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Reticulate Evolution Among the Group I *Salmonellae*: An Ongoing Role for Horizontal Gene Transfer

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1. Introduction

Salmonella enterica is responsible for 1.4 million cases of foodborne salmonellosis in the United States annually making it the number one causative agent of bacterial foodborne illnesses (CDC, 2007). Infection can occur after eating undercooked meat, poultry and eggs that have been contaminated with *Salmonella* (CDC, 2007). In recent years several outbreaks have occurred in the United States that were associated with *Salmonella* contamination of produce, the most recent being a *S. enterica* Saintpaul outbreak associated with tomatoes, jalapeño and serrano peppers that sickened over 1400 individuals (CDC, 2008). The movement of several serovars of *Salmonella* into previously naïve niches (*i.e.,* produce-growing environs) suggests that the pathogen is readily adapting to new environments. An understanding of the reticulate evolutionary mechanisms that underpin the acquisition and composition of the requisite genetic and phenotypic features of *Salmonella* is essential to more accurate risk assessment of this pathogen (Hohmann, 2001).

It is now widely accepted that horizontal gene transfer (HGT) has driven the emergence of more aggressive and virulent strains of *Salmonella* in the environment, on the farm, and in the food supply. Such assault by various salmonellae has fueled the in-depth examination of specific genotypes and conditions that permit reticulate evolutionary change and the rise of deleterious phenotypes (LeClerc et el., 1996; 1998; 1999; Cebula and LeClerc, 1997). The hypermutable phenotype represents one scheme by which reticulate evolution of the bacterial chromosome may occur (Trobner and Piechoki, 1984; Haber et al., 1988; Haber and Walker, 1991; LeClerc et al., 1996; Matic et al., 1997; Radman et al., 1999; Cebula and LeClerc; 2000; Funchain et al., 2000). Methyl-directed mismatch repair (MMR) defects, leading to a mutator or hypermutable phenotype, are found in more than 1% of the isolates within naturally-occurring populations of *Salmonella enterica* (LeClerc et al., 1996) and at even greater frequencies in the food supply where oxidative and other anti-microbial stressors are applied (Cebula et al., 2001). Up to 73% of the MMR defects found in feral settings are due to lesions within the *mutS* gene, resulting in increased nucleotide substitution rates, enhanced DNA transposition, and, perhaps most importantly, a relaxation of the internal barriers that

normally restrict homeologous recombination following HGT of foreign DNA (Cebula and LeClerc, 1997; Radman et al., 1999).

This latter role, as a major sentinel for recombination, led to a substantial focus on the genetics and evolution of the *mutS* gene and its adjacent sequences located at 63 min on the *Salmonella* chromosome (Brown et al, 2002; 2003; Kotewicz et al., 2003; 2003). Phylogenetic analyses of *mutS* alleles from strains of the SAR (*Salmonella* reference) collections (i.e., SARA, SARB, and SARC)—largely taken to represent the extent of genetic variability within the species (Boyd et al., 1993; 1996; Beltran et al., 1991)—have revealed striking levels of phylogenetic discordance between trees derived from *mutS* alleles and whole-chromosome trees of the same strains based on MLEE (multilocus enzyme electrophoresis) analysis (Brown et al., 2002, 2003). These differences were interpreted as numerous examples of HGT among *mutS* alleles in *Salmonella.* Similar observations have been made among sequences abutting the *mutS* gene in *Salmonella, E. coli,* and *Shigella* spp (Kotewicz et al., 2002; 2003; Brown et al., 2001b). Our laboratory showed previously that the 61.5 min *mutS-rpoS* region retains a novel and highly polymorphic 2.9 kb sequence in the genome of all *E. coli* O157:H7 strains, *Shigella dysenteriae* type 1, and several other *E. coli* strains (LeClerc et al., 1999) but not in *Salmonella enterica* (Kotewicz et al., 2003). This highly polymorphic stretch of DNA (previously coined the *mutS-rpoS* "unusual region") is varied in its distribution among enteric bacterial lineages and is absent in others entirely (Kotewicz et al., 2003). Sequence analysis of the region revealed an IS1 insertion element in place of the *prpB* gene in *S. dysenteriae* type 1 suggesting the existence of a recombinational crossover in the *mutS-rpoS* region for this strain (LeClerc et al., 1999). Evidence for additional crossovers in the same region were also obtained for other *E. coli* strains (Brown et al., 2001b). These findings support the notion that HGT helped forge current relationships among *Salmonella* and other enteric pathogens in this region and throughout numerous other locales in the *Salmonella* chromosome.

Indeed, as evidenced from global efforts involving whole-genome sequencing, microarray, and multi-locus sequence typing, the substantial impact that HGT has played in structuring the chromosome of *Salmonella enterica* is now indisputable (Porwollik and McClelland, 2003; Fricke et al., 2011; Kelly et al., 2009; Hall, 2010). Previous estimates indicate that at least one-quarter of the *Salmonella* genome may have been forged through HGT and reticulate evolutionary events (Porwollik and McClelland, 2003), although this number seems conservative from current views. In addition to the 61.5 min region surrounding *mutS*, HGT has played a key role in structuring many other regions of the *Salmonella* chromosome. Notably, SPI elements (*Salmonella* pathogenicity islands) have likely been acquired through HGT (Groisman and Ochman, 2000; Ochman et al., 2000; Hacker and Kaper, 2000; Baumler et al., 1997). For example, the SPI-1 pathogenicity island, comprising the genes encoding a type III secretion system, was probably acquired early in *Salmonella* evolution (Kingsley and Baumler, 2000; Li et al., 1995), yet several *inv–spa* alleles seem to have converged horizontally more recently between *S. enterica* groups IV and VII (Boyd et al., 1997; Brown et al., 2002). Additionally, type 1 pilin genes that encode fimbrial adhesins retain unusually low GC contents and aberrant DNA sequence phylogenies relative to other *fim* genes (Boyd and Hartl, 1999). Other studies focusing on numerous housekeeping gene loci have reported evolutionary histories for these genes that are strikingly decoupled from *S. enterica* strain history (Nelson and Selander, 1994; Thampapillae et al., 1994; Brown et al., 2002; Boyd et al.,

Christensen and Olsen, 1998; Groisman et al., 1992; Li et al., 1994; Liu and Sanderson, 1996; Nelson and Selander, 1994; Nelson et al., 1992; 1997).

The now incontrovertible connection between horizontal transfer and MMR gene evolution has led to the thesis that genetic exchange of *mutS* alleles could simultaneously quiet the mutator phenotype while rescuing adaptive changes from the population (LeClerc et al., 1996; Denamur et al., 2000). Consistent with this hypothesis, the *mutS* gene is evolutionarily scrambled by HGT in subspecies I *Salmonella enterica*. Our laboratories documented the prevalence of horizontal gene transfer (HGT) among strains of *Salmonella enterica* (Brown et al., 2002; 2003). In comparing across and within subspecies of *Salmonella*, a recombination gradient was noted wherein the incidence of HGT was inversely correlated with the genetic diversity separating individual strains. It appears that a genetic threshold exists that tolerates free exchange of sequences within a framework delimited by sequence variation and niche diversity of individual strains. We demonstrated this through identification of intragenic (patch-like) recombination as the primary outcome across disparate *Salmonella* subspecies and assortative (whole-allele) recombination which caused extensive reassortment of alleles among more genetically homogeneous populations of group I *Salmonella* pathogens, all sharing a common niche restricted to warm-blooded mammals.

A torrent of scientific information has accrued over the past decade to support the important role of HGT in the genetic and evolutionary diversification of *S. enterica* subspecies, serovars, and individual pathogenic clones (McQuiston et al., 2008; Octavia and Lan, 2006; Lan et al., 2009; Fricke et al., 2011). Our understanding in reconstructing the horizontal acquisitions of important features including those involved in virulence, drug resistance, and other adaptations that foster an enhanced fitness for *Salmonella* persistence in foods, animals, and people is expanding at a pace which we could not have foreseen even a decade ago (Sukhnanand et al., 2005). It is important to recall however that reticulate evolutionary pressures do not subside once selectively advantageous traits are gained. Rather, horizontal exchange likely continues to dapple the evolutionary landscape between even the most closely related salmonellae (Brown et al., 2003). Here, we provide results of several previously unreported phylogenetic studies that evidence (i) the continued role of HGT in the intra-operon shuffling of SPI-1 alleles among subspecies I *S. enterica* strains; (ii) the often under-appreciated role for HGT and recombination in the homogenization of allele structure in a closely related population of *S. enterica*; and (iii) the panmictic and reticulate nature of restriction-modification (R-M) genes among group I salmonellae. This last finding, noting free exchange of R-M (*i.e., hsd*) alleles, provides phylogenetic evidence of the compatibility of *S. enterica* subspecies I R-M complexes, likely accounting for the documented successful HGT of entire gene sequences among closely (*e.g.*, intra-subspecies) related strains as DNA exchange between strains that shared or recently shared common R-M alleles would not be subject to substantial restriction (Sharp et al., 1992).

2. Reticulate evolution in SPI-1 of *Salmonella enterica* subspecies I

Salmonella pathogenicity island 1 (SPI-1) specifies a type III secretion system essential for host cell invasion and macrophage apoptosis (Galan and Curtiss, 1989; Galan and Collmer, 1999). SPI-1 comprises a cluster of virulence genes (*e.g.*, the *inv/spa* gene cluster) that encode, in part, the "needle complex", a key delivery component for transporting virulence associated effector molecules into the host cell (Galan and Collmer, 1999). The

disparate phylogenetic distribution, lack of chromosomal synteny, and diverse base compositions of SPI-1 and its homologues indicate that these sequences were obtained independently across enteric species of bacteria. It is presumed that SPI-1 was present in the last common ancestor of all *Salmonella* lineages. Horizontal acquisition of the *inv/spa* gene cluster, however, is thought to have been a pivotal event for the emergence of *Salmonella* as a pathogenic species (Boyd et al., 1997; Groisman and Ochman, 2000). The gene complex lies adjacent to the polymorphic *mutS–rpoS* region of the chromosome. We and others previously presented phylogenetic evidence for intragenic recombination of sequences within several SPI-1 invasion loci (Boyd et al., 1997; Brown et al., 2002), primarily among *S. enterica* subspecies IV and VII. However, in order to determine the extent to which HGT may have disrupted SPI-1 evolution across the more ecologically and genetically homologous group I salmonellae, we examined nine SPI-1 invasion loci from nearly half of the SARB reference collection of strains (Boyd et al., 1993), composed exclusively of subspecies I *Salmonella* serovars.

2.1 SPI-1 gene evolution is decoupled from *Salmonella* chromosome evolution

Using a cladistic approach (Forey et al., 1992; Allard et al., 1999; Bell et al., 2011), the nucleotide sequences from nine invasion gene sequences were subjected to phylogenetic analysis. The resultant invasion gene phylogenies were then compared to phylogenetic groupings from the *mdh* gene, a chromosomal anchor locus that is taken largely to reiterate chromosome evolution within subspecies I (Boyd et al., 1994) and MLEE (multi-locus enzyme electrophoresis), also applied here as a metric of strain/chromosome evolution for the group I salmonellae (Boyd et al., 1993). As shown in Fig. 1, strains composing single SARB *mdh* and MLEE lineages were, for the most part, distributed across disparate *inv/spa* gene clades for all nine invasion genes tested indicating that many of these strains, although linked tightly in chromosome evolution, retain invasion gene alleles with unrelated evolutionary histories, presumably as a result of HGT.

Evolutionary incongruence between *inv/spa* genes and the *Salmonella* chromosome was affirmed using the ILD (incongruence length difference) test, which evaluates the likelihood of a common evolutionary history between genes (Farris et al., 1995; LeCointre et al., 1998; Brown et al., 2001a). Seven of the nine invasion genes yielded significant ILD scores ($p < 0.05$), indicating that a hypothesis of congruence could be rejected for these strains and further reinforcing the discordance evident in the clade comparisons. The only exceptions were *invB* ($p = 0.08$) and *spaP* ($p = 0.59$), albeit both still retained cladistic signatures of HGT from broken clade structures in the tree analysis.

2.2 SPI-1 gene evolution is decoupled from *mutS* gene evolution

The *mutS* gene, downstream and adjacent to SPI-1 in *S. enterica*, has been shuffled extensively by HGT (Brown et al., 2003). In order to determine whether *mutS* may have been linked in the recombination now evident among SPI-1 genes, cladistic comparisons were made between *mutS* phylogeny and *inv/spa* gene phylogeny revealing substantial incongruence between *inv/spa* trees and *mutS* trees. Six of these comparisons are shown in the form of tanglegrams (Fig. 2). Again, strains composing SARB *mutS* clades were distributed across disparate *inv/spa* gene clades for all nine invasion genes tested, and seven of nine *inv/spa* genes were further

confirmed as discordant with *mutS* based on ILD testing. Taken together, these findings indicate that *inv/spa* gene sequences and *mutS* sequences from the same strains are decoupled in their evolution. These data suggest that reticulate evolution has repeatedly forged this contiguous region of the *Salmonella* chromosome such that different strains appear to have been affected by assortative (allelic) HGT between the two loci.

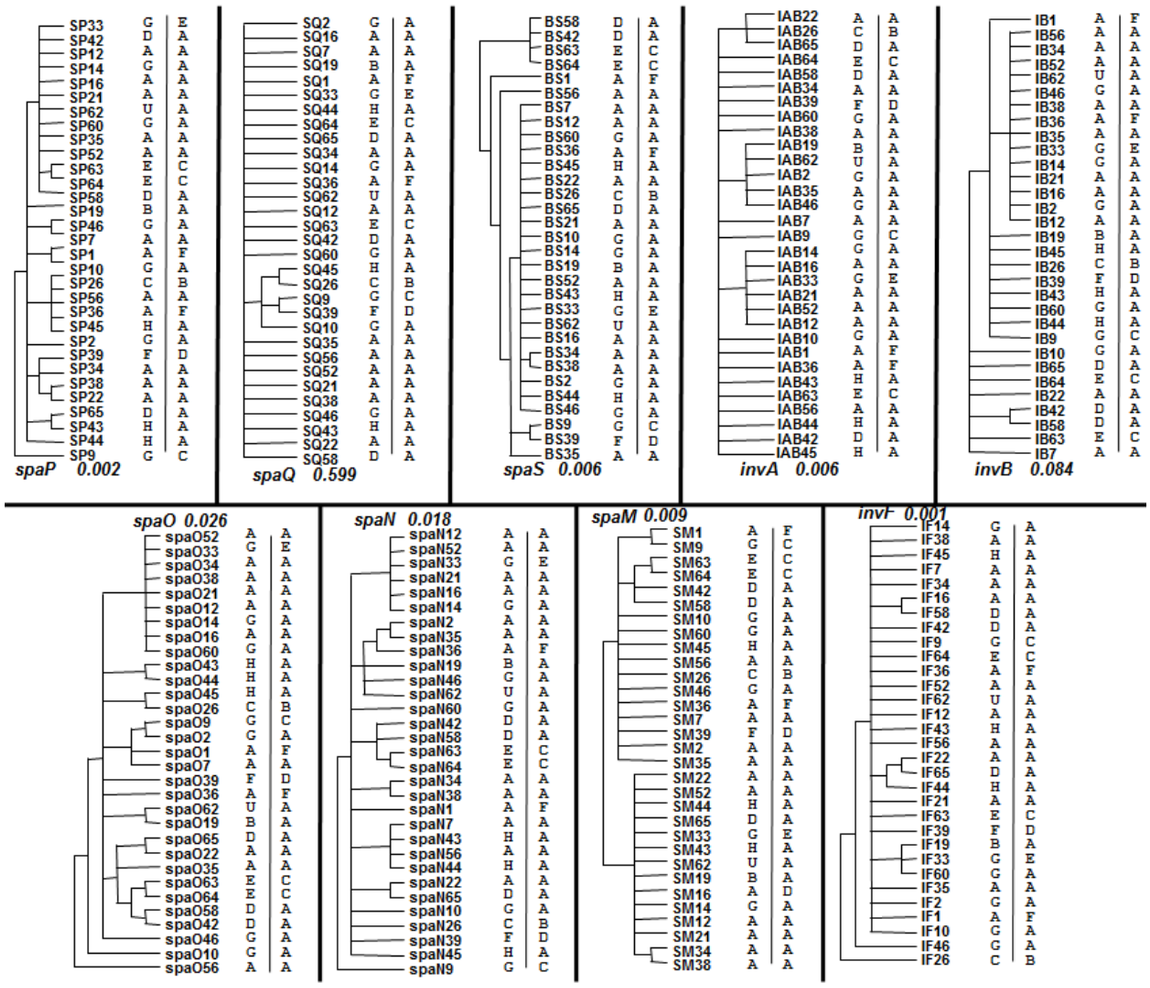

Fig. 1. Phylogenetic discordance between SPI-1invasion genes and the *Salmonella* chromosome. *mdh* and MLEE comparisons are shown to each of nine different *inv/spa* genes indicated. Identical letters denote strains from the same *mdh* or MLEE lineage. It is important to note that letters are only relevant to their respective data column and do not cross-over columns. The column to the left of the dividing line designates *mdh* clade assignments for the respective *S. enterica* strain while the column of letters to the right of the divider corresponds to MLEE clade assignments. The number at the base of each tree denotes the ILD score (p-value) relative to a comparison for congruence between the respective *inv/spa* gene and the *mdh* gene sequence alignment for the same strains. Trees shown were rooted using *S. bongori* as an outgroup. Nucleotide sequence alignments were performed using CLUSTAL X (Thompson et al., 1998). Most parsimonious trees were generated in PAUP* v.10 (Swofford et al., 2002).

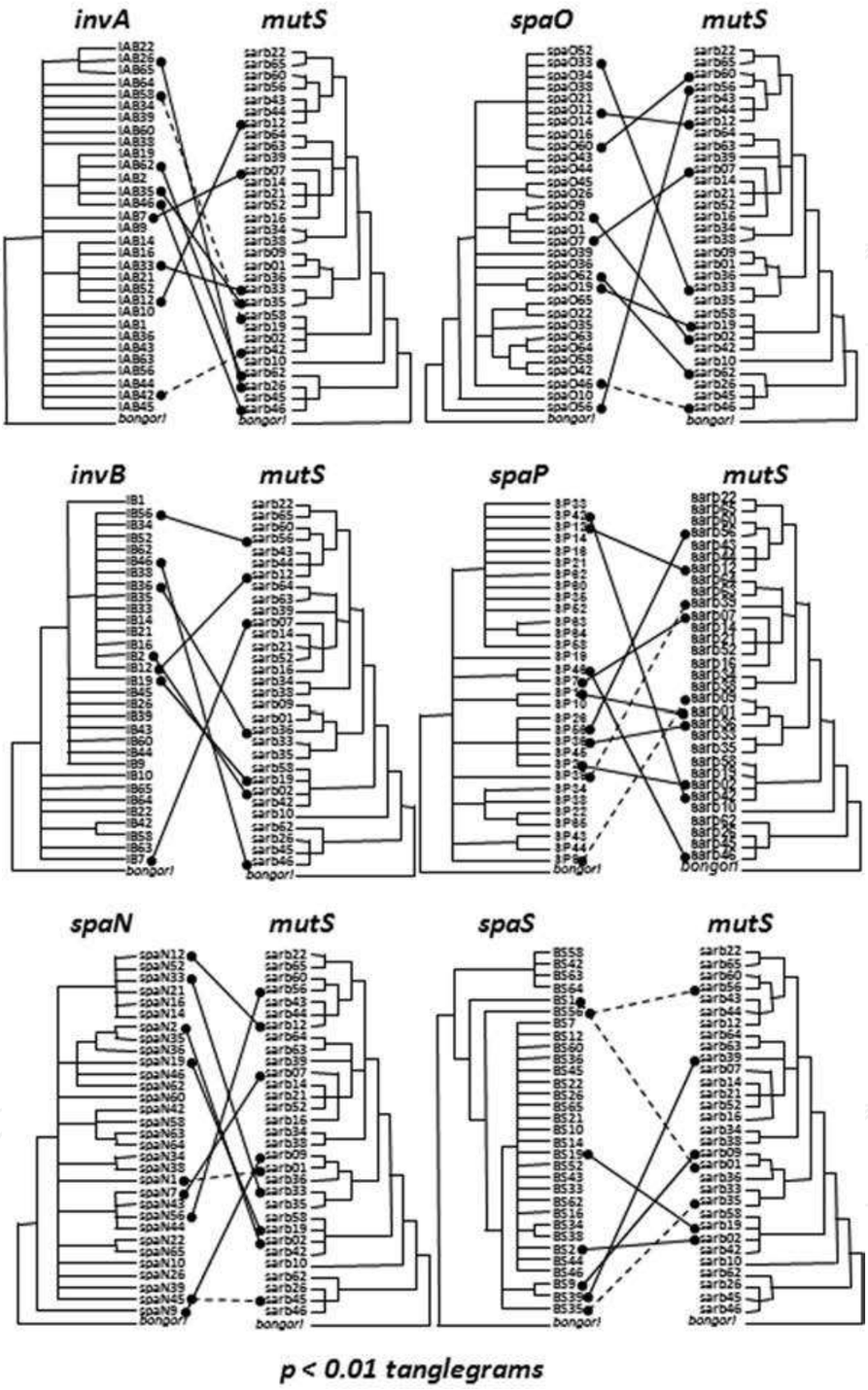

Fig. 2. Tanglegrams of several invasion gene and *mutS* revealing the phylogenetic incongruence between *inv/spa* genes and the *mutS*, which lies adjacent to SPI-1 on the *Salmonella* chromosome. Lines connect the discordant, potentially recombinagenic (incongruent) strains. *inv/spa* to *mutS* comparisons with an ILD score of $p < 0.01$ were displayed. Trees shown were again rooted using *S. bongori* as an outgroup taxa.

2.3 Intra-island HGT within the SPI-1 region of subspecies I *Salmonella* strains

In order to determine the presence and extent to which HGT has shuffled individual alleles within SPI-1 among more closely related subspecies I strains, a pairwise ILD approach was adopted wherein congruence was scored for individual comparisons of all nine of the *inv/spa* genes included in this study (Fig. 3). Several findings were noteworthy. Although no individual invasion gene showed unanimous evolutionary discordance with its neighbors, three *inv/spa* loci (*invA*, *invB*, and *spaP*) were incongruent ($p < 0.10$) with a significant majority of other genes. *invA* and *invB* showed discordance with all other loci except *spaN* and *spaQ*, while *spaP* showed discordance to all but *spaM* and *spaQ*. Conversely, with the exception of *spaQ*, no *inv/spa* gene was congruent with every other. Thus, a hypothesis of extensive intra-island shuffling begins to emerge with an evolutionary decoupling of individual invasion loci one from another. Additional tree comparisons buttressed this conclusion. Akin to the selfish operon theory (Lawrence and Roth, 1996), these data suggest that the SPI-1 region is a chromosomal mosaic, composed of *inv/spa* gene sequences that have converged within this island but with each retaining unique evolutionary paths.

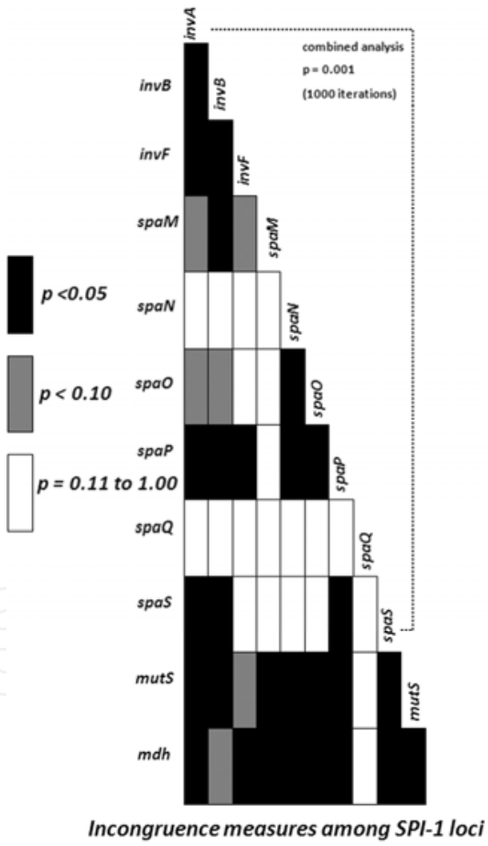

Fig. 3. ILD test results for intragenic comparisons among *inv/spa* invasion genes. ILD tests (Farris et al., 1995) were performed with 1000 partitions using the Partition Heterogeneity command in PAUP* v.10 (Swofford et al., 2002). A p-value of 0.05 or less allows for a rejection of the null hypothesis of congruence (vertical evolution) and accepts the alternative hypothesis of incongruence which is interpreted among bacterial phylogeny as evidence for HGT (LeCointre et al., 1998).

2.4 Key observations

i. The *inv/spa* complex of *S. enterica* subspecies I appears to have undergone extensive intra-island allelic shuffling due to HGT. This suggests that the SPI-1 region is a mosaic composed of SPI-1 gene sequences with distinct evolutionary origins.
ii. Invasion genes within this *Salmonella* population are not only decoupled phylogenetically from *mutS* and other flanking sequences but also from the chromosomes of group I *S. enterica* strains, suggesting that these genes have been re-assorted by HGT.
iii. Much of the recombination observed here appears to be assortative transfer, a finding that contrasts to the *inv* genes in *S. enterica* as a whole, where tree structure was largely intact with HGT limited mostly to subspecies IV and VII (Boyd et al., 1997; Brown et al., 2002).
iv. Allele shuffling appears to be most prominent within the subspecies I taxonomic boundary and not across other subspecies of *S. enterica*. This finding is consistent with a relaxed and compatible restriction-modification system among more closely related *Salmonella* strains (Brown et al., 2003).

3. HGT homogenizes the *mutS* gene among 'Typhimurium' complex strains

Here, we present phylogenetic and genetic analyses of *Salmonella* reference collection A (SARA), also known as the Typhimurium strain complex—the most homogeneous *S. enteric* reference collection, consisting solely of five closely related subspecies I serovars (Typhimurium, Paratyphi B, Muenchen, Saintpaul, and Heidelberg) (Beltran et al., 1991). Given the evolutionary similarity shared among these pathogens and trend noted previously that highlight the inverse relationship between *Salmonella* diversity and recombination, one would expect to observe an even greater role for HGT in the population structure of the *S. enterica* SARA collection of pathogens.

3.1 Cladistic evidence for horizontal exchange of *mutS* alleles among 'Typhimurium' complex strains

As was done for SPI-1 gene sequences, a phylogenetic tree was derived from 72 SARA *mutS* sequences and was compared to phylogenetic trees derived from multi-locus enzyme electrophoresis (MLEE) and *mdh* (malate dehydrogenase) gene sequences for the same strains. Phylogenies derived from horizontally exchanged sequences display evolutionary discordance (incongruence) when compared to *mdh* and MLEE trees. In the tree shown, six clades of *mutS* alleles were observed and compared to the distribution of four *mdh* and six MLEE multi-strain containing clades (Fig. 4). Two of the four SARA *mdh* clades were found to be displaced into multiple clades on the *mutS* tree. Two additional *mdh* clades were found to have converged into a single *mutS* clade, suggesting that HGT may have homogenized *mutS* diversity of these particular *mutS* lineages. Similarly, strains from five of the six MLEE lineages were displaced into separate clades on the *mutS* tree. The only exception was a single clade of MLEE SARA strains (A57, A58, A59, and A60), which was also found intact in the *mutS* tree except for the inclusion of SARA strain A56. Nonetheless, numerous examples of evolutionary discordance between the 1.1 kb *mutS* segment and the chromosome of the 'Typhimurium' complex strains indicate that horizontal exchanges of *mutS* alleles have accumulated during the rather shallow radiation of even these highly homogeneous group I pathogens. As an aside, it was

noteworthy that full-length *mutS* alleles were horizontally transferred among SARA *S. enterica* strains, lending further credence to a model for R-M compatibility among closely related *S. enterica* serovars and strains.

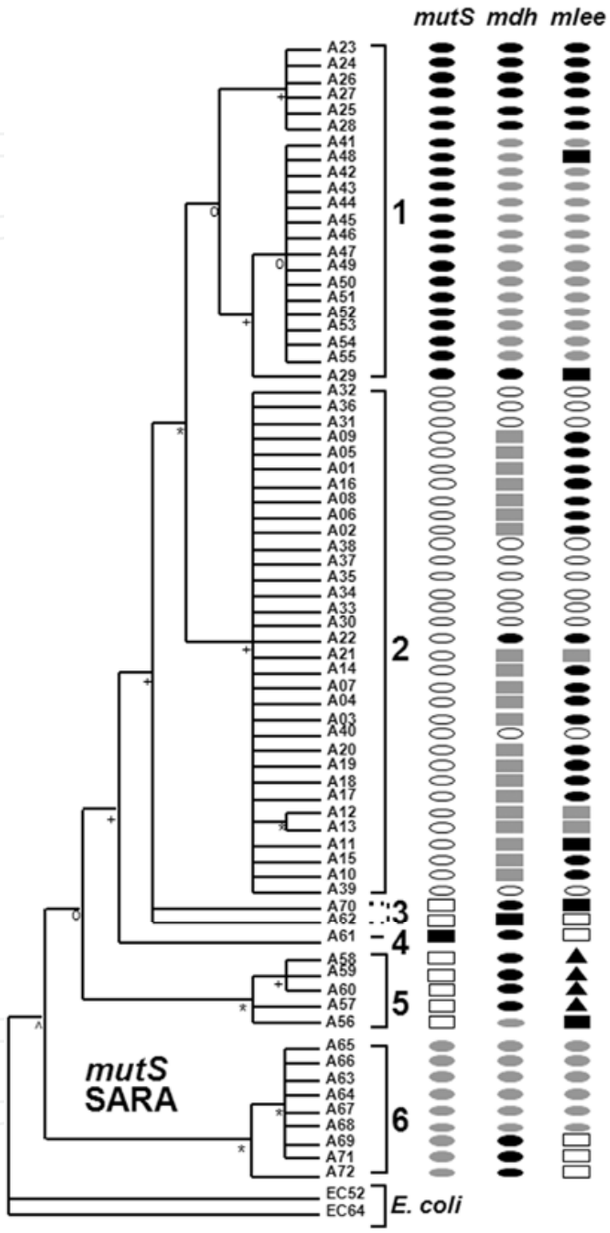

Fig. 4. Most-parsimonious relationships of SARA *mutS* alleles. *mutS* clades are bracketed and numbered to the right of the tree. Distributions of *mutS*, *mdh*, and MLEE clades are presented in column form. Note that strains originating from the same clade retain a common shape and common internal shading. Bootstrap nodal support values (Felsenstein et al., 1985) are presented on the *mutS* tree as follows: ^, 76-100%; *, 51-75%; +, 26-50%; o, 1-25%. In this case, *mdh* and MLEE are taken to represent the evolution of the strain in general (Boyd et al., 1994; Beltran et al., 1991). The tree shown is rooted with two *E. coli* outgroups.

3.2 Homogenization of *mutS* sequence diversity among *S.* Typhimurium and *S.* Heidelberg strains

Curiously, a single clade in the SARA *mutS* tree was found to comprise three distinct *Salmonella* serovars. In this clade, every strain representing *S.* Typhimurium (n=21) and *S.* Heidelberg (n=11), along with a single strain of *S.* Saintpaul, converged into a single evolutionary lineage of *mutS* alleles. In the SARA *mdh* tree (Fig. 5), *mdh* alleles for these same SARA serovars formed three disparate clades in the tree such that *S.* Typhimurium strains clustered only with other *S.* Typhimurium and *S.* Heidelberg strains only with other *S.* Heidelberg. *S.* Saintpaul strains formed a single lineage at the tip of the tree with strains of *S.* Muenchen and a single *S.* Paratyphi B. It should be noted that these distinct clades retained substantial statistical support with bootstrap values around 90% (Felsenstein, 1985). Thus, phylogenetic comparison of *mutS* and *mdh* sequences supported the notion that these serovars have converged into a single *mutS* clade, possibly as a result of the repeated HGT of only one or a few preferred *mutS* alleles.

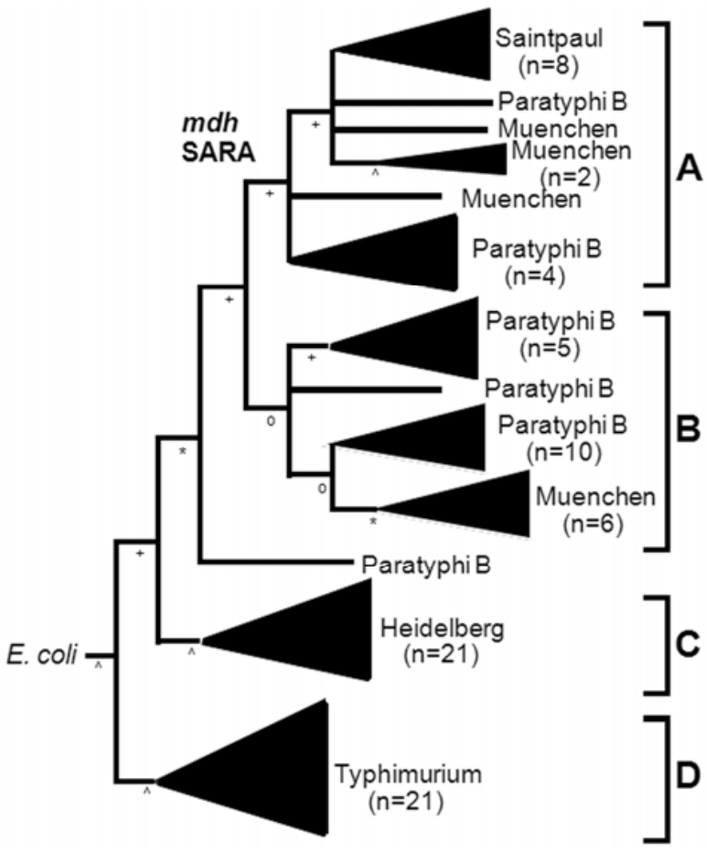

Fig. 5. Phylogenetic tree revealing the most-parsimonious relationships of SARA *mdh* alleles. *mdh* clades are bracketed and lettered while SARA serovars are labeled to the right of the tree. For sample sizes greater than one, multiple strains of the same serovar are depicted as a cone on the tree terminal nodes. Note that strains originating from the same clade are designated by a common bracket and letter. Bootstrap nodal support values are presented on the *mdh* tree as follows: ^, 76-100%; *, 51-75%; +, 26-50%; o, 1-25%. Note the bifurcations between specific clusters in the tree, signaling sequence diversity among distinct serovars using the *mdh* gene.

In order to further investigate the genetic structure of this converged clade, we examined *mutS* sequence homogeneity across the strains composing this lineage as well as the remaining *mutS* alleles of the SARA collection (Fig. 6). Evaluation of polymorphic positions in the *mutS* alignment revealed several findings consistent with homogeneous clade structure surrounding these serovars. First, five substitutions were observed across the entire 1,115 bp sequence for all 33 strains that define this *mutS* clade (#2). Second, with the exception of the polymorphism at position 913 in SARA strains 12 and 13, no clade #2 substitution was retained by more than one strain. Thus, none of the substitutions present within this clade partitioned any member serovar from another. The near structural uniformity of this clade at the nucleotide level further suggests that HGT has homogenized *mutS* alleles among these particular serovars. This is consistent with the thesis of Dykhuizen and Green (1991) who reminded that recombination can not only diversify the genome but can also homogenize it as well.

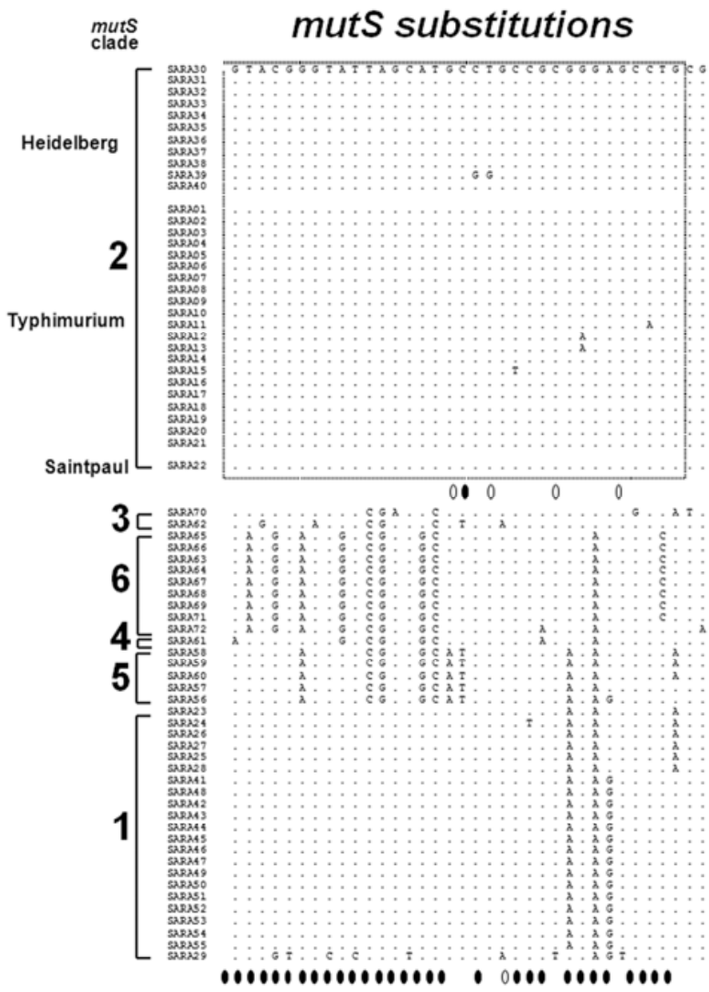

Fig. 6. *mutS* nucleotide sequence homogeneity among *S. enterica* serovars Typhimurium, Heidelberg, and a strain of Saintpaul. Periods indicate exact nucleotide identity to the reference sequence at the top of the alignment while listed nucleotides represent actual substitutions. The synonymous/nonsynonymous status (blackened ovals indicate synonymous change) of each substitution is noted below the alignment. Nucleotide sequences were generated using a PCR-based approach and automated CE-sequencing technology.

3.3 Distinct roles for HGT across various taxonomic tiers of *S. enterica*

With the inclusion of the SARA analysis reported here, we have been able to define varying roles for HGT across three taxonomically distinct populations of *S. enterica* (SARA, B, and C) (Fig. 7). Within *S. enterica* as a whole, a model for HGT begins to emerge that tolerates near-free HGT among closely-related subspecies I strains. As genetic divergence increases across serovars, however, the extent of HGT appears to decrease. The analysis reported here suggested two unique findings for SARA, the most genetically monomorphic population.

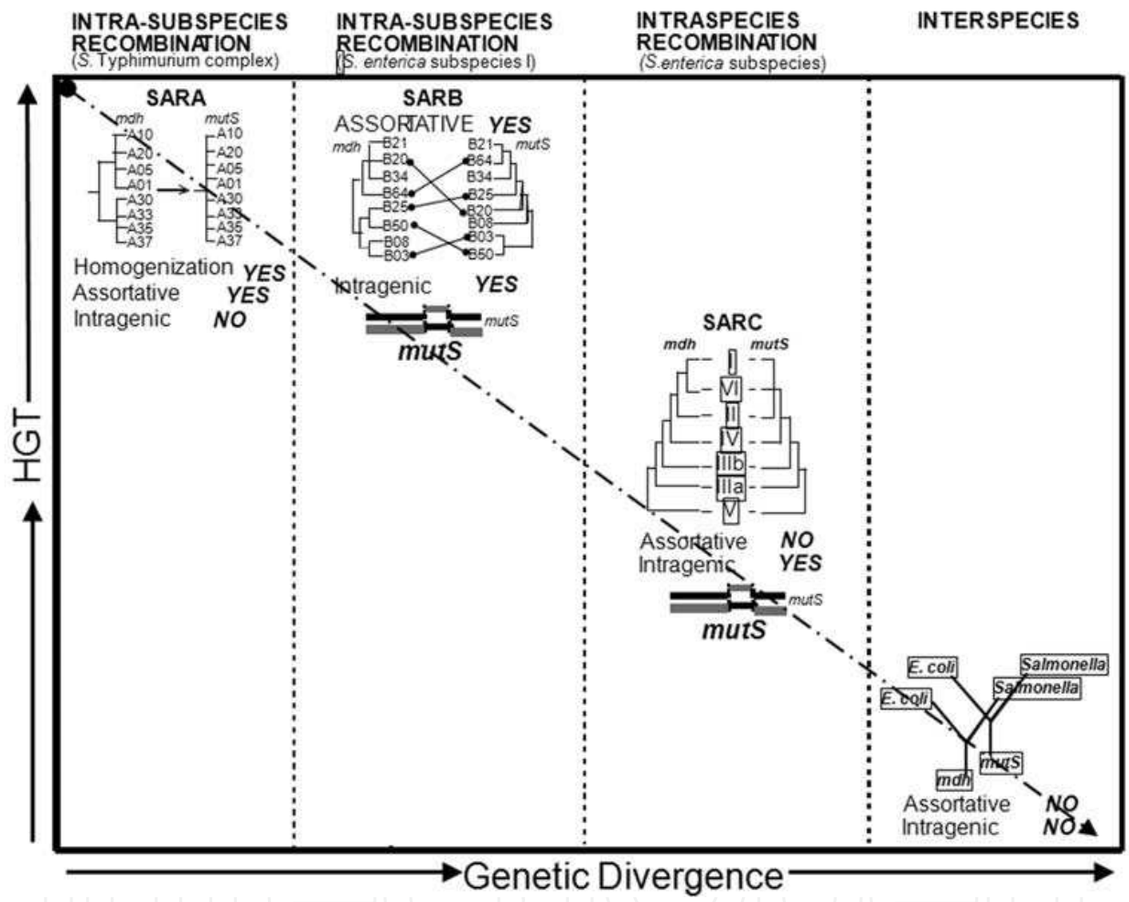

Fig. 7. Model for the frequency and effects of HGT among various taxonomic tiers of *Salmonella enterica*. Graphic representation of the various effects of HGT on the taxonomically distinct SARA, SARB, and SARC strain collections as well as an interspecies comparison. The *S. enterica* collections are plotted relative to genetic divergence versus the extent of HGT observed. Specific effects and trends associated with the HGT occurring at each taxonomic level are noted below each of the *Salmonella* populations shown.

First, SARA revealed evidence for a substantial convergence of *mutS* alleles between distinct serovars suggesting, that, recombination can have a homogenizing effect on sequence diversity in this population. Second, despite yielding numerous examples of assortative (allelic) exchange, SARA appears to be—at least from a phylogenetic perspective—refractory to intragenic (mosaic) HGT within the *mutS* gene. Thus, the SARA and SARB groups seem

to have been influenced more extensively by HGT than SARC possibly because they are not so diverged that exchange is inhibited due to extreme niche or R-M (restriction-modification) system variability. Moreover, it is also possible that much of the HGT among SARA strains have gone undetected here since identical alleles would leave no phylogenetic footprint following an exchange event.

3.4 Key observations

i. Horizontal gene transfer of *mutS* alleles in *Salmonella* appears to play a prominent role in the evolutionary structure of the five closely-related serovars representing the SARA ('Typhimurium' complex) collection, a finding consistent with extensive HGT that has been documented among subspecies I serovars in general (Brown et al., 2003).
ii. Cladistic analysis of SARA strains revealed the first example of a substantial convergence of *mutS* alleles from disparate serovars into a single clade. This suggests that HGT is homogenizing allele diversity among certain *Salmonella* strains and serovars—an observation reminiscent of allele homogenization observed for the *E. coli polA* gene (Patel and Loeb, 2000).
iii. Among closely related 'Typhimurium' complex strains, *mutS* alleles appear to have shuffled largely as single units rather than in intragenic segments. One explanation for this might be a more recent evolutionary divergence of the five serovars composing the highly homogeneous 'Typhimurium' strain complex. Alternatively, recombination of highly homologous mosaic segments of the *mutS* gene would do little to obscure phylogeny and likely go undetected in these analyses.
iv. Retrospective comparison of SARA HGT patterns with that of SARB and SARC strains yields a gradated model for HGT whereby different taxonomic tiers of *Salmonella* are subject to different HGT effects. The differences appear coupled to the extent of genetic diversity that defines these three different "tiers" of *Salmonella* population structure.

4. HGT among restriction-modification (R-M) genes of subspecies I salmonellae

The restriction and modification (R-M) system is a defense mechanism developed by bacteria to protect the bacterial genome from invasion by foreign DNA (Bullas et al., 1980). Foreign sequences entering the cell are cleaved by restriction enzyme(s), while the bacterial DNA itself is modified by methylase(s), thus providing protection from its own restriction enzyme (Murray, 2000). R-M systems are composed of genes that encode a specific restriction endonuclease and modification methylase. There are several types of R-M systems, namely type I (*e.g.*, *Eco*KI), type II (*e.g.*, *Eco*RI), and type III (*e.g.*, *Sty* LTI) (Barcus et al., 1995). Types of R-M systems are classified on the basis of their composition and cofactor requirements, the nature of the target sequence, and the site of DNA cleavage with respect to the target sequence (Murray, 2000; Naderer et al., 2002).

Compatibility of R-M systems among strains was proposed as one explanation to account for contrasting recombination rates (Brown et al., 2003). In this model, compatible R-M complexes would permit the successful transfer of larger gene segments among closely related *Salmonella* pathogens; crosses between strains with identical R-M systems would not be subject to restriction (Sharp et al., 1992). A gradation in the size limits of DNA segments exchanged would depend on the polymorphic character of R-M systems in natural strains.

Here, we investigate this model by examining the molecular evolutionary relationships of *hsd* genes encoding R-M complexes among closely related pathogenic *Salmonella* strains (*i.e.*, the 'Typhimurium' complex). If, indeed, *hsd* alleles are freely exchanged themselves among strains that display a substantial tolerance for HGT and recombination of diverged DNA sequences, then an explanation accounting for observed tolerance to extensive HGT begins to emerge for *S. enterica* group I serovars.

4.1 Evidence for HGT of R-M alleles among *Salmonella enterica* group I strains

DNA sequences from three *hsd* type I R-M genes were subjected to cladistic analysis. The resultant invasion gene phylogenies were then compared to phylogenetic groupings from the *mdh* gene and from the *Salmonella* MLEE data. Cladistic comparisons of *hsd* genes to markers of stable *Salmonella* chromosome evolution revealed several findings, and the data for *hsdS* is shown (Fig. 8). For *hsdS* section S1, SARA 56 is removed from neighboring strains when compared to *mdh* or *mutS*. For *hsdS* section S2, the collapsing of numerous clades into a single conserved clade was observed. It should be noted that such collapsing was observed in many of the trees reported here and suggests that HGT may be homogenizing *hsd* alleles. Moreover, a distinct allele that has no homology with its sister allele in a neighboring clade can be seen on the tree. Finally, the *hsdS* section S3 tree breaks up clades from both MLEE and *mdh*. In addition, this tree has three distinct allele types that can be seen phylogenetically, as in the case of S2.

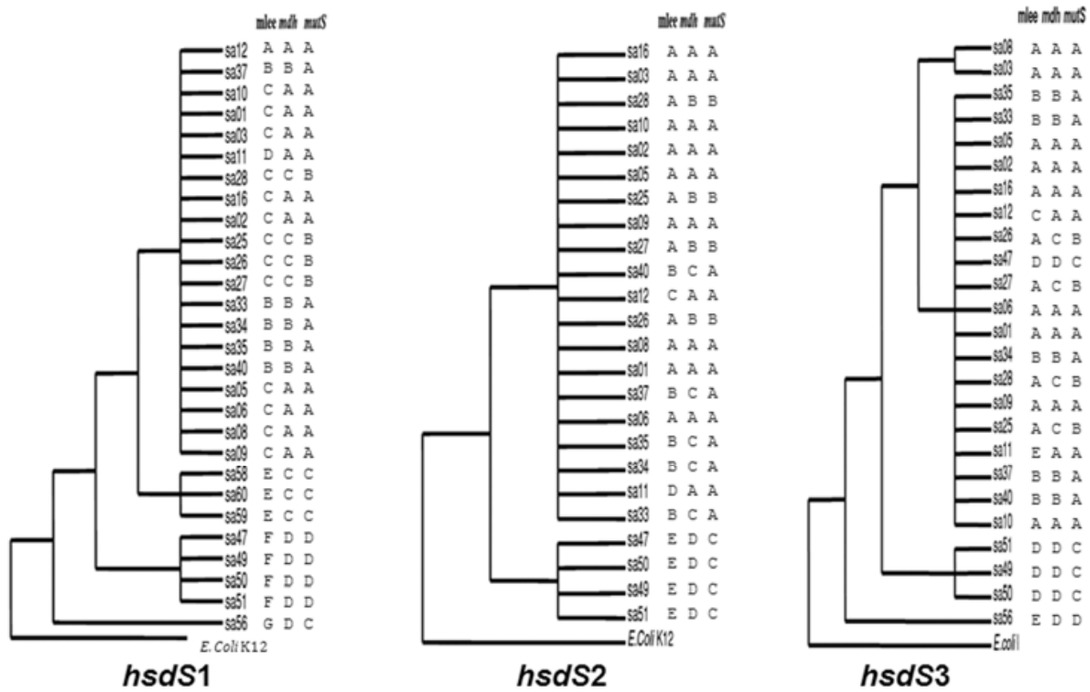

Fig. 8. Phylogenetic trees constructed for three segments comprising the *hsdS* gene, *hsdS*1, *hsdS*2, and *hsdS*3. Each respective gene tree is rooted with an *E. coli* outgroup and compared to MLEE, *mdh* and *mutS* clade patterns. Identical letters signal a common clade origin in the *mdh*, MLEE, or *mutS* datasets. Note that the letter designations from the *mdh*, MLEE, and *mutS* bar columns are independent of each other.

Compatibility among R-M systems has been proposed to account for the extensive levels of HGT documented among subspecies I *Salmonella* pathogens. Since the *mutS* gene appears to have been shuffled among this group of strains, we examined the phylogenetic relationship of *mutS* to type I R-M *hsd* genes. Incongruence was observed between *hsd* genes and *mutS* phylogeny, suggesting that patterns of HGT for *hsd* alleles differ from those for *mutS* alleles. *hsd* segments S1 and S3 each retained at least one incongruent strain between these gene phylogenies. In addition, several *hsd* genes collapsed divergent *mutS* clades into single *hsd* lineages in the trees. For instance, three *mutS* clades composed a single *hsdM* clade, a pattern that held true for other *hsd* genes, including *hsdS* segments 2 and 3. These data indicate distinct roles for HGT between most R-M genes and *mutS*. Nonetheless, observed homogenization of type I R-M loci among subspecies I *Salmonella* strains suggests they are compatible systems, allowing additional genes like *mutS* to be transferred within this population in its entirety.

4.2 Evidence for intra-operon HGT of R-M alleles

Intra-operon evolutionary incongruence between *hsd* genes was further examined using the ILD (incongruence length difference) test, which evaluates the likelihood of a common evolutionary history between genes. The ILD comparisons yielded more notable incongruence between genes than did the tanglegram analysis, suggesting that small patches of sequence within individual genes may be responsible for much of the observed incongruence. Intragenic patterns of HGT have been noted previously for more diverse subspecies (Brown et al., 2002). In the ILD comparisons (Fig. 9), eight of the ten *hsd* data set comparisons yielded significant

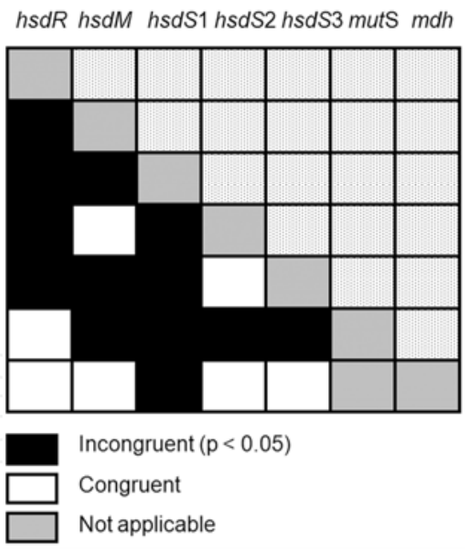

Fig. 9. Pairwise incongruence length difference (ILD) test results for several genes of the Type I Restriction – Modification system in *Salmonella*. ILD tests were performed with 1000 partitions using the Partition Heterogeneity command in PAUP*v.10 (Swofford et al., 2002). Pairwise ILD comparisons were made among the three *hsd* genes R, M, and S including the three sub-regions that were amplified from *hsdS* (*i.e.*, S1, S2, and S3). As in Fig. 3, a p-value of 0.05 or less allows for a rejection of the null hypothesis of congruence (vertical evolution) and accepts the alternative hypothesis of incongruence which is interpreted among bacterial phylogeny as evidence for HGT (LeCointre et al., 1998).

ILD scores ($p < 0.05$) such that a hypothesis of congruence could be rejected for these intragene comparisons. The only exceptions were the *hsdS2-hsdM* comparison ($p = 1.00$) and the *hsdS3-hsdS2* comparison ($p = 1.00$). It is noted that, with the exception of *hsdR*, all of the *hsd* data matrices were also incongruent with *mutS*. When examined in total, the data suggest that the Type-1 R-M operon is a mosaic comprising *hsd* gene sequences that have converged evolutionarily within this operon, but with each possessing a unique phylogenetic path.

It was also noteworthy that *hsdS* segments S2 and S3, however, retained groups of alleles that shared little or no identified homologies. That is, *hsdS2* yielded two unique sequence cassettes, one of which was found in strains of serovar *S.* Paratyphi B in the SARA complex. *hsdS3* yielded three distinct cassette types within the alignment, all of which shared no homology with their counterparts. A cassette retained by SARA strain 56 showed homology to an *hsdS* variant in *E. coli*, suggesting that this sequence has resulted from HGT between these lineages. The other unique cassette, retained by SARA strains 49, 50, and 51, showed no homology to any other *hsd* sequence, indicating that it may be been transferred into *S.* Paratyphi B from a yet unidentified source. The examples of unique cassette formation within this gene reinforce the role that HGT has played in the intra-operon and intragenic evolution of the Type-1 R-M gene system. These data also reveal the exchangeable nature of *hsd* gene sequences in these loci as a result of HGT.

4.3 Key observations

i. These findings demonstrate several instances for the three *hsd* loci encoding the type I R-M operon in *Salmonella* to be decoupled phylogenetically from the chromosomes of group I *Salmonella* strains (*i.e., mdh* and MLEE), suggesting that certain alleles from these genes have been shuffled by HGT between closely related *S. enterica* strains.
ii. The *hsd* operon of S. enterica subspecies 1 appears to have undergone intra-operon structuring due to HGT, producing an evolutionary mosaic in the *hsd* region.
iii. The lack of homology within *hsdS* indicates that these specific segments may have been acquired from distantly related bacterial species. An aberrant GC content for *hsdS* of 41%, a value far removed from an average value for enteric bacterial genomes of 56%, reinforces this conclusion.
iv. The data demonstrate that HGT has been a common occurrence in *hsd* gene evolution and point to a genetic compatibility among closely-related salmonellae for exchange of *hsd* alleles that appears to resemble a panmictic genetic structure among these closely related strains. This may explain, in part, why *Salmonella* known to share homologous genomes and common niches more freely exchange DNA.

5. Discussion and conclusions

In summary, substantial phylogenetic evidence has been presented for the horizontal transfer of *mutS* alleles within a pathogenically homogeneous group of subspecies I *Salmonella enterica* pathogens. Of note, is the observation that *mutS* clades appear to be undergoing homogenization within the 'Typhimurium' strain complex as a result of the repeated HGT of only a few preferred alleles. Moreover, examination of R-M loci revealed that subspecies I *Salmonella* readily exchange *hsd* genes. These findings support the notion that R-M compatibility may be, in part, responsible for the substantial tolerance of HGT and recombined DNA between subspecies I strains.

An overwhelming body of evidence has been compiled that documents the reticulate evolutionary nature of the *Salmonella mutS* gene and its surrounding sequences. In an analysis of nearly 200 different strains documented here and in several previous reports over the past decade (LeClerc et al., 1998; Brown et al., 2002; 2003; Kotewicz et al., 2002; 2003), our laboratory has demonstrated the extent, the chromosomal effects, and the evolutionary history of HGT events that have scrambled this part of the genome in *S. enterica*. Exhaustive phylogenetic comparisons have been brought to bear on *mutS* sequences using various chromosomal markers including MLEE, rDNA, several individual housekeeping genes, and an *a priori* prior agreement MLST data based on concatenation of a three-gene supermatrix (Brown et al., 2002). Puzzling then was a later report that argued a more nonremarkable evolutionary pattern for *mutS* stating, "*mutS* is not more recombinogenic than the other genes" (Octavia and Lan, 2006). The authors based this conclusion solely on a modest 15 strain set of subspecies I salmonellae. Albeit, it remains to be seen to what extent additional homogeneous *Salmonella* populations retain the phylogenetic vestiges of horizontally transferred *mutS* and *hsd* alleles. Whatever the final outcome, it is apparent that horizontal transfer has played a prominent role in the current evolutionary structure of *mutS* and many other genes with virulence, resistance, stress-response, and general housekeeping function, all underscoring recombination as a key mechanism in the generation of genetic diversity among these closely related salmonellae.

Roughly two decades ago, *Salmonella enterica* was regarded as one of only a few eubacterial species that maintained a "truly clonal" evolutionary structure (Selander *et al.*, 1990; 1996; Reeves et al., 1989). Today, armed with whole-genomic analysis, it is now clear that horizontal transfer has shaped and honed unique evolutionary histories for numerous genes, operons, and islands within the *Salmonella* chromosome. With the complete genome sequences of dozens of *Salmonella* now available and countless more underway, such analyses of congruence should aid in determining the extent to which recombination has disrupted clonality throughout the entire *Salmonella* chromosome. Certainly, a greater recognition of precisely how HGT has forged the genomes of *Salmonella* pathogens should enhance the accuracy of risk assessment strategies for these bacteria as well as provide avenues for better detection and characterization of this devastating foodborne pathogen.

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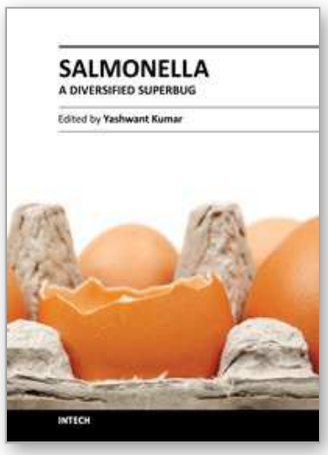

Salmonella - A Diversified Superbug
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Salmonella is an extremely diversified genus, infecting a range of hosts, and comprised of two species: enterica and bongori. This group is made up of 2579 serovars, making it versatile and fascinating for researchers drawing their attention towards different properties of this microorganism. Salmonella related diseases are a major problem in developed and developing countries resulting in economic losses, as well as problems of zoonoses and food borne illness. Moreover, the emergence of an ever increasing problem of antimicrobial resistance in salmonella makes it prudent to unveil different mechanisms involved. This book is the outcome of a collaboration between various researchers from all over the world. The recent advancements in the field of salmonella research are compiled and presented.

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