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1. INTRODUCTION

Over the last three decades, Salmonella Enteritidis (SE) has remained the cause of the food-borne salmonellosis pandemic in humans. Epidemiological source-tracking studies have identified contaminated table eggs as the primary risk factor for human infection (Gould et al., 2013). Due to this risk of human infection associated with poultry products, SE also poses a significant challenge to the commercial poultry production. Consequently, experimental SE infections in young and adult chickens have been extensively used to acquire basic information about the epidemiology and pathobiology of SE in this reservoir host. Epidemiological investigations show that, unlike other non-host adapted Salmonella serotypes such as S. Typhimurium, which is isolated from variety of food animal sources, SE is most predominantly isolated from poultry, suggesting that SE has likely evolved to gain significant predilection to the poultry host. Pathobiological investigations in chickens indicate that, unlike other non-host adapted Salmonella serotypes, SE has evolved with the unique ability to efficiently contaminate internal contents of eggs without causing discernible illness in the infected laying hens. S. Enteritidis is also antigenically, phenotypically, and genetically different from other nontyphoidal Salmonella (NTS) serotypes including the most widely studied S. Typhimurium. Nevertheless, the genetic basis of pathogenesis of SE in the chicken host has only been tangentially investigated. In the following sections, we summarize the infection kinetics of SE in the chicken host and provide an overview of the current understanding of genetic factors underlying SE infection. We also discuss the important knowledge gaps that, if addressed, will improve our understanding of the complex biology of SE in young chickens and in egg laying hens.

2. INFECTION KINETICS OF SE IN CHICKENS

S. Enteritidis infection in chickens is a complex multistep process that can be broadly categorized into a few major events that include intestinal colonization, invasion, and systemic spread to internal organs such as liver and spleen. In adult laying hens,
colonization of reproductive tract (RT) organs and contamination of internal contents of eggs is also an epidemiologically significant outcome of infection. Infection of the RT organs precedes contamination of internal contents of forming eggs before oviposition. Each step of the infection in this host is influenced by multiple host and pathogen-associated factors. It is important to first understand the infection kinetics of SE in chickens, and the host and pathogen factors that influence the outcomes of infection before dissecting the roles of genetic factors underlying these processes.

2.1 GASTROINTESTINAL TRACT COLONIZATION AND SYSTEMIC SPREAD

The primary route of infection and transmission of SE in chickens is via the feco-oral route. After ingestion, SE frequently colonizes crop (Hargis et al., 1995; Turnbull and Snoeyenbos, 1974), less frequently in proventriculus and duodenum, but most preferentially and persistently in the lower ileum, cecum, and cloaca (Turnbull and Snoeyenbos, 1974). Subsequently, SE invades intestinal epithelium and localizes in the submucosa within 4 h postinfection (Berndt et al., 2007). Invasion occurs via specialized M-cells that sample the intestinal lumen as well as nonphagocytic cells. Intestinal invasion induces inflammation, which is characterized by infiltration of heterophils, macrophages, red blood cells, and other immune cells into the lamina propria and cecal luminal exudate (Macri et al., 1997; Porter and Holt, 1993; Van Immerseel et al., 2002). As inflammation progresses, SE produces virulence factors that aid its survival within macrophages and subsequent systemic spread to colonize internal organs including liver and spleen, and in laying hens this also results in colonization of the RT organs (Berchieri et al., 2001; He et al., 2010; Higgins et al., 2007). Interestingly, the infection kinetics vary markedly depending on the age or type of chickens. Porter and Holt (1993) reported that the intestinal inflammation in the adult laying hens was confined to the cecum, whereas in broiler chickens, cellular infiltrates were also reported in the lamina propria of the small intestine (Quinteiro-Filho et al., 2012). Infection of young chickens with doses as low as 100 CFU at 1 day posthatch can induce long-term intermittent shedding of SE for up to 24 weeks postinfection (Gast and Holt, 1998; Van Immerseel, 2010). In contrast, oral infection of laying hens often results in short-term fecal shedding that may vary from 1 week to up to 6 weeks postinfection (Kinde et al., 2000; Shivaprasad et al., 1990). In very young birds, the internal organ colonization often results in gross pathological changes such as hepatomegaly and splenomegaly with foci of hemorrhages (Kinde et al., 2000). However, in adult chickens including laying hens, the internal organ colonization generally occurs without any overt clinical signs (Hogue et al., 1997; Prevost et al., 2006) and despite efficient colonization of chicken RT organs, egg production appears to remain unaffected or sometimes increases (Fan et al., 2014; Guard-Petter, 2001).

2.2 REPRODUCTIVE TRACT COLONIZATION IN LAYING HENS

Significant research has focused on the pathogenesis of RT infection in chickens because colonization of RT organs is directly associated with contamination of
internal contents of eggs. Formation of different internal components of eggs occurs at different anatomical positions in the avian RT. Thus one of the key questions that have attracted the most attention is, which anatomical part(s) of chicken RT contribute significantly to the contamination of internal contents of eggs before oviposition? Some reports show that egg yolk is frequently and heavily colonized when laying hens are challenged with SE by different routes, pointing to the ovary as the primary source for egg contamination (Bichler et al., 1996; Gast et al., 2002, 2007b). Others have reported that egg albumen is most frequently and heavily colonized, suggesting colonization of oviduct structures as the primary source for contamination of egg contents (Cogan et al., 2004; Reiber and Conner, 1995; Shivaprasad et al., 1990). Systemic spread of different phage types of SE to ovaries was demonstrated in hens experimentally infected via both oral and intravenous routes (Gantois et al., 2008b; Miyamoto et al., 1997). It was suggested that SE is deposited near the basement of the highly vascularized follicular theca wall before migrating through the perivitel- lin layer and/or attaching and invading ovarian granulosa cells to reach the forming egg yolk (Thiagarajan et al., 1994, 1996a). Ovarian follicle maturation might also play a role in susceptibility of ovarian granulosa cells to invasion (Howard et al., 2005). In one study, invasion frequency of different phage types of SE in ovarian follicles ranged from 0.016% to 0.034% compared with 0.0003% for Escherichia coli (Dawoud et al., 2011). The contribution of oviduct colonization in internal contamination of the contents of freshly laid eggs has also been extensively investigated. In general, SE is isolated more frequently and in higher counts [colony forming units (CFU) per gram] from isthmus and internal contents of eggs when compared with serotypes such as Heidelberg, Virchow, and Hadar (Gantois et al., 2008b; Okamura et al., 2001). When the laying hens were infected via the intravaginal route, the oviduct (isthmus) and uterus were more frequently colonized than the ovary, a phenotype that is likely favored by the ability of SE to attach to vaginal mucosa (Miyamoto et al., 1997; Okamura et al., 2001). In contrast, intravenous infection was reported to result in more prolific colonization of the ovary and the entire oviduct (infundibulum, magnum, and isthmus), yielding more frequent recovery of SE from egg contents and developing eggs when compared with the intravaginal, intracloacal, or oral routes (Bichler et al., 1996; Kinde et al., 2000; Miyamoto et al., 1997; Petter, 1993). In addition to vaginal epithelium, SE also attaches to glandular secretions and tubular glands of isthmus in cultured primary chicken oviduct epithelial cells (COECs), pointing to the isthmus as a colonization site (De Buck et al., 2004a). In summary, it is important to note that most published studies have reported RT organ colonization in terms of either number of SE-positive tissues or number of SE-positive eggs without really establishing bacterial burden (i.e., CFU per gram) as a baseline for comparison. Several variables such as SE strain type, phage type, inoculation route, dose, breed, and age of hens represent a significant challenge for drawing meaningful interpretations from the body of published literature. Therefore there is no clear consensus regarding which parts of RT organs may contribute more significantly to egg contamination; however, the entire RT appears to be colonized by SE. The aforementioned factors (route, dose, strain type, and age of chicken) may as well
significantly influence the ability of SE to colonize the avian RT. Thus it is imperative that these factors are carefully considered before designing studies to investigate RT organ infection in chickens.

2.3 COLONIZATION AND SURVIVAL IN THE INTERNAL CONTENTS OF EGGS

It is now widely accepted that the primary route of egg contamination is via deposition of SE from the infected RT directly into the internal contents of eggs prior to oviposition (Arnold et al., 2014; Gast and Holt, 2000; Keller et al., 1995; Shivaprasad et al., 1990). It is also known that the hen egg presents a hostile environment for efficient propagation of SE. First, the outer shell membrane and shell membrane not only act as physical barriers, but also contain chemical compounds with antibacterial properties including lysozyme (Hincke et al., 2000), ovotransferrin (Gautron et al., 2001), ovocalyxin-36 (Gautron et al., 2007), and an unknown protein extract (Gantois et al., 2009a). In addition, egg albumen, vitelline membranes, and yolk contain antimicrobial compounds such as lysozyme, ovalbumin, ovotransferrin, ovomucin, β-defensin 11, and immunoglobulins (Gantois et al., 2009a; Mageed et al., 2008; Mann, 2007; Stevens, 1991). Despite the presence of these physical and chemical barriers, certain strains of SE are efficiently able to penetrate, survive, and propagate within different egg contents. The eggshell and eggshell membrane penetration is not unique to SE because other *Salmonella* serotypes and bacterial species can also penetrate these barriers (De Reu et al., 2006). In one study, no significant differences in egg-shell penetration were found between SE and *S. Typhimurium* (Miyamoto et al., 1998). Others have compared the growth of different *Salmonella* serovars by artificially contaminating egg albumen and have reported no differences between SE (*n*=8) and *S. Typhimurium* (*n*=24) at 37°C or 42°C (Guan et al., 2006). Similarly, Messens et al. (2004) did not observe any growth advantage for SE in egg albumen when compared with other non-SE serovars including *S. Typhimurium*, Senftenberg, Stanleyville, Mbandaka, and Blockley. Interestingly, however, it was reported that *Salmonella* generally grew better in egg albumen harvested from fresh eggs than from stored eggs, presumably due to the highly alkaline environment (pH approximately 9.0) associated with storage (Messens et al., 2004). One study reported that the survival of SE (25.8%) was higher than that of *Typhimurium* (6.5%) or *E. coli* (1.8%) in egg albumen extracted from fresh eggs and incubated at room temperature (Clavijo et al., 2006). De Vylder et al. (2013) evaluated the survivability of 89 strains of *Salmonella* belonging to five different serogroups (B, C, D, E, and G) and 26 serotypes within fresh egg albumen incubated at 42°C. These authors reported that SE isolates generally displayed greater survivability within egg albumen compared with other serogroups, suggesting that both the age of egg albumen and temperature of incubation may have significant impact on the survival of SE in this system. More importantly, published data also show that there is wide interstrain variability in survival/growth of SE within egg albumen, suggesting that individual variation between strains is also an important
factor (Clavijo et al., 2006; Shah et al., 2012a; Yim et al., 2010). Finally, it is
known that SE can traverse through vitelline membrane into the egg yolk and rep-
llicate efficiently. However, this property is not unique to SE because several other
serotypes including S. Montevideo, S. Infantis, S. Heidelberg, S. Typhimurium, S.
Virchow, and S. Hadar have been reported to display a similar phenotype (Gantois
et al., 2008b; Gast et al., 2007a; Murase et al., 2006). Unlike in egg albumen,
strains of SE belonging to different phage types (4, 8, 13a, and 14b) grow equally
well in egg yolks, presumably because yolk is enriched with different nutrients that
can be utilized by bacteria for efficient growth and multiplication (Gast and Holt,
2001). In summary, it appears that SE may have the specialized ability to grow or
survive within egg albumen; however, this trait is significantly influenced by the
strain type of SE, age of egg albumen, and temperature of incubation. Therefore it
is important to carefully consider these factors while designing studies that investi-
gate genetic factors that contribute to the survivability of SE in egg albumen.

3. GENETIC BASIS OF SE PATHOGENESIS

To date, most of our knowledge of Salmonella pathogenesis in avian host has relied
on the extrapolations from the research that has utilized S. Typhimurium as a model
organism and cultured mammalian epithelial and phagocytic cells or mouse as a
model host (reviewed in Garai et al., 2012; Kaiser et al., 2012; Watson and Holden,
2010). Relatively few studies have been conducted to dissect genetic basis of SE
pathogenesis using chicken as a model host. Nevertheless, it is becoming increas-
ingly evident that the genetic mechanisms underlying SE infection in chickens may
have components that are distinct from the well-studied serotype S. Typhimurium. In
the following three sections, we attempt to summarize the current understanding of
the role of different genetic factors of SE in pathogenesis in the chicken host.

3.1 GENETIC BASIS OF GASTROINTESTINAL INFECTION IN
CHICKENS

Studies focused on the SE genetic factors that contribute to the gastrointestinal (GI)
infection in chickens have mostly revolved around the role of type-3 secretion system
(T3SS) encoded by Salmonella pathogenicity islands-1 (SPI-1), SPI-2, and flagella
and fimbriae factors. The role of SPI-1 and SPI-2 in pathogenesis of SE infection in
chickens appears somewhat contradictory and poorly defined. Published data from
our laboratory and others show that inactivation of hilA (SPI-1 invasion gene activa-
tor) in SE results in significantly reduced intestinal colonization (i.e., fewer bacteria
recovered from the ceca) and invasion (i.e., few bacteria invade the GI tract to subse-
duently colonize internal organs such as liver and spleen) in orally inoculated day-old
chickens (Addwebi et al., 2014; Bohez et al., 2006). In addition, suppression of hilA
gene expression through supplementation of medium-chain fatty acids also results in
reduced intestinal colonization and invasiveness of SE in orally challenged 5-day-old
chicks (Van Immerseel et al., 2004). Although these data suggest that SPI-1 contributes to intestinal and internal organ colonization in young chickens, Desin et al. (2009) observed no significant differences in intestinal colonization between SPI-1 mutant and wild-type parent of SE in orally challenged day-old chickens. Moreover, the SPI-1 mutant displayed varying degrees of internal organ invasiveness, which was not always significantly different from the wild-type parent (Desin et al., 2009). In another study, when 1-week-old chickens were orally challenged with an SPI-2 mutant or a mutant with deletion of the entire SPI-1 and SPI-2, the SPI-2 mutant was impaired in colonization of the cecum, spleen, and liver early during infection (days 1–3 postinfection), but by day 4 postinfection, there were no significant differences in colonization of liver, spleen, or cecum between wild type and either of the mutants (Wisner et al., 2010). In contrast, Rychlik et al. (2009) reported that SE mutant lacking SPI-1 and SPI-2 was not impaired in its ability to colonize cecum of orally inoculated day-old chickens; however, it displayed impaired ability to colonize internal organs such as liver and spleen. In addition, the colonization of the internal organs by SPI-2 mutant of SE was impaired in 1-day-, 5-day-, and 24-week-old chickens after oral, intraperitoneal, and intravenous inoculation (Bohez et al., 2008). Finally, there are also conflicting reports on the intracellular survivability of SPI-2 mutant of SE within chicken macrophages (Bohez et al., 2008; Wisner et al., 2011). Thus more research is needed to clearly define the role of SPI-1 and SPI-2 in the pathogenesis of SE in chickens.

Besides SPI-1 and SPI-2, SE genome carries at least 13 additional SPIs that have been annotated. Of these, SPI-3, SPI-4, and SPI-5 are most extensively characterized in S. Typhimurium, with some studies indicating that these may contribute to pathogenesis in non-avian models (Gerlach et al., 2007; Kiss et al., 2007; Pontes et al., 2015; Wallis et al., 1999). The role of these SPIs in intestinal and internal organ colonization of SE in chickens is currently unclear. Mutation in SPI-4 impairs the intestinal colonization of S. Typhimurium in mouse, but not in the chicken (Kiss et al., 2007; Morgan et al., 2004, 2007). Interestingly, the competitive fitness of an SPI-4 mutant of SE in orally infected mice was less affected than a similar mutant of S. Typhimurium (Kiss et al., 2007). We reported that a disruption of siiE gene of SPI-4 in SE resulted in significantly reduced survival in chicken macrophages; however, it is currently unknown if disruption of SPI-4 would result in similar effects in vivo (Shah et al., 2012b). We have also noted that disruption of pipA gene of SPI-5 significantly altered the ability to colonize the GI tract and internal organs of day-old chickens (Addwebi et al., 2014). In addition, several SPI-5 genes were upregulated in the GI tract of young chicks orally infected with SE (Dhawi et al., 2011). Interestingly, Rychlik et al. (2009) reported that SPI-3, SPI-4, or SPI-5 mutants of SE were not impaired in colonization of the cecum, liver, or spleen of orally inoculated day-old chickens; however, a reduction in the ability of SE to colonize the spleen was observed when all three SPIs were deleted simultaneously. These data suggest that SPI-3, SPI-4, and SPI-5 may contribute to pathogenesis in chickens, although their exact role during the infection process still remains elusive.
Published data from our laboratory and others suggest that SE flagellum also plays a role in pathogenesis in chickens. In one study, aflagellated SE was recovered at significantly lower numbers from the ceca of orally inoculated day-old chickens when compared with the flagellated SE (Allen-Vercoe and Woodward, 1999). These authors suggested that the presence of functional flagellum, but not the motility was important for cecal colonization of SE in chickens (Allen-Vercoe et al., 1999). We reported that impaired secretion of flagellar proteins (FlgK, FljB, and FlgL) among wild-type strains of SE was associated with a low-invasive phenotype in chicken macrophages (Shah et al., 2011). We also reported that disruption of the fljB results in reduced invasiveness of SE in cultured chicken liver cells and reduced colonization of the small intestine in orally inoculated day-old chickens (Addwebi et al., 2014; Shah et al., 2012b). Shippy et al. (2014) reported that deletion of flgC, encoding the flagellum basal body protein, resulted in significantly reduced colonization of liver and spleen in orally infected 1-week-old chickens. Finally, Parker and Guard-Petter (2001) reported that disruption of fliC and flhD negatively impacted internal organ colonization when 3-week old chickens were infected subcutaneously; however, results in orally infected chickens did not show a clear negative phenotype.

The role of fimbriae in SE pathogenesis has also been suspected but not clearly defined, and the published reports are often conflicting. Early work revealed that SefA (SEF14) and FimA (SEF21) fimbriae aid in persistence of SE in the ceca of orally inoculated 30-week-old hens (Thiagarajan et al., 1996b). In contrast, Thorns et al. (1996) observed that inactivation of SEF14 resulted in reduced recovery of the mutant from liver and spleen, but did not affect cecal colonization in orally inoculated 5-day-old chickens, especially later than 15 days post-infection. Others have reported that SE strains with inactivated SEF14 (sef), SEF17 (agf), and SEF21 (fim) fimbriae do not show significant differences in their survival within avian macrophages or colonization/persistence in ceca, spleen, and liver of orally infected 5-day-old chickens (Rajashekar et al., 2000). Similarly, lack of SEF14 in SE did not impact intestinal colonization in orally infected 1-day-old chickens (Thorns et al., 1996). Allen-Vercoe and Woodward (1999) also reported that afimbriate mutants of SE were not impaired in colonization of the GI tract or internal organs or fecal shedding in orally infected 1-day-old chickens. We reported that disruption of csgB in SE impaired invasiveness in chicken liver cells, but did not significantly impact intestinal colonization and internal organ invasiveness in orally challenged 1-day-old chickens (Addwebi et al., 2014; Shah et al., 2012b). Similarly, inactivation of stdA in SE was reported to result in reduced colonization of cecum as well as less invasion of the internal organs in orally inoculated 7-day-old chickens (Shippy et al., 2013). Finally, Clayton et al. (2008) systematically inactivated each of the 13 annotated fimbrial operons of SE and reported that only the inactivation of peg operon resulted in significant reduction in the intestinal colonization in orally inoculated 18-day-old chickens. The peg fimbriae is interesting because it is not found in other NTS, including S. Typhimurium, and has been identified as a pseudogene in an avian host adapted serotype S. Gallinarum (Addwebi et al., 2014; Clayton et al., 2008). It would be of interest to further dissect the role of peg fimbriae in SE pathogenesis in chickens.
Few studies have shown that inactivation of genes affecting SE metabolism can significantly impact infection kinetics in chickens. The *aroA* (3-phosphoshikimate 1-carboxyvinyltransferase) mutant of SE, which confers deficiency of aromatic amino acid synthesis, is significantly impaired in colonization of the cecum, spleen, and liver in orally inoculated 1- or 5-day-old chickens (Cooper et al., 1994). The *tat* operon in gram-negative bacteria transports folded enzymes across the cytoplasmic membrane to the periplasmic space. Many of the proteins predicted to be transported by this system are involved in oxidation or reduction reactions of various compounds. In one study, deletion of *tatB* in SE resulted in significantly reduced colonization of the ceca of orally inoculated 7-day-old chickens (Mickael et al., 2010). Iron metabolism genes may also play a role in the pathogenicity of SE. When SE is grown at avian body temperature, the low-pathogenic strains of SE show reduced expression of genes involved in iron metabolism, including the *suf* operon (Shah, 2014). Chickens vaccinated with purified IroN (the siderophore receptor for salmochelin) show a significant reduction in mortality when challenged intravenously with pathogenic SE strain (Kaneshige et al., 2009). Highly pathogenic strains of SE were reported to more efficiently express genes involved in protection against osmotic, oxidative, and other stresses especially when these were grown at avian body temperature (Shah et al., 2011). In addition, SE collected from ceca of young chickens showed increased expression of genes associated with ethanolamine, propanediol, sialic acid, and dicarboxylic acid metabolism (Dhawi et al., 2011). These reports suggest that SE growth in the GI tract is likely supported by diversity of metabolic substrates and that SE undergoes significant metabolic changes in the avian intestine. It is currently unknown which metabolic substrates are critical for propagation of SE in the GI tract of chickens. Finding the most critical metabolic substrates and understanding their role in SE pathogenesis in chicken may offer opportunities to find new nutritional or other alternative strategies to control SE in poultry.

There are also sporadic reports of other genetic factors that contribute to the kinetics of GI infection. For instance, the lipoprotein encoding gene, *yfgL*, was shown to affect colonization of the GI tract and spleen of orally inoculated 1-day-old chicks; specifically it was associated with lower expression of SPI-1, SPI-2, and flagellar structural proteins (Amy et al., 2004; Fardini et al., 2007). Multiple studies have reported that lipopolysaccharide (LPS) synthesis genes contribute to pathogenesis in chickens. We reported that inactivation of *rfbN* (a rhamnosyltransferase) results in a significant reduction in the colonization of the internal organs of orally challenged day-old chickens (Addwebi et al., 2014). Similarly, inactivation of *rfbM* (a mannose-1-phosphate guanylyltransferase) in SE also results in defective colonization of the GI tract and internal organs in orally challenged day-old chickens (Addwebi et al., 2014). The ribosomal maturation factor *ksgA* is under investigation in our laboratory for its contribution to pathogenicity in chickens. We have previously reported that inactivation of *ksgA* in SE results in reduced invasiveness in chicken liver cells (Shah et al., 2012b) and impaired intestinal and internal organ colonization in orally inoculated day-old chickens (Chiok et al., 2013). One study identified a few SE-specific genetic factors (*SEN1001, SEN1140, SEN1970-SEN1999,* and
SEN4290-SEN4292) that contribute to pathogenicity in mice, but their role remains to be tested in a chicken model (Silva et al., 2012; Vishwakarma et al., 2012). In addition, we reported a few SE-specific genes (pegD, SEN1152, SEN1393, and SEN1966) that contribute to the invasiveness of SE in human intestinal cells (Shah et al., 2012b). However, the role of several of these SE-specific genes in pathogenesis in chickens in general and laying hens in particular is currently unknown.

3.1.1 Knowledge Gaps and Challenges

It is clear that there are significant gaps in our understanding of the molecular basis of pathogenesis of SE in chickens partly because of the variation in the chicken model and the strains used in different studies. Efforts are needed to standardize the chicken model and experimental approaches to study genetic basis of pathogenesis in this host. When examining the host–pathogen interaction between SE and chickens, it is important to recognize that there can be significant variation in virulence between different strains/phage types of SE (Shah et al., 2011; Shivaprasad et al., 1990; Yim et al., 2010). Much of this phenotype variation is not explained at the genotypic level (Shah, 2014; Shah et al., 2011; Yim et al., 2010). In one study, the genomes of different SE isolates were reported to display thousands of SNP differences and greater than 300 variable genes (Allard et al., 2013). In this study, 21 genes from genetic lineages representing outbreak-associated isolates showed nonsynonymous mutations and this affected least five putatively virulence-associated genes. The pseudogene content among different SE strains is also known to vary (genes ratB, a known virulence gene, and mviM are examples) (Matthews et al., 2015). Virtually no studies to date have explored the basis of this inter-strain genetic variation and its association with differential virulence of SE. It is imperative that more efforts be made to compare multiple SE strains in molecular pathogenesis studies before researchers arrive at broad conclusions about this serotype. Rapid screening of thousands or more Salmonella mutants by negative selection is now possible by combining random transposon mutagenesis with next-generation sequencing using transposon directed insertion sequencing (TraDIS) or TnSeq. This technology may allow simultaneous examination of multiple SE strains to identify pathogenicity factors in both young and adult chickens.

3.2 GENETIC BASIS OF REPRODUCTIVE TRACT PATHOGENESIS

The role of genetic factors of SE that contribute to pathogenesis of RT infection has received some attention. In general, most studies have focused on the role of intrinsic bacterial structures such as type-1 fimbriae and LPS in avian RT infection. A few high-throughput screening studies have resulted in identification of genes that may play coordinated roles in RT infection in the hen and in egg microenvironments (Gantois et al., 2008a; McKelvey et al., 2014; Raspoet et al., 2014).

Type-1 fimbriae was reported to aid in interaction of SE with epithelial cell surface or extracellular matrix of the hen RT (De Buck et al., 2003, 2004a; Li et al., 2003a; Thiagarajan et al., 1996a,b). Attachment of SE expressing SefA (SEF14), a
major fimbrial protein of approximately 14 KDa (Clouthier et al., 1993), to primary chicken oviduct granulosa cells was significantly inhibited when the cells were pre-incubated with the purified fimbrial protein or with antibodies against chicken fibronectin (Thiagarajan et al., 1996a). This fimbriated strain was also able to attach to fibronectin, laminin, and collagen-IV in vitro (Thiagarajan et al., 1996a) and it was hypothesized that these interactions could aid in colonization of RT in hens. However, SEF14 fimbrial expression was not associated with ovary/oviduct colonization in orally infected hens when compared with nonfimbriated strains (Thiagarajan et al., 1996b). S. Enteritidis strains also display mannose-sensitive binding to avian isthmal tissues and secretions in vitro (De Buck et al., 2004a), a behavior consistent with mannose-sensitive type-1 fimbriae mediated attachment (deGraft-Hanson and Heath, 1990; Ghosh et al., 1994; van der Bosch et al., 1980). Adhesion through type-1 fimbriae was also demonstrated using defined fimD mutants in SE, which are unable to bind to avian isthmal secretions in vitro (De Buck et al., 2003, 2004a). Furthermore, mutation in fimD yielded less frequently contaminated eggs in 19-week-old Isa-Brown-Warren hens after intravenous challenge (De Buck et al., 2004a). Another study suggested that FimA (SEF21), a major fimbrial subunit, mediates the attachment of SE to neutral glycosphingolipids, similar to glucosylceramide and ganglioside GM3, isolated from avian oviduct mucosal epithelial cells (Rajashekara et al., 2000). These molecules could represent host-cell receptors for SE type-1 fimbriae in the hen RT (Li et al., 2003a) and in chicken intestinal mucosa (Li et al., 2003b; Thiagarajan et al., 1996b). More research is needed to clearly define the role of these fimbrial proteins in RT colonization.

A role for LPS in invasion of RT has been investigated in a few studies (Coward et al., 2013; Mizumoto et al., 2005; Parker et al., 2002). Immunohistochemistry studies revealed that attachment and invasion of vaginal epithelium may, in part, rely on LPS type. One study showed that LPS type O9 (SE) was most efficient in attaching and invading avian vaginal epithelium explants when compared with LPS type O4 (S. Agona, S. Typhimurium, and S. Heidelberg) and LPS types O7 and O8 (S. Hadar, S. Montevideo, and S. Infantis) (Mizumoto et al., 2005). It was hypothesized that the differences in molecular characteristics of LPS of different serotypes may drive such phenotype. Interestingly, wild-type strains display greater diversity in LPS O-chain length and glucosylation, producing variable degrees of glucosylated low-molecular-mass (LMM) or high-molecular-mass (HMM) LPS, whereas S. Typhimurium mostly produce the LMM-LPS type (Parker et al., 2001). This is important because HMM-LPS is commonly found in SE isolates recovered from eggs (Parker et al., 2001). The mechanism underlying favorable interaction between HMM-LPS and eggs is not completely understood, although the length of HMM-LPS in SE provides more hydrophilicity to the outer membrane and may protect SE from antimicrobial effects of egg albumen (Guard-Petter et al., 1999). In addition, SE strains expressing HMM-LPS show discrete single nucleotide polymorphisms or SNPs, but the role of such mutations in avian RT colonization is currently unknown (Parker et al., 2001). Paradoxically, the inability to make HMM-LPS by mutation of the O-chain length determinant gene (wzz) in SE results in more contaminated eggs with poor egg-shell quality and heterophilic granulomas in developing eggs.
This study suggested that HMM-LPS is important in mitigating these pathological changes in the avian RT. It is possible that HMM-LPS confers an evolutionary advantage to SE by aiding in egg contamination without causing noticeable defects in egg-shell quality and/or the hen RT (Parker et al., 2002). More evidence supporting the importance of O-chain repeat number and length in avian RT infection was provided by the generation of mutants producing either one O-antigen attached to the LPS core, long O-chains (16–35 repeated O units), or very long O-chains (>100 repeat units) (Coward et al., 2013). In this study, SE lacking O-chains or very long O-chains was less efficient in colonizing hen ovary and oviducts, and less able to survive within egg albumen. It is likely that regulation of O-chain length impacts interaction between bacterial factors and their respective cell targets in the different microenvironments found in the hen and in the egg (Coward et al., 2013).

Few high-throughput genome-wide screening studies have pointed at contribution of other genetic factors in colonization/invasion of the RT by SE. In one study, in vivo expression technology and a promoter-trap strategy was used to identify promoters of SE genes that are induced in vivo in both hen oviduct and eggs collected from intravenously inoculated hens (Gantois et al., 2008a). Some genes involved in bacterial metabolism (asnS and purA), cell membrane integrity (hflK and peg-yohN), regulation (lrp), and stress-response (uspBA and yrfI) were identified (Gantois et al., 2008a). It was also reported that expression of specific stress-response and cell membrane/wall genes might aid in survival and persistence of SE in oviduct and eggs, presumably by protecting bacteria against cell membrane and DNA damage (Raspoet et al., 2011, 2014). A high-throughput screening strategy that involved microarray-based selective capture of transcribed sequences identified several genes that were overrepresented in both COECs and avian macrophages (HD-11) (McKelvey et al., 2014). Five SPI-2 genes (all belonging to the ssa operon) were overrepresented, suggesting a potential role for SPI-2 in survival within COEC and HD-11. Overall, published reports suggest that efficient invasion of COEC and survival within HD-11 could, in part, be attributed to SPI-2 (Bohez et al., 2008; Li et al., 2009; McKelvey et al., 2014). Another high-throughput negative selection screening of a transposon-inserted mutant library identified 81 genes that impacted SE colonization of hen oviduct loops and cultured chicken oviduct tubular gland cells (Raspoet et al., 2014). Major groups of genes included SPI-1 and SPI-2, genes involved in stress response, cell-wall and LPS structure, and the region-of-difference (ROD) genomic locus 9, 21, and 40 (Raspoet et al., 2014). RODs are interesting genomic regions because these are present in SE but absent in S. Typhimurium (Thomson et al., 2008). Although the exact role of several of these RODs in pathogenesis of the hen RT infection remains elusive, one study showed that deletion of ROD9 and ROD21 did not directly impact the ability of SE to colonize avian RT (Coward et al., 2012).

### 3.2.1 Knowledge Gaps and Challenges

Although several studies have focused on identifying and characterizing the role of SE genetic factors in colonization of avian RT, follow-up studies to conclusively demonstrate their role are often lacking. Both in vitro cell culture models and in vivo
avian infection models have been utilized to identify and characterize genetic factors that contribute to RT infection in chickens. However, the outcome of different studies is not always comparable. For instance, COEC can be a useful in vitro model for high-throughput screening of mutants to identify genes and their possible role in avian RT infection, as well as to study physiological and immunological responses of RT. Nonetheless, critical aspects regarding the source and treatment of COEC vary among studies: primary cultures have been obtained from birds at different ages under different hormonal treatment schemes. In two separate studies, COEC derived from 7-week-old chicks repeatedly treated with estradiol resulted in successful intracellular replication of SE for up to 24 h postinfection (De Buck et al., 2004a,b), whereas COEC derived from 25- to 28-week-old mature hens resulted in failure to establish such intracellular replication (Li et al., 2009). The age, sexual maturation, and differential expression of immune mediators are suspected as potential underlying factors (Anastasiadou et al., 2013; Ebers et al., 2009; Withanage et al., 2003). This may also partially explain the differential susceptibility of ovarian follicles at different maturation stages (Wang et al., 2014). Thus, it is imperative to establish a standardized in vitro cell culture system to study interaction between SE and the avian RT, which may also serve as a model to study other relevant avian pathogens (e.g., avian coronavirus infectious bronchitis virus) with RT tissue tropism (Mork et al., 2014). In vivo studies also differ in several critical aspects that include age, breed, infection dose, route, and bacterial strain. Result outputs are sometimes reported as frequency of SE-positive organs or as bacterial burden (CFU per gram), complicating comparison among different studies. Development and validation of the laying hen model to study the role of different genetic factors in RT infection and contamination of internal content of eggs may help overcome some of the current challenges. In addition to identifying SE factors required for establishing infection in chickens, research is also needed to delineate the host factors. For instance, deciphering the metabolic requirements of SE in the chicken RT may provide some clues to factors that drive predilection of this bacterium to this organ system. In addition, how does SE interact with the chicken microbiota? what factors influence this interaction? and how does this interaction influence outcome of infection? These are some of the key questions that require additional research efforts (Ricke, 2003).

3.3 GENETIC BASIS OF EGG COLONIZATION AND SURVIVAL

Because the entire hen RT can be colonized by SE, contamination of internal contents of eggs could result regardless of which specific anatomic site is more heavily colonized. Consequently, there is considerable interest in understanding the genetic mechanisms underlying the ability of this serotype to colonize and survive within the internal contents of eggs, more specifically egg albumen. Early work in this field showed that nonmotile mutants of SE (ΔfliC and ΔmotAB) as well as other nonmotile serovars including S. Gallinarum and S. Pullorum are impaired in their ability to propagate in egg albumen (Cogan et al., 2004). Moreover, work conducted in our laboratory revealed that wild-type strains of SE with impaired
motility are also impaired in their survival within egg albumen when incubated at 25°C (Shah et al., 2012a). In addition, we reported that disruption of fliH and fljB in SE conferred motility impairment and these mutants were also impaired in their growth in egg albumen (Shah et al., 2012b). These reports collectively suggest that motility is associated with the ability of SE to survive and propagate within egg albumen. It is also speculated that flagella could be an important factor for moving Salmonella through albumen and toward the yolk (Baron et al., 1997).

In addition to motility, curli fimbriae production appears to be important for invasion and survival within egg contents. One study showed that inactivation of agfA (encoding curli production) invaded yolk less frequently suggesting the importance of the role of curli in survival and persistence within the egg (Cogan et al., 2004).

In addition, genes involved in DNA replication and repair have been identified in multiple studies. In one study, disruption of yafD (a member of exonuclease-endonuclease-phosphatase family) was reported to significantly impact the growth of SE and S. Typhimurium within egg albumen (Lu et al., 2003). These authors suggested that absence of yafD may affect the ability of bacteria to repair DNA damage and thereby provide a fitness disadvantage to SE in egg albumen. Subsequently, Clavijo et al. (2006) screened a library of 2850 transposon mutants of SE and identified a total of 32 SE mutants that showed defective survivability (≤10%) when grown in egg albumen at 37°C. These genes were broadly categorized into genes associated with cell-wall structure and functional integrity, nucleic acid and amino acid metabolism in SE; however, yafD gene reported earlier was not identified in this study. Interestingly, one SE-specific gene (SEN4287) putatively associated with restriction endonuclease system was identified by Clavijo et al. (2006). When SEN4287 was expressed in trans in S. Typhimurium, it conferred survival advantage in egg albumen when compared with the isogenic wild-type S. Typhimurium; however, the survivability was not as high as the wild-type SE strain (Clavijo et al., 2006). These authors suggested that SEN4287 may have an important role in providing fitness advantage to SE in egg albumen; however, factors other than SEN4278 may also be required for survival in this hostile environment.

We reported that disruption in genes involved in DNA recombination, replication, and repair (SEN1152, SEN1393, and SEN1966) and translation (ksgA) resulted in reduced growth in egg albumen (Shah et al., 2012b). LPS also appears to contribute to the survival of SE in egg albumen. In one study, expression of rfbH, an LPS biosynthesis gene, was highly upregulated during SE growth in egg albumen at room temperature (Gantois et al., 2009b). Moreover, disruption of rfbH resulted in impaired growth in egg albumen at both 37°C and 42°C. Additionally, we reported that disruption of rfbN, encoding LPS biosynthesis, resulted in reduced growth in egg albumen (Shah et al., 2012b). It is important to note that several strains of SE contain HMM-LPS, which is associated with egg contamination (Guard-Petter et al., 1999). It is possible that HMM-LPS may interfere with binding of different antimicrobial compounds including lysozyme and ovotransferrin of egg albumen with SE. Thus more research is needed to understand the specific role of the LPS in resistance to the antimicrobial compounds of egg albumen and its association
with egg contamination. Collectively, the published body of literature suggests that SE likely undergoes significant metabolic adjustments in egg albumen and that certain strains of SE are likely to be more genetically fit to propagate in egg albumen. One report shows that passage of SE in the egg yolk confers a higher rate of intestinal colonization and extraintestinal organ invasion in orally inoculated mice when compared with SE passaged in laboratory media or in mouse (Moreau et al., 2016). This raises the possibility that propagation of SE within egg contents can significantly increase the pathogenicity of SE in the host; however, the underlying mechanisms are unknown. In conclusion, multiple mechanisms may be at play to regulate bacterial physiology in the egg environment and eventually aid in contamination and survival or propagation of SE within eggs. However, based on the current literature, it has been difficult to pinpoint these mechanisms unequivocally and establish cogent pathways or genetic factors. Consequently, factors or mechanisms that enable SE to colonize and survive within eggs still remain an important knowledge gap. Further research is needed to determine the genetic factors that contribute to the interaction of SE with egg contents and their impact on gene expression and virulence of SE.

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