was only weakly attenuated both after per oral and intra-peritoneal application [50]. Although question remains to what extent *S. typhimurium* SL1344 is suitable for creating *fur* deletions since the SL1344 clone available in our laboratory, at least, is of quite an unusual morphology (Fig. 1) and since *fur* mutants are known to form filamentous cells [46], it cannot be excluded that SL1344 is naturally *fur* deficient or *fur* misregulated.

2.3. PhoPQ and the acid stress response

phoPQ is a two-component signal transduction system present not only in *Salmonella* [51] but also in *E. coli*, *Shigella* and *Yersinia* [52]. PhoQ is a membrane-bound sensor protein and PhoP is the transcriptional regulator. PhoQ senses Mg^{2+} and Ca^{2+} concentration and when these decrease to micromolar levels, it phosphorylates PhoP [53,54]. The PhoPQ regulon consists of ≈40 proteins, most of which are

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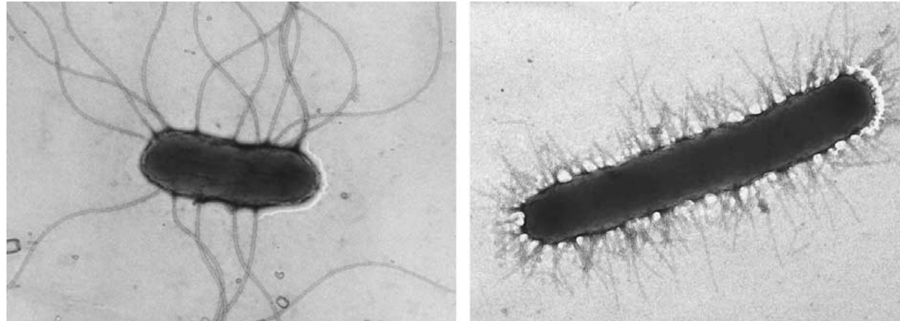

Fig. 1. Phosphotungstic acid stained 24 h old cultures of *S. typhimurium*. Left panel: typical shape of *S. typhimurium* LT2 cell. Right panel: elongated cell of *S. typhimurium* SL1344. Figures kindly provided by P. Kulich, VRI Brno, Czech Republic.

positively regulated. PhoP-suppressed genes include *prgH* and *fliC* [55–57], PhoP-activated genes include *pmrAB*, another two-component signal transduction system involved primarily in the protection of *Salmonella* to cationic antimicrobial peptides (CAMP, see below) [58], *mgtA* and *mgtCB* encoding Mg^{2+} transport systems, *phoN*, a periplasmic non-specific acid phosphatase, *pcgL* encoding a periplasmic D-Ala–D-Ala dipeptidase, or *pagL*, *pagP* and *pgtE* which contribute to increased resistance to CAMPs [59–62].

The *phoPQ* regulon is essential for *Salmonella* intracellular survival where it increases resistance to antimicrobial peptides [63], suppresses SPI-1 encoded genes necessary for *Salmonella* entry to non-professional phagocytic cells [56,57] and contributes to the expression of SPI-2 genes [64] although the SPI-2 regulation is confusing and at least some of the SPI-2 genes can be expressed independently of PhoPQ [65].

Although the main signal which controls expression of the *phoPQ* regulon is low Mg^{2+} concentration [62], moderate pH and low ionic strength are also known to influence expression of this regulon [66]. *phoP* was among the first genes shown to be involved in the acid tolerance response as a mutation in *phoP* eliminated adaption to low pH [3]. Later it was shown that the main role of the *phoPQ*-dependent acid tolerance response is *Salmonella* protection to inorganic acid stress [6]. This is consistent with PhoPQ activation within the *Salmonella*-containing vacuole, the environment of which is expected to be of relatively low complexity and in which acid stress can be caused by an increase in H^+ concentration.

2.4. OmpR/EnvZ and the acid stress response

The OmpR/EnvZ signal transduction system is usually associated with osmolarity-dependent regulation of the OmpC and OmpF porins. Under low osmolarity, OmpF is preferentially synthesised and under high osmolarity OmpC is one of the major outer membrane proteins [67]. The changes in osmolarity are sensed by EnvZ. In the presence of a signal, EnvZ autophosphorylates at histidine 243 and transfer the phosphate to aspartate 55 of OmpR [68]. Phosphorylated OmpR binds to DNA and activates transcription of target genes. *ompC* was found to be induced also upon *Salmonella* shift to low pH and such induction was dependent on OmpR [9]. This effect is also common to *E. coli* [69]. Interestingly, EnvZ was not absolutely necessary for OmpR-dependent acid induction of *ompC* and *ompF* [69] suggesting that unlike osmolarity, the EnvZ sensor is not required for full expression of the pH-dependent OmpR regulon. In *Salmonella*, *ompR* has been shown to be induced by low pH and the induction could be observed at the mRNA as well as the protein level [8]. *ompR* can be transcribed from two promoters, one being used primarily at neutral pH and the other at low pH [7]. Our results with a *ompR-luxCDABE* promoter fusion also show that in *S. typhimurium* expression of OmpR responds to increases in osmolarity only to a small extent while it is induced more than tenfold in response to acidification (Rychlik and Gregorova, unpublished). At moderate pH values of around 5.8, phosphorylation of OmpR is dependent on EnvZ whereas at lower values down to pH 4, phosphorylation becomes dependent mainly on acetyl phosphate [8]. Besides regulation of target genes, the phosphorylated OmpR also binds to its own promoter and thus further stimulates its own transcription [7]. Unlike RpoS and Fur which are important to the adaptation of log phase *Salmonella* cells to changes in external pH, OmpR is central to stationary phase-inducible acid tolerance [1]. *ompR* mutants are capable of adapting to low pH in log phase growth but are unable to improve their low pH fitness in stationary phase [8,70].

Interestingly, OmpR/EnvZ also regulates both SPI1 and SPI2 encoded genes. SPI1 genes are regulated through the regulator HilA [71] and SPI2 genes are regulated through the SsrAB regulators [72,73]. Acid shock in the stomach can be translated through OmpR into the induction of SPI1 genes essential for the invasion of epithelial cells. pH values approaching 5 inside the *Salmonella* containing vacuole can be translated by OmpR into SPI2 induction. Organic acid acidification should

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Table 1
Acid stress response regulators

Acid stress	Status of a cell	Adaptation	Central regulator	Role in
Inorganic acid			PhoPQ	Virulence
Organic acid	Stationary phase		OmpR	Virulence
	Exponential phase	20 min at pH 5	Fur	Stress
		60 min at pH 5	RpoS	Stress
?	?	?	OxrG	?

Acid induction of *phoPQ* and *ompR* influences also the regulation of *Salmonella* virulence factors.

lead primarily to OmpR activation of *hilA* while inorganic acid stress should lead to *ssrAB* activation inside the eucaryotic cell in the nutrient-deprived environment. Thus OmpR/EnvZ, in addition to PhoPQ, seem to convert pH decreases into the regulation of different virulence factors while the primary role of *rpoS* and *fur* appears to be protection against acid stress itself (Table 1).

3. *Salmonella* inside the gut

When *Salmonella* leaves the stomach it becomes exposed to several new stresses to which it must respond to survive. It is exposed to multiple external stress factors, including increase in osmotic pressure, which alters folding of proteins present in the outer membrane and periplasm. Outer and cytoplasmic membranes are subjected to the action of bile secreted by the gall bladder into the duodenum. In addition oxygen concentration gradually decreases and enteric pathogens induce anaerobic respiration [74,75]. Inside the small and large intestine, *Salmonella* has to cope increasingly with the presence of other microorganisms, components of the normal microflora. The microflora is able to passively restrict *Salmonella* growth by creating a nutrient-depleted environment, by releasing by-products of their metabolic activities such as propionate or butyrate, which can be harmful to *Salmonella*, or by production of bacteriocins. *Salmonella* is also able to sense the presence of other bacterial species via quorum sensing communication pathways.

3.1. Heat and outer membrane shock

Since the optimal growth temperature for *Salmonella* is 37 °C, the mammalian body temperature probably does not induce the expression of stress regulatory pathways. This may, however, be the case when *Salmonella* infects avian hosts, including chickens, in which the body temperature is nearer 42 °C. When *Salmonella* is exposed to temperatures exceeding 40 °C, heat shock proteins are induced. The promoters of genes coding for the heat shock proteins differ considerably from the sigma 70 promoters and contain the consensus sequence CTTGAAA at position −35 and CCCCAT at −10 relative to the transcriptional start. Such promoters are recognised by the alternative sigma factor, sigma H, the heat shock-specific sigma subunit of RNA polymerase [76–78]. Heat shock proteins belong to two main classes – chaperones required for folding and/or refolding of misfolded proteins, and proteases which degrade misfolded proteins. The former group includes GroEL/ES, DnaK/DnaJ and Ags chaperones [79–81], the latter group includes proteases such as HtrA or ClpP [82,83]. Despite the different function of chaperones and proteases, both help to maintain protein functionality under stressful conditions. Although these proteins are frequently linked with heat shock, they are in fact essential for protein repair under all stressful conditions. It is not surprising, therefore, that mutants in protein repair are frequently attenuated in virulence for mice, primarily because of their decreased ability to resist the bactericidal agents produced by macrophages [80,84–86].

Heat shock can be understood as a stress inflicted on the bacterial cell from the outside and thus occurring on the outer side of cytoplasmic membrane. Besides sigma H, *Salmonella* encodes another alternative sigma factor, sigma E which is essential for *Salmonella* survival under extra cytoplasmic stress. The origin of such stress can be different, either heat or cold shock [87], activity of antimicrobial peptides [88] or oxidative stress [89]. RpoE is also necessary for the increased stress resistance associated with stationary phase cells [90] and consistent with this, the amount of RpoE increases in stationary phase cell. The signal for this is the presence of misfolded proteins. RpoE is therefore upregulated in *dsbA* or *sodCI* mutants [89] in which greater amount of misfolded proteins are expected. *rpoE* mutants are strongly attenuated for mice, both after per oral and intra-venous application. The level of attenuation is so great that infection with *rpoE* mutants does not even raise a protective immunity against subsequent challenge with the wild-type strain [88].

3.2. Osmotic shock

When *Salmonella* is shifted to an environment under high osmotic pressure, the bacterium aims to increase internal osmotic pressure to maintain cell turgor. This happens by the cytoplasmic accumulation of solutes which are tolerated by the whole cellular machinery.

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Potassium is the preferred ion for uptake by the cell [91]. The ionic balance is then balanced by intracellular synthesis of glutamate [92–94]. If the environment is limited in potassium or if the osmotic pressure cannot be regulated by potassium alone, *Salmonella* can increase uptake or biosynthesis of other osmoprotectants such as betaine (*N*,*N*,*N*-trimethyl glycine), proline and trehalose [92,95]. In a response to increased osmotic pressure, *Salmonella* can also modify the composition of its outer membrane. This process is sensed by a two-component signal transduction system consisting of EnvZ sensor and OmpR response regulator. As a consequence, in high salt concentration, the OmpF porin is replaced by the OmpC porin which forms outer membrane pores with a smaller diameter thus decreasing the influx of solutes into the periplasm [96,97].

3.3. Response to bile

Bile is produced in the liver and consists mainly of bile salts, cholesterol and bilirubin. Due to its strong detergent action against lipids, it also has a strong antimicrobial effect. Little is known about the effects of bile on *Salmonella*. It is thought that lipopolysaccharide (LPS) and active efflux of bile components out of the cell are the primary defense barriers against its action. The importance of LPS has been demonstrated [98,99]. Besides this, only a limited number of genes are known to contribute to bile resistance. This includes *phoP* [100], *tolR* [98] and *wec* [99]. *phoP* is a member of a two-component signal transduction system which is also involved in low pH resistance and resistance to cationic antimicrobial peptides. Resistance to these peptides is mostly dependent on lipid A modification of the outer leaflet of the outer membrane [101], which probably reduces permeability not only to CAMPs but also to components of bile. The *tol* genes encode an outer membrane protein the function of which is to maintain membrane integrity [102,103]. Tol also serves as a receptor for certain phages [104] and as a colicin transport channel [105,106]. The WecA and WecB proteins are responsible for the synthesis of the Enterobacterial common antigen, a glycolipid different from LPS present in the outer membrane. These findings suggest that the main defense of *Salmonella* is based on not allowing bile components to pass through the outer membrane.

A limited amount of bile probably crosses both the outer and cytoplasmic membranes and reaches the cytoplasm. Recently a mutant in *acrAB*, defective in an efflux pump was shown to be sensitive to low concentrations of bile [107] suggesting an alternative defense mechanism against bile. Bile also serves as a signal suppressing the invasion machinery of *Salmonella* [108]. It is expected that such regulation results in *Salmonella* not expressing genes from SPI1 as long as it remains localised in the lumen of the gut. Once *Salmonella* have efficiently interacted with the mucus layer where bile concentration is expected to be lower, the SPI1-encoded type III secretion system is induced and *Salmonella* becomes capable of invading epithelial cells.

In 1–3% of *Salmonella*-infected human individuals, infection of *Salmonella* serovars Typhi and Paratyphi results in a carrier state. In these cases, one of the most important sites for *Salmonella* survival appears to be the gall bladder. *Salmonella* may colonise the surface of gallstones forming a biofilm resistant to the inhibitory activity of bile. Genes essential for such colonisation include *galE*, *luxS* and those coding for flagella [109,110]. For flagella, it is expected that these allow *Salmonella* to come into contact with the gallstone as well as enabling contact between different *Salmonella* cells forming a biofilm. The function of the other two genes is unknown in this context. Experimental work with *S*. Choleraesuis, which does not generally colonise the chicken gut also showed preferential localisation in the gall bladder in the small number of chickens where establishment had taken place [111].

3.4. Switch from aerobiosis to microaerobiosis and anaerobiosis inside the gut

As *Salmonella* passes through the intestine, oxygen availability decreases and in the large intestine the environment is essentially anaerobic although this is less likely to be the case close to the mucosa. *Salmonella* is therefore required to gradually switch from aerobic to a predominantly anaerobic metabolism, rather as a natural response to a gradually changing environment than as a stress response. There are two major regulatory circuits, dependent on Fnr and ArcAB, respectively [112]. ArcAB represents a two-component signal transduction system while Fnr is a cytoplasmic protein reacting to subtle changes of oxygen concentration in the cytoplasm. Although the Fnr and ArcAB regulatory systems can work independently, they frequently operate in a coordinated fashion to control gene expression.

ArcB is an inner membrane sensor protein monitoring changes in the redox status of membrane located quinones [113]. After oxidation of two cysteine residues, ArcB autophosphorylates, transfers the phosphate to ArcA [114] and the activated ArcA-P controls gene transcription. ArcA is most active under microaerobic and anaerobic conditions [26,115] when it suppresses genes encoding enzymes of TCA cycle, probably to decrease respiration under less favourable conditions. This has two consequences, a decrease in the production of harmful oxygen radicals and saving endogenous energy sources [116]. The ArcAB system can also act as a positive regulator by the induction of *cydAB* (cytochrome d oxidase) involved in respiration under oxygen-limiting conditions, and of the *cob* and *pdu* operons important for cobalamin-dependent utilisation of 1,2-propanediol.

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In both these cases, ArcAB regulates expression of these target genes in association with Fnr [117–120], although in the case of *pdu*/*cob* the collaborative action of ArcAB and Fnr is indirect. *Salmonella* can synthetise cobalamin de novo generally only under anaerobic conditions and under such conditions, propanediol can be utilised only with tetrathionate as a final electron acceptor in anaerobic respiration. While the *cob*/*pdu* operons are controlled by ArcAB, the *ttr* operon, which encodes enzymes essential for tetrathionate respiration, is positively regulated by Fnr [120,121]. Propanediol utilisation under anaerobic conditions is therefore dependent on both ArcAB and Fnr.

Fnr, in *Salmonella* also called OxrA, is a cytoplasmic sensor of oxygen. It is a Fe–S [4Fe–4S] cluster protein and in the presence of oxygen this cluster is oxidised in two steps into the [2Fe–2S] form [122,123]. Fnr binds at promoter sequences usually at position −41 relative to the start of transcription, although it can also bind at position −61, −71, −81 and −91 depending on the particular promoter structure [124]. The sequence recognised by Fnr is palindromic ($TTGATN_4ATCAA$). When bound to this sequence, Fnr interacts with the RpoA subunit of RNA polymerase increasing the efficiency of transcription [125]. In *Salmonella* grown under anaerobic conditions, Fnr positively regulates expression of alternative terminal acceptors [126]. However, besides its role in anaerobic respiration control, Fnr also regulates expression of, amongst other things, the aminotripeptidase *pepT* [127] and one of the major porins in outer membrane, *ompD* [128].

Both ArcAB and Fnr, due to their regulatory activities, regulate production of and defense against reactive oxygen and nitrogen intermediates [116,129,130]. This would suggest that mutants in *arcAB* or *fnr* should show reduced virulence as such reactive species are experienced by *Salmonella* in the *Salmonella*-containing vacuole in eukaryotic cells. However, inactivation of neither *arcAB* nor *fnr* reduces virulence or the ability to colonise the host by *S. typhimurium* suggesting that these proteins and their regulons probably are not highly expressed in vivo [130,131]. Inactivation of *fnr* in *S. typhi* even increased invasiveness [132].

3.5. Interaction with gut microflora

When *Salmonella* colonises the gut, it interacts with the numerically dominant and highly complex microflora. All bacteria produce metabolites which can be inimical to other bacterial species. Such metabolites may be (i) simple metabolic byproducts (e.g., short chain fatty acids), (ii) metabolites produced deliberately to reduce growth of competing bacteria (e.g., colicins produced by *E. coli* and other related bacteria) and (iii) metabolites which modify their own metabolism according to the size of their own population (quorum sensing) or the presence of other bacteria. In addition, the mere presence of gut microflora may result in *Salmonella* experiencing difficulties with nutrient uptake and induction of the stringent response.

3.5.1. Species-specific growth inhibition

It is a matter of debate whether *Salmonella* colonising the gut resembles more closely exponentially growing cells or a culture in a stationary phase. A *Vibrio cholerae* culture inoculated into ligated ileal loops was replicating exponentially for upto 8 h post-inoculation but soon after, the culture appeared to be in stationary phase [75]. Resistance to stresses associated with stationary-phase growth has been largely associated to RpoS-dependent mechanisms [21]. However, stationary-phase growth, defined as the cessation of increase in numbers, can be reached either by insufficient carbon source allocation or due to the lack of electron acceptors [26]. Actually, in nutrient-rich media in vitro, stationary-phase metabolism is usually *rpoS*-independent [133]. Instead, systems involved in nutrient uptake (*tdcC, fliM*, *yhjH, crp*) and microaerophilic respiration (*nuo* and *cyd* operons, *arcA*, *aroA*, *aroD*) are central to stationary phase as inactivation of either of these genes results in growth non-suppressive (GNS) phenotype [131,133–136]. GNS mutants are unable to suppress multiplication of the wild-type

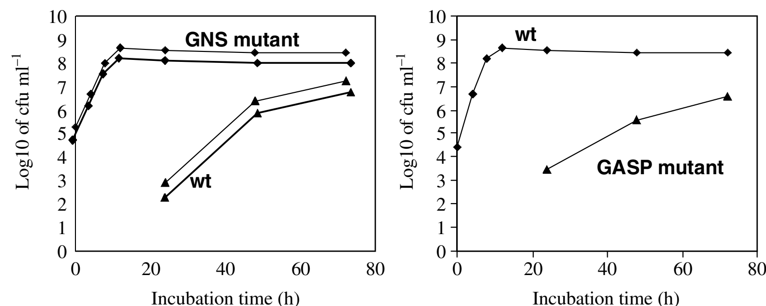

Fig. 2. Growth inhibition in stationary phase cultures of *Salmonella*. GNS (growth non-suppressive) mutants are unable to suppress multiplication of the wild-type strain when this is inoculated in their stationary phase culture (left panel). GASP (growth advantage in stationary phase) mutants are capable of growing through the stationary culture of the wild-type strain.

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strains when these are inoculated into the mutant's stationary phase culture (Fig. 2). However, most of the GNS mutants had no effect on the ability to compete with a parental strain in the intestine of newly hatched chickens [131] suggesting that the redox and nutritional conditions in the gut were different and possibly more anaerobic. Therefore, a further completely anaerobic in vitro screen for such mutants was performed which identified a role for *flhA*, *aspA* and *dcuAB* in *S. typhimurium* [137] and *dapF*, *aroD*, *sgaT* or *tatA* in *S.* Hadar [138], most of them related to nutrient uptake or anaerobic respiration. However, even in this case such mutants were fully competitive in vivo (with the exception of *dapF* in *S.* Hadar), suggesting that respiration is likely to be less important in the gut of the newly hatched chicken than substrate-level phosphorylation. A fermentative process is thus likely to be a major contributor to energy balance in the gut, as shown by the total inability of *ackA* and *pta* mutants to colonise (Barrow & Lovell, unpublished results). What therefore is the role of electron transport in the life of *Salmonella* since it is assumed, that little active growth occurs in the environment? Given the non-lactose fermenting nature of the vast majority of *Salmonella* serovars, the original source of these organisms may be reptilian. Since respiration contributes considerably to energy balance in the tissues of the warm blooded animals, it may be that this contributes to *Salmonella* stimulating an active ejection process (gastro-enteritis) after colonisation of the gut of apparently the ''wrong'' host.

3.5.2. Stringent response

When *Salmonella* experiences nutrient depletion intracellular concentrations of ppGpp increase. This metabolite serves as an alarmone and it is one of many factors involved in the expression of *rpoS*. ppGpp is produced by RelA and SpoT. The RelA protein is associated with 1–2% of ribosomes and senses the amounts of discharged tRNAs coming into contact with the ribosome. If the ratio of discharged:charged tRNAs increases, RelA catalyses ppGpp production. The SpoT protein also senses the ratio of charged:discharged tRNAs and as a result of this, produces ppGpp. SpoT is also thought to be involved in ppGpp degradation. When the concentration of ppGpp increases, synthesis of non-coding RNA (e.g., rRNAs and tRNAs) is suppressed and expression of genes coding for enzymes catalysing amino acid biosynthesis is induced [21,139]. ppGpp also stimulates expression of the alternative sigma subunit of RNA polymerase, RpoS [140]. *Salmonella relA spoT* double mutants are highly attenuated for mice [141,142].

3.5.3. SCFA

Exposure to short chain fatty acids (SCFA), namely acetate, propionate and butyrate is one of the stresses which *Salmonella* experiences when colonising the intestinal tract. *Salmonella* may experience these acids first in the crop. Unlike the intestine, the pH in the crop is relatively low (see above), and under these conditions, SCFA may induce acid tolerance [32,143] prior to entry into the gizzard (stomach). In *E. coli*, the genes induced by SCFA overlap with those of the RpoS regulon although induction of *rpoS* itself is not enough for the increased acid resistance observed since *rpoS* induced by osmotic shock by NaCl or sodium acetate did not protect *E. coli* from subsequent exposure to pH 3 [143]. PhoP, another regulatory protein involved in acid tolerance, is not involved in induction of acid tolerance by exposure to SCFA as its deletion can lead to even greater survival in *Salmonella* at low pH, consistent with its role in inorganic acid stress response described above [6].

Individual or mixed SCFA decrease *Salmonella* growth rate in vitro [144]. This effect correlates with the pH of the environment – each SCFA being more effective at pH 5 or 6 than at pH 7 [145]. Individual SCFAs differ in their effects on *Salmonella* invasion of epithelial cell lines. Butyrate suppresses while acetate seems to stimulate invasion of tissue culture cells [146,147]. Acetate, but not propionate or butyrate, was shown to induce *hilA* and *invF* regulators of SPI1 at neutral pH which may explain different invasiveness of *Salmonella* grown in the presence of different SCFAs. These in vitro studies are consistent with the results of experimental infections of birds fed different SCFAs. Feed enriched with acetate resulted in increased *Salmonella* colonisation of the host while butyrate-fed birds were more resistant to intestinal colonisation by *Salmonella*, despite the fact that such treatment did not influence *Salmonella* invasion into deeper tissue such as liver and spleen [148].

3.5.4. Bacteriocins

Bacteriocins are peptides of microbial origin which are produced by both gram-positive and gram-negative bacteria, including major components of the gut flora such as *Lactobacillus* sp., *Enterococcus* sp. and *E. coli* [149,150]. Production of bacteriocins by particular microorganisms is frequently linked with their ability to decrease *Salmonella* colonisation of experimental animals [151] although much earlier work tended to suggest that they were unimportant. Production of colicins encoded by the ColV plasmid increases colonisation ability in *E. coli* but this may be related to iron acquisition genes [152–154]. However, essentially nothing is known about *Salmonella* defense against bacteriocins. Overexpression of the *mar* locus, encoding an efficient efflux pump, results in increased resistance to microcin 24 [155]. It may be speculated that, because some of the bacteriocins utilise iron uptake receptors for their transport into a cell [156–158], expression of the Fur regulon in *Salmonella* may produce increased or decrease resistance of *Salmonella* to such bacteriocins. However, whether the presence of sublethal levels of bacteriocins

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is sensed and by what mechanisms and whether this results in increased bacteriocin resistance of *Salmonella* similarly to acid tolerance response, or whether such stress is translated into a resistance against other forms of stress or suppression of *Salmonella* virulence factors, is completely unknown.

3.5.5. Quorum sensing

Numerous bacterial species have been shown to be able to sense the density of their own population through the production and perception of specific metabolites termed autoinducers. When the autoinducer concentration reaches a threshold, when the bacterial population reaches a certain quorum, specific metabolic pathways are induced. In gram-negative bacteria two main systems are used, utilising either autoinducer 1 (AI-1) or autoinducer 2 (AI-2). AI-1 is an acyl homoserine lactone and is generally species specific. AI-2 based quorum sensing is dependent on the production of a furanone-like compound and is believed to be used for wider interspecies communication as AI-2 activity has been detected in many different bacterial species. The AI-1 dependent system typically consists of a LuxR sensor and regulator, and LuxI, the AI-1 synthase. A key protein for the synthesis of AI-2 is LuxS. Interestingly, *S. typhimurium* harbours in its genome *luxR* and *luxS* homologues but no *luxI* homologue. This suggests that quorum sensing in *Salmonella* may be different from other bacterial species.

3.5.5.1. Quorum sensing in Salmonella *and* sdiA. In *S. typhimurium* the homologue of *luxR* is *sdiA*. However, there is no obvious homologue of the AI-1 synthase *luxI* in the *S. typhimurium* genome. This, together with sequence analysis, led to the conclusion that *sdiA* was acquired by *E. coli* and *S. typhimurium* by horizontal transfer [159] which may explain some of the unusual properties of *sdiA* described below, mainly its ability to react with autoinducers produced by other bacterial species. Simultaneously, horizontal transfer without the *luxI* homologue would enable separate evolution of *sdiA* resulting in its unique characteristics in *Salmonella*.

The *sdiA* gene was first described in *E. coli* as a suppressor of the division inhibition effect in a *minCD* mutant. Suppression was obtained after overexpression of *sdiA* from a multicopy plasmid leading to overexpression of the *ftsQAZ* locus which resulted in conversion of aberrant filamentous cells back to the typical rod shape [160,161]. In parallel, a search for an *E. coli* autoinducer was made. Surprisingly, there were reports on both SdiA-dependent up- or downregulation of the *ftsQAZ* promoter by spent (conditioned) medium obtained from *E. coli* cultures [161,162]. However, these effects were quite weak and the induction or suppression was never greater than twofold. In *E. coli*, SdiA was also shown to contribute to virulence gene regulation in enterohaemorrhagic strains of serotype O157:H7 [163]. The same authors also noted that upon overexpression of *sdiA*, motility in 0.25% agar decreased.

Overexpression of *sdiA* in *E. coli* also led to increased resistance to xenobiotics through the activation of the AcrAB multidrug efflux pump [164,165]. Genome-wide microarray analysis in *E. coli* confirmed these observations showing that *sdiA* overexpression leads to increased expression of *ftsQAZ*, *acrAB* and suppressed flagella expression [166].

sdiA in *Salmonella* was first described by Ahmer et al. [167] when this group suggested a link between *sdiA* and positive regulation of ten genes on the virulence plasmid including the previously characterised *rck* gene responsible for increased resistance to complement killing and adhesion to epithelial cells [168]. Although *sdiA* in *S. typhimurium* seems to be suppressed by conditioned medium to the extent observed in *E. coli* [169], no AI-1 like metabolite was ever detected also in *S. typhimurium* conditioned media. Ahmer and his colleagues therefore suggested that SdiA may sense autoinducers produced by other bacterial species [170,171]. In parallel to these studies, we showed that a *sdiA* mutant is not defective in stationary phase survival [131] but is of increased virulence for mice [169]. The *fur* box 19 bp upstream from the *sdiA* start codon was also identified and its function in relationship to iron deprivation by dipyridyl was shown [169]. Because the Fur protein which binds the *fur* box is also involved in acid resistance [43], we extended our experiments and found that *sdiA* is induced in *S. typhimurium* when the bacterial culture is inoculated in LB at pH 4 under fully aerobic conditions [172]. SdiA in *Salmonella* and *E. coli* may therefore integrate several external stimuli. It can sense sudden reductions in pH. Upon its upregulation, the AcrAB efflux pump is induced [165,166] possibly increasing resistance of *Salmonella* to bile. When acid stress is alleviated after leaving the stomach, induced SdiA may react with autoinducers produced by other bacteria present in the gut and may induce *rck* resulting in increased attachment to epithelial cells. This would be particularly important in the distal parts of the ileum where M cells are concentrated.

3.5.5.2. Quorum sensing in Salmonella *and* luxS. Numerous gram-positive as well as gram-negative bacteria including *Salmonella* can produce AI-2 [173,174]. *luxS* gene was identified as essential for AI-2 production in *E. coli* and *S. typhimurium* [175]. The highest AI-2 production by *S. typhimurium* is observed in late exponential phase and after entry into stationary phase the AI-2 is degraded. The presence of glucose in nutrient rich media, low pH and high osmolarity stimulates AI-2 production and release from *Salmonella* cells [176,177]. The exact biological function of LuxS and

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AI-2 in *Salmonella* is unknown. In *E. coli* and *Shigella* the function of AI-2 has been investigated in greater detail. In *E. coli*, the type III secretion systems encoded by the LEE1 and LEE2 loci are stimulated threefold by the addition of conditioned medium. However, no experimental animal infections were carried out using *E. coli luxS* mutants [178,179]. In *Shigella*, conditioned medium also stimulated virulence-related genes including *virB* but invasion of a *luxS* mutant in tissue culture was unaffected. Similarly, *luxS* mutants of *Shigella* were capable of causing keratoconjuctivitis in the Sereny test in guiney pigs [180].

3.5.5.3. Biological significance of quorum sensing in Salmonella. The biological function of quorum sensing in *Salmonella* remains unclear. Initially, it was speculated that quorum sensing may prevent full expression of virulence before the size of the bacterial population becomes high enough to successfully deal with the immune system of the host [181,182]. However, recent findings in *V. cholerae* showed that quorum sensing may act also to downregulate virulence after successful colonisation of the host to decrease continued damage to the host [183]. Interestingly, it has been shown that Hha, a negative regulator of *hilA* in *S. typhimurium* [184], is induced tenfold upon exposure of *E. coli* to conditioned medium containing AI-2 [185]. Since *hilA* is a central regulator of *Salmonella* invasion into the epithelial cells, quorum sensing may act as a negative regulator of virulence also in *Salmonella*. This can be further supported by the observation of slightly increased virulence of *S. typhimurium sdiA* for mice after per oral infection [169].

It is also not clear why LuxI homologues are missing from the *S. typhimurium* genome and whether *luxS* and *sdiA* interact in *Salmonella*. Overlap of AI-1 and AI-2 signalling has been documented in *V. harveyi* in which these two systems co-regulate bioluminescence [186,187]. However, the vast majority of bacterial species utilise exclusively either AI-1 or AI-2 based quorum sensing. Our recent observations show that *sdiA* in *Salmonella* is induced in environments at pH values lower than 5 and such induction was not observed in an *luxS* mutant [172]. Interplay between *sdiA* and *luxS* was described also in *E. coli* [179]. Consistent with our observation, several reports have appeared indicating that, in different bacterial species, *luxS* is induced upon acidification [177,188,189]. Next, quorum-sensing systems have been repeatedly shown to overlap with iron metabolism or uptake [190,191]. It is therefore possible that in *Salmonella* the quorum-sensing machinery, upon horizontal acquisition of the LuxR homologue only [159], evolved into a system for sensing of acidic environments and response to iron although the reasons for and consequences of this are currently unknown but are likely to be associated with colonisation and virulence. Acid-associated *sdiA* induction is definitively not a question of a mere survival since a *sdiA* deletion mutant survives as well as does the wild-type strain in LB, pH 4, for a week (I. Rychlik, unpublished observations).

3.6. Cationic antimicrobial peptides

After *Salmonella* passage through the acid stomach environment and the small intestinal environment rich in bacterial microflora, the microorganisms finally approach epithelial cells which may be protected by antimicrobial agents including cationic antimicrobial peptides (CAMP). CAMP are produced by mammalian, bird, insect and even plant cells in response to microbial infection. In all of these living systems, CAMPs represent a central component of the innate immune system which can protect cells against microbial infection within minutes of contact with infectious agents. CAMP are found on a number of mucosal surfaces including the epithelium of the respiratory and intestinal tracts [192] and are likely to affect colonisation and infection [193]. They are also present in azurophilic granules of neutrophils where they represent the most potent non-oxidative killing mechanism [194]. The production of some of them may be induced upon contact with *Salmonella* [195].

CAMP are peptides 15–50 amino acid long. They are active against both gram-negative and gram-positive bacteria as well as enveloped viruses and some parasites. Structurally they can be classified into four major classes, α-helical, β-sheet, extended structure and looped [194]. Regardless of their structure they are known to interact with negatively charged lipid membranes. In gram-negative microorganisms they interact with lipid A of LPS on the outer leaflet of the outer membrane. After the initial electrostatic interaction they flip into the lipid bilayer [196]. Once the first molecule of CAMP is inserted into the outer membrane, others interact in a co-operative manner forming channels. Gaining access to the periplasmic space, CAMPs are thought to interact with the cytoplasmic membrane in a similar manner forming channels which results in a decrease of membrane potential, leakage of biologically active chemicals and cell death. This is the most probable mode of action of most of the CAMP although other mechanisms cannot be ruled out as some CAMPs can effectively translocate across artificial membranes [197] and therefore may affect and destroy targets inside bacterial cell. Because of a strong affinity of CAMPs for LPS, killing of bacteria does not result in massive LPS release and therefore pro-inflammatory cytokine responses are not induced, unlike the situation of antibiotic killing [198].

Salmonella, as an intestinal and intracellular parasite, has to deal with CAMP action when colonising the host. The *Salmonella* response to CAMP centres on either

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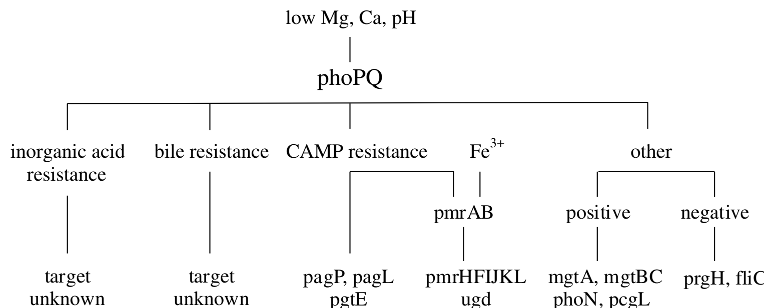

Fig. 3. Role of *phoPQ* in stress response in *S. typhimurium*. PhoPQ regulatory system is most frequently associates with response to Mg^{2+}. However, it also controls response to inorganic acid pH, and bile and CAMP resistance. In resistance to particular CAMPs, role of PhoPQ is indirect through the regulation of another two-component signal transduction system PmrAB.

modification of the lipid A structure or CAMP cleavage [101]. The most frequent modification of lipid A is its additional palmitoylation due to the activity of PagP [196] or biosynthesis of lipid A with 4-aminoarabinose due to the cooperative activity of the whole *pmrHFIJKL* operon [58,199]. The PgtE protein of *S. typhimurium* can inactivate CAMPs by proteolytic cleavage [200]. *Salmonella* is also capable of 3-*O*-deacylation of lipid A catalysed by PagL, although the biological meaning of this modification is not clear because *pagL* mutants display no obvious phenotype [201]. All of the genes involved in CAMP resistance belong to the *phoPQ* regulon although the *pmrHFIJKL* or *ugd* genes are regulated by PhoPQ indirectly through the PmrAB signal transduction system [58,202,203]. This clearly shows that the PhoPQ regulon is central to *Salmonella* protection against CAMP (Fig. 3) although PhoPQ-independent CAMP resistance has been also described [204]. The PhoPQ regulon is responsible for most of the CAMP resistance including the resistance to defensin, magainin, melittin, mastoparan or cecropin P1 [205]. PmrAB regulates only the 4-aminoarabinose modification of lipid A and therefore is responsible for the resistance to a smaller subset of CAMPs such as polymyxin [199,202]. RpoS is not involved in CAMP resistance although the presence of CAMP is sensed by RpoS but is translated into increased general stress resistance [206] and not into CAMP resistance itself.

Resistance to CAMP can be induced in *Salmonella* by its exposure to formate, succinate or sub-lethal concentrations of CAMP [206,207]. Formate can be produced by competitive microflora in the intestine and therefore contact with formate can increase *Salmonella* resistance to CAMP prior to its contact with CAMP on the surface of epithelium or in the phagolysosome of macrophages. It is no surprise that inability to resist CAMP action leads to *Salmonella* attenuation [205]. It is confusing that CAMPs can suppress SPI1-encoded type III secretion system of *Salmonella* which is necessary for the entry into epithelial cells [206]. This would suggest that CAMP resistance is mainly essential for intracellular survival of *Salmonella* in macrophages where the SPI1-encoded TTSS is not needed and can be suppressed [64,208]. This is also supported by the central role of the PhoPQ regulon in CAMP resistance as it is well described that PhoP is induced and activated in low pH, low magnesium and low ionic strength environment which is present in macrophage phagolysosomes [63]. However, at least one *Salmonella* mutant sensitive to CAMP action in vitro was attenuated after per oral infection but not after intra peritoneal inoculation [58] which suggests that there may be a differential *Salmonella* response to CAMP produced on the epithelial surfaces and those produced in the phagolysosome of macrophages and neutrophils where *phoPQ* regulon plays a dominant role (see Fig. 3).

4. Concluding remarks

Salmonella has evolved several overlapping systems that deal with stress responses, which are of particular relevance during infection of the host (Fig. 4). Despite overlapping, acid resistance operates at the top of all of the adaptations possibly because it is the very first stress which is encountered by *Salmonella* immediately after infection. The central role of acid resistance and stress can be based on the fact that acid adapted cells are resistant to a variety of other stresses such as heat or oxidative stress while heat or oxygen stressed cells are not resistant to low pH. The central role of the acid stress may also explain the results of microarray analysis of *V. cholerae* inoculated directly into ligated rabbit ileal loops. In this study, the stress regulators were not observed among the most expressed genes [75].

Salmonella cells infecting the host from the environment are usually not replicating and therefore may resemble stationary phase cells, which are naturally of increased resistance to low pH and as such, these cells are ready for infection. The natural resistance of these cells can be further potentiated by the low pH present

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environment	stress factor	regulons induced	result
out of host1	cold, lownutrients	*rpoS, csp*	general stress resistance
crop	acid pH, SCFA	*ompR, rpoS*	birds only stationary acid tolerance induced (ompR) SCFA stimulated rpoS acid resistance
stomach	extreme acid pH	*rpoS, fur, ompR, phoP*	phoP induced, bile resistance induced rpoS induced, SCFA resistance induced
duodenum	bile	*phoP*	membrane modifications, invasion suppressed
ileum	decreased O_2 supply	*fnr, arcA*	switch from aerobiosis to anaerobiosis
	SCFA	*rpoS*	acid-induced cross-resistance to SCFA SCFA induce CAMP resistance
	bacteriocins	???	???
	competitive flora quorum sensing	*sdiA, luxS*	virulence regulation, acid stress?
epithelium	CAMP	*phoP*	LPS modifications, resistance to macropahge CAMPs
out of host 2	cold shock low nutrients aerobioisis	*csp, rpoS, arcA, fnr* *oxyR, soxRS*	

flow of infection

Fig. 4. Flow chart of the different stresses experienced by *Salmonella* when colonising susceptible host. Experiencing one form of shock always make *Salmonella* of increased resistance to the stress likely to be encountered during the next step of infection, e.g., acid stress increases *Salmonella* resistance to bile and CAMPs, etc.

in the crop of birds by the induction of the stationary phase acid tolerance response. Additional defense mechanisms are induced after contact with bile and SCFA present in the intestine. These maintain activation of the low pH stress response mechanisms of *Salmonella* and in addition, induce resistance to antimicrobial peptides produced by the host cells. Sensing different adverse stimuli always allows *Salmonella* to adapt to the stress likely to be encountered and thus to successfully colonise the host. Key players in *Salmonella* stress response in intestinal colonisation are RpoS, PhoPQ, Fur or OmpR/EnvZ, which are not specific to this genus, showing that the stress adaptation was already evolved in an ancient ancestor, but whether this was also a gut coloniser is impossible to say. Although regulons of individual stress regulators may have adopted additional functions later in evolution (such as PhoPQ regulon and its role in *Salmonella* virulence), one can imagine that the basic stress resistance is likely to be similar in all microorganisms and with some caution in interpretation, knowledge from the *Salmonella* stress response may perhaps be extrapolated to other microbial species. Finally, these regulators are only partly necessary for *Salmonella* virulence. The role of oxidative stress response is confusing since mutations in *oxyR* and *soxRS* are not attenuating (Table 2), although mutations in *sodCI* or *sodCII* superoxide dismutases are [209,210]. Other stress responsive pathways such a heat shock contribute to *Salmonella* virulence mainly during its intracellular survival. Stress-related genes which are induced inside macrophages include *htrA* protease and *pgtE*. Moreover, within more than 400 ORFs of unknown function which are upregulated inside macrophage, a number of stress regulators can be expected [211]. Stress response genes are therefore suitable targets for inactivation, either with a purpose of the construction of attenuated *Salmonella* strain to be used for vaccination against salmonellosis itself, or with a purpose of the construction of attenuated carrier strain suitable for the expression of heterologous antigens. For these purposes, inactivation of genes coding for regulators of acid resistance and resistance to CAMPs seems to be the most suitable. This also shows which stress regulons are the most important for gut colonisation. Another group of target genes are those involved in stringent response, outer membrane shock and protein turnover. However, in these it is a question whether the attenuation of such mutants is due to their reduced capacity to colonise the gut or whether this is due to

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Table 2
Stress regulators and their relationship to virulence in *Salmonella enterica*: A, attenuated; V, virulent

Protein	Function	Virulence of the mutant	Refs.
RpoE	Extracytoplasmic shock	A	[88]
RpoH	Heat shock	?	
HtrA	Heat shock protease	A	[84]
ClpP	Heat shock protease	A	[85,86]
DnaK/DnaJ	Heat shock chaperone	A	[80]
GroEL/ES	Heat shock chaperone	?	
RelA/SpoT	Stringent response	A	[142]
OxyR	Oxidative and nitrosative stress	V	[212]
SoxRS	Oxidative and nitrosative stress	V	[213,214]
OmpR/EnvZ	Osmotic shock, acid response	A	[96]
RpoS	Acid pH, SCFA resistance	A	[10,37]
Fur	Acid pH, oxidative and nitrosative stress	V	[50]
PhoPQ	Acid pH, bile salts, CAMP	A	[40,215]
ArcAB	Anaerobiosis/aerobiosis	V	[130,131]
Fnr	Anaerobiosis/aerobiosis	V	[131,132]
SdiA	Quorum sensing	V	[169]
LuxS	Quorum sensing	V	Rychlik, unpublished

a reduced ability of these mutants to survive intracellularily.

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