Population heterogeneity tactics as driving force in *Salmonella* virulence and survival

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Abstract

Salmonella enterica comprises many pathogenic serovars that are able to colonize a variety of animal hosts and therefore constitute an important source of zoonotic food-borne illness. Their pathogenicity can range from gastroenteritis to typhoid fever, and depends on a series of virulence factors that are regularly located on laterally acquired genetic elements. The regulation of these virulence factors often also includes their differential expression within clonal populations. Moreover, exploitation of the resulting population heterogeneity appears to be an integral aspect of *Salmonella* virulence that could also affect its survival outside the host. This review therefore addresses how the regulation and heterogeneous expression of various virulence factors supports *Salmonella*'s success as a food-borne pathogen.

Keywords: food-borne pathogen, *Salmonella*, virulence factors, gene regulation, population heterogeneity, infection strategies

Introduction

In 2017, *Salmonella* was indicated by the World Health Organisation (WHO) as one of world's most alarming pathogens and is placed 8th on the global priority list of antibiotic-resistant bacteria in need for research and development of new antibiotics. This underscores the importance of an in depth understanding of *Salmonella*'s virulence and survival strategies to pave the way for the development of new methods to combat this pathogen or prevent its transmission. *Salmonella* largely owes its success to complex spatiotemporal gene expression patterns and phenotypically diverse subpopulations that facilitate its survival in the environment and mediate a strategic deployment of virulence factors throughout the infection process.

For *Salmonella* to successfully reach the host intestine and establish infection, the bacteria first have to survive the route through different hostile environments. Throughout evolution, *Salmonella* has evolved the necessary virulence tools and strategies to cope with these diverse surroundings. These tools comprise a series of integrated virulence factors that were often horizontally acquired in the form of plasmids, prophages or other mobile genetic elements (Mebrhatu, Cenens, & Aertsen, 2014; Wahl, Battesti, & Ansaldi, 2019). As the efficacy of *Salmonella* pathogenesis depends upon the activation of the right virulence factor at the right time, their regulation depends on a complex, hierarchal network that integrates diverse cues. The intricate coordination and cooperation of virulence factors is further achieved by extensive crosstalk between these factors. Although expression of virulence genes is tightly regulated, various factors are heterogeneously expressed at the single cell level, and often even a bifurcation into two subpopulations with the gene either in ON or OFF state occurs. This results in phenotypically distinct subpopulations that can facilitate population-level strategies, such as bet-hedging and division-of-labor, which were proven to be key for the overall success of *Salmonella* as a pathogen (Ackermann, 2015; Arnoldini et al., 2014; García-Pastor, Puerta-Fernández, & Casadesús, 2018; Stapels et al., 2018; Stewart, Cummings, Johnson, Berezow, &

Cookson, 2011). Indeed, while bet-hedging constitutes a risk-spreading tactic that allows the pathogen to respond to sudden drastic alterations in its environment by priming a subpopulation to survive those conditions (Ackermann, 2015; I. G. de Jong, Haccou, & Kuipers, 2011), division-of-labor increases the number of specialized concerted functions the (sub)population(s) can perform (Ackermann, 2015).

Given this inherent, virulence-driven population heterogeneity, even clonal *Salmonella* populations should be looked upon as a collection of individuals rather than a uniformly acting population. However, although some of the heterogeneously expressed virulence factors were found to have an influence on survival of *Salmonella* outside its host (Horstmann et al., 2017; MacKenzie et al., 2015), little research has been conducted to determine whether and how this differential expression among siblings affects their individual resistance against stresses encountered in the food production chain. Nevertheless, this heterogeneity could complicate straightforward predictions of a population's survival capacity, especially at smaller population sizes that are nevertheless still relevant for this low infectious dose pathogen. In fact, it was most recently shown that osmotic stress-induced inactivation of *Salmonella* Agona populations smaller than 100 cells could not accurately be predicted based on their bulk-level inactivation kinetics, although the underlying mechanism still needs to be resolved (Aspidou, Balomenos, Tsakanikas, Manolakos, & Koutsoumanis, 2019).

This review further discusses *Salmonella* as a food-borne pathogen, together with its key virulence factors and their regulation. Particularly those factors related to population heterogeneity will be highlighted, together with their subsequent phenotypic consequences on population-level survival and infection dynamics.

***Salmonella* as a food-borne pathogen**

93 *Salmonella* pathogenicity and related clinical symptoms depend largely on the infectious
94 serovar and the susceptibility of the infected host. The genus *Salmonella* contains a number of
95 host-adapted specialists, such as the human-adapted *Salmonella* Typhi. This pathogen
96 disseminates via the lymphatics to the blood stream to ensure the systemic spread of the bacteria
97 throughout the body (A. Bäumler & Fang, 2013; Gal-Mor, Boyle, & Grassl, 2014). In the latter
98 case, a secondary infection can then take place, with multiple organs (such as liver, spleen,
99 bone-marrow and gallbladder) becoming affected (H. K. de Jong, Parry, van der Poll, &
100 Wiersinga, 2012). Moreover, a chronic infection can be established when the pathogen settles
101 for example in the gallbladder by (i) invading of the gallbladder epithelium, (ii) forming of
102 biofilms on gallstones, or (iii) residing in the lumen of the gallbladder (Crawford et al., 2010;
103 Hernández, Cota, Ducret, Aussel, & Casadesús, 2012; Prouty, Schwesinger, & Gunn, 2002;
104 Urdaneta, Hernández, & Casadesús, 2019).

105 However, the vast majority of serovars, such as *S. Typhimurium*, are considered generalists that
106 are able to infect a broad range of hosts and tend to cause non-invasive gastroenteritis localized
107 at the terminal ileum and the colon that is usually cleared from the body within weeks (A.
108 Bäumler & Fang, 2013). These non-typhoidal serovars are a prevalent cause of food-borne
109 zoonotic disease, with tens of millions cases occurring worldwide every year, thus representing
110 a major economic burden in both developing and developed countries (overview in Figure 1).
111 In fact, on the EU list of causative agents for zoonoses, *Salmonella* is placed second behind
112 *Campylobacter*. Although the prevalence of salmonellosis steadily decreased since 2000, the
113 past 5 years progress has stalled. In 2017, a total of 91,662 confirmed human cases were
114 reported in the EU, which roughly corresponds with 19.7 in 100,000 inhabitants contracting the
115 disease (EFSA, European Centre for Disease, & Control, 2018). Furthermore, 42.5% of these
116 cases ended in hospitalization of the patient and even a fatal outcome was documented in 0.25%
117 (EFSA et al., 2018). The European Food Safety Authority, EFSA, estimates a total cost of 3

118 billion euros a year due to salmonellosis. The majority of these infections (70.5%) were caused
119 by three serovars (EFSA et al., 2018): *S. Enteritidis*, *S. Typhimurium* and a monophasic variant
120 of *S. Typhimurium* (Echeita, Herrera, & Usera, 2001). Furthermore, also less notorious serovars
121 such as *S. Infantis*, *S. Derby*, and *S. Newport* cause a significant amount of infections (Hazards
122 et al., 2019).

123 Although the majority of the cases consists of sporadic, individual patients, food-borne
124 outbreaks occur regularly. For example, in 2017, 1,241 food-borne outbreaks of *Salmonella*
125 were reported in the EU infecting 9,600 people and thereby making it the most frequently
126 detected pathogen in food-borne outbreaks (24.4% of the total number of outbreaks) (EFSA et
127 al., 2018). The food products most commonly associated with these salmonellosis outbreaks,
128 are eggs and egg derived products such as desserts (EFSA et al., 2018). *Salmonella* can infect
129 an egg via penetration through its shell or transovarially before the shell is formed (Gantois et
130 al., 2009; Whiley & Ross, 2015). Also, contaminated meat and meat products are a frequently
131 observed cause of the outbreak. The animal itself can become infected in the farm, or
132 contamination of the meat can occur during slaughtering due to contact of with the intestinal
133 content or skin of the animal (n.d.). *Salmonella* containing feces can also end up in surface
134 waters and manure, and contaminate fruit and vegetables through this way.

136 **Key virulence factors during *Salmonella* infection and their genetic regulation**

137 Starting at the ingestion of *Salmonella*, a spatiotemporally regulated expression of virulence
138 factors is unfolded. Overall, a first important virulence program that is activated during the
139 infection is the acid tolerance response that helps in countering the acidic pH found in the
140 stomach of the host and allows the bacterium to travel further into the small intestine to initiate
141 colonization (Ryan et al., 2015). This intestinal colonization of *Salmonella* is further promoted

by the expression of flagella that act as long, whip-like structures propelling the cell towards energy-rich regions (Olsen et al., 2013). A fraction of the *Salmonella* population adheres to the epithelial cells that cover the inside of the small intestine, through surface appendages called fimbrial adhesins (Baumler, Tsolis, & Heffron, 1997). From there, *Salmonella* employs the *Salmonella* pathogenicity island 1 (SPI-1) encoded type III secretion system (T3SS-1) to translocate dedicated effector proteins into the epithelial cell. In the host cell, these effector proteins induce cytoskeletal rearrangements, leading to extensive membrane ruffling engulfment of the pathogen in large vesicles termed *Salmonella*-containing vacuoles (SCVs) (Que, Wu, & Huang, 2013; Steele-Mortimer, 2008). Within these acidic vacuoles, the pathogen acidifies its own cytoplasm and this low pH is key for driving the expression of genes on a second *Salmonella* pathogenicity island (SPI-2) (Kenney, 2019). The latter is elementary in maintaining SCV integrity, localization and vacuole membrane dynamics through translocation of effector proteins via T3SS-2, encoded on SPI-2, across the membrane of the SCV and is thus essential for intracellular survival (Jennings, Thurston, & Holden, 2017). Besides in epithelial cells, *Salmonella* can also survive in SCVs of other mammalian cells such as macrophages. Next to SPI-2, also expression of the *spv*-operon located on a virulence plasmid appears to be important for intracellular survival (Fierer et al., 1993; Grabe et al., 2016; Guiney & Fierer, 2011). A few properties provided by other *Salmonella* pathogenicity islands facilitate the uptake of magnesium and iron, and resistance to multiple antibiotics (Hensel, 2004). Also, the LPS layer plays a role in preventing antibiotics or other detrimental small molecules from entering the bacteria's cytoplasm (Raetz & Whitfield, 2002). Moreover, together with flagella, fimbriae, and the T3SS-1, the LPS layer is a major determinant in the interaction with the host immune system (Kuźmińska-Bajor, Grzymajło, & Ugorski, 2015; Miao et al., 2010; Raetz & Whitfield, 2002; Stewart et al., 2011).

167 These virulence factors are generally embedded in layers of both global and local regulators in
168 order to ensure their expression at the proper stage of infection. Global regulation can be
169 executed by nucleoid-associated proteins such as H-NS, an abundant protein that tends to
170 physically insulate and thus repress laterally acquired genes or islands because of their
171 intrinsically high AT-content (Dorman, 2007; Dorman, Colgan, & Dorman, 2016; Lucchini et
172 al., 2006). Upon the right cue, such global repression can be alleviated, allowing local regulators
173 (such as PhoP/PhoQ (Perez, Latifi, & Groisman, 2008)) that sense and integrate niche-
174 indicative molecules to subsequently fine-tune expression through interaction with specific
175 DNA sites. Short chain fatty acids such as acetate and formate, for example, signal the distal
176 ileum environment as *Salmonella*'s site of invasion (Jones, Ghorri, & Falkow, 1994)), and
177 stimulate SPI-1 expression (Martinez et al., 2011). In turn, SPI-1 repression takes place by
178 compounds found in the large intestine such as propionate and bile acids (Eade et al., 2016;
179 Hung et al., 2013). Also to prepare the transition from an extracellular to an intracellular life
180 style and shift the cellular machinery towards other functionalities, *Salmonella* has multiple
181 sensing systems that incorporate the detection of low levels of ions into the regulatory scheme
182 of virulence factors. For example, magnesium, iron and phosphate are omnipresent in the lumen
183 of the small intestine, but occur in much lower amounts inside host cells. The presence of iron
184 will positively influence the expression of SPI-1 (J. R. Ellermeier & Slauch, 2008; Troxell et
185 al., 2011) while fine-tuning SPI-2 regulation inside macrophages, indicating that *Salmonella*
186 uses the intra- and extracellular iron concentration to timely regulate the level of SPI-expression
187 (Choi et al., 2014). Moreover, limiting amounts of Mg^{2+} will downregulate SPI-1 (M. Aaron
188 Baxter & Jones, 2015), while inducing SPI-2 (Bijlsma & Groisman, 2005; Gal-Mor, Elhadad,
189 Deng, Rahav, & Finlay, 2011). Additionally, low amounts of phosphate in macrophages
190 reduces expression of invasion genes while turning on expression of genes that help *Salmonella*
191 to survive intracellularly (M. Aaron Baxter & Jones, 2015). Other systems react to osmotic and

membrane stresses, affecting SPI-2 (Wang, Zhao, McClelland, & Harshey, 2007) and *spv* expression (Mariscotti & García-del Portillo, 2009). Also stresses to the cell envelope are known to inhibit operons involved in the production of flagella and fimbriae and has now been proven to negatively influence SPI-1 and SPI-2 expression (De la Cruz et al., 2015).

Regulation also further extends into cross-talk between virulence factors. SPI-1 is capable of regulating SPI-2 thereby mediating the transition from SPI-1 to SPI-2 when entering the late stationary phase in rich medium (Bustamante et al., 2008). Additionally, mapping the targets of SPI-1 associated transcription factors suggested a more extensive crosstalk between the two pathogenicity islands (Smith, Stringer, Mao, Palumbo, & Wade, 2016). Oppositely, also SPI-2 regulators have been identified to mediate crosstalk between the two virulence regions, with SPI-2 downregulating SPI-1 genes upon invasion of macrophages, thereby facilitating a successful switch to an intracellular lifestyle (Perez-Morales et al., 2017). Also co-regulation of SPI-1 with the expression of flagella occurs and both operons upregulate each other (Chubiz, Golubeva, Lin, Miller, & Slauch, 2010; Singer, Kuhne, Deditius, Hughes, & Erhardt, 2014). Furthermore, expression of the type-1 fimbriae affects SPI-1 with the latter consequently being downregulated (M. A. Baxter & Jones, 2005). Type-1 fimbriae also decrease expression of flagella and vice versa (Clegg & Hughes, 2002; Saini, Slauch, Aldridge, & Rao, 2010). Coupling of the synthesis of flagella with that of type-1 fimbriae ensures that these appendages are oppositely regulated (Clegg & Hughes, 2002). Moreover, co-regulation of the *std* fimbrial operon displays several similarities with that of type-1 fimbriae as also in *std* expressing bacteria, SPI-1 and flagellar expression are downregulated. Although the primary site of SPI-1 mediated epithelial host cell invasion remains unclear, studies suggest the distal ileum and proximal colon as site of invasion (Crum-Cianflone, 2008; Darwin & Miller, 1999), whereas, type-1 and Std-fimbriae result in attachment to the small intestine and the cecal mucus, respectively (Chessa, Winter, Jakomin, & Baumler, 2009; Lindquist, Lebenthal, Lee, Stinson,

& Merrick, 1987). This suggests a tendency to oppositely regulate flagella and fimbriae in order to inhibit expression of the costly type three secretion system as long as the cells are not attached at the right place. Furthermore, biofilm production is connected with the expression of other virulence traits as the SPI-2 regulator has been shown to induce biofilm formation (S. K. Desai et al., 2016). On the other hand, the major biofilm regulator presumably also affects SPI-1, by indirectly inhibiting T3SS-1 effector protein secretion (Ahmad et al., 2011) whilst planktonic cells exhibit an increase in SPI-1 expression compared to cells in aggregates indicating one subpopulation is intended for survival and one for infection (MacKenzie et al., 2015).

Despite many sensing systems and cross-talk mechanisms that could in principle sequentially turn the proper virulence genes on and off when required, several virulence genes seem to be subjected to heterogeneous expression as well. In fact, a series of feedback and feedforward loops often present in the regulatory circuit of virulence genes seem to ensure a non-homogeneous expression. This differential gene expression allows for bet-hedging and division-of-labor strategies that support virulence and survival tactics. In the following paragraphs, we will more closely focus on the genetic regulation and phenotypic impact of those virulence factors that are closely relating to population heterogeneity (Figure 2).

Flagella

Flagella work as molecular motors for bacterial mobility, and thereby enable chemotaxis (Kojima & Blair, 2004). Moreover, flagella also play a role in adhesion and invasion (Haiko & Westerlund-Wikström, 2013), and serve as antigens that trigger the host immune response (Hayashi et al., 2001). *S. Typhimurium* can bear ca. 6 to 10 flagella per cell, each composed of a basal body serving as anchor, a transmembrane motor, and a large whip-like filament that is built up of either the FliC or FljB flagellin protein (Aizawa, 1996; Bonifield & Hughes, 2003). A bistable expression pattern of flagellar genes is observed that divides the population in

242 flagellated and non-flagellated cells (overview in Figure 3)(Cummings, Wilkerson,
243 Bergsbaken, & Cookson, 2006; Freed et al., 2008; Koirala et al., 2014; Stewart & Cookson,
244 2014). Regulation of flagellum synthesis is complex and the genes involved are arranged into
245 three classes (Koirala et al., 2014; Stewart & Cookson, 2014). The class I genes encode the
246 master regulator FlhD₄C₂ complex that activates class II genes and can be repressed by YdiV.
247 The class II genes encode the basal body and two additional regulators FliA and FliZ. The latter
248 forms a positive feedback loop through (i) increase of the FlhC protein level and (ii) direct
249 inhibition of *ydiV* expression, whilst FliA activates class III genes, encoding the chemotaxis
250 pathway and the flagellin proteins. The production of YdiV and FliZ varies from cell-to-cell
251 and presumably multiple inputs from different pathways generate variation in FlhD₄C₂ activity
252 between isogenic cells, which in turn results in bistable expression of flagellar genes (Stewart
253 & Cookson, 2014).

254 During infection, the bistable expression of flagella appears to contribute significantly to
255 *Salmonella*'s success as a pathogen. Flagella trigger the host to initiate an antibacterial response
256 through Pathogen Associated Molecular Patterns (PAMPs). These PAMPs alarm the human
257 immune system leading to a response that will eventually result in the formation of reactive
258 oxygen species (ROS) and reactive nitrogen species (RNS) in the gut (Behnsen, Perez-Lopez,
259 Nuccio, & Raffatellu, 2015; Fang, 2004). These reactive intermediates give *Salmonella* a
260 competitive advantage during gut colonization as the generated oxidative environment reduces
261 the number of obligate anaerobes competing for this niche, while *Salmonella* is better equipped
262 to thrive in the presence of these radicals (Stecher et al., 2007; Uzzau, Bossi, & Figueroa-Bossi,
263 2002). Next to their intrinsically higher resistance to these radicals, *Salmonella* has developed
264 strategies to directly exploit products formed in the presence of ROS (such as nitrate or
265 tetrathionate) by using them as electron acceptors in its anaerobic respiration (Lopez et al.,
266 2012; Winter et al., 2010). These respiration opportunities accommodate *Salmonella* with an

important energetic advantage for further outcompeting the resident microflora that typically uses a slower fermentative metabolism (Stecher et al., 2007). Additionally, *Salmonella* uses these flagellar structures in chemotaxis towards nutrient rich micro-environments (Rivera-Chávez et al., 2013). Thus, the presence of flagella during infection first stimulates the inflammatory response and afterwards enables the cells to swim to zones rich in ROS-generated terminal electron acceptors thereby optimizing the use of the newly created environment. However, whereas flagellated cells are motile, bacteria without flagella are able to evade caspase-1 mediated host defenses that normally induce programmed cell death (pyroptosis) in macrophages, thereby enabling intracellular life (Stewart et al., 2011). A failure to repress flagellar genes has been reported to attenuate *Salmonella* virulence severely in systemic tissues (Stewart et al., 2011).

On top of this bistability in flagellar expression, also the flagellin protein making up the filament of the flagellum can bistably switch between either the FljB or FliC protein in *S. Typhimurium*. The mechanism driving this flagellar phase variation requires a site-specific DNA inversion event (Zieg, Silverman, Hilmen, & Simon, 1977), mediated by the Hin recombinase (Silverman & Simon, 1980) as the promoter for the FljB flagellin protein is flanked by the *hin* recombination sites (Johnson & Simon, 1985). In one orientation, the *fljBA*^{ON} state, the *fljBA* promoter allows transcription of *fljB*, encoding the FljB flagellin protein, and *fljA*, a transcriptional and translational inhibitor of *fliC* (Bonifield & Hughes, 2003). In the *fljAB*^{OFF} state, no FljB nor FljA is produced and transcription of the *fliC* gene takes place.

Interestingly, FliC expressing bacteria outcompete those expressing FljB for intestinal tissue colonization due to the use of a different swimming mode that is characterized by stopping more often and longer lasting near-surface swimming (Horstmann et al., 2017). This strategy promotes the search for invasion sites and thereby enhances the invasion of FliC expressing bacteria. *Salmonella* thus gears up to respond to sudden changes in environment by creating

two different flagellated subpopulations: one FliC expressing subpopulation that is ready for invasion and a FljB subpopulation that fosters survival and motility in other environmental contexts (Horstmann et al., 2017).

Fimbrial adhesins

The *Salmonella* genome harbors various regions encoding fimbriae, which are proteinaceous, fibrillary structures that extend from the membrane into the extracellular space. Among these is the *lpf* operon (required for attachment to Peyer's patches) (Baumler, Tsolis, & Heffron, 1996), the *pef* operon (for adhesion to intestinal epithelial cells) (A. J. Bäumler et al., 1996), the *std* operon (for attachment to cecal epithelium) (Chessa et al., 2009; García-Pastor, Sánchez-Romero, Gutiérrez, Puerta-Fernández, & Casadesús, 2018), and the *fim* operon encoding the type-1 fimbriae. These fimbriae operons are known to be subject to phase variability (Clegg, Hancox, & Yeh, 1996; Old & Duguid, 1970).

The *lpf* operon was shown to be bistably expressed although little is known about the underlying mechanism (Kingsley, Weening, Kestra, & Baumler, 2002). Induction of the *pef* operon depends upon the methylation pattern of the three GATC sites upstream of the promoter, that is controlled by the Dam methylase and the regulators Lrp and PefI (Nicholson & Low, 2000). To date, the regulation of the bistably expressed *std* operon is not fully elucidated, although its expression seems to be repressed by Dam methylation (Balbontin et al., 2006). The *fim* gene cluster consists of 6 different structural genes, 3 regulatory genes (*fimZ*, *fimY* and *fimW*) and a tRNA, encoded by *fimU*, specific for rare arginine codons (Blomfield & van der Woude, 2007; Saini, Pearl, & Rao, 2009). The regulators FimZ and FimY both independently activate expression of the structural operon. Additionally, FimY and FimZ activate their own promoter as well as each other's promoter (Saini et al., 2009). FimY also induces expression of the third

regulator, FimW (Saini et al., 2009), that in turn exerts a negative effect on type-1 fimbriae expression (Saini et al., 2009; Tinker, Hancox, & Clegg, 2001). Finally, *fimU* affects the production of these regulators as they all contain rare arginine codons in their sequence (Swenson, Kim, Six, & Clegg, 1994; Tinker & Clegg, 2001). Although multiple positive and negative feedback loops are present in this circuit, the exact mechanism of bistable expression has not been elucidated yet. The origin of *fim* phase variation presumably also involves posttranscriptional mechanisms that have not yet been documented (Saini et al., 2009). These operons, their regulation, and bistable expression are more elaborately described by (García-Pastor, Puerta-Fernández, et al., 2018).

Salmonella adherence to epithelial cells during the first phase of infection is mediated by type-1 fimbriae (Baumler et al., 1997). These fimbriated cells can aid in triggering the immune response via PAMPs, which will facilitate intestinal colonization by *Salmonella*. For *E. coli* it has been proven that some phagocytes have fimbriae-specific receptors that trigger internalization of the fimbriate population, which also exhibits an increase in survival inside macrophages, consequently promoting antibiotic evasion (Baorto et al., 1997; Vizcarra et al., 2016). For *Salmonella* it has been proven that adhesive cells are better at being internalized by and surviving in leukocytes (Althouse, Patterson, Fedorka-Cray, & Isaacson, 2003). Next to type-1 fimbriae, the bistable expression of the other fimbrial operons (*lpf*, *pef*, and *std*), each responsible for adhesion in different tissues, might aid in bacterial movement through the gastro-intestinal tract, by consecutive adhesion (Nicholson & Low, 2000). Moreover, it has also been hypothesized for the *std*-operon, necessary for adhesion in the cecal epithelium, that *std*^{OFF} cells permit shedding of the bacterial cells, whereas *std*^{ON} bacteria serve in colonizing the intestine (García-Pastor, Sánchez-Romero, et al., 2018).

Salmonella pathogenicity island 1 (SPI-1)

The horizontal acquisition of the SPI-1 pathogenicity island has been a major deterministic event for *Salmonella* and is considered a phylogenetic branching point between the genera *Escherichia* and *Salmonella* from their common ancestor (P. T. Desai et al., 2013). SPI-1 comprises a ca. 40 kb region including 35 genes (Mills, Bajaj, & Lee, 1995), encoding several effector proteins and a T3SS through which effectors are secreted into the intestinal cells. These effectors trigger localized membrane ruffling of the epithelial cells, leading to efficient internalization of the pathogenic bacterium (Zhou & Galán, 2001). However, not all effectors secreted through T3SS-1 originate from SPI-1, indicating that on top of a complex regulation network within the pathogenicity island also coordinated regulation between SPI-1 and other chromosomal determinants is required to efficiently synchronize secretion events. The T3SS-1 secreted SopE protein, for example, is important for membrane ruffling of epithelial cells and is located on the temperate phage sopE ϕ (Hardt, Chen, Schuebel, Bustelo, & Galan, 1998).

A key regulator for the SPI-1 circuit is HilD, a SPI-1-encoded transcription factor for which expression and activity are controlled by several pathways that sense environmental signals linked with invasion (Smith et al., 2016). HilD assembles in homodimers (Paredes-Amaya, Valdes-Garcia, Juarez-Gonzalez, Rudino-Pinera, & Bustamante, 2018) and activates the expression of the transcription factors HilA and InvF, which in turn induce expression of more SPI-1 genes (Overview in Figure 4). Next to HilD, HilA and InvF, other SPI-1-associated transcription factors have been described, of which HilC and RtsA (C. D. Ellermeier & Slauch, 2003) are well-studied examples. HilD, HilC and RtsA regulate the expression of similar genes and are capable of independently activating each other's transcription, yielding three coupled positive feedback loops (Saini, Ellermeier, Slauch, & Rao, 2010). However, the HilD output is the dominant one and this is reflected in the many environmental and intracellular signals inducing SPI-1 expression through modification of HilD activity and in the drastic reduction of HilA expression in its absence (Lucas et al., 2000; Saini, Ellermeier, et al., 2010). Finally, HilE,

a protein encoded outside SPI-1, exerts a negative effect on SPI-1 gene expression by interacting (i) with the central region of HilD, preventing the latter to dimerize, and (ii) with the C-terminal region, inhibiting HilD from binding DNA, thereby preventing HilD dependent activation (Paredes-Amaya et al., 2018). The regulatory architecture of the SPI-1 gene circuit actually functions as a signal amplifier (subjected to positive feedback loops) with an activation threshold, which enables the system to transform diverse input into a binary output when the feedback is sufficiently strong (Saini, Ellermeier, et al., 2010).

In the small colon two subpopulations of *S. Typhimurium* are therefore present with a different invasion potential based on the ON or OFF state of SPI-1 expression. Through T3SS-1, SPI-1^{ON} cells are able to translocate effectors to the epithelial cells, leading to their engulfment in large SCVs (Steele-Mortimer, 2008). A subpopulation of the invading bacterial cells (~ 10-20%) is able to lyse these vacuoles, thereby escaping into the cytosol of the infected epithelial cells where they can hyperreplicate (≥ 100 bacteria/cell) (Knodler, Nair, & Steele-Mortimer, 2014; Wrande et al., 2016). SPI-1^{ON} is suggested to play a role in this escape to the cytosol or in cytosolic replication as these bacteria were proven to all be SPI-1^{ON} (Knodler et al., 2010). These infected epithelial cells will eventually die due to pyroptosis and will be extruded to the apical side of the colonic epithelial cell layer. Consequently, the *Salmonella* cells are released again into the gut lumen and can thus contribute to further spreading of the pathogen within or between hosts (Knodler et al., 2010). Moreover, this epithelial cell death caused by the SPI-1^{ON} subpopulation will further instigate the existing mucosal inflammation already primed by the PAMPs recognition.

SPI-1^{OFF} cells fail to engage with the epithelial cells and play an important role in colonization of the gut. As the SPI-1^{OFF} bacteria remain in the gut lumen, they suffer less from the antibacterial defenses and can benefit from the inflammation triggered by the SPI-1^{ON} subpopulation that resulted in the emergence of inflammation-borne respirable compounds

(Stecher et al., 2007). Together with *Salmonella*'s resistance against antimicrobial peptides and radicals, lumen-*Salmonella* can further outcompete resident microflora by utilization of these anaerobic electron acceptors (Lopez et al., 2012; Winter et al., 2010). As mainly SPI-1^{OFF} cells can benefit from the SPI-1^{ON} orchestrated inflammation, this is an example of division-of-labor. A recent study analyzing epithelial cell invasion proved that a low number of SPI-1^{ON} cells is already sufficient to confer invasiveness, making it even a very flexible division-of-labor strategy (Sánchez-Romero & Casadesús, 2018).

Strangely, optimal invasion of epithelial cells appears to require the presence of both SPI-1^{ON} and SPI-1^{OFF} cells (Sánchez-Romero & Casadesús, 2018). Although it remains speculating on why a mixed population is better suited for invasion than a SPI-1^{ON} population alone, epithelium invasion by SPI-1^{OFF} cells may extend the cooperative virulence tactics described above (Sánchez-Romero & Casadesús, 2018). When internalized by macrophages, it has been proven that SPI-1^{OFF} cells transition smoothly to intracellular life whilst SPI-1^{ON} cells induce macrophage death after their internalization. The latter results in the release of signal molecules and subsequent recruitment of phagocytes to the site of infection that in turn are able to internalize SPI-1^{OFF} cells, allowing them to be transported throughout the lymph system (Stewart & Cookson, 2012).

Interestingly, SPI-1 bistability seems to be advantageous in other ways as well. In fact, cells expressing the T3SS-1 injectisome and effectors proteins have to pay a cost for their increased virulence and exhibit slower cellular growth (Arnoldini et al., 2014; Sturm et al., 2011). Since SPI-1^{ON} expression thus coincides with significant growth retardation, the SPI-1^{ON} subpopulations could in principle easily be outcompeted by avirulent defector mutants that benefit from the triggered inflammatory response without contributing to it. However, the rise of such cheater mutants is slowed down by the SPI-1^{OFF} population that grows as fast as potential defectors (Diard et al., 2013; Sturm et al., 2011). In addition, the associated slow

growth of SPI-1^{ON} cells has been linked to increased tolerance to antibiotic exposure, a feature that might in turn support persistent infections. In this regard, it was shown that the SPI-1^{ON} population suffered from a growth penalty compared to the SPI-1^{OFF} cells but also had a more than 1000-fold higher probability to survive exposure to a clinically relevant ciprofloxacin treatment (Arnoldini et al., 2014). This tolerance could be the cause of relapsing *S. Typhimurium* infections after treatment. Therefore, aside from enabling division-of-labor, the formation of SPI-1^{ON/OFF} subpopulations also promotes a bet-hedging strategy, which has its benefits when *Salmonella* is exposed to antibiotics (Arnoldini et al., 2014).

Salmonella pathogenicity island 2 (SPI-2)

SPI-2 was initially characterized in the mid 90's as a key component to survive and replicate inside epithelial cells and macrophages (Hensel et al., 1995; Ochman, Soncini, Solomon, & Groisman, 1996). The SPI-2 effector proteins are translocated across the membrane of the SCV within the infected host cell via the SPI-2 encoded T3SS-2, and are implicated in processes such as maintaining SCV integrity and localization, vacuole membrane dynamics, host cell immune functions and host cytoskeleton remodeling (Jennings et al., 2017). SPI-2 encodes around 42 open reading frames (Hensel, 2000). A majority of the genes are required for the functioning of T3SS-2, and they can roughly be categorized into four classes: the *ssa*-genes encode the T3SS-2 injectisome; the *ssr*-genes encode the regulators; the *ssc*-genes encode the chaperones that facilitate secretion and function of the effectors; and the *sse*-genes encode the effectors themselves (Figueira & Holden, 2012). Just as T3SS-1, the T3SS-2 is also able to translocate effectors encoded outside of SPI-2. T3SS-2 expression is regulated by the SPI-2-encoded two-component signal transduction system (Worley, Ching, & Heffron, 2000). This system consists of SsrA, a predicted integral membrane sensor, and SsrB, the cognate response

regulator capable of binding promoters of the SPI-2 functional gene clusters (Fass & Groisman, 2009). SsrB can activate genes by relieving H-NS repression but can also directly stimulate transcription (Walthers et al., 2011). Moreover, SsrB both regulates its own and *ssrA* (located directly upstream and in the same orientation of *ssrB*) expression (Feng, Oropeza, & Kenney, 2003; Feng, Walthers, Oropeza, & Kenney, 2004). Also a cluster of three structural components of the anaerobic tetrathionate reductase (*ttrABC*) is present in SPI-2 (Hensel, Hinsley, Nikolaus, Sawers, & Berks, 1999), although these genes are regulated by another two component regulatory system encoded by an adjacent gene cluster (*ttrRS*) (Hensel et al., 1999).

Although SPI-2 has not been reported to be heterogeneously expressed, survival in the SCV seems to give rise to another phenotypic bifurcation. Recently, it was shown by Helaine *et al.* (Helaine et al., 2014) that immediately after SCV installation in macrophages, some of the invaded *Salmonella* cells reach a non-replicative state while the others keep on proliferating. This dormant state is reversible, and is instigated by toxin/antitoxin modules (Helaine et al., 2014). While the replicative subpopulation allows intracellular proliferation of the bacteria, the non-replicative subpopulation is expected to better withstand long-term host-induced damage and treatment with antibiotics. Cells of this latter subpopulation are therefore called persisters and could provide a reservoir for relapsing infection (Helaine et al., 2014). Persister cells that resume growth afterwards were shown to remain metabolically active during their dormant phase. Moreover, these persisters adjust macrophages using SPI-2 effectors translocated via T3SS-2, that repress the proinflammatory response while increasing an anti-inflammatory reaction. This likely allows non-growing cells to survive for longer time periods in the host (Stapels et al., 2018).

Salmonella plasmid virulence genes (spv)

464 The best studied virulence plasmid of *Salmonella* is the plasmid of *S. Typhimurium* (pSLT)
465 which contains a highly conserved 8 kb region of five genes: *spvRABCD* (*Salmonella* plasmid
466 virulence) (overview in Figure 5). SpvR has been established as an essential activator of the
467 *spvABCD* operon (Fang, Krause, Roudier, Fierer, & Guiney, 1991), whilst the SpvB, SpvC and
468 SpvD proteins have been proven to be virulence factors with specific roles in infected host cells
469 (Browne, Hasegawa, Okamoto, Fierer, & Guiney, 2008; Grabe et al., 2016; Haneda et al., 2012;
470 Mazurkiewicz et al., 2008; Rolhion et al., 2016; Tezcan-Merdol, Engstrand, & Rhen, 2005). In
471 particular, SpvB activates host cell actin degradation thereby mediating cytotoxicity in
472 macrophages (Browne et al., 2008) while SpvC leads to attenuation of the intestinal
473 inflammatory response (Haneda et al., 2012). Also, the SpvD protein was found to
474 downregulate pro-inflammatory responses which contributes to systemic infection of
475 *Salmonella* (Grabe et al., 2016; Rolhion et al., 2016). It has been demonstrated that SpvB and
476 SpvC rely on the T3SS-2 for translocation into the host cell cytoplasm (Mazurkiewicz et al.,
477 2008). Also *in vitro* translocation of SpvC via T3SS-1 was shown (Browne, Lesnick, & Guiney,
478 2002; Mazurkiewicz et al., 2008). These results further underline the importance of
479 synchronizing expression of different (horizontally acquired) virulence genes in order to
480 achieve efficient infection.

481 Recently, it was shown that the *spvABCD* operon is expressed in a bimodal fashion, resulting
482 in a *spv*^{ON} and *spv*^{OFF} subpopulation, of which the percentage of *spv*^{ON} cells depends upon
483 environmental conditions (Passaris, Cambré, Govers, & Aertsen, 2018). The ON or OFF state
484 of the operon was determined by SpvR that itself displayed a bimodal expression pattern.
485 Moreover, it was shown that SpvA can negatively regulate *spvABCD* without influencing the
486 expression of SpvR, suggesting that SpvA might function to restrain the positive feedback loop
487 of SpvR (Passaris et al., 2018). The bistable expression of the *spv*-operon has only recently been

documented, and the biological implications of this phenotypic heterogeneity have not yet been discovered.

LPS modifications

The lipopolysaccharide (LPS) layer is known to stabilize the outer membrane and forms a permeability barrier that prevents unwanted molecules from entering the bacteria's cytoplasm (Raetz & Whitfield, 2002). Functional interference with this layer thus severely attenuates *Salmonella* virulence in multiple model systems (Hoare et al., 2006; Ilg et al., 2009; Morgan et al., 2004; Nevola, Stocker, Laux, & Cohen, 1985). A single LPS molecule consists of a lipid A anchor in the outer leaflet of the outer membrane, followed by a core oligosaccharide that in turn is linked to the O-antigen (Steimle, Autenrieth, & Frick, 2016). One of the operons that is able to modify the O-antigen is *opvAB*, that consists of two cytoplasmic membrane proteins (Overview in Figure 6) (Cota, Blanc-Potard, & Casadesus, 2012). These proteins, when expressed, can reduce the length of the O-antigen to 3 – 8 units (Cota et al., 2012), compared to the standard 16 – 100 units (Murray, Attridge, & Morona, 2003). The regulatory region in front of the operon contains 4 GATC methylation sites, and the expression of the operon is bistably regulated by the corresponding Dam methyl transferase. In the ON state, OxyR proteins bind near the second and fourth GATC site, preventing their methylation, whilst the first and third site are provided with a methyl group. In OFF state, the opposite methylation pattern is observed (Cota et al., 2016). Similar to the *opvAB* operon in regulation is the *gtrABC* operon of the temperate bacteriophage P22, which is also able to modify the O-antigen of *S. Typhimurium* upon lysogenisation of the cell (Overview in Figure 6). The *gtrABC* operon codes for a flippase (GtrA), a bactoprenol glucosyltransferase (GtrB), and also a glucosyltransferase (GtrC). The first two genes add glucose to a carrier lipid and flip it to the periplasm, whilst the latter uses this glucose to glycosylate the O-antigen side chain during its assembly (Allison & Verma,

2000). Expression of the operon is bistable and regulated by OxyR and Dam methylation. Here, the ON state is defined by methylation of the first and second GATC site while methylation of the third and fourth site lead to OFF-switching of the operon (Broadbent, Davies, & van der Woude, 2010).

Just as fimbriae and flagella, LPS molecules are PAMPs used by *S. Typhimurium* to trigger an inflammatory reaction which will support competitive gut colonization due to creation of an oxidative environment. However, when antibodies are raised, the antigenic O-chain becomes a point of recognition, and altering this chain then supports immune evasion (Murray, Attridge, & Morona, 2006).

Since several phages use the O-antigen as a receptor, and additional advantage of O-chain modification is resistance development against phages. As such, reducing the length of the O-antigen by *opvAB* results in resistance against bacteriophage P22, 9NA and Det7. However, this *opvAB*-mediated LPS trimming simultaneously results in (i) reduced virulence in *in vitro* mouse models, (ii) less survival in serum and (iii) less proliferation in macrophages. Typically, the majority of the *Salmonella* population consists of *opv^{OFF}* cells, and only a small part expresses the LPS-modifiers. As an example of bet-hedging, this small *opv^{ON}* population could then survive the potential calamity of a phage infection, after which the survivors can again spawn a *opv^{OFF}* population (Cota et al., 2015). Also bacteriophage P22 infection of *S. Typhimurium* starts with the specific interaction between the phage and the repeating units of the O antigen. Expression of *gtrABC* from the prophage residing in the bacterial chromosome leads to glycosylation of the O-antigen, thereby preventing superinfection by P22 and other phages using the same receptor (Davies, Broadbent, Harris, Thomson, & van der Woude, 2013).

Biofilm formation

Expression of the master regulator, CsgD, of biofilm development in *S. Typhimurium* also occurs in a bistable fashion (MacKenzie et al., 2015). Biofilms are organized structures of microorganisms, adhered to a biotic or abiotic surface and encased in a self-produced extracellular matrix (Steenackers, Hermans, Vanderleyden, & De Keersmaecker, 2012). It is known that *Salmonella* can form biofilms which consist of a three-dimensional extracellular matrix of curli fimbriae, cellulose, proteins and extracellular DNA, encasing clusters of bacteria. As these are more resistant to stresses such as desiccation, disinfectants, drugs, and the host immune system, the formation of biofilms plays an important role in the survival strategy of *Salmonella* inside as well as outside host environments. MacKenzie et al. demonstrated that subpopulations of aggregated and planktonic cells arise in a *Salmonella* population due to bistable expression of the CsgD master biofilm regulator (MacKenzie et al., 2015). CsgD activates the *csgBA* operon that encodes the proteins required to produce curli fibers (Arnqvist, Olsen, & Normark, 1994).

As single cells are necessary for host cell invasion, spread and replication while multicellular aggregates are beneficial for persistence in the environment and causing infections at a later time, it was hypothesized that this CsgD dependent differentiation into either virulent or stress-resistant subpopulations is a form of bet-hedging, allowing *Salmonella* to be better prepared for transitions between non-host and host environments.

Myo-inositol utilization

S. Typhimurium is able to grow in minimal medium with only myo-inositol as carbon source due to the presence of the myo-inositol degradation pathway, that comprises enzymes, transporters and regulators (Kröger & Fuchs, 2009; Kroger, Stolz, & Fuchs, 2010). The expression of the pathway displays a long lag time. Moreover, on poor medium supplemented

with myo-inositol, bistable expression of the genes is observed resulting in the creation of two subpopulations of which one can proliferate whilst the other is non-growing (Kröger, Srikumar, Ellwart, & Fuchs, 2011). Although the precise mechanism causing the ON vs. OFF bifurcation is unknown (Kröger et al., 2018), Kröger *et al.* (2011) proposed a mechanism in which IolR, the main regulator of the degradation pathway, represses expression in the absence of myo-inositol. However, upon *Salmonella* encountering the carbon source, small concentrations of degradation products of myo-inositol will be formed in the bacterial cells, thereby inhibiting IolR functioning. Consequently, induction of the metabolic degradation pathway in some cells occurs, leading to the bistable expression of the operon in the population. However, due to a positive-feedback loop, the majority of *Salmonella* eventually reaches the ON state (Kröger et al., 2011).

It was shown that expression of the *iol* genes is a burden for the bacteria as the ON cells displayed a growth disadvantage during competitive growth assays (Hellinckx, Heermann, Felsl, & Fuchs, 2017). However, the *iol* genes allow *S. Typhimurium* to face conditions in which carbon sources are limited, such as survival in soil, in food products, or in certain niches in the host.

Conclusion

It is clear that virulence tactics have driven a complex cooperative heterogeneity within clonal populations of *Salmonella*, and other pathogens as well (Davis & Isberg, 2019). In fact, taking into account the combinatorial effect of different heterogeneously expressed virulence factors, it seems that *Salmonella* populations consist of highly specialized individuals rather than a uniformly acting population. However, neither the full functional heterogeneity, nor the underlying mechanisms causing it are always fully elucidated yet. It is hypothesized that much

more factors are heterogeneously expressed in *Salmonella*, and future research using single cell RNA-seq (reviewed in (García-del Portillo & Pucciarelli, 2017)) or fluorescence microscopy should further reveal these phenomena. Moreover, to fully interpret the biological function of this phenotypic heterogeneity and understand the accompanying virulence benefits, studies have to focus on population dynamics rather than on cell-to-cell variation alone (Martins & Locke, 2015).

Unfortunately, for many of the above described virulence factors it remains unknown whether and how their differential expression would affect the individual survival chances of clonal cells within a *Salmonella* population exposed to stresses relevant in the food production chain. While it can be anticipated that *Salmonella* heterogeneity could impact its behavior in the food industry as (i) aggregation propensity and biofilm formation are relevant for the persistence and spread in food production environments, (ii) differential fimbriae expression can vary adhesion to food and stainless steel surfaces (Dufresne & Daigle, 2017), (iii) bistable expression of the myo-inositol degradation pathway might play a role *Salmonella* survival in various food matrices, the ramifications of many other forms of heterogeneity in this context still remain unclear. Moreover, it has been noticed that the behavior of smaller *Salmonella* populations under stress becomes more variable and less predictable (Aspridou et al., 2019), which could be a consequence of its heterogeneous composition and behavior. An improved understanding and consideration of cellular individuality in the future will therefore benefit predictions and risk assessment in food safety.

611 Declarations of interest: none.

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