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REVIEW
Host adapted serotypes of *Salmonella enterica*

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INTRODUCTION

Salmonella constitutes a genus of zoonotic bacteria of worldwide economic and health importance. The current view of salmonella taxonomy assigns the members of this genus to two species: *S. enterica* and *S. bongori*. *S. enterica* itself is divided into six subspecies, *enterica*, *salamae*, *arizonae*, *diarizonae*, *indica*, and *houtenae*, also known as subspecies I, II, IIIa, IIIb, IV, and VI, respectively [1] . Members of *Salmonella enterica* subspecies *enterica* are mainly associated with warm-blooded vertebrates and are usually transmitted by ingestion of food or water contaminated by infected faeces. The pathogenicity of most of the distinct serotypes remains undefined, and even within the most common serotypes, many questions remain to be answered regarding the interactions between the organism and the infected host.

Salmonellosis manifests itself in three major forms: enteritis, septicaemia, and abortion, each of which may be present singly or in combination, depending on both the serotype and the host involved. Although currently over 2300 serovars of *Salmonella* are recognized, only about 50 serotypes are isolated in any significant numbers as human or animal pathogens [2, 3] and they all belong to subspecies *enterica*. Of these, most cause acute gastroenteritis characterized by a short incubation period and a predominance of intestinal over systemic symptoms. Only a small number of serotypes typically cause severe systemic disease in man or animals, characterized by septicaemia, fever and/or abortion, and such serotypes are often associated with one or few host species [4–6].

It is the intention of this review to present a summary of current knowledge of these host-adapted serotypes of *S. enterica*. The taxonomic relationships between the serotypes will be discussed together with a comparison of the pathology and pathogenesis of the disease that they cause in their natural host(s). Since much of our knowledge on salmonellosis is based on the results of work on Typhimurium, this serotype will often be used as the baseline in discussion. It is hoped that an appreciation of the differences that exist in the way these serotypes interact with the host will lead to a greater understanding of the complex host–parasite relationship that characterizes salmonella infections.

Definitions

Salmonella serotypes are normally divided into two groups on the basis of host range; host adapted and ubiquitous (non-adapted). Host-adapted serotypes (Table 1) typically cause systemic disease in a limited number of related species. For example, Typhi, Gallinarum and Abortusovis are almost exclusively associated with systemic disease in humans [7], fowl [8] and ovines [5] respectively. However, some host

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Table 1. *Examples of* Salmonella *subspecies I serotypes adapted to higher vertebrates*

Serotype	Natural host	Other host(s) rarely infected
Host adapted serotype		
Choleraesuis	Swine	Human
Dublin	Bovine	Human, ovine
Host restricted serotype		
Typhi	Human	—
Paratyphi A	Human	—
Paratyphi C	Human	—
Sendai	Human	—
Abortusovis	Ovine	—
Gallinarum	Poultry	—
Typhisuis	Swine	—
Abortusequi	Equine	—

Table 2. *Nutrient requirement(s) of HA and HR serotypes*

	Requirement		
Serotype	Amino acid	Vitamin	Ref.
Typhi	Tryptophan		[19]
Typhisuis	Cystine		[237]
Gallinarum	Cystine, Leucine, Aspartic	Thiamine	[237]
Abortusovis	Cystine	Nicotinic acid, Thiamine	[237]
Paratyphi A	Cystine, Arginine		[237]
Dublin		Nicotinic acid	[17]

adapted serotypes can also cause disease in more than one host species: Dublin and Choleraesuis, for example, are generally associated with severe systemic disease in cattle and pigs respectively but may also infrequently cause disease in other mammalian hosts including humans [9–11]. Animals infected with such serotypes frequently become clinically asymptomatic carriers. These infected animals, the so-called 'symptomless excreters', constitute a reservoir and represent a health hazard since they contaminate their environment and increase the number of infected individuals [12–14]. Clearly the degree of host adaptation of *Salmonella* serotypes can vary widely. To avoid confusion and contradiction within the literature, we propose the adoption of the following terms to describe salmonella host-adaptation: *Salmonella* serotypes which are almost exclusively associated with one particular host species, for example, Typhi, Abortusequi, Gallinarum, Typhisuis, and Abortusovis, will be referred to as host-restricted (HR) serotypes. Serotypes which are prevalent in one particular host species but which can also cause disease in other host species, for example Dublin and Choleraesuis, will be referred to as host-adapted (HA) serotypes. Ubiquitous serotypes, for example Typhimurium and Enteritidis, although capable of causing systemic disease in a wide range of host animals, usually induce a self-limiting gastroenteritis in a broad range of unrelated host species, and these serotypes will be referred to as un-restricted serotypes (UR).

Host-adapted and host-restricted *Salmonella* serotypes have special nutritional requirements

In the past, special attention has been dedicated to nutritional requirements and distinct biochemical characters of *Salmonella* serotypes. In particular, *Salmonella* strains had been divided in 'ammonium weak' and 'ammonium strong' strains on the basis of their ability to assimilate nitrogen from ammonia in a defined media that contained simple carbon compounds such as citrate (Simmons citrate agar) or other sugars as sole source of carbon and energy [15]. Kauffmann noted that although most serotypes of *Salmonella* were 'ammonium strong', i.e. Typhi-

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murium and Enteritidis, others did not grow or grew poorly in such media [16]. Serotypes noted as 'ammonium weak' were all host-adapted (Dublin, Rostock, and Choleraesuis) or host-restricted (Paratyphi A, Abortusovis, Typhisuis, Typhi, and Sendai) [15]. It is important to note that a negative result of this test might be due also to the failure of the organism to grow in absence of other substances that were not provided with such minimal media.

Fierer and colleagues have examined the biochemical features of several Dublin strains and found them all unable to grow in Simmons citrate agar [17]. In the presence of supplemental nicotinic acid, however, all strains were able to utilize citrate. Similarly, we found Abortusovis strains able to utilize citrate in a minimal defined medium only when cystine and nicotinic acid were supplemented (Uzzau and colleagues, unpublished results). Detailed analysis of the nutritional requirement of *Salmonella* spp. has led to the observation that whereas ubiquitous Typhimurium and Enteritidis were able to grow in relatively simple defined media, certain amino acids and vitamins must be supplied for most strains of Typhi, Typhisuis, Abortusovis, Gallinarum, Paratyphi A, and Dublin [18, 19]. Auxotrophy therefore, seems to be a characteristic of HR and HA serotypes (Table 2).

A relationship between auxotrophic characters and bacterial virulence has been described for *Staphylococcus aureus*. In its wild type form, this pathogen also fails to grow on defined media in absence of one (or more) amino acid, purine or vitamin. In particular, *S. aureus* strains, often auxotrophic for tryptophan (trp^-), carry the gene for the toxic shock syndrome toxin-1 (TSST-1) on a mobile element that may be found to be inserted in either one of two different loci on the chromosome. A screen for trp^- *S. aureus* revealed that in all such strains the TSST-1 element was inserted in the tryptophan operon region [20]. Since TSST-1 expression is not influenced by tryptophan concentration [21] and a sufficient concentration of this amino acid is available in the host to enable bacterial growth, it is conceivable that auxotrophy does not play a role in *S. aureus* pathogenesis, and that this character is maintained only because of the physical linkage with the TSST-1 element. Correspondingly, the evolution of *Salmonella* HR and HA strains towards a defined ecological niche might have involved genetic rearrangement(s) that in turn induced specific nutritional requirement. Alternatively, as proposed by Fierer and colleagues, the high content of specific amino acids and/or vitamins in the host might have supported the growth of *Salmonella* serotypes with auxotrophic characters that were otherwise growth impaired in a broad range of hosts [17].

Taxonomy and population genetics

The taxonomic classification of salmonella has been continually revised over the years. Beyond the level of subspecies, serotyping is used for differentiation, and serotypes have been described within *S. enterica* subspecies *enterica* on the basis of somatic (O), flagellar (H), and capsular (Vi) antigens [1]. Within subspecies *enterica*, some serotypes are polyphyletic; identical serotypes occur among isolates of distantly related clones that also differ in pathogenic potential and host range. This can be attributed to horizontal genetic transfer and recombination of antigen genes between lineages, an event that has been proposed to happen with relatively high frequency [22]. However, overall the subspecies remain clonal [23].

The host-restricted/host-adapted serotypes all belong to subspecies *enterica*, but studies of population structure do not indicate that they should be regarded as a single bacterial lineage. Selander and colleagues employed multilocus enzyme electrophoresis analysis (MLEE) to study the evolutionary relationship of many *Salmonella* serotypes. This technique revealed allelic variations in multiple chromosomal genes encoding metabolic enzymes. These authors demonstrated that HA and HR serotypes exhibit fewer numbers of electrophoretic types than UR serotypes, indicating significantly less diversity among HA and HR serovars than among UR [24].

Among *Salmonella* HR serotypes, Typhi (antigenic formula O: 9,12,Vi:d:—) presents a very low genetic heterogeneity in the natural populations. Typhi has been shown in one study to be monoclonal, i.e. all strains belong to the same electrophoretic type [25]. The monophyletic nature of Typhi has been also demonstrated by common DNA fingerprint patterns and by sequence analysis of the 5′ end of the *fliC* region [26]. A more extensive study distinguished two clones by MLEE: Tp1 that was predominant worldwide and Tp2 that was present in Africa only [24]. However, genetic and phenotypic polymorphism was observed among isolates from Indonesia, with biphasic (d:z66) and monophasic (d:— or j:—) isolates, suggesting that Typhi evolved in an isolated human population in the Far East [26]. These studies

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indicated that Typhi has evolved separately from the other serotypes of *S. enterica* subspecies *enterica*, and that it does not show any close resemblance to other HA or HR serotypes. The chromosome of Typhi shows considerable evidence of frequent DNA rearrangement compared to other serotypes for which a genetic map has been constructed [27–31] but it is uncertain whether this plasticity of the chromosome has played a major role in development of the host restriction.

Gallinarum (antigenic formula O: 1,9,12: − : −), belongs to the same serogroup (D1) as Typhi, as well as the HA Dublin (antigenic formula O: 1,9,12[Vi]: g,p: −), but no close association was demonstrated between these three serotypes [24]. The two biotypes of Gallinarum, i.e. *gallinarum* and *pullorum* both avian-restricted, are phylogenetically related and part of a common clonal lineage with En1, of the polyphyletic serovar Enteritidis [32]. Gallinarum biotypes *gallinarum* and *pullorum* form separate subclones in analysis based on MLEE, sequence of *fliC* [32] and by most RFLP-based typing methods [33]. A careful selection of the enzymes used for ribotyping has been reported to separate the two biotypes completely [34].

Abortusovis (antigenic formula O: 4,12: c: 1,6), belongs to serogroup B. To our knowledge, no population genetic analysis has been performed with this serotype. One collection of Abortusovis examined for the presence of the insertion element IS*200* showed 3–4 IS*200* copies that efficiently discriminated between clonal lines [35]. However, whether this polymorphism is an indication of diversity within the serotype is doubtful as Typhi also shows a high diversity by IS*200* typing [36].

Serotypes of antigenic formula O: 6,7: c: 1,5 belong to the serogroup C1. This group of serotypes includes the swine-adapted Choleraesuis, the swine-restricted serotype Typhisuis, and the human-restricted Paratyphi C. Typhisuis has been reported to be polyphyletic by MLEE analysis, with clone Ts3 genotypically related to Decatur (also with serotype O: 6,7: c: 1,5) and very different from Ts1 and Ts2 [24, 37]. However, in a more recent study based on ribotyping, IS*200* fingerprinting, and biochemical analysis, Ts3 was reclassified as Decatur [38]. Thus, Typhisuis is strongly homogeneous and supports the argument that HR serotypes are less diverse than those that are pathogenic for a wide variety of animal hosts. Furthermore, Typhisuis appeared to be allied to Choleraesuis and to certain clones of Paratyphi C [37, 38]. It has been suggested that these serotypes evolved from a common ancestor that was already capable of systemic infection and adapted to swine and that the acquisition of the Vi antigen by Paratyphi C allowed this serotype to adapt to humans [24].

Choleraesuis clones listed in the *Salmonella* reference collection B (SARB) comprise a predominant, widely distributed clone, of electrotype Cs1, and 3 other (Cs6, Cs11, and Cs13) much less common electrotypes [37]. Uzzau and colleagues observed that Choleraesuis and its Kunzendorf variant had related but distinct ribotypes [38]. Application of ribotyping to Choleraesuis showed that Cs1 electrotype consists of Choleraesuis var. Kunzendorf strains (of ribotype I) and that Cs11 electrotype of Choleraesuis non-Kunzendorf strains (ribotype II). These authors also proposed to reassign clone Cs6 (SARB 5), as Decatur and showed that electrotype Cs13 (SARB 7) should be excluded from the Choleraesuis group. In conclusion, Choleraesuis var. Kunzendorf appears to be monophyletic and distributed world-wide, whereas Choleraesuis non-Kunzendorf strains are mostly from South East Asia and as with the Indonesian isolates of Typhi, they show a certain degree of polymorphism by ribotype analysis [38].

Dublin consists of three closely related electrophoretic types with 95% of strains belonging to a clone that is present world-wide [39, 40]. Dublin strains showed the same RFLP-patterns when hybridized with the insertion sequence IS*200* while restriction polymorphism can be demonstrated by ribotyping [41]. The clonal nature of Dublin has been confirmed by analysis of molecular variation in the serotype specific plasmid of this host-adapted serotype [42] and also showed that the geographically restricted clone (Du3) harboured a serotype specific plasmid more closely related to that of the UR serotypes. This has been further corroborated by incompatibility studies of the plasmids in both Du3 and the widespread clone Du1 (D. J. Platt, unpublished). Finally, Dublin, as well as Gallinarum, is closely related to certain strains of Enteritidis, a polyphyletic serotype that is pathogenic for a variety of host species [32, 41].

In conclusion, HR and HA serotypes are genotypically less diverse than UR from which they may have arisen. Furthermore, there are cases where no close genetic relationship exists between serotypes adapted to the same host (e.g. Typhi and other human adapted serotypes), and conversely, serotypes that share the same antigenic formula and are geno-

typically closely related, but are adapted to different hosts (i.e. Choleraesuis and Paratyphi C). Based on these observations, Selander and colleagues pointed to the possibility for convergence of both host adaptation and virulence factors between these serotypes [24]. This may hold true for most of the serotypes discussed in the present review. A search for, and an understanding of the mechanisms that underlie host-restriction and host-adaptation in salmonella is therefore most likely a search for a unique set of mechanisms in each of the different serotypes.

The host-restricted serotypes

Each of the serotypes described in this section produce systemic infection in the natural host with various degrees of systemic involvement and different clinical signs. In general there is limited or no evidence of enteritis. These *Salmonella* serotypes appear to migrate rapidly from the intestine to the reticuloendothelial system (RES) of their natural host, where they may find niches that increase the probability of induction of a carrier state.

Typhi

Typhi is the causative agent of typhoid fever, a protracted and debilitating febrile illness that remains a major public health concern in many developing countries, particularly in tropical regions. World wide, more than 16·6 million typhoid cases have been estimated each year, with an annual incidence of $> 0{\cdot}5\%$ of the population, causing 600000 deaths [43]. Transmission of the disease occurs via the faecal–oral route.

We have defined this serotype as host-restricted since it exhibits a very narrow host-range, limited to humans and chimpanzees [7]. As for the other serotypes described below, infection is characterized by predominance of septicaemic over intestinal symptoms. After ingestion of Typhi in contaminated food or water, the bacteria pass through the stomach and then colonize the mucosa of the distal ileum without undergoing any significant multiplication within the lumen of the intestine. Mills and Finlay showed that Typhi and Typhimurium used similar mechanisms of invasion and intracellular trafficking in human epithelial cells [44]. Although this demonstrates a clear similarity in some aspects of invasion strategy between Typhimurium and Typhi, some specific host-related mechanisms of invasion are present. Comparative analysis of the early steps of pathogenesis has led to the conclusion that interaction of Typhi with intestinal epithelial cells shows striking differences from that of other serotypes, particularly with those that are capable of eliciting gastroenteritis in a broad range of animal hosts [45–48]. For instance, a chromosomal region of Typhi confers the ability to enter epithelial cells to a non-invasive strain of *E. coli*, whereas a homologous region of the Typhimurium chromosome did not [49]. Also, rough strains of Typhi lost the capability to enter epithelial cells [50] while rough mutants of Typhimurium remained invasive [51]. Epithelial cell adhesion and invasion may not be uncoupled in Typhi, since all Typhi invasion mutants isolated in recent studies [47, 52] were also adhesion-defective, whereas mutants obtained from UR serotypes like Typhimurium and Enteritidis were found to adhere to cell monolayers but invaded significantly less [53–55]. In particular, Weinstein and colleagues have recently shown that null mutations of *invA* and *invE* genes, identified as necessary for invasion but not for adhesion in Typhimurium, abolished both properties in Typhi [47]. Finally, Altmeyer and colleagues found that adherence to and invasion of cultured cells by Typhimurium was affected by a null mutation in the gene *invH*, a component of the invasion associated type III secretion system, and that this impairment was most remarkable in the HA/HR serotypes including Typhi [56]. Ileal loop infection analysed in mice has shown that Typhi penetrated the intestinal wall preferentially via the M cells which overlie Peyer's patches (PP) [45, 57], but the number of bacteria internalized was significantly lower than that observed for Typhimurium and bacteria were cleared soon after, apparently with minor damage to M cells and enterocytes [45]. Furthermore, Kops and colleagues demonstrated that Typhi transmigrated through polarized human epithelial cell monolayers earlier and in larger numbers than Typhimurium [46]. Pier and colleagues showed that Typhi used the cystic fibrosis transmembrane conductance regulator (CFTR) to enter intestinal epithelial cells, a property that was not shared by Typhimurium [58]. In conclusion, Typhi interaction with the small intestine epithelia appeared to have evolved towards the establishment of a transient infection of the small bowel without significant inflammation, in contrast to serotypes that cause acute enteritis in humans.

Infection by *Salmonella* serotypes, as well as other enteropathogenic Gram negative bacteria (e.g. *Vibrio cholerae*, diarrhoeagenic *Escherichia coli*, etc.) that

induce diffuse enteritis, is characterized by diarrhoea and therefore, bacterial dissemination in the environment. Instead, a high level of transmissibility is ensured in systemic infection produced by Typhi due to the potential of this serotype to develop the carrier state [59, 60].

Once the intestinal epithelial layer is crossed, Typhi enters the blood stream, surviving within macrophages, and disseminates to the liver, spleen, bone marrow, and other organs rich in phagocytic cells. In order to reach the reticuloendothelial system, Typhi gives rise to a bacteriaemia that appears to be rather atypical. In fact, Typhi infected patients exhibit very low numbers of bacteria in the bloodstream, endotoxin-negative sera, and specific bactericidal activity [61]. A number of clinical studies have shown that Typhi can be more readily isolated from the bone marrow than from peripheral blood [62–64]. These data suggest that Typhi survives and replicates within phagocytic cells of the bone marrow, spleen, and liver, but the genetic basis of this capability remains largely unknown. The expression of a capsular polysaccharide, the Vi antigen, appears to be crucial for Typhi to survive in mouse and human macrophage cell lines and to resist to lysis by serum complement [65–67], whereas it does not appear to be necessary for epithelial cell invasion [68]. This antigen is also known to be associated with the virulence of the organism *in vivo* and to confer immunity against typhoid fever, when injected alone, in areas with a high incidence of this disease [69, 70]. This virulence factor is not uniquely found in Typhi but also in Paratyphi C and rarely, in Dublin and *Citrobacter freundii*, suggesting that horizontal gene transfer have occurred in the evolution of these bacteria [15, 71–73].

Non-typhoidal *Salmonella* serotypes have been reported to require a large molecular mass virulence plasmid (i.e. the *spv* operon) for systemic infection of the reticuloendothelial organs [74]. Different virulence determinants, chromosomally encoded, should be responsible for reticuloendothelial infection by Typhi and other typhoidal serotypes, since these strains do not harbour the *Salmonella* virulence plasmids (with the exception of Paratyphi C).

Other salmonellae which are primarily or exclusively restricted in host range to humans are Paratyphi A and C and Sendai, all of which cause enteric fever. Some strains of Paratyphi B cause human enteric fever, whereas others, designated as Java, produce gastroenteritis in both humans and animals. Miami, which is serologically related to Sendai, is largely limited to humans but causes gastroenteritis rather than enteric fever in animals [24].

Gallinarum

Gallinarum is divided into two biotypes, *gallinarum* and *pullorum*, which can be differentiated biochemically [75] and genotypically [76]. We define Gallinarum as host-restricted since all reported cases of systemic disease are from avian hosts [8].

Biotypes *gallinarum* and *pullorum* are the causative agents of two different disease syndromes, fowl typhoid and pullorum disease. Although largely eradicated from the commercial poultry industry in many developed countries, outbreaks have occurred [77], and the prevalence of the disease in poultry in areas such as Eastern Europe, Africa and South America, where the poultry industry is undergoing rapid expansion, remains high [78]. Cases of Gallinarum food poisoning have been reported in humans [15], but these cases may possibly represent misclassification of strains by serotyping.

Fowl typhoid generally presents as septicaemia, affecting birds of all ages although mainly those over 3 months, whereas pullorum disease tends to be restricted to an enteric infection of birds under 6 weeks of age. Infection occurs via the faecal–oral route or by means of vertical transmission. The course of Gallinarum infection varies greatly depending on the breed, nutritional and immune status and age of the birds involved [79], and mortality can be up to 100% in some infected flocks. The acute disease is characterized by rapid onset, weakness, diarrhoea, respiratory distress and loss of weight with sudden death. Peritonitis, enteritis and localization of the organisms in the myocardium and the ovary accompany swelling of the liver, kidneys and spleen. Infection with biovar *gallinarum* often induces the development of haemolytic anaemia [80].

Gallinarum is the only non-flagellated, and therefore non-motile serotype of *S. enterica*. Despite its phenotypic non-motility, Gallinarum contains the gene *fliC* which encodes the phase 1 structural flagella protein. The *fliC* sequence in strains of biotype *gallinarum* has been shown to be identical to that of Enteritidis (antigen g) except for a subtype which contains a stop codon internal in the sequence. Strains of *pullorum*, on the other hand, contain 3–4 non-synonymous substitutions in the *fliC* gene [32]. In recent studies, growth of *pullorum* strains on solid medium containing iron, thiosulphate and 100 mM

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hexoses and amino-acids has been reported to induce flagellation and to confer motility [81, 82]. The flagella described on *pullorum* would be encoded by *fliC* since antisera to 'g' flagellar antigen strongly react against motile *pullorum* strains. Although these data generated some controversy [83], they would explain the presence of a conserved *fliC* gene in these serotypes and are suggestive of differential control over its expression compared to all the other *Salmonella* serotypes. It is tempting to speculate that Gallinarum adaptation to the avian host required the development of such control on flagellation. Furthermore, the finding that Enteritidis and Typhimurium isolated from birds are frequently non-motile [84, 85] supports this hypothesis and suggests that the interaction with the avian host may select for a specific restriction of flagellar expression, although the reason for such negative control is not clear. Pathogenesis of avian infection by Gallinarum begins with intestinal colonization and bacteria can be detected in the distal ileum, and mostly, in the caecal mucosa [8]. Gallinarum restriction to the avian host and, in particular, the low level of virulence for the mouse, appears to reside on the differential interactions with the intestinal epithelia and the associated lymphoid tissue. It is noteworthy that Gallinarum cannot successfully infect the mouse by oral administration [8]. Therefore, the murine intestinal mucosa represents a primary barrier to this serotype. Pascopella and colleagues have discovered that this phenotype corresponds to the lack or a very low frequency of invasion of the mouse M cells or enterocytes [45]. Thus, a differential development of mechanisms of invasion may be proposed based on the observation that mutagenesis of the *invH* gene leads to a more pronounced effect on the *in vitro* invasiveness of Gallinarum than that observed for Typhimurium [56]. Moreover, Typhimurium virulence for chicks was not affected by mutation of *invH* [86] whereas the same mutation greatly reduced Typhimurium virulence in cattle and in the mouse.

From these primary sites of infection, Gallinarum reaches the reticuloendothelial system. *In vitro* evidence indicated that Gallinarum enters macrophages in smaller numbers than Typhimurium and without induction of ruffling, micropinocytosis, and spacious phagosome (SP) formation [45].

An 85 kb virulence plasmid is commonly found among field isolates of both Gallinarum biotypes [87]. Three restriction profiles have been identified with this plasmid and among these, two different RFLP profiles are seen when the common virulence gene *spvC* is used as a probe [77]. Elimination of the virulence plasmid completely abolished mortality in chickens and re-introduction of the virulence plasmid fully restored virulence [87, 88]. In conclusion, Gallinarum host specificity for the avian host, as seen for Typhi, appears to be due to chromosomally encoded virulence determinants whose expression may be crucial during the interaction with the intestinal mucosa and for survival within the reticuloendothelial organs.

Abortusovis

We define this serotype as host-restricted since it has been isolated only from ovine sources under natural conditions [5]. Abortusovis ranks among the main causes of ovine abortions in Europe and western Asia [89, 90], where it represents a major pathological and economic problem in countries with a sheep-based economy.

The typical signs of Abortusovis infection are the induction of abortion and mortality of newborn lambs, whereas adult sheep naturally exposed to Abortusovis produce no symptoms. The disease tends to produce an endemic pattern with a cyclic rhythm in the frequency of abortion. In endemic areas, abortion occurs in 30–50 % of sheep in a flock, generally during the first pregnancy, and mainly during the last stages of gestation [90]. Three months after abortion specific antibody titres drops to the level of non-infected animals [90]. Nonetheless, there are usually few abortions in the year following an outbreak of Abortusovis in a flock, suggesting that the animals develop an acquired resistance to the disease [90]. Following abortion, bacteria can be isolated from placental and foetal tissues (liver, spleen, brain and stomach), which are the principal sites of multiplication [90]. Infected ewes that do not abort deliver weak lambs that generally develop bacteriaemia and die within a few days. Lambs may also be born strong and become infected and die within the first 2 weeks after birth. Abortusovis could also be isolated from the ewe vaginal discharges for up to 10–12 days following abortion [90], and further contributed to increased transmission during the lambing season. Ingestion of contaminated pasture represents the most probable route of infection. Abortusovis that survive the passage through the four stomachs, colonize the small intestine. It is noteworthy that Abortusovis, as generally seen in Typhi and Gallinarum, is able to cross the mucosal barrier and reach systemic sites,

without producing enteritis (i.e. diarrhoea). The examination of Abortusovis mechanisms of pathogenicity in the mouse model (BALB/c) has led to the observation that orally administered strains are able to colonize the murine ileum but infect the Peyer's patch (PP) at a much lower level than Typhimurium (Uzzau and colleagues, unpublished results) [91]. Abortusovis adaptation to the ovine host may have favoured the loss of gene functions that are related to the capability to induce enteritis, toward the establishment of a systemic, asymptomatic, and well tolerated infection of the adult host. Host factors may also be involved in the process of adaptation. In fact, the presence of enteritis in the lamb but not in the ewe, parallels the differences in lymphoid tissue in the intestine between animals of different ages [92]. At about 150 days gestation, foetal PP are histologically mature and lymphopoiesis is more intense than anywhere in the body. Newborn lambs possess PP in the jejunum, in the proximal ileum and a single 2·5 m long ileocecal Peyer's patch. From 12 weeks of age this lymphoid tissue began to involute and only few follicles are still detectable by 18 months of age. Thus, a possible explanation of the different outcome of infection in the adult and the foetal/newborn host is that Abortusovis infects the ewe small intestine transiently, but small numbers of bacteria disseminate systemically, predominantly in the reticuloendothelial organs, and strongly stimulates the immune system [93]. During pregnancy, the tropism for the foetal PP might allow intense colonization of the foetus and leads to abortion or delivery of a weak lamb that will generally experience enteritis and septicemia.

A virulence plasmid is present in strains of Abortusovis. The molecular mass varies between 50 and 75 kb, and restriction polymorphisms are relatively frequent in this serotype [94]. The role of this plasmid has only been determined in the murine model, where an Abortusovis plasmid cured strain was avirulent following oral challenge [94a].

Typhisuis

This serotype does not naturally infect animals other than the pig and, for this reason, is considered host-restricted to swine. Typhisuis is antigenically almost identical and genetically related to Choleraesuis [38, 95]. Although these serotypes also share the same natural host, they differ significantly in several phenotypic characters and in the clinical aspects of the disease.

Typhisuis is the causative agent of chronic paratyphoid, a progressive and lethal disease: death usually occurs within several weeks. Following infection that probably occurs via the oral route, the bacteria are found associated with the lymphoid tissue along the alimentary tract. In these sites, Typhisuis grows slowly and progressively produces proliferative and caseous lesions [96]. In particular, such lesions are consistently found in the palatine tonsils, the caecum, the colon, and, generally, in the ileum, and they account for the dehydration, emaciation, and the intermittent diarrhoea frequently associated with this infection. Systemic dissemination of the bacteria is also characterized by formation of granulomas and necrosis in infected tissues (i.e. liver) [96]. As seen with the other HR serotypes above, Typhisuis does not produce acute enteritis and, based on the histopathological characters of inflammation observed in infected pigs, it appears that this serotype may induce the secretion of a different pattern of chemokines by the pig intestinal epithelia compared to other swine associated serotypes (e.g. Choleraesuis and Typhimurium).

The host-adapted serotypes

Host-adapted *Salmonella* serotypes produce both enteritis and systemic infection in their natural hosts. The major exemplars are Dublin and Choleraesuis. Both serotypes have been described to infect silver foxes [15] in addition to their natural hosts (respectively bovine and porcine). Although rare, natural transmission of these serotypes may occur to human, due to close contact with infected animals or to consumption of contaminated products.

Choleraesuis

This serotype is defined as host-adapted on the basis that 99% of incidents are associated with pigs. However, it does naturally infect other host species, including man, in which the disease can be severe [15, 97, 98]. Over the last 20 years the incidence of Choleraesuis in Europe has remained low whereas the incidence in the US has increased and Choleraesuis is responsible for 95% of salmonella outbreaks in the pig industry and represents a major swine disease that costs an estimated $100 million per annum [99, 100]. Choleraesuis and Choleraesuis var. Kunzendorf have also been isolated from the mesenteric lymph nodes of

apparently healthy pigs [15, 100], and it has been suggested that Choleraesuis can establish a symptomless infection that becomes clinically apparent upon stress of the animal [101]. Before 1920, non-Kunzendorf strains were predominant among choleraesuis in the US and Choleraesuis infection in man was more common than now [102]. Human infections were well known for severity with 10–40% case mortality and the majority of isolates were from non-intestinal sites (i.e. blood-stream, bones, joints). Later, Kunzendorf variety became the most frequent serotype associated with disease in swine in both Europe and US, where it caused swine paratyphoid. This serotype was also frequently isolated in China, where Choleraesuis, predominantly the non-Kunzendorf variety, seemed to have a higher incidence in man than in the rest of the world [97, 98]. Potential differences in pathogenicity and host adaptation between the two varieties have not been evaluated to date. On the other hand, geographic differences in the prevalence of human infection by Choleraesuis and its Kunzendorf variant may be due to the large consumption of porcine products, culinary customs, and, perhaps, a high frequency of Choleraesuis infection in the local swine. Human infections by Choleraesuis, although uncommon, tend to present as septicaemia and affect mostly young and debilitated persons. The bacteria are isolated more frequently from blood, bone marrow, and vascular lesions than from faeces [98, 103]. Infection may also be complicated by pneumonia, arthritis, aortitis, and endocarditis [104–106].

Choleraesuis causes severe systemic salmonellosis in weaned pigs, 2–4 months of age. The major clinical manifestations are septicaemia, fever, and chronic wasting [100]. Systemic salmonellosis is often fatal due to the rapid onset of the disease and may lead to abortion in pregnant sows. Infection is thought to be by oral ingestion or by inhalation. Experimental and natural infections with Choleraesuis in pigs support this hypothesis [99, 107, 108]. Infected animals showed positive faecal, tonsil, and nasal swabs for up to 4 weeks following infection and shed sufficient numbers of bacteria to infect naturally exposed pigs. Colonization of the pig intestine and invasion of the intestinal mucosa does not generally produce a severe enteritis and it is followed by systemic dissemination. Interstitial pneumonia, congested mesenteric lymph nodes, and hepatic necrosis are the most common systemic lesions [100, 109, 110]. The understanding of the pathogenesis is limited.

Serotype Typhimurium induces watery diarrhoea in pigs as a typical sign of the disease but is rarely isolated from other organs, whereas Choleraesuis results in septicaemia followed by necrotic lesions and button-shaped ulcers in the colonic mucosa [100, 109]. The steps in pathogenesis that may account for such differences are not yet clear. Altmeyer and colleagues found sequence divergence of the *invH* downstream region between Choleraesuis and Typhimurium [56]. Accordingly, the two serotypes may have acquired some diversity in genes involved in epithelial cell invasion and/or in its regulation. Curtiss and colleagues have also reported that Choleraesuis and Typhimurium appear somewhat different in their mechanisms of epithelial cell invasion, since they are differently affected by deletion mutations in the genes for adenylate cyclase (*cya*) and the cyclic AMP receptor protein (*crp*) [111, 112]. The Crp-cAMP complex controls carbon catabolite repression in salmonella by inducing positive and negative transcriptional regulation in a variety of genes, a mechanism that is restricted to enteric bacteria and other closely related bacteria [113]. Comparison of epithelial cell monolayer adhesion and invasion by a Typhimurium Δ*cya* Δ*crp* strain with that of a Choleraesuis Δ*cya* Δ*crp* strain, revealed that while the former retained a wild type phenotype, the latter showed reduced adherence and greatly reduced (i.e. more than tenfold) invasion [112]. These authors therefore suggested that capability of Choleraesuis to invade may be characterized by a unique control of common salmonella genetic sequences by catabolite repression and/or by Choleraesuis specific genetic information. These observations may account for the different intestinal lesions produced by Choleraesuis compared to Typhimurium [109].

Choleraesuis harbours a virulence plasmid that shows extensive homology with that of other serotypes investigated [114, 115]. Although curing of this plasmid leads to loss of virulence in the mouse model [116], the importance of the virulence plasmid genes in the natural host of Choleraesuis has not been yet determined.

Dublin

Dublin is host-adapted to bovine and affects both young and adult cattle causing enteritis and/or systemic disease. Acute disease is characterized by fever, anorexia and abruptly reduced milk yield, often in association with firm faeces, rapidly followed by

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severe diarrhoea and high levels of mortality. Milder cases of disease sometimes occur which result in acute diarrhoea and abortion in the pregnant cow, or sometimes abortion in the absence of other clinical signs. Other clinical manifestations of Dublin infection may include a typhoid-like illness and often results in a chronic carrier state [117–119]. Natural infection by this serotype may also occur in other animals including man and, in particular, sheep and goats [15, 17, 120]. In humans Dublin infection generally occurs in patients with underlying chronic diseases, and arises from contact with animals or via the food chain. In these patients, Dublin may cause diarrhoea but more often, as seen for Choleraesuis, it produces a systemic infection with metastatic abscesses [121–123]. Sporadic outbreaks of disease in sheep flocks have often been recorded in the UK and USA and in some farms, a link with infection in cattle has been observed [124–126]. Clinical symptoms, except for abortion in pregnant ewes, are variable and apparently non-specific [127, 128]. McCaughey and colleagues have carried out experimental infections in sheep with Dublin and found that this serotype can successfully infect this host by the oral route and cause both enteritis and systemic dissemination [128]. In pregnant ewes, abortion occurred following infection with high doses of bacteria and it was always associated with death of the ewe. This is in contrast to observations in the natural and experimental infection of sheep with HR Abortusovis, where enteritis was not seen and abortion occurred without apparent illness in the ewe [124, 129].

Several investigations on the molecular basis of Dublin pathogenicity based on both in vivo and in vitro studies have been reported [130–134]. Dublin virulence plasmid genes (*spv*) have been found to be strongly expressed when the organism is within eucaryotic cells and to be regulated by both SpvR and RpoS [135, 136]. The *spv* genes are required for survival and replication of Dublin within bovine monocytes [131, 137], and experimental Dublin infections have shown that these virulence genes are necessary for the development of systemic salmonellosis in both the bovine and murine hosts but do not affect the capability of Dublin to colonize the intestine, to invade PP or to cause diarrhoea in cattle [138, 139]. Alternatively, enteric inflammation appears to be due to Dublin adhesion to the epithelial cells and is mediated by activation of the proinflammatory genes transcription factor NF-kB and chemokine (i.e. IL-8, IL-6, MCP-1, GM-CSF and TNFα) expression [140–142]. Invasion of the bovine intestine also plays a role in the induction of enteritis and it is strongly affected by null mutation of the *invH* gene in Dublin as it is in Typhimurium [143, 144]. With respect to the mechanisms of pathogenicity, therefore, no significant differences in the interaction with the bovine intestinal tissues have been reported so far between HA Dublin and UR Typhimurium strains. Wallis and colleagues obtained compelling evidence from cross challenge of cattle and pigs with strains of Dublin and Choleraesuis, where a striking difference in the infection pattern between the HA and the non-HA serotype in both animal models was the recovery of high CFUs from internal sites 7 days post infection [145; Wallis and colleagues, unpublished data]. However, in another study Dublin was recovered from both bovine and porcine ileal loops invasive assays in numbers comparable to Choleraesuis 3 h after challenge. The systemic dissemination and prolonged colonization of extraintestinal sites might be partially or completely restricted to non-HA serovars.

Immunity and host resistance to salmonella

Levels of susceptibility to salmonella infection differ between animal species, and both natural and acquired immunity play an important role in the development of systemic salmonellosis [146]. Immediately following the invasion of the organisms beyond the intestinal mucosa, more than 90% of the organisms are destroyed at, or close to, the site of inoculation, primarily by resident phagocytic cells. Surviving organisms disseminate, and bacterial growth occurs in the cells of the reticuloendothelial system. The crucial phase occurs when bacterial multiplication is either controlled or continues in an uncontrolled fashion.

Polymorphonuclear leukocytes (PMN) are the first phagocytic cells to be attracted towards infected tissues by means of salmonella-induced cytokines secretion [147, 148]. PMNs have been recognized for many years as having a function in the inflammatory response, and recently PMNs have also been implicated in the modulation of the other immune cells [149]. Salmonella has adapted to grow inside macrophages where it is relatively sheltered from PMN [150]. Macrophages play a dual role in the salmonella infection process. Once activated, they can kill salmonella, but macrophages are also the site of bacterial multiplication. Infected macrophages are therefore responsible for the dissemination of the

infection via the lymphatic ducts to other organs [151].

Other non-specific defense mechanisms have been considered in relation to host specificity in salmonella. The serotypes capable of causing systemic disease may withstand complement mediated lysis (serum resistance) better in their adapted host, hence gaining a selective advantage in the extracellular environment. Collins [152] demonstrated that even undiluted serum from chickens could not kill strains of Gallinarum, whereas Enteritidis was more sensitive. A recent testing of several strains of different HA, HR and UR serotypes in serum from different animals have, however, demonstrated a large strain variation and no correlation between serum resistance and host adaptation/restriction (Brown, unpublished data).

The humoral immune response towards salmonella begins with a specific IgM response, and intraperitoneal infection of mice with attenuated or killed salmonella induces protection by means of specific IgG and IgM against the O-antigens [153]. The response time of 1 week following *i.p.* and more than 3 weeks following *p.o.* challenge [154] however, suggests that humoral immunity is not an important factor in determining host adaptation/specificity.

Cell mediated immunity is supported by T cells, but also by the cytokines excreted during the activation of the different cells involved in the inflammatory immune response. γ/δ T-lymphocytes were described to be efficient during the early phase of infection with *Listeria monocytogenes* and *Mycobacterium bovis* [155, 156]. A similar role in control of salmonella infection was suggested based on investigation with Choleraesuis, where the induction of γ/δ T-cells was correlated with the endogenous production of stress proteins (Heat Shock Protein, HSP) by macrophages [157]. With Enteritidis, resistance against oral infection was greatly supported at the mucosal epithelial level by γ/δ T-cells [158]. Associated to activation of all these cells by bacteria, cytokines play an important role both in the development of the immune response and the protection. Pro-inflammatory cytokines as TNF seemed to be essential in the early phase, and also during the specific immune response [146, 159]. IFNγ was also shown to be important, as neutralization of this cytokine with antibodies lead to the death of Typhimurium infected mice [159].

When *Salmonella* serotypes colonize in the intestinal mucosa of mammals, before progression to a systemic infection in the body, they face to an effective barrier of macrophages that line the lymphatic sinuses of lymph nodes. The granuloma formations caused by the accumulation in inflamed tissue of polynuclear granulocytes in mammals, also exist in avian hosts where it is the heterophiles that are involved, and are morphologically similar to inflammatory lesions in reptiles [160]. Therefore, one of the first steps in the salmonella development towards being a systemic, facultative intracellular pathogen may have been to enter the macrophage in order to escape from the aggressive environment. It is tempting to speculate that it is the ability of HA and HR serotypes to escape cellular defences that has led to the development of host specificity. That pathogens have adopted different tactics to escape immune systems is well known. Immune evasion of virus and helminth parasites related to cytokine activities is beginning to be explored [161], and also bacteria can be supposed to contain and produce a large number of diverse molecules, which can selectively induce the synthesis of cytokines, as LPS does [162]. Unfortunately, little evidence has been accumulated to date with respect to salmonella.

In conclusion, very few investigations have explored the possibility that the immune system of mammals and birds positively selected for changes in the *Salmonella* phylum leading to adaptation to their new hosts. We can hypothesize that macrophages were the first target for a host adaptation and that the ability of a pathogen to survive and even replicate within phagocytic cells is a potent method of evading the host defence mechanisms. Thereafter a discontinuous equilibrium was established between the bacteria and the animals. One example is that pathogens that lack host specificity, such as Typhimurium and Enteritidis, tend to be more pathogen in young animals than in adults, suggesting that they may not be optimally adapted to overcome the fully mature immune system and that serotypes that are host-specific have acquired the ability to bypass these defence mechanisms in adult animals.

Pathogenesis and virulence factors: potential role in host-adaptation and host-restriction

Salmonella has long been considered to be a facultative, intracellular pathogen. However, the precise sites of invasion, its persistence and multiplication *in vivo* are yet to be identified and remain controversial [163]. The usual route of entry for salmonella infections is by means of the faecal-oral route. As such, the organisms are faced with an impressive array of non-specific host defences, such as the acidic

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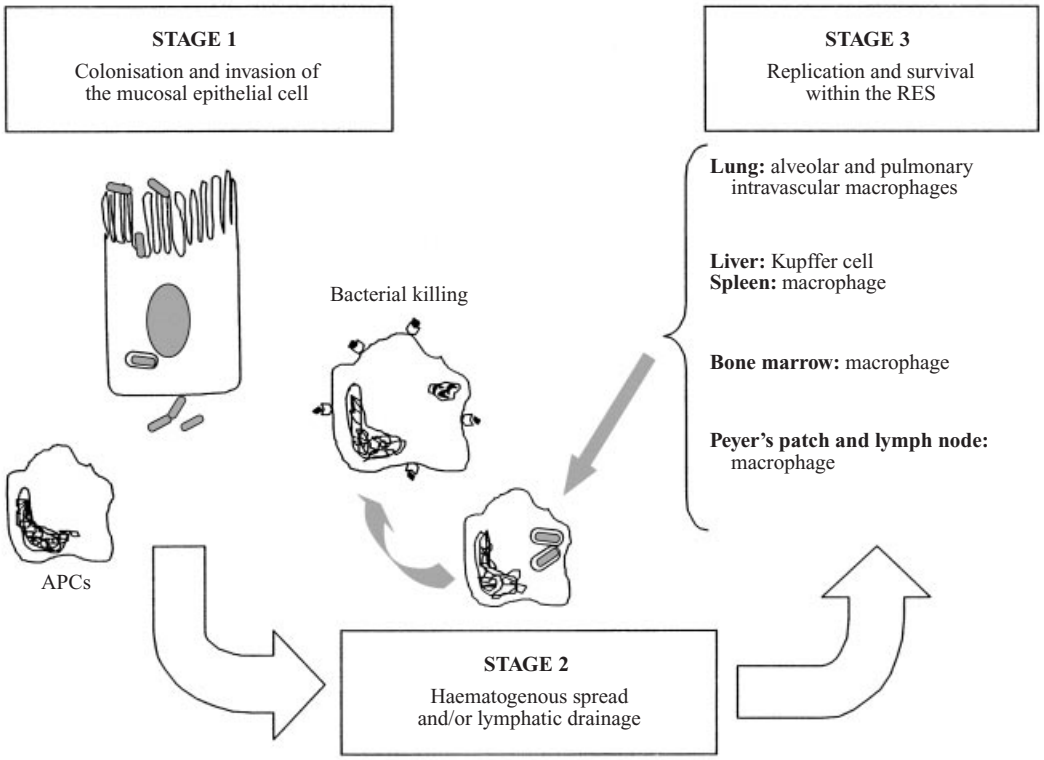

Fig. 1. Principal steps in *Salmonella* pathogenesis with potential for involvement in the development of host restriction and adaption. (APC, antigen presenting cell, the final 'activated macrophage' is shown coated with processed salmonella antigen.)

environment of the stomach, intestinal mucus, and the normal gut microflora. During the course of infection, salmonellae may well have to endure both intra- and extra-cellular environments, and the relative ability of strains of each serotype to disseminate and proliferate within these different niches could well determine the nature and severity of the disease.

Possible pathogenic mechanisms that mediate salmonella host-adaptation and host-restriction are poorly defined, and study in this area is complicated by the complex pathogenesis of salmonella infections. Key stages in the pathogenesis, and therefore stages which are potentially instrumental in determining the host-adaptation of *Salmonella* serotypes are summarized in Figure 1; these include colonization and invasion of the intestine, dissemination of the salmonella throughout the body, and replication and survival of salmonella within professional phagocytes. The possible involvement of these different stages in salmonella host specificity is considered below.

Colonization and invasion of intestinal epithelium

The intestinal epithelium represents the largest interface (more than 2000000 cm^2) between the external environment and the internal host milieu and, therefore it is a major route of entry of microbial pathogens into the host. In this environment, *E. coli* and *Salmonella* sp. have diverged and developed numerous serotypes with various degrees of pathogenicity and host adaptation. Concomitant evolution of the animal host has been characterized by the development of a set of conserved intestinal epithelial cell functions that are activated by the interaction with a broad range of pathogens, including salmonella, and are likely to play a significant role for host survival.

Rate of colonization and infection

Several studies have indicated that mucus constitutes an important site of colonization by salmonella [164, 165]. Studies of growth kinetics of the HR and HA serotypes Gallinarum, Dublin and Choleraesuis, compared to the un-restricted serotype Typhimurium in diluted mucus isolated from poultry, cattle, pig, and mouse have not demonstrated significant differences in growth rate between the serotypes, suggesting that they are all capable of utilising mucus as a growth medium (Skov, personal communication). The ex-

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perimental approach is artificial, but the conclusion which can be drawn from the results, i.e. host specificity is not determined at the level of interaction with mucus, is consistent with conclusions from invasion studies in intestinal loop assays, where the mucus layer of the animal remained intact (see below).

The host intestinal microenvironment (i.e. low oxygen tension, osmolarity, and pH) is likely to modulate bacterial antigen expression that in turn, triggers a variety of host responses in a contact dependent fashion between salmonella and the enterocyte apical membrane [52, 166, 167]. Modulation of salmonella protein expression by *in vitro* conditions that simulate the intestinal environment has been extensively studied [168, 169], but the different serotypes overall appear to respond similarly.

Salmonella invasion of the host intestinal mucosa does not seem to play an important role in determining the host specificity seen with the HR and HA serotypes. Microscopic observations in pigs suggests that the HA serotype Choleraesuis has a higher tendency to invade the intestinal mucosa of pigs compared to Typhimurium and, furthermore, that it has a predilection to invade the intestinal mucosa overlying the PP [109]. However, in extended studies where the invasion of Dublin and Choleraesuis strains in bovine and porcine ileal mucosa was assessed in 3-h ligated ileal loops, both serotypes were recovered in comparable numbers from both host tissues, suggesting a similar ability to invade and survive intracellularly despite the adaptation to one host only. The Dublin strains induced more severe intestinal lesions in both tissue types, and no difference was observed between loops with or without PP [145]. A loop-invasion model has recently been developed using chicken intestinal loops. Surprisingly, but in support of the conclusions drawn from the above studies, Dublin was better at invading the chicken intestine than Gallinarum, which in turn did not invade to any greater extent than Choleraesuis. The rate of invasion was lower than that seen for Typhimurium (Aabo and colleagues, unpublished results), again indicating no correlation between the early intestinal invasion and host specificity. Similarly, comparative analysis of the rate of invasion showed by Abortusovis, Gallinarum, Choleraesis, Dublin, and Typhimurium in sheep ileal loops, demonstrated that Abortusovis was less invasive than the other four serotypes tested in the ovine intestine (Uzzau and colleagues, unpublished results). These findings are of particular interest since for many years salmonella invasiveness has been considered the major virulence attribute in evaluating the pathogenicity of *Salmonella* strains.

Site of invasion

The investigation of differential mechanisms of invasion displayed by host specific serotypes is further complicated by the fact that the infection may occur by a route(s) other than the oral one (i.e. faecal–nasal) and the primary site of infection may be represented by non-intestinal tissues such as the tonsil and the lung as hypothesized in case of Choleraesuis infection [13].

Salmonella interactions with specific parts of the host epithelium might play an important role in the host-restriction and tissue-restriction of the infection. HA and HR serotypes may be permitted to give raise to a systemic infection provided they interact with a unique character of a specialized tissue of the host epithelium. This hypothesis is supported by the observation that, although the majority of salmonella epithelial cell interactions occur at the apical membrane of the enterocytes [147], early association and entry of salmonella appears to be via the M cells lining the small intestine PP [57, 170–173]. Cytokine secretion by M cells and activation of subepithelial phagocytic cells may regulate both intestinal inflammation and salmonella persistence in deeper tissues. This specialized lymphoid tissue possesses different physiological and anatomical characteristics among host species, but the role of this in determining outcome of HA and HR infections remains to be investigated [174–178]. Moreover, organized mucosal lymphoid follicles, morphologically and functionally similar to PP, are found in the pharynx (palatine tonsils), in the respiratory tract, and in the appendix [179]. These lymphoid tissues appear well developed in swine [13, 180] where the total number of M cells was highest in lymphoglandular complexes in the rectum and lowest on domes of the ileal Peyer's patch (IPP) [181]. In this host bronchus-associated lymphoid tissue (BALT) is found as follicle-like aggregations with lymphocytes infiltrating the epithelium, which shows specialized epithelial cells and a structure similar to that seen in the PP of the small intestine [182, 183]. BALT is not a constitutive structure in all species, i.e. in humans [183, 184], and the interaction and colonization of these sites by Choleraesuis and Typhisuis may be preferential in the pig host where interstitial pneumonia is frequent and typical lesions

Table 3. *Specialised lympohid tissues in ovine and pig: correlation with major sign of HA salmonellosis*

Host lymphoid tissue	Serotype	Major anatomo-pathological and clinical signs
Sheep	Abortusovis	None or abortion
Involuted PP	Dublin	Septicemia, abortion
Lamb	Abortusovis	Septicemia, diarrhoea
Mature PP	Dublin	Septicemia, diarrhoea
Pig		
Constitutive BALT;	Choleraesuis	Pneumonia, tonsil enlargement, colitis, proctitis
highly reactive palatine tonsil;	Typhisuis	Pneumonia, tonsil enlargement, colitis, proctitis
high number of rectal M cells;	Typhimurium	Watery diarrhoea

of the alimentary tract are localized at the level of the palatine tonsils, cecum, and rectum [96, 109]. The characteristics of these lymphoid tissues in the sheep and pig hosts and their possible influence on the diseases caused by the most frequently encountered *Salmonella* serotypes are summarized in Table 3.

Transmembrane signalling

Salmonella adhesion to and invasion of epithelial cells is accompanied by a cascade of events, including chemokine secretion and PMN migration that are possibly responsible for the development of systemic infection and/or gastroenteritis. Secretion of the chemokine IL-8 has been correlated with the migration of PMNs to the site of bacterial infection, small bowel inflammation and therefore, diarrhoea. McCormick and colleagues found that the bacteria-enterocyte contact dependent signalling to PMNs may represent an essential virulence determinant which underlies the development of enteritis in the mammalian host, and that such signalling is differentially modulated in *Salmonella* serotypes that give raise to systemic infections and are host specific, compared to those that elicit enteritis in a broad range of animal hosts [48, 140, 185]. Recent studies have elegantly shown that both Typhimurium and Typhi, as well as other human adapted serotypes (Paratyphi A and Paratyphi C), were able to induce basolateral secretion of IL-8 by T84 polarized cells (resembling the human small intestine enterocytes), but only Typhimurium induced apical secretion of the pathogen-elicited epithelial chemoattractant (PEEC) [48]. These results may account for the migration of PMNs to the small intestine, due to IL-8 and observed during both Typhi and Typhimurium infection, and to the lack of PMN observed in the lumen of Typhi infected gut, but not in that of Typhimurium infected gut, due to PEEC [186]. Similar results were observed with Gallinarum. This serotype too, has been described to exert a different control over the secretion of chemokines by human intestinal epithelial cell compared to Typhimurium, i.e. Gallinarum was not able to promote PMN migration across T84 monolayers [140]. The induction of chemokines and consequently, PMNs migration and inflammation, was not due to salmonella internalization (i.e. Typhi, Paratyphi A. Paratyphi C, and Gallinarum biotype *pullorum* invaded the enterocytes but did not elicit PMN transepithelial migration) and was not blocked by inhibition of invasion [48]. This is in contrast to the results of others [187] who observed that Dublin-induced IL-8 secretion and Typhimurium-induced IL-7 receptor expression required bacterial entry. The secretion of another chemokine, IL-6, has been shown to be implicated as a physiological molecule in several epithelium-parasite interaction systems [188, 189], and may also be regulated differently by different *Salmonella* serotypes. In fact, Typhi induced significantly greater quantities of IL-6 than Typhimurium or Dublin in murine and human small intestine cell lines [47, 190].

Bacterial-induced apoptosis of intestinal cells

In the recent years, an increasing number of studies have focused on bacteria-induced apoptosis of eucaryotic cells (for review see Moss and colleagues [191]). In the intestine, apoptosis occurs spontaneously to counterbalance the enterocytes proliferation in the crypt compartment and results in the elimination of cells without release of cellular contents and therefore with no inflammation.

Salmonella-induced apoptosis of the host macrophage has been clearly demonstrated by several studies [191] and more recently, extended to include intestinal epithelial cells [192]. Epithelial apoptosis appears to be delayed compared to that induced in macrophage

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and requires salmonella invasion of and replication within the epithelial cell. The apoptotic programme may function in order to eliminate salmonella infected cells and restore integrity of the infected intestinal epithelium and, therefore, might represent a defense mechanism. Kim and colleagues have noted that apoptosis might be beneficial for both salmonella and the intestinal cells, since the time delay (at least 6 h) observed after interaction with the bacteria may be sufficient to generate signals for the activation of mucosal inflammation and non-specific immunity, but also for the bacteria to adapt to the new environment before invading deeper tissues [192]. Although epithelial intestinal apoptosis appears to be a conserved response toward bacterial pathogens with different strategies of invasion and intracellular trafficking (e.g. Dublin and enteroinvasive *E. coli*), it may contribute to the complex interaction established by HR and HA *Salmonella* strains and their natural host, and thus overall to the pathogenesis of the host specific salmonellosis.

In conclusion, the rate of invasion does not correlate with host-specificity and is not considered important for determining the HA and HR phenotypes. The interaction between the HR serotypes and their respective natural hosts, contrary to what is seen with UR serotypes, appears to be aimed to reach the deeper tissues while inducing minor damage to the host mucosa. Rather, the major difference observed so far in respect to the outcome of the salmonella-intestinal epithelial cell contact, concerns those *Salmonella* serotypes (UR) that elicit diarrhoea (i.e. enteritis) and those serotypes (HR) that do not.

Dissemination route

Following the penetration of the intestinal epithelium, the exact course of salmonella invasion is not entirely known. It is generally agreed that salmonellae are carried by cells of the reticuloendothelial system via the mesenteric lymph nodes to systemic sites. Bacteria samples by the Peyer's patches M cell induce their own uptake by macrophages and, perhaps, by B- and T-cells, exploiting these cells trafficking for lymphatic dissemination [193, 194]. Alternatively, *Salmonella* serotypes capable of disseminate in a particular host may utilize alveolar macrophages and pulmonary intravascular macrophages (PIM) for translocation [195]. Calves, sheep, goats, and pigs, but not man or small rodents, possess PIM densities and clearance capacity in the lung parenchyma similar to that of human and murine Kupffer cells in the liver [196]. Pigs rooting behaviour and the ovine and bovine grazing allow salmonella in the environment an easy access to the nasal cavity and thus to the lungs. *Salmonella* serotypes (i.e. Dublin, Abortusovis, Choleraesuis, and Typhimurium) able to produce systemic infection in these animals might have developed specific mechanisms to take advantage of both the intestinal and the pulmonary route of entry and dissemination. It is worth noting that salmonella infection of these hosts is often characterized by pneumonia and that bovine-adapted Dublin may cause pneumonia as a major sign of infection in sheep [110, 124, 197, 198]. Experimental infections of lambs challenged intravenously further support this hypothesis, since Dublin, Abortusovis, and Choleraesuis, but not Gallinarum, infected the lungs significantly more than the liver, suggesting convergence of adaptation for the first three serotypes toward the lung lymphoid tissues (Uzzau and colleagues, unpublished results).

In the process of host adaptation, salmonella may have learned how to capitalize on M cell sampling and monocyte/macrophage trafficking in order to reach systemic sites. It is conceivable that, due to the complex interaction between these lymphoid cells and the cells encountered in the tissues where their circulation occurs, *Salmonella* serotypes have developed virulence determinants capable of interaction specifically with such eucaryotic signalling. Wiant and colleagues recently obtained results that support this hypothesis [194]. Human macrophages were stimulated to produce TNF-α, IL-6, IL-1b, and IL-10 by whole-cells of Typhi or Typhi purified flagella (STF). Furthermore, lymphoproliferative responses to mitogens were decreased, as the result of STF-mediated decreased uptake of soluble antigens and decreased expression of the adhesion molecule CD54 and of the LPS-receptor CD14 [194]. Although the mechanisms involved in STF-mediated suppression of lymphoproliferative responses has yet to be fully elucidated, these data suggest that STF modulates the human monocyte/macrophage to differentiate toward dendritic cells (DC) in their migratory stage [194, 199]. The clear advantage for intracellular Typhi resides in the induction of a host cell phenotype (i.e. by suppression of adhesion molecules expression) that allows, like a 'Trojan horse', the systemic dissemination of the microorganisms throughout the reticuloendothelial system within a few hours. In particular, the observation that the flagella *per se* may trigger

such a cascade of events raises the question of whether serotype specific flagellins are required to effectively modulate antigen presenting cells in the natural hosts.

Extraintestinal infection: interaction with the reticulo-endothelial system (RES)

Comparison of infection kinetics in mice and chicken by strains of Gallinarum, Dublin, Typhimurium and *Escherichia coli* shows that the three *Salmonella* serotypes can all be isolated from the intestinal mucosa of both animals, but only Gallinarum propagate in chicken to be reisolated in any significant number form internal organs such as liver and spleen [8]. This suggests that the role of invasion of the RES is important in determining the host-restriction and host-adaptation.

The ability of salmonella to resist killing by macrophages is undoubtedly important in salmonella virulence and therefore possibly influential in host-specificity [8]. Several lines of evidence suggest this hypothesis for Typhi human-restriction. Vladoianu and colleagues [200] demonstrated that Typhi strains persisted in human monocyte-derived macrophages, but not in murine macrophages, confirming that the macrophage control over salmonella infection could influence host specificity. On the other hand, Typhimurium, which causes typhoid in mice and diffuse enteritis and, less frequently, bacteraemia in humans, is able to survive in mouse macrophages and in human macrophages [200]. Furthermore, Typhi induces ruffling and micropinocitosis in murine macrophages, but internalization is followed by formation of fewer numbers of SP than Typhimurium, an indicator of reduced capability to survive within macrophages that is essential in pathogenesis [201, 202]. However, the exact role of the survival of salmonella in macrophages is not known and is made more difficult to rationalize by the reported ability of salmonella to lyse macrophages both *in vitro* and *in vivo* [203–205]. Recently a lack of correlation between the animal species from which macrophages were isolated and the ability of HA-serotypes and *S. typhimurium* to survive within these macrophages has been published. Also there were significant differences in the level of pro-inflammatory cytokines induced by these serotypes [144a].

In vitro studies have demonstrated that host-specific pathogenesis of *Salmonella* serotypes may depend on the selective recognition of complement receptor (CR) types on the macrophages membrane [206]. Typhi and Typhimurium induced their own uptake by micropinocytosis in both human and murine macrophages, but only Typhi was capable of growth in human macrophages. Conversely, Typhimurium survived in murine macrophages whereas Typhi did not. The molecular basis of such restriction has been hypothesized based on the fact that intracellular survival and replication is only made possible by recognition, in the presence of serum opsonin, of the CR type 1 (CR1) but not of CR type 3 (CR3). Strikingly, Typhi and Typhimurium recognized, respectively, CR1 and CR3 on human macrophages, whereas they recognized, respectively, CR3 and CR1 on murine macrophages. Baker and Morona have recently observed that phorbol myristate acetate (PMA) differentiated U937 (PMA-U937, human) cells restricted the net growth of Typhi but not Typhimurium *phoP* mutants, suggesting that the *phoP/Q* locus may control expression of genes involved in host specificity, particularly affecting differential effects on Typhi and Typhimurium LPS [206]. The relevance of the *phoP/Q* regulatory system in host specificity can also be recognized by the recent finding that *pagK*, a positively regulated gene in a trasposon-like element, is found only in broad host range *Salmonella* spp. [207].

Active phagocytosis by the RES is due to macrophages that are resident in all organs and connective tissues and have been given special names in different locations (e.g. Kupffer cells in the hepatic sinusoid, alveolar macrophages in pulmonary airways, and osteoclasts in the bone). Survival and replication of salmonella within these phagocytic cells may vary in the different sites and organs. In pigs, Choleraesuis [99], Typhisuis [96], but also Typhimurium [195, 208] have been frequently observed to persist in the lungs, presumably within the alveolar macrophages, whereas Typhimurium lung infection in other mammals is far less frequent. Typhimurium grows better inside splenic and bone marrow-derived murine macrophages than in peritoneal murine macrophages [203]. In humans, typhoid and non-typhoid *Salmonella* strains that provoke systemic infection persist in macrophage-rich organs for example in the liver, where they may rarely cause salmonella hepatitis, and in the bone marrow [209]. Infection may be responsible for thrombocytopoenia, anaemia, leucopaenia, or pancytopaenia [62, 64, 210], and it is found associated with granulomatous inflammation [210] and bone abscesses [97, 98, 211]. Bone marrow localization of salmonella in other animals frequently affected by this micro-

organism, such as cattle, sheep and poultry have not been described, although only few reports are available [212]. Rather, localization of salmonella from poultry occurs mostly in the lower intestinal tract, associated with granulomatous nodules, and in the bursa of Fabricius [213, 214].

Interestingly, the bone marrow in humans and small rodents share physiologically important properties with the bursa of Fabricius in poultry and the PP in ovine foetus/lamb. In fact, in addition to being preferred sites of infection in the natural hosts by the respective *Salmonella* host-restricted serotypes [215, 216] (S. Uzzau and colleagues, unpublished results), they all represent primary lymphoid organs for the B-cell repertoire of that particular animal species. It is tempting to speculate, therefore, that a convergence exists between the evolution of *S. enterica* subspecies I strains able to colonize internal organs of warm blood animals, and the acquisition by these hosts of primary lymphoid tissues for B-cell maturation that are more sophisticated than those of the *S. bongori* and *S. enterica* subspecies II, IIIa, IIIb, IV, VI, and VII natural hosts, i.e. cold blooded vertebrates [115].

Another outcome of the salmonella-RES interaction that might be involved in host-specificity, is represented by salmonella induced apoptosis of phagocytic cells [217, 218]. Recently, *Salmonella* spp. were shown to trigger apoptosis of monocyte-macrophages and apoptosis or anergy in T cells, whereas PMN spontaneous apoptosis can be delayed by interaction with the bacteria [219–222]. Modulation of this mechanism in the cells encountered in the various tissues of the RES may provide an excellent strategy to evade the immune system. In particular, macrophage apoptosis induced by salmonella signals, appears to require SipB translocation into the cytoplasm via the salmonella type III secretion system [223] and prior activation of the macrophage [217]. Therefore, salmonella hides itself inside the monocyte-macrophage but it may 'decide' to kill this host cell when it become activated, inducing a pathway, apoptosis, that limits inflammation and allows the bacteria to remain intracellular. At the present, investigation of salmonella-induced apoptosis in monocyte-macrophage using host adapted HA Dublin and HS Typhi and Gallinarum serotypes and murine macrophages, indicated that this a common mechanism of pathogenicity among *Salmonella* spp. and may not be involved in host-restricted pathogenesis [224]. Conflicting results have been published [144a], and further studies are required to test this hypothesis.

The virulence plasmid

The importance of the salmonella plasmid virulence (*spv*) genes in the systemic infections caused by certain serotypes of *S. enterica* is well established. The *spv* operon contains five genes (*spvR*, *A*, *B*, *C*, and *D*) [225] which are apparently highly conserved and are present on a family of serotype associated plasmids (SAP) commonly found in isolates of Typhimurium, Enteritidis, Dublin, Abortusovis, Choleraesuis and Gallinarum, these last four serotypes being HA or HR.

The *spv* genes are expressed during the stationary growth phase and during the intracellular stages of infection [226]. Curing of the SAP or deletion of the *spv* region abolishes the virulence of these serotypes in animal models [87, 227–229]. The introduction of these genes, however, into serotypes not associated with the SAPs does not contribute to any enhanced virulence, and these other serotypes cannot express the *spv* gene products (D. J. Brown and colleagues, submitted for publication).

The role of the virulence plasmid in the pathogenesis of salmonellosis remains an enigma. At least 11 serotypes are known to carry virulence plasmids, which share common and unique sequences [230]. Typhi does not carry virulence plasmids and not all isolates of those serotypes associated with the plasmids do. The role of the virulence plasmid in pathogenesis has been mainly studied using the mouse model of salmonellosis. In mice, plasmid genes are not required for the translocation of salmonella through the intestinal mucosa but have been implicated in controlling the growth rate inside cells of the RES [74]. The presence of the virulence plasmid in Dublin is needed for systemic but not enteric disease in cattle and does not influence intestinal invasion and dissemination to systemic sites [231].

In relation to HA/HS, the virulence plasmids seem to be functionally interchangeable. The *spv*-genes derived from the Typhimurium virulence plasmid can restore mouse virulence fully in all cured strains of serotypes normally carrying a virulence plasmid (Brown and colleagues, submitted for publication), and Gallinarum remains virulent to chicken when its resident plasmid is exchanged with that of Typhimurium [232]. However, it cannot be ruled out that sequences outside the functional *spv*-genes contribute to host-specificity.

Recently developed molecular methods with application to studies of host-adaptation in salmonella

Three major approaches for the identification of genetic determinants of host specificity are: (i) the isolation of mutants with altered host-specificity, (ii) the construction of hybrid strains carrying heterologous host-specificity determinants, and (iii) the detection of genes with differential expression *in vivo*.

Transposon mutants are usually preferred over chemically-induced mutants because tagging a gene of interest with a dominant marker can help in many genetic manipulations. In P22-sensitive serovars, transposon mutagenesis can be achieved using the procedures developed for Typhimurium LT2. Phages SE1 and PIL4 can be alternative transducing phages for P22-resistant strains. Conjugal delivery of transposons may replace transduction in phage-resistant serovars. A sophisticated procedure for the identification of genes involved in bacterial pathogenesis, developed by Hensel and colleagues, can be also applied to the detection of host adaptation genes [233]. The so-called Signature-Tagged Mutagenesis (STM) employs a mini-Tn*5* element that contains a variable DNA sequence tag. The variable DNA region is designed such that each bacterium present in a transposon pool carries a unique DNA sequence. Animals are infected with pools of insertion mutants and, after *in vivo* infection, bacteria recovered from the animal are screened by DNA hybridization; clones which were present in the inoculum but are absent from the recovered population can then be isolated and identified. This procedure can circumvent the problem that insertion in a gene of interest can cause loss of viability of the mutant *in vivo*. STM can be applied to the search for mutants that cannot be recovered from a natural host but are still virulent in other animals. Mutants unable to proliferate in specific host locations (but able to proliferate in others) can be likewise identified.

Chromosomal hybrids can be obtained using two different methods: replacement of partially homologous regions and addition of fragments to the chromosome. One potential problem for hybrid construction is that regions with partial homology undergo low frequencies of recombination. Disruption of mismatch repair genes can be expected to increase recombination [234]. Insertion mutations in mismatch repair genes such as *mutL* and *mutS* are available in Typhimurium; because these genes show evolutionary conservation, insertion mutations in mismatch repair genes should be easily transferred to any *Salmonella* serovar, provided that a delivery system is available. If host-specificity is associated with chromosomal DNA divergence, hybrid construction should be feasible by using mismatch-repair mutants. Addition of fragments to the chromosome can be performed upon circularization of transduced fragments and recombination with a chromosomal target [235]. This procedure might be helpful for the introduction of heterologous genes in a given serovar when homology does not exist (e.g. if 'islands' of host-specificity were found).

A strategy which allows the detection of salmonella genes that are specifically induced in host tissues has been developed by Mahan and colleagues [236]. The strategy, named *in vivo* expression technology (IVET), takes advantage of the fact that purine auxotrophy greatly attenuates growth of Typhimurium in the animal host; this deficiency provides a strong selection for a $PurA^+$ phenotype in animal tissues. If a promoterless *purA* gene is used, any gene rearrangement that provides a strong promoter to *purA* will permit growth in the mouse. The IVET system employs a plasmid, pIVET1, carrying a promoterless *purA-lacZ-lacY* operon constructed *in vitro*. The recipient strain contains a deletion of the chromosomal *purA* gene, to prevent plasmid integration in this region. Cloning of *Sau3A* fragments in pIVET can generate fusions of Typhimurium promoters to the chimaeric operon *purA-lacZ-lacY*. The plasmid contains the origin of replication of R6K and thus can only replicate in the presence of π protein. Since the latter is not naturally synthesized in salmonella transfer of pIVET1 derivatives to Typhimurium selecting Ap^r permits the isolation of transconjugants that have integrated the pIVET derivative into the salmonella chromosome. Whenever a strong promoter is provided, expression of *purA* will permit survival and enrichment of those cells that carry an active promoter driving the *purA-lacZ-lacY* operon. Because the chimaeric operon also contains *lacZ* and *lacY*, the fusion strain must be also Lac^+, a phenotype that can be easily scored on appropriate media. However, isolates which are Lac^- on plates (in spite of having been enriched in the animal) are of particular interest, because their Lac-phenotype may indicate that the cloned fragment carries a promoter that is only active in host tissues. This simple and imaginative method has proved useful for the isolation of a number of *ivi* (for '*in vivo* induced') genes of salmonella. Sequencing of 200–400 bp upstream of

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the *purA* gene is usually enough to establish whether the *ivi* promoter detected belongs to a previously known gene or identifies a novel locus.

The IVET strategy might become helpful for the identification of host specificity determinants, particularly if host specificity were a multigenic trait, and the corresponding genes were scattered on the chromosome, the virulence plasmid or both. In such an undesirable situation, IVET might provide a breakthrough. Genes induced *in vivo* can be expected to include host-specificity determinants. Thus IVET screenings in various host-adapted serovars might be able to detect determinants unique to each of them.

CONCLUSION

Although the precise mechanisms of host adaptation have not been yet elucidated, it is conceivable that they operate at different stages of salmonella infection and in an interdependent manner. During the development of host adaptation, each HA and, in particular, each HR serotype would have been faced with the evolution of an impressive array of specific and non-specific mechanisms of immunity of the animal host. To be successful pathogens, *Salmonella* host-specific serotypes, therefore, have adapted to the physiology of their natural hosts and developed the means to capitalize on it. In particular *S. enterica* subspecies I serotypes acquired the ability to overcome the mechanisms of immunity of warm-blooded animals. Modulation of physiological pathways in the natural host(s), such as intracellular trafficking, apoptosis, antigen sampling by M cells, and macrophages and/or lymphocytes circulation throughout the RES, would have been the key in the development of adaptation. Due to the nature of these pathways and the diversity of the lymphoid tissues among the different warm-blooded hosts, the consequences of a successful interaction with a specific host could also have meant the inability to carry out the same modulation as efficiently in other animal hosts that are distantly related.

Further observations suggest that specific pathways might be exploited by HR strains in their natural host(s). In particular, Typhi, Gallinarum, and Abortusovis show a striking tissue tropism toward their host primary lymphoid organs for B cell development (respectively, bone marrow, bursa of Fabricius, and Peyeris patches). Since the outcome of such interactions, particularly in the adult animals, appears to be the establishment of a chronic infection, the dissemination of microorganisms may occur through shedding a relatively low bacterial load for an extended period of time. In this situation, the induction of a severe enteritis is not necessary and may even be detrimental to the efficient spread of the organism. On the other hand, dissemination of ubiquitous and HA serotypes take more advantage from the ability to induce a strong enteritis.

Each HR/HA serotype has probably evolved independently, since no close genetic relationship occurs among these groups of bacteria. Nonetheless, these strains are all auxotrophs and the same nutrient requirement is frequently found in different serotypes (e.g. cystine, thiamin, nicotinic acid). The lack of specific metabolic pathways, therefore, may have favoured adaptation. Alternatively, 'hot spots' in the chromosome may have been rearranged, due to the acquisition of new clusters of genes, resulting in deletion of these metabolic genes. This hypothesis may be considered also in view of the 'mosaic' structure of the salmonella chromosome, where several pathogenicity islands and phage insertions carring virulence determinants have been identified to date. Further studies are warranted to define if differences in the distribution and/or control over these clusters of genes occurs in HA/HR serotypes.

In conclusion, although important observations have been made by studying salmonella HA/HR serotypes pathogenicity in *in vitro* models, new insights on the complex interaction of such serotypes with their natural host(s) may only be achieved if the host is considered as a whole and by a comparative analysis of the anatomy and the physiology of different hosts. Furthermore, the few examples of bacteria-host interaction outlined in this review suggest that we can gain new insights on the host cell biology by studying those serotypes that have acquired the ability to communicate with their warm-blooded host.

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