Salmonella Pathogenicity and Host Adaptation in Chicken-Associated Serovars

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SUMMARY

Enteric pathogens such as *Salmonella enterica* cause significant morbidity and mortality. *S. enterica* serovars are a diverse group of pathogens that have evolved to survive in a wide range of environments and across multiple hosts. *S. enterica* serovars such as *S.* Typhi, *S.* Dublin, and *S.* Gallinarum have a restricted host range, in which they are typically associated with one or a few host species, while *S.* Enteritidis and *S.* Typhimurium have broad host ranges. This review examines how *S. enterica* has evolved through adaptation to different host environments, especially as related to the chicken host, and continues to be an important human pathogen. Several factors impact host range, and these include the acquisition of genes via horizontal gene transfer with plasmids, transposons, and phages, which can potentially expand host range, and the loss of genes or their function, which would reduce the range of hosts that the organism can infect. *S.* Gallinarum, with a limited host range, has a large number of pseudogenes in its genome compared to broader-host-range serovars. *S. enterica* serovars such as *S.* Kentucky and *S.* Heidelberg also often have plasmids that may help them colonize poultry more efficiently. The ability to colonize different hosts also involves interactions with the host's immune system and commensal organisms that are present. Thus, the factors that impact the ability of *Salmonella* to colonize a particular host species, such as chickens, are complex and multifactorial, involving the host, the pathogen, and extrinsic pressures. It is the interplay of these factors which leads to the differences in host ranges that we observe today.

INTRODUCTION

Enteric pathogens are a major source of morbidity and mortality throughout the world. It has been estimated that there are more than 3 million deaths associated with Gram-negative enteric pathogens worldwide due to diarrhea and enteric fever each year (1). Bacteria of genera such as *Escherichia*, *Campylobacter*, *Vibrio*, *Brucella*, *Shigella*, *Yersinia*, and *Salmonella* are responsible for causing enteric diseases. Infections by these pathogens manifest as several disease syndromes, including secretory/noninflammatory diarrhea, inflammatory diarrhea, and enteric fever (2). The differences in disease manifestations are related to the different virulence factors present in the bacteria and the altered phenotypes

that these virulence factors allow the organisms to employ during disease pathogenesis.

Noninflammatory or secretory diarrhea is characterized by watery stools without the presence of white blood cells in feces. Patients generally do not develop a high fever. The diarrhea is associated with bacterial release of enterotoxin or their superficial adherence to the lumen of the proximal small intestine (3). Bacteria that are associated with secretory diarrhea include *Vibrio cholerae* and enterotoxigenic, enteroaggregative, or enteropathogenic pathotypes of *Escherichia coli* (2, 4–6). Diarrheal disease is typically associated with the interaction of the bacterial toxins with the host epithelium, leading to disruption of ion transport systems and subsequent fluid secretion that is the hallmark of diarrhea (7).

In contrast to secretory diarrhea, inflammatory diarrhea typically involves invasion of the colonic epithelium by bacterial pathogens, which may produce cytotoxins. Stool samples typically contain white blood cells, especially neutrophils, and patients often have fever associated with the illness (3). Bacteria associated with causing inflammatory diarrhea include *Campylobacter jejuni*, *Shigella* spp., nontyphoidal *Salmonella enterica*, and enteroinvasive *E. coli* (2). These organisms generally utilize mechanisms such as type III secretion systems (T3SSs) (described in detail below) to interact with the host cell's cytoskeletal system to facilitate invasion (8, 9). The cytoskeletal arrangements lead to the pathogens being internalized, where they reside predominantly in either a protected vesicle (*Salmonella* and *Campylobacter*) or the host cell cytoplasm (*Shigella* and enteroinvasive *E. coli*) (9, 10). During the attachment and internalization processes, the innate immune system can become activated through the recognition of pathogen-associated molecular patterns (PAMPs) on the surface of the pathogens (11, 12). These PAMPs include molecules such as lipopolysaccharide (LPS) and flagella that are recognized by Toll-like receptors (TLRs) on the surface of the host cells, which leads to an antibacterial response that is characterized by increased vascular permeability and the attraction of neutrophils to the site of infection (2). This response leads to the development of inflammatory diarrhea, in which the watery stool contains elevated levels of neutrophils.

Enteric fever is associated with invasive organisms that typically initially invade the distal small intestine and spread to underlying tissues and other parts of the host. Patients generally develop a high fever, and fecal samples contain white blood cells, which are predominantly monocytes and lymphocytes (3). Bacteria associated with enteric fever include typhoidal *S. enterica* and *Brucella* spp. (2). These organisms generally cause severe disease that is characterized by a disseminated infection in different parts of the body. The immune response to enteric fever is characterized by interstitial inflammation associated with mononuclear cell infiltration rather than a strong neutrophil response (3, 13). In *S. enterica* serovar Typhi, the presence of *Salmonella* pathogenicity island 7 (SPI-7) and factors encoded in its *viaB* locus, including the Vi capsular antigen, prevents recognition of PAMPs on the cell surface, such as LPS and flagella, by TLR4 and TLR5, respectively. Activation of these TLRs is associated with increased vascular permeability and neutrophil attraction associated with inflammatory diarrhea (14). *Brucella* spp. employ a distinct approach from that employed by *S.* Typhi to evade recognition by TLR4 and TLR5, in that *Brucella* spp. have modified LPS and flagellin structures that are not efficiently recognized by TLRs (15, 16). The ability to evade

recognition by the innate immune system allows the organisms to establish systemic infections associated with enteric fever (2).

Of these enteric pathogens, one of the most commonly detected, in terms of both numbers of human infections and severity of disease (numbers of hospitalizations and deaths associated with infections), is *Salmonella enterica* (17). Salmonellosis can be manifested through different syndromes, including gastroenteritis, enteric fever (typhoid fever), and bacteremia, and as asymptomatic carriage in animals and humans (18). There are approximately 1.03 million cases of nontyphoidal *Salmonella* that occur each year in the United States, costing the economy approximately $3.31 billion due to premature mortality, disability, and medical and productivity costs, with an annual loss of 16,782 quality-adjusted life years (QALYs) (19). Compared to cost-of-illness figures (medical, employment, and death), the QALY losses also incorporate "psychometric scales" to quantify adverse health status due to people's discomfort and their abilities to engage in routine day-to-day activities following infection (19). *Salmonella* has adapted to survive in a wide range of different environments, such that a large number of human infections are associated not only with food animal sources but also with pets, reptiles, fruits, vegetable, legumes, and other humans (20–22). Among these sources, poultry and poultry-associated products are widely recognized as being among the most important vehicles for human *Salmonella* infections (20, 23). Therefore, *S. enterica* associated with chickens is likely a good model to explore bacterial host adaption and pathogenesis, which is a key focus of this review.

OVERVIEW OF *SALMONELLA* COLONIZATION AND PATHOGENESIS

The genus *Salmonella* is broken up into three different species, *S. bongori*, *S. enterica*, and *S. subterranea* (24, 25). Previously, the genus was broken up into many more species, with each individual serotype being considered its own species. The genus was subsequently divided into seven subgenera (I, II, IIIa, IIIb, IV, V, and VI) based on biochemical and genetic properties (24). As more advanced genetic techniques such as DNA-DNA hybridization were used to analyze the members of the genus, it was discovered that many of the serotypes shared a high degree of genetic similarity. Consequently, the genus was divided into two species, *S. enterica* and *S. bongori*, with *S. bongori* containing the members of subgenus V and *S. enterica* containing the members of the remaining six subgenera (24). *S. subterranea* was described as a species in 2005 (25). Serotypes in what is now *S. enterica* subspecies *enterica* (subspecies I) are the predominant pathogens associated with birds and mammals (24).

Within subspecies I, there is a diversity of *Salmonella* serotypes that infect different animal hosts. The ability of different *Salmonella* serotypes to survive and thrive in different host environments involves a number of interconnected factors, including differences in host environments (pH, temperature, and sites of attachment, etc.), the host immune system and its response to different serotypes, the commensal organisms present, and the genetics of the pathogen itself (26, 27). Many serotypes, such as those most commonly associated with human infections, including *S.* Enteritidis, *S.* Typhimurium, *S.* Newport, and *S.* Heidelberg, have a broad host range, with the ability to infect multiple host species (28). Conversely, other serotypes, such as *S.* Typhi, *S.* Paratyphi, *S.* Gallinarum, *S.* Choleraesuis, *S.* Abortusovis, and *S.* Dublin, have restricted host ranges and are associated primarily with

one or a few hosts (29). *S.* Dublin and *S.* Choleraesuis are typically associated with cattle and swine, respectively; however, they also cause infections in humans (30–33). Human infections caused by these narrow-host-range serotypes tend to be fewer (with the exception of human-associated serotypes such as *S.* Typhi and *S.* Paratyphi) yet typically more severe in disease outcome. Data from the Centers for Disease Control and Prevention (CDC) FoodNet Program indicate that over 50% of human cases of *S.* Typhi, *S.* Dublin, *S.* Paratyphi A, and *S.* Choleraesuis infections are invasive rather than limited to gastroenteritis (34). Lower percentages of infections by the broader-host-range serotypes, such as *S.* Typhimurium (6%), *S.* Enteritidis (6%), and *S.* Heidelberg (11%), are invasive in nature (34).

Initial Colonization of the Host

The primary route of infection in humans and animals is through fecal-oral transmission of *Salmonella*. *Salmonella* pathogenesis has been studied mostly as it relates to human infections, while there is more limited information about the mechanisms of colonization and pathogenesis in food animals such as chickens. In general, when food contaminated with *Salmonella* is ingested, the bacteria have to pass through the alimentary system and survive the acidic environment of the stomach. *Salmonella* has been found to respond to the acidic environment through a complex adaptive system, called the acid tolerance response, which requires the synthesis of over 50 acid shock proteins, including the RpoS σ-factor, PhoPQ, Ada, and Fur (35, 36). Bearson et al. reported that *S.* Typhimurium RpoS and PhoPQ provided protection against inorganic acids, while RpoS and FurR offered protection against organic acids (35).

Those *Salmonella* organisms that survive the low-pH environment proceed to the lumen of gastrointestinal tract (GIT) organs, including the small intestine, colon, and cecum (in poultry). Epithelial and immune cells lining these GIT organs provide the initial protective barrier against *Salmonella* in the gut. *Salmonella* competes with the gut microflora to make the initial contact with enterocytes or M cells in order to colonize the GIT (37, 38). Adhesion to the GIT epithelium by *Salmonella* is facilitated by flagella and fimbriae present on the bacterial cell surface (39, 40). Studies have shown that *Salmonella* serovars employ both conserved and host-specific factors that facilitate colonization in the host GIT (41). Signature-tagged mutagenesis studies have reported the ability of multiple *S.* Typhimurium transposon mutants to colonize intestinal tracts of mice, calves (2), chickens, and pigs (42, 43). In addition to the oral-GIT route of invasion, *Salmonella* bacteria have been reported to invade and disseminate in swine and cattle following uptake in the tonsils and respiratory system (44, 45).

Invasion of Host Cells

Once attached to the intestinal epithelium, *Salmonella* bacteria typically express a T3SS, which is a multiprotein complex that facilitates endothelial uptake and invasion (28, 46). The T3SS apparatus acts as a "molecular syringe" (a conduit between the bacterial cytoplasm and the host cell membrane) to transport toxins and other effector proteins into intestinal cells and is associated with over 20 structural and regulatory proteins (47–49). Several effector proteins, such as InvJ, SpaO, PrgI/J, SipA/B/C/D, SptP, AvrA, SopA/B/D/E/E2, SlrP, and SspH1, are also secreted through the T3SS (47, 50, 51). The T3SS machinery is associated with SPI-1, which harbors virulence genes involved in *Salmonella* ad-

hesion, invasion, and toxicity (see below for details on SPIs). The structural apparatus of the SPI-1 T3SS has been reported to be vital for invasion of bovine and porcine ileal mucosa by *Salmonella* (41).

Among the effector proteins that are translocated through the T3SS, SopB plays an important role in the activation of secretory pathways, facilitating inflammation (by attracting neutrophils to the sites of infection) and altering ion balances within cells, leading to secretion of fluids in the GIT and consequent diarrhea (52, 53) in humans (54) and cattle (55). Other translocated proteins, such as SopA, SopD, SopE2, and SipA, have been found to play a role in *Salmonella* gastroenteritis (53). Effector proteins such as SipA, SipC, and SopB can interact with the actin cytoskeleton, causing the host cell membrane to extend outwards, a process referred to as membrane ruffling (50). This ruffling process facilitates engulfment of the *Salmonella* cells by the host cell membrane and internalization in a membrane-bound compartment termed a *Salmonella*-containing vacuole (SCV) (56–58). As the SCV matures, it migrates from the luminal border to the basal membrane, thus avoiding destruction by the phagolysosomal processing pathways (59).

The SCVs play key roles in survival and proliferation of *Salmonella* in intestinal cells and macrophages (60). Once internalized into host cells, *Salmonella* cells express a second T3SS encoded on SPI-2 (SPI-2 T3SS) within the SCV that plays a critical role in causing systemic infections and intracellular pathogenesis (61). The SPI-2 T3SSs are responsible for secreting effector proteins in the SCV, and these proteins interact with the cytoskeleton and motor proteins, leading to the formation of *Salmonella*-induced filaments (SIFs), which project out of the SCV (28). Furthermore, SPI-2 T3SSs have been reported to modulate trafficking of the SCV to avoid fusion with the lysosomes (41). Several effector proteins, such as SpiC, PipB, SseF/G/I/J, SifA/B, SspH1/H2, SlrP, SopD2, and phage-located SrfA/B/C/D/E/G/I/J/K/L/M, are secreted through the SPI-2 T3SS (62). The SIF facilitates fusion of SCVs with other vesicles in the cell and may play a role in *Salmonella* replication (58, 61, 63); the precise role of SIFs in *Salmonella* infections remains unclear (28).

Survival in Macrophages and Dendritic Cells

In a small percentage of cases, *Salmonella* cells are able to replicate within host cells, evade immune responses, and develop invasive and systemic infections (2, 37). These severe manifestations of salmonellosis usually occur when *Salmonella* cells invade macrophages or dendritic (migratory phagocyte) cells. *Salmonella* cells have been shown to be able to multiply within macrophages (64) but do not appear to replicate within dendritic cells, even though they remain viable (60). The exact mechanisms for the differences in *Salmonella* responses within different immune cell types are not entirely clear, as several pathogen and host factors may play roles (65). Dendritic cells are widely distributed in the lymphoid and nonlymphoid tissues and can rapidly facilitate the spread of *Salmonella* cells to various organs of the host body (66). Researchers have shown that the SPI-2 T3SS in *Salmonella* harbors genes that can suppress antigenic presentation in dendritic cells, which limits the host immune response to infected cells (62). In general, the ability of *Salmonella* to cause an infection in humans or animals depends on the innate ability of the bacteria to encode and express a set (or combination) of virulence genes that can evade and neutralize host defenses. These factors are associated with pathogenic-

ity islands, virulence plasmids, toxins, fimbriae, and flagella (28, 40, 67–69).

Salmonella Pathogenicity Islands

The ability of *Salmonella* to efficiently colonize the host has been attributed to gene clusters, such as SPIs, encoding virulence factors that are distributed in the *Salmonella* genome (28). Several major pathogenicity islands have been reported for different serovars, with SPI-1 to -5 being present in most serovars and others being less widely distributed (28, 69–71). In general, SPI-1 is required for invasion of host cells and induction of macrophage apoptosis, SPI-2 is required for systemic infection and replication within macrophages, SPI-3 is required for survival in macrophages and the ability of *Salmonella* to grow in low-magnesium environments, SPI-4 is required for intramacrophage survival and harbors genes for toxin secretion and apoptosis, SPI-5 has been found to cluster genes that encode multiple T3SS effector proteins, and SPI-6 has been found to transport proteins into the environment or host cells in response to external stimuli (28, 40, 41, 69, 70, 72, 73). Amavisit et al. reported genetic variations among SPI-1, -3, and -5, while SPI-2 and -4 were well conserved among 13 different *Salmonella* serovars isolated from warm-blooded animals (bovine, porcine, avian, and equine), the environment, and human patients in their study (73). Those authors found that, with the exception of *S.* Typhimurium, all isolates within the same serovar were identical with regard to the five SPIs that were tested. SPI-1 and SPI-2 have been found to play a role in *Salmonella* persistence and enteritis in chickens (43, 74), cattle (75–77), pigs (42), and humans (37). Fibronectin-binding proteins, encoded by SPI-3, facilitate host-specific *Salmonella* colonization. For example, MisL contributes to *Salmonella* colonization in mice, chickens, and pigs but does not play a significant role in calves (42, 43), while ShdA influenced *S.* Typhimurium persistence in mice but not in pigs (78, 79). SPI-4 has been reported to mediate adherence to and invasion of bovine ileal mucosa, possibly in combination with the SPI-1 T3SS, but not in chickens and pigs (41, 43, 80).

Virulence Plasmids

Strains from several *Salmonella* serovars have serotype-specific virulence plasmids (81). These are low-copy-number plasmids (1 to 2 copies per cell) and range from 50 to 100 kb, depending on the serovar (40). Each of the plasmids contains the *Salmonella* plasmid virulence (*spv*) locus, whose expression has been reported to be important for multiplication of *Salmonella* in the reticuloendothelial system, including the liver and spleen (40, 82). Other plasmids, in addition to the serotype-associated virulence plasmids, also likely contribute to the observed resistance among *Salmonella* bacteria. Recent studies from our laboratories have identified several different plasmids that potentially contribute to virulence in serovars such as *S.* Heidelberg, *S.* Kentucky, and *S.* Typhimurium (83, 84). A more in-depth exploration of plasmid-associated virulence is presented below.

Toxins

Salmonella pathogenicity has also been attributed to the production of endo- and exotoxins. Endotoxins have been found to elicit a wide range of biological responses, while exotoxins, comprising cytotoxins and enterotoxins, have the ability to kill mammalian cells (85). Ashkenazi et al. reported that *Salmonella* serovars Chol-

eraesuis, Enteritidis, and Typhi produced heat-labile, trypsin-sensitive cytotoxins with various molecular masses, including 56 kDa (*S.* Typhi), 70 kDa (*S.* Typhimurium), and 78 kDa (*S.* Choleraesuis) (85). A *Shigella dysenteriae* 1-like cytotoxin has been detected in *Salmonella* serovars Enteritidis, Kapemba, and Thompson (86). Two other types of exotoxins, salmolysin (encoded by the *slyA* gene) and *Salmonella* enterotoxin (Stn; encoded by the *stn* gene), have been identified in *Salmonella* serovars Typhi, Typhimurium, and Enteritidis (87, 88).

Fimbriae

Fimbriae (pili) are filamentous surface structures that contribute to the colonization of the epithelium by *Salmonella* (89). Each of the fimbrial operons contains multiple genes (typically 8 to 11) that encode the structure and assembly of fimbrins (fimbria proteins) (90). Several fimbrial operons, ranging from 7 to 9 kb in size, have been identified in *Salmonella*. The sequenced strain *S.* Enteritidis PT4 has 13 fimbrial operons (91). Some examples of fimbrial operons include the *agf* and *sef* operons, which encode the *S.* Enteritidis fimbria SEF17 (92, 93); the *pil* operon (located in SPI-7) in *S.* Typhi CT18 (94); and the *lpf* (long polar fimbriae) and *pef* (plasmid-encoded fimbriae) operons in *S.* Typhimurium (95, 96). The SEF14 fimbriae have been found to be expressed by *S.* Enteritidis, *S.* Dublin, and poultry-associated *Salmonella* serovars Berta and Gallinarum, where they appear to be important for adhesion of these serovars to tissues of the reproductive tract (97, 98). Type I fimbriae contribute to *Salmonella* colonization of pigs (99), while 13 major fimbrial subunits of *S.* Enteritidis PT4 have been found to play a role in adherence and colonization of the bacteria in chicken gut (100, 101); the loci on which these subunits were detected were conserved in *S.* Paratyphi and *S.* Gallinarum. A detailed distribution of fimbrial operons among *Salmonella* subspecies and serovars was highlighted in a review by van Asten and van Dijk (40).

Flagella

The majority of *Salmonella* serovars possess up to 10 randomly positioned flagella on their cell surface, which confer motility to these bacteria (40). The ability of certain serovars to display flagellin phase variation provides a potential means for the organisms to minimize the host immune response by creating phenotypic heterogeneity of the flagellar antigens (40). The *fliC* gene, encoding the phase 1 flagellin protein, has been found in *Salmonella* serovars Gallinarum and Enteritidis (102). However, the exact role of flagella (motility and direction of rotation) in *Salmonella* pathogenesis and their possible role in adhesion and invasion of mammalian cells remain unclear (40).

Other Virulence Factors

Virulence factors such as surface polysaccharides may also play a role in persistence of *Salmonella* in the intestinal tract. Multiple mutants affecting lipopolysaccharide (LPS) biosynthesis have been identified in *Salmonella* strains isolated from calves and chickens (41–43, 103). For example, the virulence of the LPS *rfbK*, *dksA*, *hupA*, *sipC*, and *ptsC* mutants and *clpB* and *rfaY* transductants was studied in 1-day-old chicks by Turner and coworkers (103). That study showed that all but the *ptsC* and *rfaY* mutants were attenuated for virulence in chickens. Signature-tagged mutagenesis showed that LPS *S.* Typhimurium mutants (*rfaK*, *rfaB*, *rfaG*, *rfbP*, *rfbN*, *rfbU*, *rfbH*, and *rfbA*) were unable to colonize calf

intestines, suggesting a role of surface polysaccharides and cell envelope proteins as virulence factors contributing to *S.* Typhimurium colonization of calves (43). LPS has been found to contribute to the ability of *S.* Enteritidis to survive in egg albumen (104). That study showed that a mutant strain unable to produce LPS (Δ*rfbH*) was not able to multiply in eggs at room temperature and did not survive in egg whites at 42°C. Those authors concluded that attenuation increased susceptibility of the Δ*rfbH* mutant to undefined antibacterial components of egg albumen (104).

IMPACT OF HORIZONTAL GENE TRANSFER ON GENOME EVOLUTION AND HOST RANGE

There are two major, seemingly opposite, evolutionary approaches in bacteria that impact the range of hosts which an organism can infect and in which it can survive; these approaches are gene acquisition through horizontal transfer and gene or gene function loss (105, 106). *Salmonella* serotypes have expanded their host ranges through the acquisition of new genes through horizontal gene transfer of plasmids and pathogenicity islands, which in turn provide genetic factors that increase the ability to infect new hosts. The addition of these pathogenicity islands to the *Salmonella* genome led to the ability to invade host cells and persist in causing disease. Some transfer events happened more recently, and thus, not all *Salmonella* serovars have acquired the clusters, while other events happened more distantly, and thus, the gene clusters are present in multiple lineages of *S. enterica* (73). There are five SPIs (SPI-1 to -5) that have been found to be important for the ability of *Salmonella* to become pathogenic relative to the commensal *E. coli* (107). It is estimated that 5 to 7% of the genes in the *Salmonella* genome play a role in virulence; interestingly, many of these genes are also present in the commensal *E. coli*; however, they alone are not enough to cause disease (108, 109).

It has been hypothesized that *Salmonella* virulence evolved in a multistep fashion, starting with the acquisition of SPI-1 by all *Salmonella* bacteria and acquisition of additional SPIs distinguishing *S. enterica* from *S. bongori*, followed by expansion of the host range to warm-blooded vertebrates (110). The SPIs, with the exception of a few, are highly conserved across members of the species *S. enterica* but are absent from closely related species such as *S. bongori* and *E. coli* (111). While most of the SPIs are not likely to retain their mobility, the ability of some *Salmonella* genomic islands to excise and transfer has been demonstrated. This excision is also inducible for some regions upon exposure to host conditions, such as macrophage survival and oxidative stress (112).

Integrative conjugative elements (ICEs) are also present in *Salmonella*. ICEs appear to be major players in the horizontal transfer of genetic material between bacterial species, as they carry genes required for their own excision and transfer to recipient bacteria (113). This is exemplified in *Salmonella* by SPI-7, an ICE that is present in *Salmonella* but also among a variety of other *Enterobacteriaceae* (114). SPI-7 ICEs have been found in both *S. enterica* and *S. bongori* and in other bacterial species, and they appear to be evolutionarily related, suggesting that both inter- and intraspecies ICE transfer occurs and further enhances ICE diversity (115).

Role of Plasmids in *Salmonella* Genome Evolution

Considerable research has focused on the plasmids that circulate among *Salmonella* populations of humans and animals, and there is no doubt that plasmids are indigenous to *Salmonella*. Evidence for this exists through a straightforward analysis of the historical

ECOR and SAR reference collections, which reveal a great deal of diversity among their plasmids and an abundance of plasmids in strains of both collections (116). Indeed, a variety of plasmid types have been found among *Salmonella* isolates, including those of incompatibility (Inc) types IncA/C, IncF, IncH, IncI, IncL/M, IncN, IncR, and IncX (117–122). What seems to have changed over time is the accessory gene content that these plasmids carry. It can be argued that genes encoding antimicrobial resistance are omnipresent in microbial populations and that they surface under selective pressure. While this is likely true, it is difficult to argue against the notion that multidrug resistance has increased among clinical *Salmonella* isolates. Historical isolates generally lack phenotypic resistance patterns but still contain plasmids. In contrast, contemporary *Salmonella* isolates still harbor plasmids, but many seem to have evolved in the face of selective pressures to acquire resistance-associated gene arrays. The purpose of this section is not to document the hundreds to thousands of articles that have demonstrated the presence of plasmids among *Salmonella* but to instead present insights into the impact of their presence on genome evolution.

Salmonella virulence plasmids have been considered a defining trait of *Salmonella* serotypes involved in systemic disease. These plasmids range in size from approximately 50 to 100 kb and are found in numerous serovars, including *S.* Typhimurium, *S.* Enteritidis, *S.* Dublin, *S.* Choleraesuis, and *S.* Gallinarum (123). The *Salmonella* virulence plasmid harbors several key virulence factors, including the *spvABCD* system and its regulator *spvR*, which are essential for systemic virulence and are induced upon exposure to nutrient-limited conditions and host cells, particularly macrophages (124–126). These genes are also sufficient to restore systemic virulence in plasmid-cured strains (127). These genes appear to be horizontally acquired on an IncFIIA/IncFIB plasmid backbone, since they have a lower G+C content than the core plasmid (71). There are multiple lineages of the *Salmonella* virulence plasmid that are correlated with a respective serovar, and the virulence plasmids among these lineages have uniquely evolved (Fig. 1). The plasmids sequenced and analyzed thus far can be broadly distributed into three major lineages, and it appears that they have evolved through a combination of gene acquisition and deletion events.

Several possible scenarios for the evolution of *Salmonella* virulence plasmids have been proposed (123, 128). It is possible that an ancestral IncFIIA *spv*$^+$ plasmid present in serogroup D isolates diverged into other serogroups and acquired additional traits. A more likely scenario based upon recent work is that an ancestral plasmid type possessed an IncFIIA/IncFIB backbone containing *spv*, *prf*, and *pef* and separated following the divergence of serogroups and that the plasmid lineages subsequently underwent recombination events, resulting in the current virulence plasmid profiles. Interestingly, there is little evidence of extensive transfer of virulence plasmids among the *Salmonella* serovars, even though their transfer *in vivo* has been documented (129). The horizontal transfer of these plasmids is likely a neutral trait in *Salmonella*, and thus, transfer between serovars is not strongly selected (130, 131). In fact, not all strains of serotypes that typically contain virulence plasmids have them; this lack of virulence plasmids is likely reflective of differences in the strain's genetic background that impact the ability to utilize the plasmid for establishing systemic disease (132).

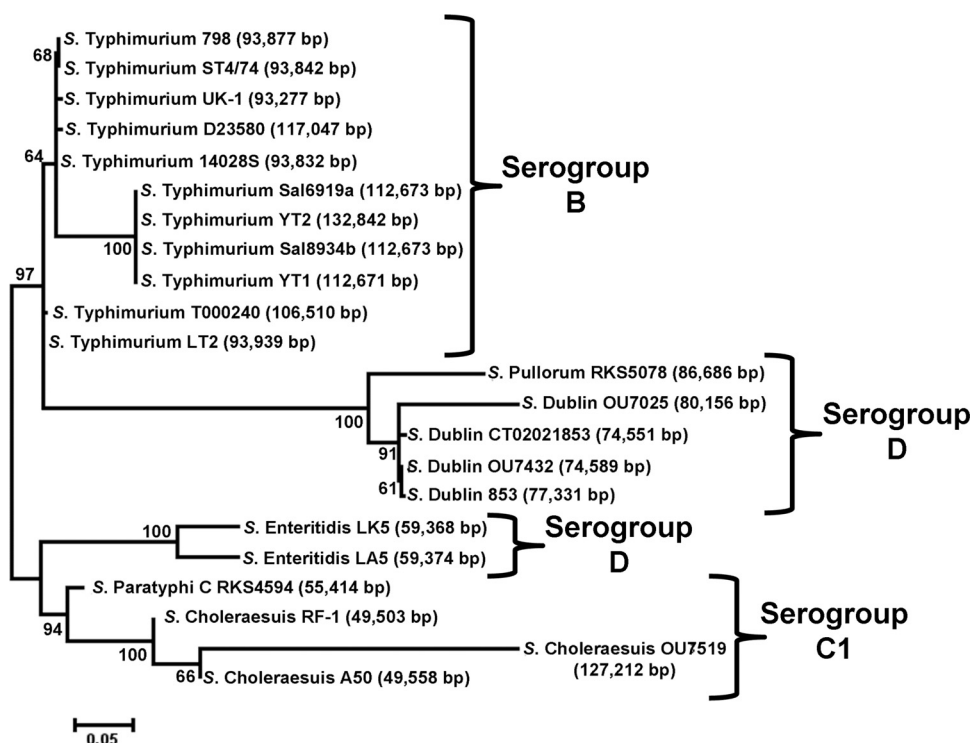The *S.* Typhimurium virulence plasmid has also been shown to

FIG 1 Evolutionary history of *Salmonella* virulence plasmids inferred by using the maximum likelihood method, based on the Hasegawa-Kishino-Yano method. The bootstrap consensus tree was inferred from 100 bootstrap replicates. Analysis was based on 441 SNPs identified from the conserved sequences of 22 plasmids. Analysis was conducted with MEGA5 (403).

be capable of carrying resistance-associated integrons and transposons (133, 134). Cointegrated plasmids have surfaced in *S.* Dublin which include regions of the *Salmonella* virulence plasmid and plasmids of other incompatibility groups, such as IncX, encoding multidrug resistance (135). This could aid in the expansion of the host range of this plasmid to additional *Salmonella* serovars (136). Cointegrated IncFIB-IncI1 plasmids have also been identified among sequenced *Salmonella* genomes, and they have been found to belong to a unique clade of IncI1-type plasmids and harbor a unique subset of putative virulence genes (115). Certainly, cointegrated plasmids offer an increased potential to harbor more diverse arrays of resistance-associated genes and to expand host range and transmissibility.

In addition to virulence plasmids, *Salmonella* bacteria also harbor a variety of other plasmid types. It seems that the spread of such plasmids, in contrast to the *Salmonella* virulence plasmid, occurs primarily via horizontal gene transfer (137). Resistance-associated plasmids are found among a wide range of *Salmonella* serovars, in a variety of shapes and sizes (138–140). Plasmids belonging to groups such as IncI1, IncH, IncN, and IncX are not considered emergent in *Salmonella*, as they have been isolated from this genus for many years (118, 141). However, they appear to be actively evolving to acquire resistance genes with relevance to therapeutic antibiotics used in humans and animals. In contrast, IncA/C plasmids are not found in historical collections of *Salmonella* isolates but have recently emerged among *Salmonella*, *E. coli*, and *Klebsiella pneumoniae* isolates (142). The emergence of IncA/C plasmids among *Salmonella* isolates has often coincided with the emergence of dominant clonal types, such as serotype 4,[5],12:i:− in Spain (143). In some cases, IncA/C plasmids appear to have integrated into the *Salmonella* genome. A case in point was highlighted by a study by Shahada et al., which identified a unique 125-kb genomic island among isolates of a dominant clone of *S.* Typhimurium from cattle in Japan. This island, named GI-VII-6, displayed striking similarities to portions of IncA/C plasmids containing three copies of bla_{CMY-2} (144).

Other resistance plasmids are also emerging, with increased concern to health. IncN plasmids found in *Salmonella* and *E. coli* were recently associated with possession of genes encoding quinolone resistance (122, 145, 146). IncHI1 and IncHI2 plasmids have been linked to quinolone resistance-encoding genes, extended-spectrum beta-lactamase (ESBL) genes, heavy metal resistance genes, and carriage of typical complex class 1 integrons (119, 147–149). IncI1 plasmids are widespread among *Salmonella* isolates and are notorious for their carriage of bla_{CMY} and bla_{TEM} genes (137–139). As mentioned above, IncA/C plasmids have been identified more recently among *Salmonella* isolates, and they carry large numbers of resistance-associated genes, including bla_{CMY-2}, on a conjugative transposon encoding resistance to third-generation cephalosporins; a gene module containing *floR-strAB-tetAR*, encoding resistance to chloramphenicol-florfenicol, streptomycin-spectinomycin, and tetracycline; and a complex class 1 integron harboring a variety of resistance genes (121, 150, 151).

Small plasmids harboring quinolone resistance genes have been identified in both *Salmonella* and *E. coli* (122, 152, 153). More importantly, it exemplifies a mechanism for plasmid gene swapping via Xer- and *oriT*-mediated site-specific recombination. This mechanism of recombination can occur independently of conjugative transposons or integrons and could represent an additional means of genetic recombination involving plasmids. Other mech-

anisms of additional recombination potential exist on the *Salmonella* chromosome. For example, variant regions of *Salmonella* genomic island 1 (SGI-1) have been identified, which include additional resistance genes driven by IS*6100*-mediated recombination (154). A number of variants of SGI-1 that confer resistance to a variety of classes of antimicrobial agents have been identified (155).

In addition to plasmids being mobile themselves, they harbor a variety of mobile genetic elements within them, including integrons and conjugative transposons. Class 1 integrons are the most common type among *Salmonella* isolates and can be localized to the bacterial chromosome or plasmids. A large number of novel gene cassette arrays have been identified among class 1 integrons harbored by *Salmonella*, and some appear to have been derived recently from other bacterial species (156). These accessory regions are actively evolving on plasmids, as evidenced by recent comparative genomics-based studies of closely related plasmids with a variety of resistance island variations (149).

SGI-1 is a 43-kb accessory region of the *Salmonella* genome that contains a class 1 integron (In*104*) encoding resistance toward ampicillin, chloramphenicol-florfenicol, tetracycline, sulfamethoxazole, and streptomycin-spectinomycin, also known as classical "pentaresistance." This region is found in many *Salmonella* isolates and, as mentioned above, is mobilizable. This alone enables a multidrug resistance phenotype, but the carriage of additional plasmids by SGI-1-containing strains is common (157). Interestingly, some plasmids have also been shown to enhance excision and variant formation of *Salmonella* genomic islands, such as the enhanced excision of SGI-1 by plasmids belonging to the IncA/C type (158, 159). In addition to enhancing the excision of chromosomal islands, these plasmids may also harbor receptor sites for phage recognition. For example, plasmids such as IncN and IncX have been shown to convert phage types among *Salmonella* strains (160, 161), and IncX plasmids seem to harbor phage recognition sites (162). Thus, when considering the role of plasmids in genome evolution, it is important to consider not only the accessory gene repertoire that they carry but also the modulations that they confer to the *Salmonella* host.

Horizontal transfer of individual mobile elements between different plasmids also occurs. There are numerous examples of this, but one of recent concern involves an emergent gene known as bla_{NDM-1}, encoding the New Delhi metallo-beta-lactamase (163). The bla_{NDM-1} gene was originally identified among *Klebsiella pneumoniae* isolates from infections originating in India. However, it was quickly realized that this gene was plasmid borne, was present on multiple plasmid types (164–167), and was rapidly spreading between bacteria (168). The first report of bla_{NDM} in *Salmonella* involved a patient with a *Klebsiella pneumoniae* infection that was resistant to carbapenems (169). Subsequent culturing identified NDM-positive *Klebsiella* and *Salmonella* within the patient, with bla_{NDM} apparently residing on different plasmids in each bacterial isolate. There are many additional examples of genetic transfer between *Salmonella* and other bacterial species. For example, heavy metal resistance operon transfer from *Klebsiella* spp. and environmental bacteria to *Salmonella* has also been hypothesized (170). Certainly, plasmid transfer and plasmid gene transposition play a major role in the evolution of *Salmonella* (171).

Role of Bacteriophages in *Salmonella* Evolution

Bacteriophages are among the most diverse and abundant forms of microbial "life" on earth and have played important roles in bacterial evolution. Phages integrate into the host chromosome as a prophage, where they can impact expression of host factors as well as facilitate the transfer of DNA among bacteria through transduction (172, 173). There are at least 170 known phages that infect *Salmonella*, and these generally fall into five categories based on their genetic sequences, P27-like, P2-like, P22-like, lambdoid, and T7-like, along with outliers such as ε15, KS7, and Felix O1 (174). There is a large amount of diversity that is increasingly being revealed among phage families, which reinforces the idea that recombination is occurring frequently between subgroups of phages and between unrelated groups of phages (175).

The host ranges of *Salmonella* bacteriophages are variable, with some being highly host specific and others being generalists. An example of a generalist is ΦX174, which has been demonstrated to lyse >99% of *Salmonella* isolates tested (176). Thus, the susceptibility of an organism to phage lysis (often used to describe the phage type of the organism) does not necessarily correlate with clonality in all *Salmonella* strains. In contrast, the host specificity of other phages to lyse a narrow range of bacteria has been utilized for subtyping of *Salmonella*. Phage typing has historically been a method of choice for characterizing *Salmonella*. The method is still used for typing of bacteria but typically in conjunction with molecular techniques. For example, single-nucleotide polymorphism (SNP) typing has been used in combination with phage typing to explore relationships between host genetics and phage susceptibility in *S.* Typhimurium (177). This work demonstrated that the majority of isolates of particular phage types, such as definitive type 104 (DT104), had a single evolutionary origin, while *S.* Typhimurium isolates belonging to other phage types, such as DT9 and DT135, seemed to have originated from multiple lineages, indicating a broader host range of the typing phages (177). Phage typing has also identified potential geographical differences among isolates; for example, in the United States, isolates of phage type 8 are the most commonly detected *S.* Enteritidis isolates, compared to phage type 4 in Europe (178).

Phages have played significant roles in *Salmonella* virulence through the regulation of expression of virulence factors and the introduction of a number of effector proteins that are implicated in pathogenesis (179). A detailed understanding of the roles that bacteriophages play in *Salmonella* pathogenesis has been complicated by phage diversity and *Salmonella* strain diversity; however, it is becoming increasingly clear that phages do indeed play major roles in terms of delivering functional effector molecules and adding to the *Salmonella* virulence regulatory networks. For example, SopE2 and SspH2 are effector proteins encoded adjacent to phage-like sequences in *Salmonella*. Others, such as SopE, GogB, SseI, SspH1, and SseK3, are found directly on various prophages of *Salmonella* (179). The distribution of these phage-encoded effector molecules is often serovar and even strain specific, suggesting that they are indeed incorporated via transducing phage (180). In some cases, phage-mediated acquisition of traits may confer a fitness benefit in certain niches. For example, a SopE-encoding phage was identified primarily in cattle-associated epidemic isolates, suggesting that SopE may confer a reservoir advantage in cattle (181–183). Lambdoid phages such as Gifsy-1 and Gifsy-2 have been shown to be important for modulating virulence in *S.*

Typhimurium. The Gifsy-1 prophage carries several potential virulence-associated genes, including *gipA* and *gogB*, which may play a role in colonizing the small intestine (184, 185). The Gifsy-2 prophage also carries multiple virulence factors, including *gtgE*, *gtgB* (*sseI*), and *sodC1*, which have been shown to be important for overall *Salmonella* virulence (184, 186).

Plasmids have been shown to alter the susceptibility of *Salmonella* to bacteriophages. In *S.* Enteritidis, IncN plasmid acquisition resulted in conversion of the phage type from type 4 to type 24 (160). Acquisition of IncX plasmids by *S.* Enteritidis has been show to induce resistance to multiple bacteriophage types, thereby altering the phage type profile and facilitating phage conversion (161). Low-molecular-weight plasmids have been shown to exert similar effects on *S.* Enteritidis as well (187). In contrast, the acquisition of some plasmids can result in increased phage susceptibility (188). The precise mechanisms of phage conversion relative to plasmid acquisition have not been intensively investigated, but there is certainly a relationship between the two. The clustered regularly interspaced short palindromic repeats (CRISPRs) may also play an important role in the defense of *Salmonella* against foreign DNA from incoming plasmids and phages. A comparative analysis of 28 sequenced *Salmonella* genomes demonstrated that CRISPR-mediated immunity among specific strains might control short-term phenotype changes and mediate long-term sublineage evolution (189). Thus, it is evident that bacteriophages reshape the *Salmonella* genome not only through their direct contributions to the host bacterium but also through indirect interactions with other mobile elements and cellular regulatory networks.

IMPACT OF THE LOSS OF GENES OR THEIR FUNCTION ON GENOME EVOLUTION AND HOST RANGE

The loss of genes or gene function can also impact genome evolution and host range due to the inactivation of genes needed to infect multiple hosts (106). Host-adapted strains of *Salmonella* have a relatively large number of pseudogenes and chromosomal rearrangements associated with recombination of the 16S ribosomal gene (*rrn*) operons that impact their host range (190, 191). Host-adapted serovars often demonstrate a high degree of *rrn* rearrangement within their genome, which is likely the result of the adaptation to the specific host niche that the serovar occupies. This view was consistent with the finding that host-adapted and host generalist strains demonstrated similar rates of *rrn* rearrangement, and as such, the differences were due to adaptation rather than having a more highly mutable genotype (192). Additionally, host-adapted serovars such as *S.* Typhi, *S.* Paratyphi A, *S.* Dublin, *S.* Choleraesuis, and *S.* Gallinarum have a relatively high percentage of pseudogenes in their genomes, especially compared to the broad-host-range *Salmonella* serovars Enteritidis and Typhimurium (193). Pseudogenes are genes that have been inactivated by insertion, deletion, or nucleotide substitution, leading to a failure to express the gene product associated with the original gene. For example, the genomes of *Salmonella* serovars Typhi and Paratyphi, which are human-adapted serovars, have >4% of the coding sequences in their genomes as pseudogenes, while for the non-host-restricted *Salmonella* serovar Typhimurium, only about 0.9% of the coding sequences are pseudogenes (194). A key consideration related to host adaptation is whether the presence of pseudogenes and the corresponding loss of function led to the host restriction or whether the increased number of pseudogenes

was due to the serovars adapting to their new niches. Indeed, the accumulation of pseudogenes is likely due to host adaptation and subsequent genetic drift following adaptation to the new host niche (194). Approximately one-quarter of the genomes of *Salmonella* serovars Typhi and Paratyphi A have a high degree of nucleotide similarity to one another, while the remaining portions are more divergent, similar to the differences between the other serovars. It is likely that the region of higher similarity is due to recombination between the two human host-adapted serovars (194). Several (22) of the pseudogenes identified between *Salmonella* serovar Typhi and Paratyphi A isolates were outside the recombination region and thus likely arose independently in each the two serovars. However, since the pseudogenes were present in both serotypes, they may be important for survival in the human host niche. The pseudogenes identified included *safE* and *sefD*, which are fimbrial genes, and *shdA*, *ratB*, and *sivH*, which are involved in intestinal colonization and persistence, pathological features not typically associated with typhoid and paratyphoid infections and, as such, not essential for *Salmonella* serovars Typhi and Paratyphi A (194).

Based on genome analyses, it appears that *Salmonella* pseudogenes are typically removed from the genome relatively early following their formation. The removal is potentially associated with the fitness costs associated with the replication of now noncoding DNA in the bacterial genome: the expression of partial, nonfunctional genes or toxicities associated with partially expressed genes (195). This selective pressure to remove the non-fully active genes is evidenced in that the host-restricted serovars have higher K_a (number of nonsynonymous substitutions per nonsynonymous site)/K_s (number of synonymous substitutions per synonymous site) ratios than broad-host-range serotypes (195, 196). The higher K_a/K_s ratio is indicative of positive evolution selective pressure for genome change (195). Other studies with *S.* Typhi and *S.* Choleraesuis have found that the majority of pseudogenes are not expressed; however, some pseudogenes appear to have undergone recoding, such that there were secondary mutations that allowed expression of a functional protein (193). In contrast to full recoding, partial expression of truncated, non-fully functional proteins can potentially bind to receptors blocking the fully functional proteins from eliciting their roles in cellular physiology, thereby harming the bacterium (195).

Other factors also likely play a role in host range variability. These factors include the genetic adaptation of organisms to particular nutrients that are abundant in the host organism but limited in other environments (106). For example, in *S.* Gallinarum, there are multiple pathways associated with carbon and energy metabolism that are not functional, including the catabolism of D-glucarate and long-chain maltodextrins, which may limit their ability to survive in certain environments outside the avian host (191). Loss of gene function can also be associated with an increased ability to cause disease in some instances. A good example is *Shigella* species, where OmpT is absent, which reduces the early immune response to infection (105). *S.* Typhi is also able to modulate the early immune response through the repression of *fliC* expression, which limits the activation of TLR5 by the flagellin FliC (197). In both examples, the loss of the immune response facilitates the intracellular spread of the organism rather than being rapidly cleared with a stronger initial immune response.

EXAMPLES OF *SALMONELLA* HOST RANGE IN CHICKEN-RELATED SEROVARS

Because the diversity of the *Salmonella enterica* species is quite expansive, this section focuses primarily on the host range adaptation of four particular serovars (*S.* Enteritidis, *S.* Heidelberg, *S.* Kentucky, and *S.* Gallinarum) that are associated very commonly with chickens and to various extents with other food animal species and human infections. According to the CDC, in the United States, *S.* Enteritidis was the serovar most commonly implicated in human illness, overtaking *S.* Typhimurium as the most common serovar (198). Likewise, when data from the National Veterinary Services Laboratory of the USDA and from other studies examining the prevalence of *Salmonella* serovars were compared, *S.* Enteritidis was associated most commonly with chickens and eggs and to a much lesser extent with other food animal species (198–202). *S.* Heidelberg is found in most of the major food animal species, eggs, and retail meat samples and is among the top five most common serotypes associated with human illnesses (203, 204). Conversely, *Salmonella* serovars Kentucky and Gallinarum rarely cause human infections in the United States (although *S.* Kentucky is an emerging serovar in Europe and North Africa) (205). *Salmonella* serovar Gallinarum is a host-adapted serovar that is presently made up of two biovars, Gallinarum and Pullorum (which were previously considered two separate serotypes) (206). This serotype was associated with severe losses to the poultry industry in the United States until it was targeted for eradication by the National Poultry Improvement Plan (NPIP) starting in 1935 (207). After implementation of the NPIP, *S.* Gallinarum was eradicated from commercial poultry flocks in the United States by the mid-1960s (26).

S. Kentucky is currently one of the most common serotypes isolated from broiler chickens in the United States and is detected fairly often in dairy cattle as well (198). The increase in the rate of isolation of *S.* Kentucky in broilers is likely related to a number of factors, including management practices, flock immunity, and genetic changes in the organism (26). When *S.* Kentucky was compared to other serovars with respect to virulence, it was found that the *S.* Kentucky isolates grew more rapidly than other serovars under moderately acidic conditions (pH 5.5) but worse under more highly acidic conditions (pH 2.5) (208). The enhanced ability to grow under these moderately acidic conditions may provide an advantage over other serotypes in environments such as the chicken cecum. This serovar exhibited greater invasiveness in *in vitro* assays using chicken embryo hepatocytes than serovars such as *S.* Enteritidis and *S.* Typhimurium (208). Many *S.* Kentucky isolates also have plasmids with factors such as those associated with antimicrobial and disinfectant resistance, iron acquisition, bacteriocin production, and complement resistance, which may enhance their abilities to survive in birds (83).

It is interesting that *S.* Kentucky has not become a larger public health problem for consumers in the United States, as *Salmonella* from poultry is predicted to be the fourth most important pathogen-food combination associated with food-borne illnesses in the United States (20). It is quite probable that *S.* Kentucky strains most common in poultry are not overly virulent to humans. In analyzing the data from the CDC National *Salmonella* Surveillance Program (http://www.cdc.gov/ncidod/dbmd/phlisdata/salmonella.htm), the states with the largest numbers of human *S.* Kentucky infections were generally those associated with higher rates of dairy production rather than poultry production (198, 209). Likewise, several studies have utilized pulsed-field gel electrophoresis (PFGE) for the molecular characterization of *S.* Kentucky isolates from various sources (chickens, dairy cattle, and human infections); the most common profiles associated with human infections were similar/visually indistinguishable from common profiles from cattle but typically distinct from those of poultry (205, 210–212). Likewise, a majority of *S.* Kentucky strains isolated from poultry-associated sources fall into the sequence type 152 (ST152) clonal complex, while the majority of those isolated from human patients were of ST198 (205). Therefore, a plausible explanation for *S.* Kentucky-associated human infections could be due to occupational exposure to cattle or due to consumption of contaminated products such as raw (unpasteurized) milk or raw-milk cheeses. *S.* Kentucky has been one of the most commonly detected serotypes isolated from prepasteurized milk in the United States (213), and consumption of raw milk and raw-milk cheese has been associated with multiple outbreaks of salmonellosis (214–216).

For *S.* Heidelberg, there does not seem to be a strong association of subtypes with a particular food animal host and subsequent human infection. When *S.* Heidelberg isolates from human patients were compared to those of the major food animal species, there was extensive overlap in PFGE profiles, plasmid types, and antimicrobial susceptibility profiles, indicating a lack of host restriction among *S.* Heidelberg genotypes (203, 217, 218). In a study examining the core genomes of the population structure of many of the prominent serovars, it was concluded that the genomes of *S.* Heidelberg isolates were likely shaped by a high degree of horizontal genetic transfer (219). Consequently, the *S.* Heidelberg strains resided in a lineage distinct from that of the avian-associated *Salmonella* serovars Enteritidis and Gallinarum, based on genomic comparison. Additionally, the members of *S.* Heidelberg fell outside the lineage containing *Salmonella* serovars Typhimurium and Saintpaul, yet *S.* Heidelberg isolates shared a relatively high proportion of sequence similarity with the lineage (219).

S. Heidelberg isolates also often contain plasmids. While they lack a serotype-specific virulence plasmid, a common feature in serovars such as *S.* Typhimurium, *S.* Enteritidis, and *S.* Gallinarum (81), they often contain plasmids with virulence genes (83, 84). Interestingly, a number *S.* Heidelberg strains isolated from poultry-associated sources were found to harbor IncFIB plasmids similar to those previously recognized as being important for extraintestinal survival in avian-pathogenic *E. coli* (83, 220–222). Similar plasmids have been found very commonly in *S.* Kentucky isolates from poultry as well. Many IncFIB plasmids contain genes for iron acquisition (aerobactin operon and Sit iron transport systems), colicin production, and serum survival, which likely play a role in increased fitness in the avian environment (83). In many cases, these IncFIB plasmids also contain genes that encode resistance to multiple antimicrobials (often including resistance to tetracycline, streptomycin, chloramphenicol, and sulfonamide). This presents the possibility for coselective pressure, with antimicrobial use selecting for enhanced virulence, or conversely, the increased ability of these bacteria to survive iron-limited environments in the host could select for resistance to one or more antimicrobials (83, 84). *S.* Heidelberg plasmids also contain genes encoding disinfectant and heavy metal resistance, which may provide a selective advantage for survival in the avian production environment where pathogen control strategies are employed (84, 218).

Studies have shown that *Salmonella* serovars Gallinarum and Enteritidis are closely related in both their gene content and their antigenic formula (191, 223–225), suggesting that these two serovars originated from a relatively recent common ancestor (226, 227). Because of their close genetic similarity, they are valuable examples to explore host adaptation. Genomic comparisons of members of these two serovars have indicated that there is a high degree of genetic similarity between the two serotypes, with average nucleotide identities among orthologous genes being 99.7% (191). The genomes of sequenced isolates shared 4,179 predicted coding sequences (CDSs). The differences in CDS content between the strains were associated primarily with bacteriophages incorporated into the respective bacterial genomes (191). The lineage of *S. enterica* that contains these serotypes tends to be one of the most conserved among the species, with an estimated 4% of their core genome sequences originating from recombination with genes from other *Salmonella* serovars (219). Both serovars share the O1, O9, and O12 antigens (223). However, even though *S.* Enteritidis and *S.* Gallinarum are relatively closely related on the genomic level, they are diverse in their numbers of pseudogenes. *S.* Gallinarum isolates are unable to carry out mannose-sensitive hemagglutination and do not express flagellar genes, leading to an observed lack of mobility, while the majority of *S.* Enteritidis strains are motile (110). This lack of motility observed for *S.* Gallinarum isolates is in part associated with mutations in genes associated with flagellar biosynthesis and chemotaxis, including *flhA*, *flhB*, *flgI*, *flgK*, or *cheM* (191). Mutations in *fliC* may also contribute to the lack of motility of isolates of this serovar (226).

One study found that *S.* Enteritidis has 21 pseudogenes, compared to 147 for *S.* Gallinarum, but there are only 5 pseudogenes shared between the two serovars (195). Of these five pseudogenes, three are likely ancestral in origin, and the other two were likely independently acquired. Similarly, the genetically related *S.* Dublin had 212 identified pseudogenes, 177 of which are active genes in *S.* Enteritidis. Many of these functional genes encode surface structures or are involved in the central metabolism of *Salmonella*; thus, their inactivation in *S.* Dublin likely contributes to its host restriction in cattle (91). Likewise, certain pseudogenes are common to multiple host-restricted serotypes; for example, *mglA* and *shdA* are transcribed in *S.* Enteritidis but are present as pseudogenes in *S.* Choleraesuis, *S.* Dublin, *S.* Gallinarum, *S.* Paratyphi A, and *S.* Typhi (91). MglA is a small GTP-binding protein subunit of a binding-protein-dependent galactose transport system (228, 229) that likely plays a role as a virulence regulator in intracellular pathogens such as *Francisella* spp. (230) and motility in *Myxococcus xanthus* (231). ShdA is a protein that is involved in the colonization of Peyer's patches by *S.* Typhimurium and the shedding of the bacteria following infection (232, 233). The presence of a larger number of functional genes in broader-host-range serovars such as *S.* Enteritidis likely contributed to the ability to colonize and infect a greater number of hosts (234).

There are also several differences in the numbers of the fimbrial genes between *S.* Gallinarum and *S.* Enteritidis. Several fimbrial operons, including *lpf*, *bcf*, *stb*, *stc*, *std*, and *sth*, are important for long-term carriage and shedding of *Salmonella* (37). The *S.* Enteritidis genome has 13 fimbrial operons, 12 of which are present in *S.* Gallinarum (191). However, several of the CDSs within these common operons were identified as pseudogenes due to mutations that potentially prevent the expression of the functional gene product, which consequently impacts the overall function of the operons. These include mutations in *stiC*, *stfF*, *safC*, *stbC*, *cheM*, *flhB*, *flhA*, *flgK*, *flgI*, *pegC*, *lpfC*, *sefC*, *sefD*, *stbE*, *sthB*, and *sthA* (191). Additionally, the virulence plasmids in the two serotypes contain fimbrial operons; however, they are unique, with the *S.* Enteritidis plasmids carrying the plasmid-borne fimbria (*pef*) operon, while the *S.* Gallinarum plasmids have fimbria genes similar to those of *E. coli* K88 (40, 81, 123, 191, 235).

The sequenced *S.* Gallinarum isolate also lacked some of the T3SS effectors that are present in the *S.* Enteritidis genome, and *sopA* had an early stop codon that likely prevents the expression of a functional SopA protein (191). In addition, there was a mutation in *bscG* of *S.* Gallinarum, whose gene product is predicted to be important for cellulose biosynthesis, which likely contributed to the observed deficiency in its ability to form biofilms (191). Taken together, these mutations may negatively impact the ability of *S.* Gallinarum to colonize mammalian hosts, thereby limiting its ability to cause illnesses in a broad range of host species.

In addition to mutations in *sopA*, several studies have demonstrated the influence of other T3SS effectors on the ability of *Salmonella* serovars to colonize different hosts (206, 236, 237). The T3SSs are multiprotein complexes composed of structural and regulatory elements that transfer functional effectors from the bacterium into host cells, thereby facilitating invasion of and survival within host cells (48, 49). The T3SSs play vital roles in the interaction of *Salmonella* with the host (238). Variations in the gene sequences encoding T3SS factors for both the SPI-1 and SPI-2 systems have been associated with differences in the abilities to invade different hosts. Some of the main variability associated with host specificity is with the secreted effectors and the SipD T3SS tip protein rather than other structural components of the respective T3SSs (206, 236). The SPI-1 T3SS tip protein SipD is important for cell invasion; studies showed that antibodies directed at SipD block the ability of *S.* Enteritidis to invade intestinal epithelial cells (239). Effectors secreted by the SPI-I T3SS may also play a role in host specificity/range; sequence variability in the genes encoding the effector proteins SipA, SopA, and SopE and the chaperones SicP and InvB showed close evolutionary similarity in *S.* Enteritidis and *S.* Gallinarum compared to other serovars not as closely associated with avian sources (206). Thus, these SPI-1-associated factors may play a role in specificity in the initial invasion of the intestinal epithelium or immune cells in birds. The inflammatory responses to infections by *S.* Enteritidis and *S.* Gallinarum are different, with *S.* Enteritidis eliciting a stronger inflammatory response in chickens, which may prevent a more systemic spread, while *S.* Gallinarum elicits a weaker initial immune response and typically leads to systemic fowl typhoid (240).

On the SPI-2 side, at least 13 SPI-2-associated genes in *S.* Enteritidis and *S.* Gallinarum show close evolutionary similarity to each other compared to other serovars (206). It was hypothesized that the sequences may have coevolved for survival in the avian host, since survival in the reticuloendothelial system has been shown to be important for host specificity in chickens (241). The SPI-2 T3SS plays an important role in survival in the SCVs of macrophages (58). Genetic variability in SPI-1 factors, such as SopE and/or SodC, along with SPI-2 factors, such as SseC, SseD, and/or SseF, may lead to differences in the abilities to survive in different host cells (236). The factors SseG and, possibly, SseF appear to impact the migration of the SCVs to the Golgi apparatus in the host cell, which may serve as a potential source of nutrition for *Salmonella* (242). Hence, differences in the effector proteins

may impact the ability to survive in different host cells and to be transported to sites of systemic infection. In addition to changes in the gene sequences themselves, posttranslational modifications of T3SS effectors can impact the targeting of *Salmonella* or the SCVs to different parts of the host cells. Observed posttranslational modifications, such as lipidation, phosphorylation, and ubiquitylation, can occur following translocation into host cells (243).

THE *SALMONELLA*-CHICKEN HOST INTERACTION

Each of the serovars discussed above are quite proficient at colonizing chickens; this section provides a more in-depth examination of the interaction of chicken-associated serovars and the avian host environment. One issue that continues to complicate attempts to understand host responses to *Salmonella* infection is the potential variability in host cell interactions among the different serovars. Several studies have been undertaken to gain knowledge of these interactions. Methner et al. conducted a series of colonization inhibition studies in young chicks gavaged with *Salmonella* strains belonging to the same or different serovars. The results demonstrated that single strains did not prevent colonization by other serovars, but mixtures of different serovars were effective against heterologous serovars (244). He et al. compared five poultry-associated serovars (*S.* Typhimurium, *S.* Enteritidis, *S.* Heidelberg, *S.* Kentucky, and *S.* Senftenberg) for their impact on an MC29 virus-transformed chicken macrophage cell line, HD-11, to assess *Salmonella* cell invasion, intracellular survival, and modulation of phorbol myristate acetate (PMA)-stimulated oxidative burst and nitric oxide (NO) activity. Neither *S.* Enteritidis nor *S.* Typhimurium generated detectable NO production by the macrophages, while the other 3 serotypes elicited production of over 100 µm NO. All of the serotypes repressed PMA-mediated oxidative burst and were equally able to invade HD-11 chicken macrophages; however, *S.* Enteritidis was more resistant to intracellular killing than the other 4 serovars (245). Those researchers speculated that these findings, particularly the intracellular survival ability of *S.* Enteritidis, may account for its association with systemic invasion in chickens (245). However, when newly hatched chickens were challenged with each of the 5 serovars, all but *S.* Senftenberg exhibited high rates of organ invasion (>85%, versus 14% for *S.* Senftenberg), which were different than the *in vitro* intracellular survival outcomes (245). These findings were consistent with studies on *S.* Enteritidis strains classified as displaying either low- or high-invasive phenotypes in Caco-2 human colon tissue *in vitro* assays (246, 247). Those studies demonstrated that when mice were orally challenged, low-invasive strains were as virulent as high-invasive strains, even though not all *S.* Enteritidis isolates recovered from poultry are equally pathogenic. Some of the infectivity differences between *S.* Enteritidis and other serovars in avian model systems may be related to the seemingly high epidemiological frequency and long-term association of *S.* Enteritidis with egg-related salmonellosis and a potentially unique evolutionary relationship with laying hens (248–256). This relationship may be evidenced by the limited genetic diversity observed among *S.* Enteritidis strains based on random amplified polymorphic DNA plasmid profiling and phage typing (257). Work with laying hens suggested that because *S.* Enteritidis infects reproductive tissues, namely, the oviduct and ovaries, these tissues represent critical avenues for egg contamination; additionally, this serovar may also have evolved the means to survive the hostile environment of internal egg contents (252, 258–267).

More recently, attempts have been made to identify unique genetic traits which distinguish *S.* Enteritidis from other serovars and lead to its propensity for laying hens and eggs. Methner et al. demonstrated that *S.* Enteritidis strains exhibited substantial colonization inhibition of other *S.* Enteritidis strains but not of other serovars in young chicks, suggesting that there may be differences in colonization targets (244). However, infection studies comparing *S.* Enteritidis and *S.* Typhimurium in adult laying hens by Keller et al. indicated only minimal differences among strains from these two serovars in intestinal, hepatic, splenic, and reproductive tract colonization (261). Likewise, it appears that *S.* Enteritidis genes associated with intestinal colonization are similar to those observed for other *Salmonella* serotypes. Bohez et al. constructed a deletion mutant of the SPI-1 regulator gene *hilA* in *S.* Enteritidis and observed decreases in shedding and cecal colonization to nearly nondetectable levels after 4 weeks in young chicks. Those authors concluded that the HilA regulatory protein was required for continued cecal colonization and long-term shedding (268). Subsequent studies by these researchers demonstrated that the initial colonization by the *S.* Enteritidis *hilA* mutant was sufficient to prevent long-term fecal shedding and cecal and internal organ colonization by the respective *S.* Enteritidis wild-type strain (264, 269). This linkage of *hilA* with systemic infection is supported by the general trends of increased liver, spleen, and ovary colonization observed in adult laying hens when *S.* Enteritidis *hilA* expression levels were increased (270).

However, studies based on infection of young chicks by *S.* Enteritidis deletion mutants for individual SPIs (SPI-1 to -5) revealed that colonization of the liver and spleen was dependent on SPI-1 and SPI-2 but that cecal colonization was not (271). Desin et al. observed less invasion by *S.* Enteritidis SPI-1 mutants of polarized Caco-2 human intestinal epithelial cells and chicken cecal and small intestinal explants but no differences in cecal colonization of 1-week-old chickens, with the exception of an infection delay in livers and spleens (272). Mixed results have also been reported for other serovars and other hosts. *S.* Typhimurium *hilA* deletion mutants were recovered from infected mouse intestinal contents, intestinal tissues, and systemic tissues at a lower frequency than the parental wild-type strain, while SPI-1 deletion mutants were recovered at frequencies similar to those of the wild-type strain (273). Morgan et al. reported a similar lack of an impact on chicken cecal colonization by *S.* Typhimurium SPI-1 mutants, but SPI-1 was required for colonization of calf intestines (43). Dieye et al. used a mixed-infection approach by combining isogenic SPI-1 *S.* Typhimurium deletion mutants with their respective wild-type counterparts and administering them to 1-week-old chicks instead of younger birds. For the most part, the wild-type strain outcompeted the SPI-1 deletion mutant over the 14-day infection period. When cecal and spleen samples were recovered, the level of cecal colonization by the wild-type was substantially higher than that by the mutant by day 14 (74). More recently, Clark et al. used green fluorescent reporter gene constructs for comparison of various *S.* Typhimurium strains in tissue culture infection assays and discovered that only subpopulations expressed SPI-1 genes. Those researchers speculated that genetic heterogeneity of invading *S.* Typhimurium populations could lead to differential SPI-1 expression and heterogeneous *Salmonella* population behavior during infection (274).

There appear to be serovar differences in pathogenicity gene expression as well. Silva et al. constructed an *S.* Enteritidis trans-

poson library and used a microarray-based negative-selection screening approach to isolate mutants deficient for liver and spleen colonization of BALB/c mice. Many of the colonization-deficient strains were identified as having mutations in SPI genes already known to be responsible for *Salmonella* systemic infection; however, mutants that were associated with genes and genomic islands not identified in other *Salmonella* serovars, including *S.* Typhimurium, were also isolated (275). Shah et al. also identified genes and genomic islands in *S.* Enteritidis that were not present in *S.* Typhimurium after screening *S.* Enteritidis mutants generated from a random Tn*5* transposon library for their ability to invade Caco-2 and chicken liver (LMH) cells and for survival in chicken macrophages (HD-11) (276). Strains within individual serovars also display differences in the ability to express different genes. Shah et al. screened six *S.* Enteritidis isolates and were able to phenotypically differentiate them as stress sensitive (acid and oxidative stress, survival in egg albumen, and impairment in intestinal colonization and systemic infection) versus stress resistant, even though no changes in genomic content were detectable with genomic hybridization microarrays. Sequencing of the stress-regulatory gene *rpoS* in one of the stress-sensitive strains revealed an insertion mutation and truncated protein, leading those authors to conclude that even minor polymorphisms in certain genes would be sufficient to impact stress or virulence gene responses (277).

As more becomes known about the role of host-pathogen interactions in *Salmonella* pathogenesis, some of the seemingly contradictory pathogen responses may become clear. For example, Knodler et al. demonstrated that hyperreplicating *Salmonella* subpopulations in the cytosol of GIT epithelial cells that are distinct from the SCV populations can mediate their release back into the lumen by inducing inflammation accompanied by epithelial cell death and subsequent shedding (58). Since T3SS-based invasion gene systems are involved, it may be that pathogenicity alterations occurring with different mutants and serovars could be attributed the intracellular subpopulations that are impacted, thereby impacting the abilities to sustain long-term fecal excretion of the respective subpopulation (58, 278). This may partially explain the emergence of individual *Salmonella* carrier chickens within poultry flock populations as well as in other animal species.

In addition, the complexity of the indigenous GIT microflora, interaction of the immune system, and host factors such as age, stress level, and overall health status may play important roles in host-pathogen interactions (279–281). Identifying overarching themes for *Salmonella* pathogenesis and designing effective cross-protective control measures that can be applied across different serovars will require application of combinations of high-throughput omics analyses; animal model responses to sort out shared signals, such as common immune signatures across animal species; along with subsequent mathematical modeling (282–286). Particularly, in poultry, the potential roles of host genes that may impact infection and carrier states of *Salmonella* have been studied for a number of years and have been extensively discussed recently in a comprehensive review by Calenge and Beaumont (283) and will not be discussed here. The remainder of the current review focuses on *Salmonella* serovar responses to the poultry host.

SALMONELLA AND STRESS RESPONSES IN THE CHICKEN GIT

Prior to infecting a potential host, *Salmonella* encounters numerous environmental conditions that represent sufficient stress to influence the organism's ability to grow optimally and in some cases even survive. These stressors can originate as a wide range of physical and chemical challenges either in the environmental niches that *Salmonella* occupies or as manufactured barriers, such as those incorporated during food production and processing, such as antimicrobials, disinfectants, biocides, and preservatives. These intervention approaches have been reviewed extensively elsewhere (273, 287–291).

As noted above, when *Salmonella* spp. come into contact with a prospective host and gain access to its GIT, there are a number of potential barriers that the organism encounters as it travels through the GIT. Ahmer and Gunn classified these host barriers into broad categories of physical, chemical, enzymatic, and immune hurdles that must be surmounted by *Salmonella* spp. (292). These include, in addition to specific host-generated immune- and non-immune-based defenses, iron limitation, acidic and pH shifts, oxidative and osmolarity stresses, and limited nutrient availability that leads to starvation (35, 36, 293–301). Although these barriers are manifested either as a function of the host's defense mechanisms or as a consequence of high population densities and a metabolically active indigenous GIT microbial population, in reality, there is a substantial interaction between the two that makes it difficult to separate them experimentally. For example, the field of avian genetics has advanced sufficiently such that a range of host-innate, cell-mediated, and humoral immune responses that resist and clear pathogens such as *Salmonella* have been identified. The extent of the immune involvement is determined by microbial challenge severity as well as the health and nutritional status of the host (280, 283, 302). The immune system also interacts with the indigenous microflora, and the presence of this microflora is now considered essential for proper immunological development (303). Consequently, nonpathogenic bacteria can be considered active players in modulating immune recognition of pathogens (304, 305). Given the diversity of the GIT microflora, an understanding of the symbiotic relationship between the microbial consortia and the immune system is extremely complex and has been reviewed elsewhere (303, 306, 307). Here the focus is on the microbial metabolic interactions that occur in the GIT that appear to influence *Salmonella* and countermeasures employed by this pathogen to overcome these challenges.

Salmonella Acid Tolerance Response and Organic Acids

The compounds collectively referred to as organic acids are comprised of the saturated straight-chain monocarboxylic acids and their respective derivatives (unsaturated, hydroxylic, phenolic, and multicarboxylic organic acids) and have a long history as antifungal and antimicrobial agents in perishable animal feeds and human foods (308–310). Traditionally, organic acids have been added to elicit bacteriostatic and bactericidal impacts on undesirable organisms in potentially contaminated food products. A wide range of antimicrobial mechanisms, including osmotic stress, anion toxicity, and membrane disruption, have been proposed over the years, but inhibition is generally believed to be closely tied to pH because of its influence on the formation of undissociated organic acids, which can easily cross the cell membrane and dis-

sociate into anions and protons in the presence of the cytoplasmic neutral pH (309–313). The acids not only result in the acidification of the cytoplasm and the accompanying difficulties for cellular constituents to perform essential metabolic functions in the cell but also lead to an energy drain due to the consumption of ATP to actively export protons (310).

Salmonella can encounter short-chain fatty organic acids (SCFAs) (primarily acetate, propionate, butyrate, and lactate) in a variety of environments, because they are used as animal feed additives and antimicrobials in food and meat processing and can be generated by certain starter cultures used for food fermentations (308, 309, 314–317). Once in the GIT, particularly in the lower regions of monogastric hosts and the foregut in ruminants, *Salmonella* must be able to survive the presence of variable SCFA concentrations (mM ranging from <1 to hundreds for particular organic acids) depending on the host animal and digestive tract site associated with fermentation by the indigenous microflora in the GIT (309, 311, 314, 318–321).

Traditionally, it was believed that *Salmonella* spp., much like other bacteria, were sensitive to organic acids and that this sensitivity was dependent on pH (322–325). However, Leyer and Johnson demonstrated that HCl acid-adapted *S.* Typhimurium could also resist organic acids typically found in cheeses (326). Baik et al. induced stationary- and exponential-phase acid (pH 4.4) tolerance responses in *S.* Typhimurium after exposure to a series of organic acids and concluded that both physiological states were protected, but the stationary-phase protection occurred with only some of the organic acids (327). The protection against organic acids did not occur if organic acids were used for the initial acid shock. When Kwon and Ricke exposed *S.* Typhimurium to SCFA at neutral pH for 1 h, they observed that acid tolerance was greater for cells exposed to acetate or propionate than for cells exposed to butyrate or valeric acid and was enhanced under anaerobic incubation conditions (328). Calhoun and Kwon observed substantial enhanced acid tolerance in *S.* Enteritidis when exposed to 100 mM propionate at neutral pH over a longer period of time (16 h) (329). The enhancement may not be surprising since *Salmonella* cells cultured under anaerobic growth conditions can produce fermentation acids (330, 331), suggesting that some of the resistance to SCFA may be related to protection from their own end products. It is also known that *Salmonella* can directly catabolize SCFA. For example, *S.* Typhimurium utilizes the 2-methylcitric acid cycle to convert propionate to pyruvate for reentry into the tricarboxylic acid cycle (332–334).

S. Typhimurium exposure at pH 7.0 to high (mM) levels of SCFA typical of large intestine concentrations induces not only resistance to extreme acidic conditions (pH 3.0) but also cross-protective resistance to high osmolarity (2.5 mM NaCl) and reactive oxygen (20 mM H_2O_2) (335). Similar cross protection against salt stress (KCl and NaCl) has been reported for acetic acid-induced acid tolerance in *S.* Typhimurium but not lactic acid-induced acid tolerance, which appears to be related to sensitivity to hydrogen peroxide (336, 337). Initial pH appears to be a factor, as both growth and survivability are limited when *S.* Typhimurium cells are exposed to propionate at pH 5.0 versus pH 7.0 (328). It appears that even more subtle fluctuations in pH can influence *Salmonella* growth metabolism. For example, Dunkley et al., using anaerobic continuous cultures, demonstrated that *S.* Typhimurium glucose- and ATP-based microbial cell yields (calculated as mg of cell protein synthesized per ATP formed or glucose con-

sumed) were decreased when the pH in the growth vessels was shifted from near-neutral pH to levels approaching pH 6.0 (330). Other stress combinations appear to have more of an impact on SCFA inhibition of *Salmonella*. Milillo and coworkers demonstrated not only that the combination of heat (50°C to 60°C) and organic acids was more effective than single intervention applications when applied to *S.* Typhimurium but also that heated acidified solutions caused substantial membrane damage and leakage of intracellular ions (338, 339). There may also be potential serovar differences. Joerger et al. concluded that *S.* Kentucky was more acid sensitive (pH 2.5) than other *Salmonella* serovars after growth in the presence of acetic acid (340). Gene expression microarray comparisons between *S.* Kentucky and *S.* Enteritidis indicated that regulatory genes associated with acid response (*rpoS*, *fur*, and *phoPQ*) were similar between the two serovars, as were most global transcriptional responses, leading Joerger et al. to speculate that phenotypic differences were perhaps more a function of posttranslational or small undetectable transcriptional differences (340).

Salmonella Virulence Response and Organic Acids

While acid tolerance is considered a component of pathogenesis, the presence of SCFA may also directly impact *Salmonella* pathogenesis and virulence gene expression. Durant et al. conducted a series of tissue culture studies with an epidermal carcinoma cell line (HEp-2) to determine the influence of *S.* Typhimurium after growth in either a mixture or separate intestinal concentrations of SCFA (acetate, propionate, and butyrate at pH 6.0 or 7.0) on its subsequent ability to adhere to and invade tissue culture cells (341–343). For *S.* Typhimurium incubated for 4 h in the presence of SCFA, acetate at pH 6.0 and 7.0 did not alter attachment, and only slight increases in invasion occurred, while propionate and butyrate dramatically decreased attachment and invasion at pH 6.0 but not at pH 7.0. There were also indications that these *S.* Typhimurium responses were growth dependent, particularly in the presence of propionate and butyrate at pH 6.0, which reduced the growth rate considerably (341). However, when late-logarithmic-phase (12 h) and stationary-phase (18 h) *S.* Typhimurium cells were compared (342, 343), all SCFAs reduced cell attachment at pH 6.0 and 7.0 but only marginally influenced invasion for late-log-phase cultures of *Salmonella*. The stationary-phase *Salmonella* cells exhibited reduced attachment only in the presence of SCFA at pH 6.0, while actual increases in invasion occurred for lower concentrations of acetate (342, 343). Van Immerseel et al., using avian intestinal and cecal epithelial cell lines, also observed increases in invasion of *S.* Enteritidis exposed to acetate for 4 h and decreases with propionate and butyrate (265, 344). Gantois et al. reported that exposure of *S.* Enteritidis and *S.* Typhimurium to butyrate for 4 h resulted in the lowest percentage of invasion of HeLa cells (345).

In follow-up studies to determine the genetic elements associated with the SCFA influence on *Salmonella* pathogenesis, Durant et al. used *S.* Typhimurium strains constructed to carry chromosomal *lacZY* transcriptional fusions within either *hilA* or *invF* to assess the impact of pH and individual SCFAs on their expression (346). The study concluded that growth of *S.* Typhimurium in the presence of acetate, but not propionate and butyrate, stimulated β-galactosidase expression for both genes, even though growth was inhibited only at pH 6.0. When the *S.* Typhimurium *hilA-lacZY* fusion was incorporated into an *S.* Enteritidis isolate from

poultry, increasing lactate concentrations along with lowering pH reduced expression levels of β-galactosidase (347). Lawhon et al. concluded that acetate provided a signal for invasion through the production of cytoplasmic acetyl-phosphate, while propionate and butyrate repressed invasion genes (348). Gantois et al. conducted comparative transcriptomic analyses of *S*. Enteritidis and *S*. Typhimurium and reported that butyrate downregulated SPI-1 genes, including the regulatory genes *hilD* and *infV*, while Huang et al. identified formate as a diffusible signal to induce SPI-1 genes and invasion (345, 349). Virulence gene responses to organic acids may vary in different serovars as well. Using quantitative reverse transcriptase real-time PCR, Gonzalez-Gil et al. reported that expression of the virulence gene regulator *hilA* was both serovar and strain dependent when isolates were exposed to either mild acetic or HCl acid stress. Responses were dependent on the type of acid used and were time dependent, with *hilA* being upregulated after a 2-h exposure to the respective acid (350). When Milillo et al. compared *S*. Typhimurium transcriptome responses to sodium acetate and sodium propionate exposure after 1 min (55°C at pH 4.0), the respective gene expression patterns were similar, with no increases in expression levels of *rpoS*, *phoPQ*, or virulence factors despite their established roles in organic and inorganic acid stress (35, 327, 328, 338). Conversely, genes related to attachment and/or motility functions were induced, which has been observed previously for *S*. Typhimurium attachment genes after exposure to 42°C (351). There was also a repression of several heat shock response genes, which, in conjunction with cell membrane damage, indicates that membrane integrity is an important component for *Salmonella* cells to accurately assess their extracellular environmental conditions and respond accordingly (351). Clearly, more in-depth genetic analysis is needed to sort out the interactions that occur among different stresses in *Salmonella* versus its collective virulence response capabilities as well as how these may shift over time after initial encounters with the cell's surrounding environment. Serovar and strain differences will also need to be examined to determine whether some of the serovar dissemination patterns are due to the ability of some serovars, but not others, to effectively counter external stresses in their environments.

Salmonella Virulence Response and Competition with GIT Microflora

The GIT systems in most hosts, including food animals and humans, harbor highly complex and metabolically interactive microbial consortia. Particularly in the lower GIT of monogastric animals (colon or cecum) and the foregut (rumen) of ruminants, microbial populations consist of large numbers of mostly saccharolytic anaerobic microorganisms along with methanogenic archaea, which together result in a highly reduced ecosystem with minimal oxygen (318, 320, 352–359). Given the complexity of diets in general and the recalcitrant nature of some of the fiber-containing components (particularly those consumed by herbivores), the GIT microbial consortia are characterized not only by their anaerobic physiology but also by their ability to hydrolyze fiber polymers into soluble carbohydrates that are readily fermentable (360–364). Since energy generation for ATP formation is anaerobic and, therefore, much more limited per carbon source, GIT saccharolytic microorganisms must extract maximum ATP through a multitude of limited-substrate-level and anaerobic respiration phosphorylation pathways (318, 365–368). Given the complexity of the GIT microflora and the limited energy generation capabilities for microbial growth, each species must catabolize similar carbon sources, necessitating a hierarchy for substrate preferences and competition among microorganisms based on maximum growth, energy efficiency, and substrate affinity (369, 370). If these capabilities are combined with the constant GIT turnover that occurs due to the passage rate of digesta, GIT microbial ecosystems are characterized by their exceptional ability to metabolically scavenge and extract maximum nutritional value from incoming dietary sources.

Consequently, pathogens entering the GIT, in addition to combating the hostile environment derived from fermentation activities of the indigenous microflora and the host barriers, must also directly compete with the GIT microflora for scarce nutrients (298, 371). Pathogen metabolism and virulence are often interconnected to the point that it becomes difficult to distinguish which functions are occurring simultaneously. Rohmer et al. suggested that not only do pathogens acquire virulence factors to access new niches, they may also require new metabolic pathways to sequester available nutrient sources, as evidenced by the presence of metabolic functions encoded on pathogenicity islands (371, 372). In the GIT, pathogens entering the lumen during times of nutritional stress could conceivably employ this strategy by either inducing virulence to escape the GIT altogether or manipulating the GIT environment to outcompete indigenous microflora.

Starvation, malnutrition, or abrupt changes in diet can alter the composition of indigenous GIT microflora and potentially impact the host's immune system and the host's general physiology and well being (373–375). Changes in nutritional status and dietary alteration have also been known for some time to alter the relationship between the GIT and pathogens, such as attachment to the epithelial lining (376). Emptying of the GIT content represents one of the more drastic alterations that occurs either naturally or as part of a prescribed treatment in humans or a management option in animals. Increased populations of *Salmonella* have been consistently observed in a variety of animals undergoing emptying of the GIT due to feed removal, including chickens (377–381), mice (382), and ruminants (383–385). Although the indigenous GIT microflora were not characterized in these early studies, it was speculated that feed removal/GIT emptying potentially influenced the microbial populations or activities, and susceptibility to *Salmonella* colonization was presumed to be related to decreases in levels of antimicrobial organic acids generated during fermentation (250).

One of the most extensively studied host-pathogen systems, which represented a nutritional management strategy linked with *Salmonella* dissemination, was the use of dietary restrictions to induce artificial molting in laying hens. In the U.S. egg industry, laying hens can be induced to molt in order to cease egg production and allow the reproductive tract to undergo a rest phase to allow flock rejuvenation, feather renewal, and a second, higher-production egg-laying cycle (386). Historically, this was accomplished by complete removal of diet for anywhere from 5 to 14 days until cessation of egg production was completed (386, 387). However, *S*. Enteritidis was closely linked with egg contamination, and research conducted during the 1990s with laying hens as the infection model suggested that management practices such as feed removal-induced molts might be partially responsible for the contamination (388, 389). In the following decade, more detailed

studies on *S.* Enteritidis infection in susceptible laying hens concluded that complete removal of feed created a GIT environment in laying hens that not only was easily colonized by *S.* Enteritidis but also led to systemic infection of internal organs, including the ovaries (250, 331, 390). Increased expression levels of the *S.* Enteritidis *lacZY-hilA* fusion gene in cells incubated in crop contents from birds that had undergone feed removal demonstrated that enhanced systemic invasiveness could be linked with increased expression levels of *hilA* (391). These findings were later replicated and confirmed *in vivo* by quantitative PCR (392). This increase in *S.* Enteritidis colonization and invasiveness also coincided with diminished numbers of lactic acid bacteria in the crop and reduced organic acids in the crop (391) and cecum (270, 393) and led to major shifts in fecal and cecal bacteria compared to fully fed birds (270, 394, 395). Ricke hypothesized that the key to limiting *S.* Enteritidis during molt was to develop low-nutrient dietary strategies, such as formulations with high fiber content, that would still induce molt due to low energy density but were sufficiently fermentable to support an active GIT microflora that would serve as a barrier to pathogen establishment (255). Several molt diets with fermentable substrate combinations have been screened *in vitro* and shown to support optimal avian cecal fermentation (396–398) and retain similar cecal microbial molecular profiles (397). *In vivo* application of some of these diets was shown to retain fermentation and GIT populations during molt while limiting the ability of *S.* Enteritidis to colonize the GIT and become invasive (255, 270, 392, 399, 400). The overall results of the studies demonstrate that there is a complex and important relationship between the microflora of the avian GIT and *Salmonella*, which is key to limiting the pathogen.

CONCLUDING REMARKS

Salmonella serovars display a range of abilities to colonize different animal hosts and cause illnesses in humans. Many of the serovars most commonly implicated in food-borne human infections have a broad range of animal hosts that they can colonize, while others, especially some that cause severe human infections, have much narrower host ranges. Some serovars, such as *S.* Typhi and *S.* Paratyphi A, are human-restricted serovars and can cause severe disease manifestations such as enteric fever. The factors that impact *Salmonella* pathogenesis and host range are complex and are associated with differences in variables such as the genetics of the bacterium and expression of these genetic factors, the host immune status and specific pathogen responses, the host environment, and interactions with intestinal microflora.

A great deal of work has been undertaken to understand the genetics of *Salmonella* and explore the factors that contribute to the evolution of pathogenesis. Based on genome sequencing results and pangenome analyses of *Salmonella* serovars, it is evident that two distinct, yet potentially complementary, evolutionary approaches that impact host range and virulence are occurring. These approaches include gene acquisition through horizontal transfer (associated with SPIs, transposable elements, phages, and plasmids) and the loss of genes or a loss of their function, which impacts host range (105, 106). As the species *S. enterica* evolved, its members' genomes acquired the multiple SPIs that allowed the organism to become pathogenic. Several serovars also acquired specific virulence plasmids that harbor the *spv* operon, which increases virulence (126). Other serovars, while not having serovar-specific virulence plasmids, have other plasmids that may encode

factors that are important for survival in a potential host niche. Plasmids have played a significant role in the evolution of host specificity in some *Salmonella* serovars. A key example includes some of the plasmids of *S.* Kentucky and some *S.* Heidelberg isolates that contain genes associated with extraintestinal survival in poultry, which may provide a selective advantage in poultry (83). Many of these plasmids also contain genes for antimicrobial resistance, which is concerning because of the possibility that antimicrobial use could select for enhanced virulence or conversely that the selective advantage to survive in one environment/host may select for resistance to one or more antimicrobials (83, 84). While our knowledge of the genetic composition of these plasmids has greatly increased with the advent of next-generation sequencing, our understanding of the evolution and dissemination of these plasmids and their interactions with one another remains limited. There is not a clear correlation between carriage of the plasmid and the establishment of systemic disease in humans, further suggesting that it may be a requirement of some strains but not others (401).

On the other side of the spectrum of factors that impact virulence and host range is gene loss or the loss of gene function due to mutation, insertion, or deletion in the gene or regulatory element. Many of the host-restricted serovars have a large number of pseudogenes, which inactivate genes that are often involved in aspects of virulence, nutrient utilization, and metabolism (91). The impact of pseudogenes on host restriction seems fairly clear; however, it is less clear whether gene inactivation leads to the host-restricted phenotypes or whether the organisms settled in the niche and those genes not essential for survival are undergoing the process of genome reduction. Interestingly, *Salmonella* serovar Typhi demonstrates aspects of functional gene loss (a high percentage of pseudogenes) (193) and the impact of horizontal gene transfer on pathogenicity (14). *S.* Typhi isolates have mutations in several of the fimbrial genes that are associated with intestinal attachment, which is important for gastroenteritis but not enteric fever (194). Members of the serovar also acquired SPI-7, which harbors genes for the Vi capsule, which prevents recognition of PAMPs on the surface of *S.* Typhi, thereby limiting the initial immune response following infection (2).

Despite the wealth of knowledge associated with *Salmonella* pathogenesis mechanisms in host animals, several key questions remain on the exact roles and extent of involvement of the respective virulence genes and genomic islands of particular serovars and their characteristic pathogenic phenotypes expressed in animal models. Not only do *in vitro* models, such as tissue culture adhesion and invasion assays, sometimes differ from *in vivo* results, there may also be differences in responses depending on the type of cell line used (402). It is also becoming apparent, as more sophisticated genetic analyses are being applied to animal infection studies, that *Salmonella* serovars vary in their virulence gene capacities, and given the tremendous number of serovars identified and the wide range of corresponding strains isolated for some serovars, there is the potential for almost endless nuances of perhaps subtle but different serovar- and even strain-specific genetic strategies for overcoming host barriers.

An in-depth understanding of the mechanisms of *Salmonella* pathogenesis is important for developing intervention strategies to limit their prevalence and spread and aid in the development of newer drugs and vaccines which could result in better treatment and/or prevention of salmonellosis in animals and humans. The

continued use of whole-genome sequencing, gene expression, and bioinformatics analyses of *Salmonella* serovars from host-restricted and host-specific serovars should continue to provide insights into the specific genetic traits that could contribute to symbiotic and/or disease-causing potential in humans and animals and lead to an overall reduction in disease burden associated with *Salmonella*.

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