Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 7252–7256, August 1995
Genetics

Relationship between evolutionary rate and cellular location among the Inv/Spa invasion proteins of *Salmonella enterica*

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ABSTRACT **For 21 strains of *Salmonella enterica*, nucleotide sequences were obtained for three invasion genes, *spaO*, *spaP*, and *spaQ*, of the chromosomal *inv/spa* complex, the products of which form a protein export system required for entry of the bacteria into nonphagocytic host cells. These genes are present in all eight subspecies of the salmonellae, and homologues occur in a variety of other bacteria, including the enteric pathogens *Shigella* and *Yersinia*, in which they are plasmid borne. Evolutionary diversification of the invasion genes among the subspecies of *S. enterica* has been generally similar in pattern and average rate to that of housekeeping genes. However, the range of variation in evolutionary rate among the invasion genes is unusually large, and there is a relationship between the evolutionary rate and cellular location of the invasion proteins, possibly reflecting diversifying selection on exported proteins in adaptation to variable host factors in extracellular environments. The SpaO protein, which is hypervariable in *S. enterica* and exhibits only 24% sequence identity with its homologues in *Shigella* and *Yersinia*, is secreted. In contrast, the membrane-associated proteins SpaP, SpaQ, and InvA are weakly polymorphic and have >60% sequence identity with the corresponding proteins of other enteric bacteria. Acquisition of the *inv/spa* genes may have been a key event in the evolution of the salmonellae as pathogens, following which the invention of flagellar phase shifting facilitated niche expansion to include warm-blooded vertebrates.**

The ability of the pathogenic bacterium *Salmonella enterica* to invade host cells is determined by a large number of genes, including the *inv/spa* cluster of 15 or more loci in the 59-min region of the chromosome (1–5). The *inv/spa* loci are not present in the genome of *Escherichia coli* K-12 (6), but groups of similarly organized genes with related sequences occur on the virulence plasmids of the invasive enteric pathogens *Shigella* and *Yersinia* and in the genomes of certain plant and animal pathogens of the genera *Erwinia, Pseudomonas*, and *Xanthomonas*. And there are similarities between certain *inv/spa* genes and loci involved in biogenesis of flagella in a variety of bacteria (7, 8).

Functional analyses of the *inv/spa* genes and their homologues have identified their products as an unusual (type III) export system dedicated to the secretion or surface presentation of proteins that interact with host cells (2, 3, 7, 9). Among proteins encoded by homologous genes of the *Salmonella inv/spa* complex and the *Shigella mxi/spa* cluster, there is a wide range of variation in degree of amino acid sequence identity (Fig. 1). Pairs of some proteins are >60% identical, and for mutants at the loci encoding two such proteins—*invA* and *spaP*—invasive ability in *S. enterica* serovar Typhimurium can be restored by introduction of the corresponding gene from *Shigella* (2, 10). In contrast, the product of *spaN* has only 19% amino acid identity with its *spa32* counterpart in *Shigella*. These findings have been interpreted as evidence of interlocus variation in the strength of selective constraints on amino acid substitution (2), but, alternatively, accelerated rates of evolution could result from diversifying selection on those proteins that directly interact with the host environment, as postulated for antigens and other cell-surface components in diverse bacteria (11–13).

To identify factors that generate polymorphism and determine evolutionary rate in invasion determinants within and among enteric pathogens, we have analyzed sequence variation in *spaO, spaP*, and *spaQ* among 21 strains of representative serovars of the eight subspecies of *S. enterica*.¶ We report here that *spaO* is hypervariable within *S. enterica* and present evidence that its product is secreted.

MATERIALS AND METHODS

Bacterial Strains. Seven strains of *S. enterica* subspecies I and two isolates each of subspecies II, IIIa, IIIb, IV, V, VI, and VII were selected for study. Sixteen of the 21 strains were previously examined for sequence variation in the housekeeping genes *gapA* (14), *putP* (15), *mdh* (16), *gnd* (17), and *aceK* (K.N., unpublished data).

PCR and Nucleotide Sequencing. Primers for PCR were designed from the published sequence of Typhimurium LT2 (2). For each of the 21 strains, a 1976-bp segment that includes the complete coding regions of *spaO, spaP*, and *spaQ* (Fig. 1) was amplified and sequenced in both orientations (18). In addition, we have compared the sequences of the *invA* locus for the same sample of strains (E.F.B., unpublished data).

Cellular Localization of SpaO. A *spaO* derivative incorporating a segment encoding a C-terminal DYKDDDDK epitope tag was constructed by PCR amplification. The PCR product was cloned downstream of the *lac* promoter in plasmid pUHE21-21acIq, resulting in plasmid pEG7238. The cellular location of the tagged SpaO protein (molecular weight, 34,766) was studied in pEG7238 transformants of both wild-type Typhimurium 14028s and the isogenic Δ*spa*LMNOPQRS noninvasive mutant EG5537 (2), with Typhimurium 14028s harboring pUHE21-21acIq as a control.

Single colonies grown overnight in 5 ml of Luria broth containing ampicillin (50 μg/ml) and $CaCl_2$ (5 mM) were used to inoculate 100 ml of Luria broth containing ampicillin (50 μg/ml) and $CaCl_2$ (5 mM), with or without 0.5 mM isopropyl β-D-thiogalactopyranoside to induce expression of *spaO*. After growth for 8 hr, without shaking, bacteria were harvested and cell fractions were prepared (19). To extract proteins from culture supernatants, trichloroacetic acid was added to a final concentration of 10%, the mixture was centrifuged at 69,000 ×

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¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U29345–U29365).

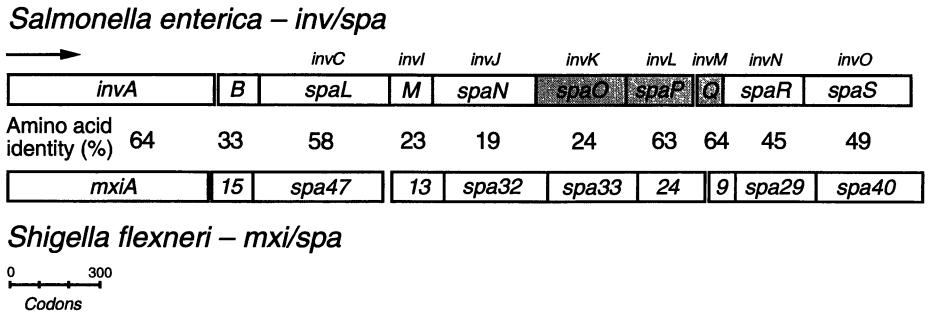

FIG. 1. Organization of the *inv/spa* genes of *S. enterica* and homologous *mxi/spa* genes of *Shigella flexneri*. Gene designations are those of Groisman and Ochman (2); for the *spa* genes of *S. enterica*, alternative *inv* designations are also shown. Arrow indicates direction of transcription.

g, and the resulting pellet was rinsed in cold acetone, dried, and resuspended in 15 μl of 10 mM Tris·HCl (pH 8.0).

Protein concentrations were determined by a modification of the Lowry procedure with the use of bicinchoninic acid (Sigma). Protein fractions were electrophoresed in SDS/12% polyacrylamide gels and then transferred to BioBlot-NC nitrocellulose membranes (Costar) with an LKB Multiphor II electroblotter. Western blots were made with anti-FLAG M2 monoclonal antibodies (IBI) diluted 1:2400 in Tris-buffered saline containing Tween (0.05%) and polyvinylpyrrolidone 40 (2%) and developed with ECL Western blotting detection reagents (Amersham) before exposure to film.

RESULTS

Gene Organization and Variability. The three invasion genes analyzed are arranged on the *S. enterica* chromosome in the order *spaO, spaP*, and *spaQ* (Fig. 1), with an 11-bp overlap of the coding regions of *spaO* and *spaP* and a 25-bp noncoding sequence between *spaP* and *spaQ*.

The most notable feature of sequence variation is a very high incidence (21%) of polymorphic amino acids in the SpaO protein, which contrasts with unusually low frequencies in SpaP (3.1%) and SpaQ (2.3%). Correspondingly, in comparison with the other invasion genes and five housekeeping genes, the mean number of nonsynonymous (replacement) nucleotide substitutions per nonsynonymous site in the *spaO* gene is very large (d_N = 0.0355), although the corresponding value for synonymous (silent) sites (d_S = 0.2430) is only slightly increased (Fig. 2).

Variation in *spaO*. The distribution of polymorphic amino acids in SpaO is nonrandom (Fig. 3). Of the last 38 amino acids (amino acids 266–303), only two (amino acids 294 and 303) are polymorphic; yet, despite relatively strong conservation in this terminal segment of the protein, the incidence of substitutions at synonymous nucleotide sites in the corresponding region of the *spaO* gene is roughly the same as the average for the locus as a whole (Fig. 3).

There is a second highly conserved region of SpaO involving amino acids 184–214; in this case, there is a low frequency of synonymous as well as nonsynonymous site polymorphisms in the gene. In this interior 93-bp segment, substitutions at only two nonsynonymous and five synonymous sites were detected. The G+C content is only 35.8%, as compared with an average of 52.4% for the entire gene and 46.0% for the terminal 38 codons (Fig. 3).

Variation in *spaP* and *spaQ*. Levels of sequence diversity in these genes are similar to those of housekeeping genes (Fig. 2), and both products exhibit relatively high (>62%) amino acid sequence identity to their *Shigella* homologues. An absence of polymorphic sites in the 5′ end of *spaP* may be attributable in part to the 11-bp overlap with *spaO*, but in both genes the conserved segment extends beyond the overlap region. In the *spaQ* gene, there are 28 polymorphic nucleotide sites, only two of which involve replacement substitutions.

Hydropathy analysis (20) revealed the presence of putative membrane-spanning domains in SpaP and SpaQ, which have also been identified in their *Shigella* and *Yersinia* homologues (9, 21), but such domains are not present in the SpaO protein (data not shown).

Evolutionary Relationships. An evolutionary tree for the combined nucleotide sequences of *spaO, spaP, spaQ*, and *invA* (Fig. 4*A*) is similar in topology and branch length to a tree based on the sequences of five housekeeping genes (Fig. 4*B*), with the notable exception of the relationship between subspecies IV and VII. In each of the housekeeping genes, as well as in multilocus chromosomal genotype as indexed by enzyme electrophoresis (13), these subspecies are distinct, whereas in the case of the invasion genes, they are closely similar. This points to the occurrence of at least one horizontal exchange of a DNA segment containing most or all of the *inv/spa* genes.

Cellular Localization of SpaO. To identify SpaO in various cell fractions, Western blot analysis was performed with a SpaO derivative carrying a C-terminal tag recognized by monoclonal antibodies (Fig. 5). SpaO was detected in crude extracts from both wild-type and noninvasive Δ*spa* mutant cells but not in those of control bacteria carrying the plasmid vector without the tagged gene. In both the wild-type and Δ*spa* strains, SpaO was present in the cytoplasmic and inner-membrane fractions, but it was not detected in the periplasmic

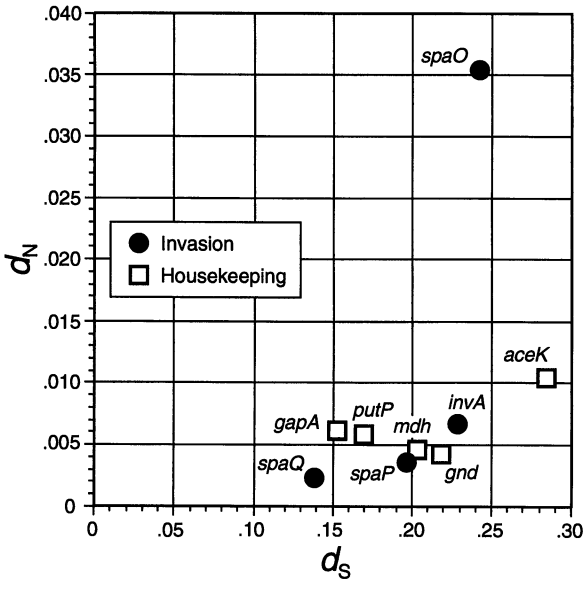

FIG. 2. Estimated average pairwise numbers of synonymous substitutions per synonymous nucleotide site (d_S) and nonsynonymous substitutions per nonsynonymous site (d_N) for four invasion genes and five housekeeping genes in 16 strains of *S. enterica*.

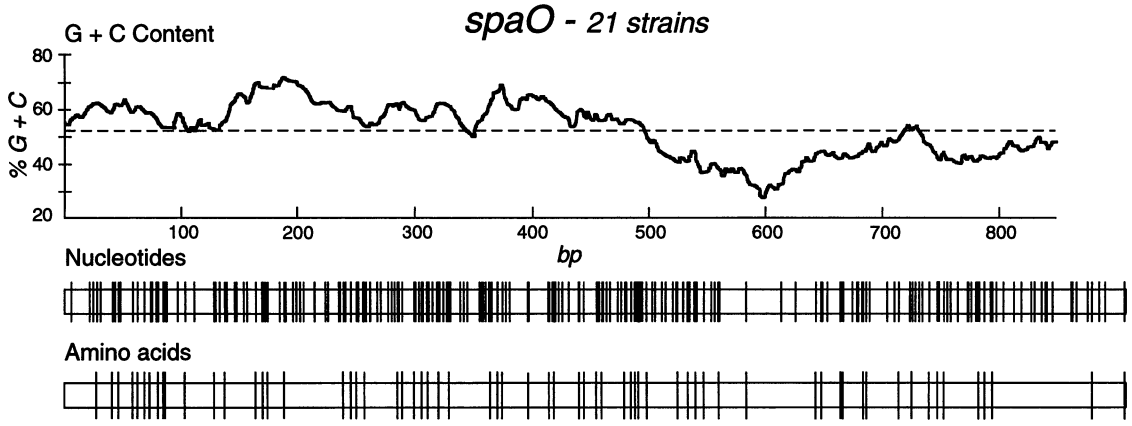

FIG. 3. Variation in the *spaO* gene and SpaO protein among 21 strains of *S. enterica*. (*Top*) Regional variation in percentage G+C content of *spaO*, based on a sliding window of 60 nucleotides. (*Middle*) Positions of polymorphic nucleotides. (*Bottom*) Positions of polymorphic amino acids.

or outer-membrane cell fractions. However, in culture supernatants, SpaO occurred only in the wild-type strain. The absence of SpaO in the supernatant of the Δ*spa* mutant cannot be attributed to a low level of gene expression, because similar amounts of the protein were present in the crude cell extracts and in the cytoplasmic and inner-membrane fractions of both wild-type and mutant strains.

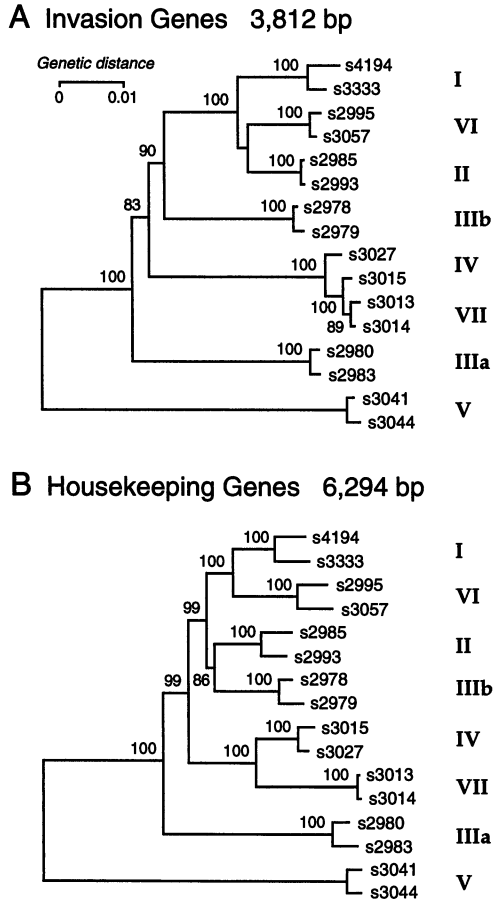

FIG. 4. Neighbor-joining trees (22) for 16 strains of the 8 subspecies of *S. enterica* based on variation in the combined coding sequences of four invasion genes (*A*) and five housekeeping genes (*B*). Pairwise genetic distances were estimated from numbers of substitutions (23, 24). Subspecies are designated by roman numerals, and bootstrap values based on 1000 computer-generated trees are indicated at the nodes.

DISCUSSION

Ancestry of the Invasion Genes. Homologues of the *inv/spa* genes of *S. enterica* occur in several other enteric pathogens, but in view of the base compositions, genomic locations (chromosomal in *S. enterica* but plasmid borne in *Shigella* and *Yersinia*), and phylogenetic distribution of these genes, it is unlikely that the complex, as such, was ancestral in the Enterobacteriaceae. Because of their relatively low G+C content in *S. enterica* (46%), it has been suggested that the *inv/spa* genes were horizontally transferred from *Yersinia* (25), but their occurrence in all the subspecies of *S. enterica* and the overall similarity of their pattern of evolutionary diversification to that of housekeeping genes (Fig. 4) indicate that they were already present in the last common ancestor of the contemporary lineages of the salmonellae. All things considered, it is likely that *Yersinia, Salmonella*, and *Shigella* independently acquired these genes from another source.

Certain strains of the *S. enterica* serovars Senftenberg and Litchfield reportedly lack *invA* sequences (26), but these apparently represent sporadic cases of secondary loss. In a sample of 40 strains of Senftenberg from natural populations that were tested by PCR amplification of *invA* and the *spaO–spaP–spaQ* segment, we found no case in which these genes were absent.

Diversification of *inv/spa* Genes. The general equivalence of the branch lengths of the trees for the invasion and the housekeeping genes (Fig. 4) indicates that the rates of evolution of these two groups of loci have, on average, been roughly the same. However, the range of variation in the frequency of nonsynonymous substitutions (d_N) among the invasion genes greatly exceeds that shown by the housekeeping genes, largely because of the hypervariability of *spaO*. While the incidence of polymorphic amino acids is high over most of the length of the SpaO protein, two segments (amino acids 184–214 and the last 38 amino acids, 266–303) are strongly conserved. Because the conservation of the internal segment of the *spaO* gene involves both synonymous and nonsynonymous nucleotide sites, it is most readily attributable to horizontal transfer and intragenic recombination among several of the subspecies. But in any event, conservation of this segment apparently is specific to *S. enterica*, since it exhibits only 9.6% amino acid identity to the corresponding sequences of the homologous proteins of *Shigella* and *Yersinia*. Although the terminal segment of SpaO is all but invariant in amino acid sequence, the *spaO* gene exhibits a normal level of synonymous substitution in this region. This strongly suggests that the similarity in amino acid sequence among the subspecies reflects selection against replacement mutations rather than the horizontal exchange of a common segment, an interpretation supported by the observation that

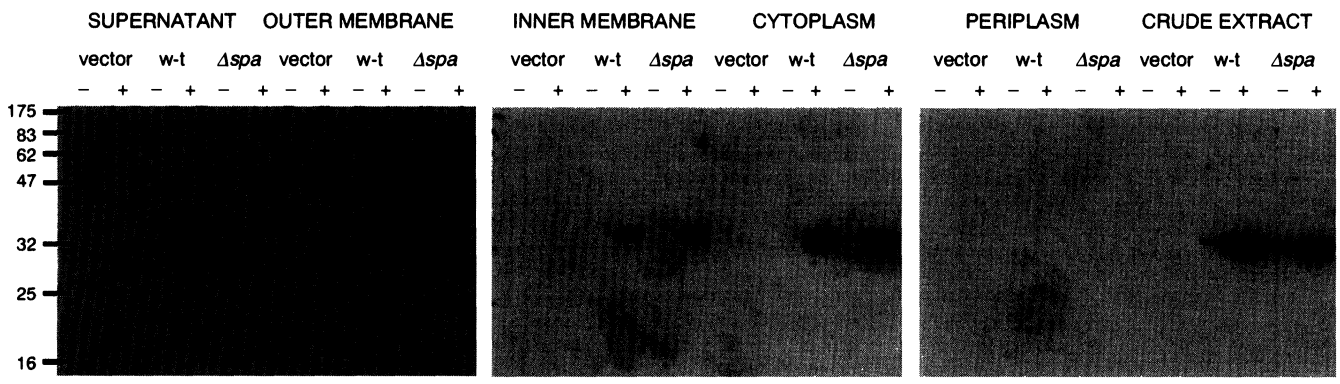

FIG. 5. Cellular localization of SpaO. Cell fractions and culture supernatants were prepared from wild-type Typhimurium 14028s/pEG7238 (w-t), the isogenic Δ*spa*LMNOPQRS mutant EG5537/pEG7238 (Δ*spa*), and 14028s/pUHE21-21acIq (vector) grown under conditions that promote invasion of host cells. Plasmid pEG7238 harbors a *spaO* derivative with a C-terminal epitope tag under control of the *lac* promoter in plasmid pUHE21-21acIq. Fractions [supernatant (10 μg), outer membrane (6 μg), inner membrane (6 μg), cytoplasm (6 μg), periplasm (6 μg), crude extract (10 μg)] were separated, transferred to nitrocellulose, and blotted with anti-FLAG M2 antibodies. Numbers denote positions and sizes (kDa) of molecular mass standards. + and −, Cells grown in the presence and absence of isopropyl β-D-thiogalactopyranoside.

the terminal part of SpaO shows 40–45% amino acid identity to the homologous regions of Spa33 in *Shigella* and YscQ in *Yersinia* versus average identities of only 24–25% for the protein as a whole.

Protein Cellular Location and Sequence Variability. The SpaO protein was present in the culture supernatant of a wild-type Typhimurium strain but absent from the supernatant of a noninvasive mutant. The inference is that the mutant strain is unable to export SpaO, which does not have a signal sequence, and that in normal strains the protein is exported by the dedicated type III secretion system encoded by the *inv/spa* genes.

The *spaO* gene is preceded by *spaN* and *spaM*, both of which also exhibit low levels of sequence identity to their *Shigella* counterparts (Fig. 1) and are hypervariable among the subspecies of *S. enterica* (J.L., unpublished data). Collazo *et al.* (5) demonstrated that SpaN is secreted to the culture supernatant and that the process requires functional *invG* and *spaL* genes. However, they reported that SpaM was not detected in the supernatant of cells grown under conditions that allowed export of SpaN.

Epithelial cell invasion by enteric bacteria is believed to involve the stimulation of host-cell receptors that initiate a signal transduction cascade. Contact between Typhimurium and host cells has been reported to promote phosphorylation of host-cell proteins (27) and the transitory appearance of surface appendages in the microorganism (3). As secreted proteins, SpaO and SpaN could be involved in stimulating host-cell factors that are involved in internalization of the bacterium. In *Shigella*, invasion requires the participation of several secreted proteins, including IpaB, IpaC, and IpaD (28–30). The genes encoding these invasion antigens are located several kilobases upstream of the *mxi/spa* cluster, and, to date, counterparts of the Ipa antigens have not been recovered from *S. enterica*. SpaM has been reported to be homologous to IpaB (5), but this is unlikely given the low level of sequence similarity between these proteins, the fact that SpaM could not be detected in culture supernatants, and the similarity in size, position, and sequence of *spaM* to *spa15* of *Shigella*.

The available evidence indicates a relationship between the cellular location of the products of the *inv/spa* genes and evolutionary rate, as reflected in both the level of polymorphism within *S. enterica* and the degree of variation among homologues in various types of bacteria. Thus, the secreted proteins SpaO and SpaN are hypervariable within *S. enterica* and exhibit <25% amino acid sequence identity with their homologues in *Shigella* and *Yersinia*. In contrast, InvA, which is located in the inner-cell membrane (10, 31), and SpaP and SpaQ, for which there is structural evidence of membrane association, are highly conserved in *S. enterica* and have >60% identity with the corresponding genes in these other bacteria.

Because an unusually high level of polymorphism is characteristic of a variety of bacterial genes encoding or mediating the structure of cell-surface components that directly interact with factors of the extracellular environment (13), it is tempting to speculate that the hypervariability of the secreted products of the *inv/spa* complex reflects the action of diversifying selection in adaptation to variable aspects of the environment encountered by the bacteria in their hosts. One possibility is selection for antigenic diversity to escape host immune systems. Another is selection for attributes that determine the host specificity of particular serovars, but our results do not support this hypothesis, since SpaO is invariant in sequence among strains of the strongly host-adapted serovars Dublin (cattle) and Gallinarum (fowl), as well as Enteritidis, which has a moderately broad host range. Finally, there is the possibility that hypervariability merely reflects a relaxation of selective constraints on amino acid substitution in the secreted proteins.

Evolution of Pathogenicity and Host Range. The topologies of the evolutionary trees for invasion and housekeeping genes (Fig. 4) are similar and consistent with evidence from genomic DNA hybridization experiments (32, 33) and may, therefore, be indicative of the actual evolutionary relationships of the subspecies of *S. enterica*. The observation that subspecies I, II, VI, and IIIb—the serovars of which are predominantly diphasic in flagellar expression (34)—cluster apart from the monophasic subspecies provides information on the evolutionary history of the species and suggests the following scenario. After the divergence of *S. enterica* and *E. coli* from a common ancestor 120–160 million years ago, coincident with the origin of mammals (35), *E. coli* evolved as a commensal and opportunistic pathogen of mammals and birds. The four nominal species of *Shigella*, which are actually clonal lineages of *E. coli* (36–38), evolved through the acquisition of a virulence plasmid to become invasive pathogens of primates. Meanwhile, the lineage ancestral to the salmonellae remained associated with reptiles (which are still the primary hosts of the monophasic subspecies) and presumably evolved as an intracellular pathogen only after acquiring the chromosomal segment containing the *inv/spa* genes. Subsequently, by providing increased ability to circumvent host immune systems, the invention of the mechanism of flagellar antigen phase shifting (diphasic condition) in the lineage ancestral to subspecies I, II, IIIb, and VI may have been a critical factor in the expansion of ecological

range to include warm-blooded vertebrates, but as a pathogen rather than a commensal—a niche already long occupied by *E. coli*.

This research was supported by grants from the National Institutes of Health (AI-22144, GM-48407, and AI-29554). E.A.G. is a recipient of a Junior Faculty Research Award from the American Cancer Society.

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