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Review

Emergence, Distribution, and Molecular and Phenotypic Characteristics of *Salmonella enterica* Serotype 4,5,12:i:–

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Abstract

Salmonella spp. represent one of the most common causes of bacterial foodborne illnesses around the world. The species *Salmonella enterica* contains more than 2500 serotypes, and emergence of new human pathogenic *Salmonella* strains and serotypes represents a major public health issue. *Salmonella enterica* subsp. *enterica* serotype 4,5,12:i:– represents a monophasic variant of *Salmonella* Typhimurium, which has rarely been identified before the mid-1990s. The prevalence of this serotype among human salmonellosis cases has increased considerably since the mid-1990s and *Salmonella* 4,5,12:i:– currently (i.e., the first decade of the 2000s) represents one of the most common serotypes among human cases in many countries around the world. This paper discusses our current knowledge of the global ecology, epidemiology, transmission, and evolution of this emerging *Salmonella* serotype.

Introduction

Salmonella spp. represent a well-recognized foodborne bacterial pathogen, which causes a considerable number of illnesses and deaths worldwide. For example, in the United States *Salmonella* was estimated to represent the leading cause of foodborne illnesses due to bacterial pathogens in 2006 (CDC, 2007a). In addition, *Salmonella* has been estimated to cause more deaths due to foodborne illnesses than any other known pathogen in the United States (Mead *et al.*, 1999). Symptoms of human nontyphoidal salmonellosis include enteritis as well as, less commonly, systemic manifestations, including septicemia (Mead *et al.*, 1999). While the majority of human *Salmonella* infections appear to be foodborne, salmonellosis can also be acquired through contaminated drinking water, contact with infected animals, and direct human-to-human transmission. In addition to humans, *Salmonella* can also infect a variety of animals species, including mammals, birds, and reptiles.

The genus *Salmonella* currently includes two species, *S. enterica* and *S. bongori*. *S. enterica* is divided into subspecies I (*enterica*), II (*salamae*), IIIa (*arizonae*), IIIb (*diarizonae*), IV (*houtenae*), and VI (*indica*). Traditionally, characterization of *Salmonella* isolates uses serotyping, based on the Kauffmann–White scheme, for subtyping and strain differentiation (Kauffmann, 1965b; CDC, 2003; Foley *et al.*, 2007); over 2500 *Salmonella* serotypes are currently known. Serotyping is based on antigenic variability of lipopolysaccharides (O antigen), flagellar proteins (H antigen), and capsular polysaccharides (Vi antigen). Most *Salmonella* strains are motile by means of peritrichous flagella, which can be encoded by two different flagellin genes on the bacterial chromosome (*fli*C and *flj*B); *fli*C and *flj*B expression is regulated through a mechanism called "phase variation." The majority of the *Salmonella* serotypes are biphasic, meaning that they can express both genes (phase 1 and phase 2). Some *Salmonella* isolates and strains are monophasic though and may lack either phase 1 or phase 2 expression. For example, *Salmonella* serotype 4,5,12,i:– lacks expression of phase 2 flagella.

While the overall incidence of human salmonellosis appears to be fairly stable, e.g., in the United States (CDC, 2003), the incidence of infections caused by different serotypes and subtypes appears to change considerably over time (CDC, 2005). For example, the proportion of human *Salmonella* Typhimurium isolates in the United States that show the drug resistance phenotype ACSSuT (i.e., resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline), which is typical for *Salmonella* Typhimurium phage type DT104, increased from 9% in 1990 to 33% in 1996 (Hogue *et al.*, 1997). Similarly, the frequency of multidrug-resistant (MDR) Newport among human *Salmonella* isolates in the

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United States appears to have increased from 1998 to 2001; in 1998 only 1% of Newport isolates characterized by the National Antimicrobial Resistance Monitoring System showed the MDR-AmpC phenotype, while 26% of Newport isolates from 2001 showed this phenotype (CDC, 2002). In addition to changes in the frequency of drug-resistant *Salmonella* subtypes, the frequency of different serotypes among human isolates also seems to change. For example, the frequency of *Salmonella* Newport among human isolates in the United States increased from 5% in 1997 to 10% in 2001 (CDC, 2002). On the other hand, the frequency of serotype *Enteritidis* among human clinical isolates in the United States decreased considerably from 21.9% in 1993 to 14.5% in 2003 (CDC, 2005). As detailed in this review, the monophasic serotype *S. enterica* subsp. *enterica* serotype 4,5,12:i:– appears to represent an emerging serotype with apparent worldwide distribution. Serotype 4,5,12:i:– currently is among the 10 most common serotypes associated with human infections in a number of countries, including the United States (based on data from 2005) (CDC, 2005) and Spain (based on data from 1998 to 2000) (Echeita *et al.*, 2001). In addition, this serotype has been responsible for human outbreaks in California in 2004 (Norton *et al.*, 2004), Luxemburg in 2006 (Mossong *et al.*, 2007), and most recently, a 2007 multistate outbreak with more than 272 cases in the United States (CDC, 2007c).

Serological Characterization of *Salmonella* 4,5,12:i:–

While a number of *Salmonella* serotypes are named (e.g., serotype Typhimurium, Newport), newly isolated serotypes are now typically designated by antigenic formulae with four components, including (i) subspecies designation (e.g., I), (ii) O antigen, (iii) phase 1 H antigen, and (iv) phase 2 antigen; the three antigen designations are separated by colons (Brenner *et al.*, 2000). According to this scheme, *Salmonella* Typhimurium would be described as I 4,5,12:i:1,2, indicating that this serotype belongs to subspecies I and carries the "4,5,12" O antigens, the "i" phase 1 H antigen, and the "1,2" phase 2 H antigens. *Salmonella* 4,5,12:i:– thus shares all O antigens and phase 1 H antigens with *Salmonella* Typhimurium. *Salmonella* Lagos (4,5,12:i:1,5) also has the same O antigens and phase 1 H antigens as *Salmonella* 4,5,12:i:–. Molecular subtype data showed considerable similarities between *Salmonella* 4,5,12:i:– and *Salmonella* Typhimurium, but not between *Salmonella* 4,5,12:i:– and *Salmonella* Lagos (Guerra *et al.*, 2000; Echeita *et al.*, 2001). The observations that *Salmonella* 4,5,12:i:– and *Salmonella* Typhimurium are genetically highly similar and have identical serotypes, except for the lack of phase 2 flagella in 4,5,12:i:–, lead to the hypothesis that *Salmonella* 4,5,12:i:– is a monophasic variant of *Salmonella* Typhimurium (Echeita *et al.*, 2001). Importantly though, O factors 1, 5, and 27 may sometimes not be expressed in a given strain, suggesting that other serotypes that share O antigens 4 and 12 and phase 1 antigen i with *Salmonella* 4,5,12:i:– may be alternative ancestors of this serotype. Serotypes with related antigen profiles include Agama (4,12:i:1,6), Farsta (4,12:i:e,n,x), Tsevie (1,4,12:i:e,n,z_{15}), Gloucester (1,5,12,27:i:l,w), Tumodi (1,4,12:i:z_6), and an unnamed subspecies II serotype (4,12,27:i:z_{35}) (Holt, 1984; Grimont and Weill, 2007). Unfortunately, isolates with these serotypes have not been included in comparative subtype and evolutionary studies on serotypes Typhimurium and 4,5,12:i:– (e.g., Echeita *et al.*, 2001; Garaizar *et al.*, 2002; Zamperini *et al.*, 2007); and it is thus not currently possible to exclude these other rare serotypes as ancestors of *Salmonella* 4,5,12:i:–.

Worldwide Distribution of *Salmonella* 4,5,12:i:–

While there have been few reports of serotype 4,5,12:i:– in the peer-reviewed literature before the 1990s, isolates that appear to represent this serotype have occasionally been reported since at least the middle of the 20th century. For example, Edwards and Brunner (1946), both located at the Kentucky Agricultural Station in the United States, reported three *Salmonella* Typhimurium isolates that contained only phase 1 antigens; these isolates would now be designated as serotype 4,5,12:i:–. Unfortunately the country of isolation was not specifically detailed for these three isolates. In 1965, Kauffmann also reported a monophasic *Salmonella* Typhimurium isolate in a paper entitled "Monophasic *Salmonella* cultures for the preparation of H-serum" (Kauffmann, 1965a). While the rare isolation of this serotype before the 1990s may reflect a recent expansion and/or emergence of this serotype, it is important to acknowledge that 4,5,12:i:– isolates have been and still may be misclassified as *Salmonella* Typhimurium, leading to underreporting of this serotype. Serotype 4,5,12:i:– isolates also appear to often have been reported as "group B" or "subspecies I" (CDC, 2003).

Since the 1990s, isolation of *Salmonella* serotype 4,5,12:i:– has been reported in a variety of countries (Table 1). In Asia, serotype 4,5,12:i:– isolates obtained in 1993 in Thailand (Boonmar *et al.*, 1998) included 52 isolates from humans patients with clinical illness and eight isolates from frozen

Table 1. Selected Peer-Reviewed Reports on Worldwide Isolation of *Salmonella enterica* Serovar 4,5,12:i:–

Year(s) of isolation	*Country*	*Source*	*Reference*
1986–1987	Portugal	Chicken	Machado and Bernardo, 1990
1993–1994	Thailand	Human, chicken meat	Boonmar *et al.*, 1998
1997	Spain	Human, food	Echeita *et al.*, 1999
1991–2000	Brazil	Human, food, animals	Tavechio *et al.*, 2004
1998–2000	United States	Human, raw chicken meat	Agasan *et al.*, 2002
1998–2000	Spain	Swine	de la Torre *et al.*, 2003
2000–2001	Thailand	Human, frozen meat, foods	Amavisit *et al.*, 2005
2000–2003	Taiwan	Human	Chiu *et al.*, 2006
2003–2004	Portugal	Pig carcasses	Vieira-Pinto *et al.*, 2005
2004	United States	Human, bovine	Alcaine *et al.*, 2006
Not available	United States	Bovine, poultry, nondomestic birds	Zamperini *et al.*, 2007
2006	Luxembourg	Human, food, porcine	Mossong *et al.*, 2007

chicken meat. Another study of *Salmonella*, isolated between 1991 and 1995 from patients with septicemia in Thailand, also found that *Salmonella* 4,5,12:i:– represented 8.2% of the 741 isolates from human blood samples that were characterized (Komolpis *et al.*, 1999). In Asia, serotype 4,5,12:i:– has also been reported among human isolates in Taiwan (Chiu *et al.*, 2006). Asian countries that are listed in the World Health Organization (WHO) Global *Salmonella* Surveillance system as having reported *Salmonella* 4,5,12:i:– include Thailand (2004 data) and Japan (2007 data) (WHO, 2008).

One of the first of *S. enterica* subsp. *enterica* serovar 4,5,12:i:– isolates from Europe reported in the peer-reviewed literature was obtained from a chicken carcass in Portugal in 1986/87 (Machado and Bernardo, 1990). Subsequently, a considerable number of 4,5,12:i:– isolates have been reported from Spain, with the first reported isolation in this country in 1997 (Echeita *et al.*, 1999). Since then, serotype 4,5,12:i:– appears to have become the most frequently encountered serotype in swine and the second most frequently encountered serotype in pork products in Spain (based on data from 2000 as reported by de la Torre *et al.*, 2003); this observation has lead to the hypothesis that pigs may be a reservoir of this serotype (de la Torre *et al.*, 2003). Isolation of serotype 4,5,12:i:– has also been reported for Luxemburg (Mossong *et al.*, 2007), Portugal (Vieira-Pinto *et al.*, 2005), and Germany (Guerra *et al.*, 2004a) as well as Denmark, Bulgaria, and Slovakia, which are all listed in the WHO Global *Salmonella* Surveillance system as having reported *Salmonella* 4,5,12:i:– (WHO, 2008). In Luxembourg, serotype 4,5,12:i:– caused at least two *Salmonella* outbreaks in 2006, which appear to have been linked to consumption of contaminated pork (Mossong *et al.*, 2007).

In the United States, serotype 4,5,12:i:– represented 0.2% of human clinical isolates in 1995; in 2004, 2.1% of human clinical isolates were classified as serotype 4,5,12:i:– (Grenne *et al.*, 2006). Based on U.S. Centers for Disease Control and Prevention (CDC) reports, serotype 4,5,12:i:– was the 18th and 6th most common serotype recovered from human illness cases in 2002 and 2005, respectively (CDC, 2005). In the United States, serotype 4,5,12:i:– has also been isolated from different foods (e.g., raw ground chicken [Zamperini *et al.*, 2007], chicken enchiladas [Norton *et al.*, 2004]) and a variety of animal species, including chickens (Zamperini *et al.*, 2007), cattle (Alcaine *et al.*, 2005, 2006), nondomestic birds (Zamperini *et al.*, 2007), and turtles (CDC, 2007b). Salmonellosis outbreaks in the United States have also been caused by *Salmonella* serotype 4,5,12:i:–, including a multistate outbreak in 2007 (linked to consumption of frozen poultry pie [CDC, 2007c]). Some salmonellosis cases in the United States caused by *Salmonella* serotype 4,5,12:i:– in 2006 (including two cases in Ohio and one case in Tennessee [CDC, 2007b]) were also linked to exposure to turtles infected with this serotype. Based on the WHO Global *Salmonella* Surveillance system (WHO, 2008), Canada has also reported isolation of *Salmonella* 4,5,12:i:– from human cases (2004 data) and Barbados has reported isolation of this serotype from animals and humans (2006 data).

Serotype 4,5,12:i:– has also been reported in South and Latin America. Among *Salmonella* isolated in the Brazilian state São Paulo between 1991 and 2000, 8.8% of human clinical isolates and 1.6 % of nonhuman isolates (representing predominantly food and animal isolates) were classified as serotype 4,5,12:i:– (Tavechio *et al.*, 2004). Based on these data, serotype 4,5,12:i:– appears to have been one of the five most common *Salmonella* serotypes associated with human infections in São Paulo between 1991 and 2000 (Tavechio *et al.*, 2004). According to the WHO Global *Salmonella* Surveillance WWW database (WHO, 2008), Chile and Costa Rica have also reported isolation of *Salmonella* 4,5,12:i:– from human cases (2007 data).

In summary, *Salmonella* serotype 4,5,12:i:– has been identified in a number of countries around the world since the early and mid-1990s. This serotype appears to specifically be responsible for a considerable number of human salmonellosis cases in different countries and has also been responsible for salmonellosis outbreaks in different continents. This serotype has also been isolated from a number of animal species (e.g., chickens, cattle, swine, and turtles) and food items (raw poultry, pork, and pork sausages). While at least some European studies suggest a common link of human infections with this serotype to pork and pork products, worldwide, serotype 4,5,12:i:– appears to be widely distributed and not characterized by a single reservoir, as supported by outbreaks and cases linked to poultry products and direct contact with turtles.

Genetic Characterization of *Salmonella* 4,5,12:i:–

Molecular subtyping and phylogenetic characterization of serotype 4,5,12:i:–

A number of studies have used different molecular subtyping methods (including pulsed-field gel electrophoresis [PFGE], multilocus sequence typing [MLST], and phage typing) to characterize *Salmonella* 4,5,12:i:– isolates (e.g., Echeita *et al.*, 2001; Agasan *et al.*, 2002; de la Torre *et al.*, 2003; Amavisit *et al.*, 2005). Findings from most of these studies indicate that *Salmonella* 4,5,12:i:– isolates are closely related to *Salmonella* Typhimurium, suggesting that 4,5,12:i:– is a monophasic variant of serotype Typhimurium. Phage types found among 4,5,12,i:– isolates from Spain include U302, DT 208, and DT 193, all phage types typical for *Salmonella* Typhimurium (de la Torre *et al.*, 2003). Characterization of thirteen 4,5,12:i:– isolates from Spain (Echeita *et al.*, 2001) found that these isolates, along with two *Salmonella* Typhimurium phage type DT104 and two U302 isolates, allowed for amplification of a 1000-bp *fliB-fliA* polymerase chain reaction (PCR) product (indicating the presence of an IS200 element downstream of *fliB*), while the same primers amplified a 250-bp fragment in *Salmonella* Lagos and a selection of isolates representing other serotypes (indicating the absence of the IS200 element downstream of *fliB*). Only the 4,5,12:i:– and Typhimurium DT104 and DT302 isolates yielded PCR products with another set of primers targeting a DT104 and U302 specific region; these primers did not yield a product with *Salmonella* Typhimurium LT2 or other *Salmonella* serotypes (Echeita *et al.*, 2001). In a subsequent study, 16 of 23 serotype 4,5,12:i:– isolates from Spain were classified as phage type U302 (de la Torre *et al.*, 2003); in addition at least some *Xba*I and *Bln*I PFGE types were found to be shared between 4,5,12:i:– and Typhimurium isolates, even though these two serotypes never shared the same combined *Xba*I/*Bln*I PFGE type (de la Torre *et al.*, 2003). These data suggested that serotype 4,5,12:i:– isolates from Spain represent a variant of *Salmonella* Typhimurium and indicate that it may have emerged from an ancestor representing *Salmonella* Typhimurium U302 or a close relative to this phage

type (Echieta *et al.*, 2001; de la Torre *et al.*, 2003). This is consistent with the observation that most Spanish 4,5,12:i:– isolates are phage type U302 (Echeita *et al.*, 2001).

MLST-based characterization of 335 *Salmonella* isolates collected in New York state (USA), including 15 *Salmonella* Typhimurium and 18 *Salmonella* 4,5,12:i:– isolates, showed that all but one serotype 4,5,12:i:– isolate had the same sequence type 6 (ST6) that also represented the predominant ST among the characterized *Salmonella* Typhimurium isolates (Alcaine *et al.*, 2006). ST6 was unique to serotypes Typhimurium 4,12:i:– and 4,5,12:i:–, supporting the initial findings based on characterization of Spanish isolates (de la Torre *et al.*, 2003), that 4,5,12:i:– appears to have emerged from a *Salmonella* Typhimurium ancestor. More recent characterization of 32 serotype 4,5,12:i:– isolates from poultry, bovine, and nondomestic birds in Georgia (USA), along with characterization of selected Typhimurium isolates, also revealed a close genetic relationship between 4,5,12:i:– and Typhimurium isolates. Specifically, a number of 4,5,12:i:– isolates had *Xba*I and *Bln*I PFGE patterns that were identical or closely related to PFGE patterns found among *Salmonella* Typhimurium isolates (Zamperini *et al.*, 2007). Characterization of different *Salmonella* isolates from Thailand (Amavisit *et al.*, 2005) showed that 30 serotype 4,5,12:i:– isolates were positive in a duplex PCR assay that included a set of serotype Typhimurium specific *mdh* primers as well as a set of primers that target a Typhimurium phage type DT104 and U302 specific region. These data further support emergence of serotype 4,5,12:i:– from an ancestor representing *Salmonella* Typhimurium U302 or a close relative to this phage type.

In conclusion, characterization of *Salmonella* 4,5,12:i:– isolates from various countries consistently supports the hypothesis that this serotype has recently emerged from a *Salmonella* Typhimurium ancestor. At least for the *Salmonella* 4,5,12:i:– isolates from Spain and Thailand, the Typhimurium ancestor for 4,5,12:i:– appears to most likely be a strain closely related to Typhimurium phage type U302. Interestingly, most studies using discriminatory subtyping methods, such as PFGE, found that *Salmonella* 4,5,12:i:– isolates represent a considerable diversity of subtypes, even among isolates from a single given country. For example, at least 13 different *Xba*I PFGE types were found among 32 serotype 4,5,12:i:– isolates from Georgia (as determined by visual analysis of the PFGE patterns shown in Fig. 2 in Zamperini *et al.*, 2007) and at least 11 *Xba*I PFGE types were found among 23 Spanish serotype 4,5,12:i:– isolates (de la Torre *et al.*, 2003). These findings suggest considerable diversification of serotype 4,5,12:i:– after emergence (e.g., from a *Salmonella* Typhimurium ancestor) or possibly multiple independent emergence events.

Genetic basis of the monophasic phenotype in Salmonella 4,5,12:i:–

More than 50 genes are required for flagellar formation and function in *Salmonella*, these flagellar genes constitute at least 14 different operons. Most of operons are clustered in four regions on the chromosome. According to the relative position in the transcriptional hierarchy, the flagellar operons are grouped into three classes, including class 1 (represented by the *flhD* operon whose products are required for class 2 expression), class 2 (which contains the operons responsible for formation of basal structure and the hook-basal-body complex), and class 3 (which contains the operons responsible for filament formation, flagellar rotation, and chemotaxis) (Ikebe *et al.*, 1999). Flagellar phase variation in *Salmonella* Typhimurium involves genes in the operon class 3 (Ikebe *et al.*, 1999) and entails transcription of either *fliC* or *fljB*, which both encode flagellin proteins. Cellular expression of FliC is called phase 1 and cellular expression of FljB is called phase 2. Flagellar phase variation is caused by the reversible inversion of a DNA segment (i.e., the H segment), which contains the promoter for *fljB* (Yamamoto *et al.*, 2006). The H segment is flanked by inverted repeat sequences, *hixL* and *hixR* (Fig. 1), between which site-specific recombination occurs, leading to

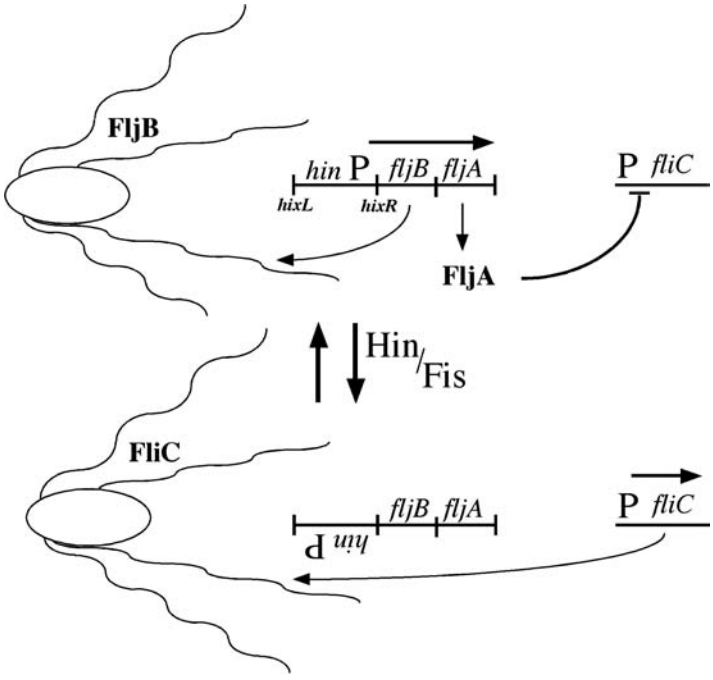

FIG. 1. Flagellar phase variation in *Salmonella* (this figure is reproduced, with permission, from Aldridge *et al.* [2006]). The reversible, Hin-mediated inversion of the H segment (located between *hixL* and *hixR*) results in the inversion of a promoter driving the expression of *fljB* (which encodes the phase 2 flagellin) and *fljA*. *fljA* encodes an inhibitor of *fliC*, which in turn encodes phase 1 flagellin. The Hin recombinase in conjunction with the Fis protein catalyzes a site-specific recombination reaction between the *hixL* and *hixR* recombination sites. The upper part of this figure shows the promoter configuration during phase 2 flagella expression; *fljBA* is transcribed allowing for production of FljB and FljA, which inhibits *fliC* transcription. The bottom part of this figure shows the promoter configuration during phase 1 flagella expression; the promoter for *fljBA* is inverted and thus cannot facilitate *fljAB* transcription; *fliC* is transcribed because FljA is not produced and thus cannot inhibit *fliC* transcription.

H inversion. This recombination event is catalyzed by a DNA invertase encoded by *hin*, which is located within the H segment. The gene *fljA*, which encodes a negative regulator for *fliC* expression, is located downstream of *fljB*. When the H segment is in the "on" orientation, both *fljB* and *fljA* are transcribed, and *fliC* is consequently repressed by FljA (Fig. 1). When the H segment is in the off orientation, both *fljB* and *fljA* genes are not transcribed, the *fliC* gene is expressed (Aldridge *et al.*, 2006; Yamamoto *et al.*, 2006). This phase switching occurs at a rate of 10^{-3} to 10^{-5} per cell generation (Yamamoto *et al.*, 2006).

Isolates of serotype 4,5,12:i:– are phenotypically characterized by a lack of phase 2 flagella expression. Genetic characterization, using microarrays, of four Spanish 4,5,12:i:– isolates revealed a large chromosomal deletion, which spanned 16 genes including *hin*, *fljB*, and *fljA* (Garaizar *et al.*, 2002). These findings provided the initial identification of the genetic basis for the lack of phase 2 flagella in serotype 4,5,12:i:–. Characterization with PCR-based and colony blot approaches of 30 serotype 4,5,12:i:– isolates from poultry, bovine, and nondomestic birds in Georgia (USA) also found that a number of these isolates had partial or complete deletions of *fljB*, the structural gene for phase 2 flagella. Interestingly, the *fljB* deletions in these 4,5,12:i:– isolates from Georgia represented different deletion patterns, including apparent deletion of the entire *fljB* in some isolates as well as partial deletion of *fljB* in other isolates (Zamperini *et al.*, 2007). All but one of these isolates were characterized by presence of *hin* though. Recently, a genome sequence of a 4,5,12:i:– animal isolate (CVM23701) has become available (Rosovitz *et al.*, 2007); a preliminary analysis (Soyer *et al.*, unpublished data) of this sequence (GenBank accession number ABAO010000014) suggests a deletion of a multigene fragment, including *fljA* and *fljB* with retention of an intact *hin*, which is 100% identical to the *hin* sequence in *Salmonella* Typhimurium.

Overall, the findings of studies summarized above suggest that different mutations and deletions can be responsible for the lack of phase 2 flagella expression in naturally occurring 4,5,12,i:– isolates. Specifically, at least some of the 4,5,12,i:– isolates from Spain appear to be characterized by deletion of a large fragment (between STM2757 and STM2774), including *hin* (Garaizar *et al.*, 2002), while most of the isolates from the United States characterized thus far seem to be typified by deletions that eliminate *fljB* but maintain *hin* (Zamperini *et al.*, 2007; Soyer *et al.*, unpublished data). These findings may support a model that suggests multiple independent deletion events that led to emergence of 4,5,12,i:– from *Salmonella* Typhimurium ancestors in different locations. Alternatively, the evolution of 4,5,12,i:– may represent a single emergence event, followed by subsequent rearrangements and/or additional deletions in the region surrounding the *fljAB* operon, resulting in different 4,5,12,i:– lineages.

We are not aware of any comparative phenotypic experiments characterizing the virulence of wild-type Typhimurium and 4,5,12,i:– isolates. Genetically engineered *Salmonella* Typhimurium mutants missing *fljB* (having flagellar expression patterns similar to serotype 4,5,12,i:–) have shown a reduced ability to induce IL-8 secretion in tissue culture cells (Gewirtz *et al.*, 2001). In another study, *Salmonella* mutants expressing FliC (but not FljB), i.e., constructs with flagellar expression patterns similar to serotype 4,5,12,i:–, were recovered in greater number from blood and spleen of infected mice and cause higher mortality, as compared to strains expressing FljB (but not FliC) (Ikeda *et al.*, 2001). While there is thus some evidence that genetically engineered *Salmonella* Typhimurium strains lacking phase 2 flagella show virulence-associated phenotypes distinct from parent strains expressing phase 2 flagella, future experiments with wild-type Typhimurium and 4,5,12,i:– isolates are needed to further probe the virulence characteristics of serotype 4,5,12,i:– strains.

Drug Resistance of *Salmonella* 4,5,12:i:–

Drug resistance patterns

Interestingly, the drug resistance profiles of the *Salmonella* 4,5,12:i:– isolates recovered around the world range from pansusceptible to multidrug resistance. Resistance phenotypes in this section will be reported using abbreviations for the main antibiotics, including ampicillin (A), chloramphenicol (C), kanamycin (K), streptomycin (S), sulfamethoxazole (Su), tetracycline (T), trimethoprim (Tm), gentamicin (G), and nalidixic acid (NA).

Characterization of 122 serotype 4,5,12:i:– isolates from humans (114 isolates) and chicken meat (8 isolates) collected in 1993 and 1994 in Thailand found a number of MDR isolates, along with pansusceptible isolates (38% of human and 75% of food isolates were pansusceptible). The most common multidrug resistance patterns among these isolates from Thailand included ACKGSuTm (22 isolates) and ACKGSuTm with additional resistance to nalidixic acid (NA) (20 strains) (Boonmar *et al.*, 1998); these two resistance types are very similar with resistance to nalidixic acid typically conferred by point mutations in *gyrA* and *gyrB* (Giraud *et al.*, 2006). Some of the earliest reported isolates (i.e., those isolated in Thailand in 1993 and 1994) thus represented a mix of MDR and pansusceptible types. Characterization of 271 human and 17 food isolates with serotype 4,5,12:i:– that were collected in Spain in 1998 and 1999 revealed that all of these isolates showed a multidrug resistance phenotype (generally ACSuGSTTm, with a few isolates showing sensitivity to tetracycline [T]) (Echeita *et al.*, 1999). Serotype 4,5,12:i:– isolates from two human outbreaks in Luxemburg in 2006 also showed a multidrug resistance phenotype (ASSuT) (Mossong *et al.*, 2007).

Among 369 serotype 4,5,12:i:– isolates collected in Brazil between 1991 and 2000, 8% of human and 5% of nonhuman isolates (i.e., isolates from foods and animals) were susceptible to all the tested agents (Tavechio *et al.*, 2004). A total 55% and 62.5% of human and nonhuman isolates, respectively, were reported as showing intermediate resistance to one or more antibiotics (typically tetracycline and streptomycin). A total of 37% and 31% of human and nonhuman isolates, respectively, showed resistance to between 1 and 13 of the antimicrobial agents tested; a considerable number of isolates (18.5% and 13.6% of human and nonhuman isolates, respectively, were resistant only to tetracycline). In total, 27 of the 4,5,12,i:– isolates from Brazil displayed multiresistance to three or more antimicrobials, two of these isolates were reported as resistant to 13 antimicrobial agents, including netilmicin, tetracycline, chloramphenicol, gentamicin, kanamycin, ampicillin, cephalothin, sulfonamides, sulfamethoxazole-trimethoprim, amoxicillin-clavulanic acid, streptomycin, amikacin, and nalidixic acid (Tavechio *et al.*, 2004). Among 114 human serotype 4,5,12:i:– isolates obtained in the United States between 1996

and 2003, 82% were pansusceptible and 18% were resistant to at least one antimicrobial agent, three isolates showed an ACSSuT resistance type, and four isolates showed ceftiofur resistance (Grenne *et al.*, 2006). Another study, which evaluated 68 human serotype 4,5,12:i:– isolates collected between 1998 and 2000 in New York city, found that 38% of these isolates were susceptible to all antimicrobial agents tested; 34% of isolates showed intermediate resistance to one or two antibiotics (streptomycin, sulfamethoxazole, tetracycline). A total of 28% of these isolates from New York city showed resistance to one or more antimicrobial agents, with only four isolates (5.9%) showing resistance to four or more antimicrobial agents (Agasan *et al.*, 2002).

Overall, based on the data published to date, the majority of serotype 4,5,12,i:– isolates from Europe (i.e., Spain and Luxemburg) appear to show a multidrug resistance phenotype, while the majority of 4,5,12,i:– isolates from North and South America (i.e., United States, Brazil) appear to be pansusceptible or resistant to only a few antimicrobial drugs.

Mechanism of antimicrobial resistance among serotype 4,5,12,i:– isolates

Salmonella spp. isolates can carry a number of different antibiotic resistance genes, which may be located on either the chromosome or on plasmids (Michael *et al.*, 2006; Miriagou *et al.*, 2006; Alcaine *et al.*, 2007). In addition, point mutations in chromosomal genes can confer resistance to selected antimicrobial agents (e.g., fluoroquinolones, nalidixic acid). A number of studies have identified the specific resistance genes and genetic mechanisms associated with antimicrobial drug resistance phenotypes in different *Salmonella* serotypes (Alcaine *et al.*, 2007).

While only a few studies exist so far on antimicrobial resistance genes found among antimicrobial drug–resistant *Salmonella* serotype 4,5,12:i:– isolates, the available data may help to develop an initial understanding of the evolution and emergence of serotype 4,5,12:i:–, including 4,5,12:i:– MDR strains. While initial characterization of serotype 4,5,12:i:– isolates from Spain reported the presence of multiple (two to four) small cryptic plasmids as well as either a 140-kb *spv*C (*Salmonella* plasmid virulence gene)-positive or 120-kb *spv*C-negative plasmid (Guerra *et al.*, 2000; Echeita *et al.*, 2001), these studies did not test either these plasmids or the chromosome for the presence of antimicrobial resistance genes. Further characterization of MDR 4,5,12:i:– isolates from Spain with an ACGSSuTSTm resistance phenotype revealed the presence of multiple previously described resistance genes, including $bla_{\text{TEM-1}}$ (encoding a broad spectrum β-lactamase that provides resistance to penicillin and amino-penicillin such us ampicillin), *aac(3)*-IV and *aad*A2 (encoding modified aminoglycoside enzymes that can inactivate gentamicin and streptomycin by modifying different residues in the active sites of these drugs), *cmlA* (encoding an efflux pump that mediates resistance to chloramphenicol), *sul1* and *sul2* (both encoding an dihydropteroate synthase that is resistant to sulfonamides), *dfrA12* (encoding an dihydrofolate reductase that is resistant to trimethoprim), and *tetA* (encoding an efflux pump that mediates resistance to tetracycline) (Guerra *et al.*, 2001, 2004b). These studies (Guerra *et al.*, 2001, 2004b) further found that *dfrA12* and *aadA2* appear to be located on a class 1 integron in the 4,5,12:i:– isolates characterized, while $bla_{\text{TEM-1}}$, *cml*A, *aac(3)*-IV, and *tet*A were mapped to the large 120- or 140-kb plasmid previously described in Spanish 4,5,12:i:– isolates (Guerra *et al.*, 2000). Guerra *et al.* (2004b) found evidence that the class 1 integron carrying *dfr*A12 and *aad*A2 is also located on a plasmid in the Spanish 4,5,12,i:– isolates characterized. Interestingly, Guerra *et al.* (2004b) did not find the same combination of resistance genes found in the two 4,5,12,i:– isolates they characterized among any of the eight MDR *Salmonella* Typhimurium isolates (including four DT104 isolates) they characterized.

While $bla_{\text{TEM-1}}$, *cmlA*, *aadA2*, *dfrA12*, *sul1*, and *sul2* have all previously been found in *Salmonella* Typhimurium isolates (Michael *et al.*, 2006), $bla_{\text{TEM-1}}$, *cmlA*, and *dfrA12* have not been typically found in the MDR *Salmonella* DT104 (which typically carries $bla_{\text{PSE-1}}$, *floR*, *tetG*, *aadA2*, and *sul1*) suggesting that the antibiotic resistance gene clusters found in the Spanish serotype 4,5,12,i:– isolates are unlikely to be related to the resistance genes in the pandemic DT104 strain (Boyd and Hartl, 1998; Guerra *et al.*, 2004a, 2004b).

While MDR *Salmonella* DT193 typically carry $bla_{\text{TEM-1}}$ along with *sul1* and *sul2*, they carry *dfrA1* and *aadA1* and *tetB* (Gebreyes and Altier, 2002; Miriagou *et al.*, 2006) and thus are also unlikely to represent the source of the antibiotic resistance gene clusters found in the Spanish serotype 4,5,12,i:– isolates. MDR *Salmonella* Newport, another MDR subtype that appears to have emerged in the North America in the late 1990s (Bird *et al.*, 2002; Poppe *et al.*, 2005), has been reported to carry the resistance genes bla_{CMY}, flo_{st}, *strA*, *strB*, *sul2*, and *tetA* on a plasmid (Poppe *et al.*, 2005). Except for *sul2* and *tetA*, these genes are different from those identified in the Spanish 4,5,12,i:– isolates, and MDR *Salmonella* Newport thus also appears to be unlikely to be the source of the resistance gene cluster reported in the Spanish 4,5,12,i:– isolates. Interestingly, the resistance gene profile most closely related to the resistance gene found in the Spanish 4,5,12,i:– isolates was found in a human *Salmonella* Cholerasuis isolate for which a complete genome sequence was determined (Chiu *et al.*, 2005). This isolate carried a number of resistance genes on a plasmid, including $bla_{\text{TEM-1}}$, *aad*A2, *cml*A, and *sul1*, which have been found in 4,5,12,i:– isolates, as well as other resistance genes that have not yet been reported in 4,5,12,i:– isolates. It is thus unlikely that the specific plasmid in *Salmonella* Cholerasuis isolate was transferred to the ancestor of the Spanish MDR 4,5,12,i:– isolates. The resistance clusters in this Cholerasuis isolate and in the Spanish MDR 4,5,12,i:– isolates may be related though and share a common ancestor.

Overall, the limited data available on the antimicrobial resistance genes found in serotype 4,5,12,i:– isolates constrain our ability to understand the evolution of MDR 4,5,12,i:– strains and the natural history of the antimicrobial resistance gene clusters found in these strains. While characterization of the antimicrobial resistance genes in additional 4,5,12,i:– isolates, including from countries other than Spain, is needed, the data available to date suggest that the resistance gene clusters found in the Spanish 4,5,12,i:– isolates originated from an ancestor other than an MDR *Salmonella* Typhimurium. Whether acquisition of the resistance genes in the Spanish 4,5,12,i:– isolates occurred before or after the loss of the phase 2 flagella expression also remains to be determined.

Conclusions

Salmonella 4,5,12:i:–, which has only rarely been reported among *Salmonella* isolated before 1993, has been found in human clinical cases, different animal species, and foods in countries located on different continents, including Europe, Asia, and South and North America. Molecular subtyping data and phylogenetic analyses consistently support *Salmonella* 4,5,12:i:– being closely related to *Salmonella* Typhimurium and most likely representing a monophasic variant that emerged from a *Salmonella* Typhimurium ancestor through deletions and/or mutations of the genes responsible for phase 2 flagella expression. Other rare *Salmonella* serotypes (e.g., Agama, Farsta, Tsevie, Tumodi, Gloucester, and unnamed subspecies II) (Grimont and Weill, 2007) do share many O antigens and the phase 1 H antigens with both *Salmonella* 4,5,12:i:– and Typhimurium though and may thus have been ancestors for some emergence events. Comparative molecular subtype characterization of isolates representing all these serotypes will be necessary to further clarify the emergence and evolution of *Salmonella* 4,5,12:i:–.

While the deletions and mutations linked to the loss of phase 2 flagella appear to differ among most Spanish and North American 4,5,12:i:– isolates (most North American isolates apparently retained a copy of *hin*, while this gene apparently is absent from most Spanish isolates), it is not yet clear whether the natural history of 4,5,12:i:– represents a single emergence event followed by subsequent diversification and pandemic spread or whether it represents multiple independent emergence events. The observation that European 4,5,12:i:– isolates appear to predominately represent an MDR phenotype, while Asian and North and South American isolates appear only rarely to be MDR may support the hypothesis of two independent emergence events leading to an MDR "European" lineage and a non-MDR lineage that may be more commonly found in Asia and the Americas. Further studies are thus clearly needed to better understand the evolution and emergence of *Salmonella* 4,5,12:i:–.

Regardless of the specific events leading to the evolution and emergence of *Salmonella* 4,5,12:i:–, it is clear that this serotype is evolutionary successful as supported by its worldwide distribution and the fact that it has become one of the 5–10 most common *Salmonella* serotypes responsible for human infections in different countries. Thus, a critical question is whether this emerging serotype has specific characteristics that facilitate its rapid spread and ecological success. While the MDR phenotype found among some strains (e.g., those apparently predominant in Europe) may provide one selective advantage for this emergent subtype, serotype 4,5,12:i:– isolates also are commonly reported among human cases in the United States and Brazil, despite the observation that most 4,5,12:i:– isolates in these countries are not MDR. It is thus tempting to speculate that characteristics other than an antimicrobial drug resistance phenotype at least partially account for the ecological success of serotype 4,5,12,i:–. For example, while phase variation between two flagellin types generally is believed to be an important virulence mechanism allowing the pathogen to evade the immunity of the host, by producing a subpopulation with different flagellin antigens (Ikeda *et al.*, 2001), monophasic variants lacking the second flagellar phase may have a selective advantage; for example, by completely silencing expression of flagellar antigens that may be recognized by the immune system. Further studies are also needed to test this hypothesis and to identify other genetic and phenotypic characteristics that may provide a selective advantage for serotype 4,5,12,i:– isolates.

Importantly, initial genomic microarray studies (Garaizar *et al.*, 2002) have already identified gene deletions other than those in the region responsible for phase 2 flagella expression in selected 4,5,12,i:– isolates, which may contribute to phenotypic characteristics impacting transmission and virulence. Further analyses of the recently completed genome sequence for a 4,5,12,i:– isolate from the United States (Rosovitz *et al.*, 2007) will provide additional opportunities to probe the evolution of *Salmonella* 4,5,12:i:–.

Finally, it will be critical for public health systems worldwide to continue and expand systems that monitor emergence and frequency of different *Salmonella* serotypes, including 4,5,12,i:–. These continued efforts will be critical not only to identify emerging new *Salmonella* strains, but also to allow for an improved understanding of various factors that may be responsible for or contribute to emergence of new strains of *Salmonella*, which continues to be responsible for most deaths due to known foodborne pathogens in many countries around the world.

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