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Differential patterns of acquired virulence genes distinguish *Salmonella* strains

(pathogenesis/horizontal transfer/*in vivo* expression technology/*ivi*/*Salmonella typhimurium*)

CHRISTOPHER P. CONNER, DOUGLAS M. HEITHOFF, STEVEN M. JULIO, ROBERT L. SINSHEIMER, AND MICHAEL J. MAHAN*

Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, CA 93106

Contributed by Robert L. Sinsheimer, February 17, 1998

ABSTRACT **Analysis of several *Salmonella typhimurium in vivo*-induced genes located in regions of atypical base composition has uncovered acquired genetic elements that cumulatively engender pathogenicity. Many of these regions are associated with mobile elements, encode predicted adhesin and invasin-like functions, and are required for full virulence. Some of these regions distinguish broad host range from host-adapted *Salmonella* serovars and may contribute to inherent differences in host specificity, tissue tropism, and disease manifestation. Maintenance of this archipelago of acquired sequence by selection in specific hosts reveals a fossil record of the evolution of pathogenic species.**

Microbial speciation is driven by the loss or acquisition of genes that allow an organism to occupy a niche unavailable to its relatives (1–3). Within the context of pathogenesis, these acquired sequences reflect a pathogen's unique lifestyle in nature, comprising functions favorable to growth in the host and the environment (4). Many virulence functions that contribute to *in vivo* fitness are encoded on pathogenicity islands, sequences presumed to have been acquired by horizontal transfer as evidenced by their atypical base composition and codon usage (5). Here we have employed *in vivo* expression technology (IVET; refs. 6 and 7) to identify *in vivo i*nduced (*ivi*) acquired sequences within *Salmonella typhimurium* that are distinct within and between *Salmonella* serovars and contribute to its fitness within its host.

MATERIALS AND METHODS

Bacterial Strains and Phage. All *S. typhimurium* strains used in this study were derived from strain ATCC 14028 (CDC 6516–60). The high-frequency generalized-transducing bacteriophage P22 mutant HT 105/1, *int*-201 was used for all transductional crosses (8), and phage-free, phage-sensitive transductants were isolated as described (9). The isolation of all *ivi* fusions used in this study was described (10).

Media and Chemicals. Luria broth (11) was the laboratory media used in these studies. Final concentrations of antibiotics (Sigma) were as follows: ampicillin (50 μg/ml), kanamycin (50 μg/ml), tetracycline (20 μg/ml).

Hybridization Conditions. Probes used for hybridization were obtained by restriction digestion or amplified by PCR. Typically, 1 μg of genomic DNA was spotted on a nylon membrane and probed overnight with 100 ng of DNA. Membranes were washed at 42°C in 0.5× SSC plus 0.4% SDS and 6 M urea. Hybridization was detected by enhanced chemiluminescence (Amersham).

Competitive Index (CI) Assay in BALB/c Mice. Mutant and wild-type cells were grown overnight in Luria broth. BALB/c mice were intraperitoneally infected with a total of 10^3 cells of a 1:1 ratio of deletion mutant: wild type (MT2057). The mice were killed after 5 days and the bacterial cells were recovered from the spleen; the CI is the ratio of the number of mutant/wild-type bacteria recovered.

RESULTS

Serovar-Specific Sequences. A collection of more than 100 *ivi* genes (10) was screened for those that reside in regions of atypical base composition ($\leq$49% or $\geq$57%) and for the absence of sequence homology in the DNA database. A subset of 30 *ivi* genes that fit these criteria were used as molecular probes to hybridize against genomic DNA isolated from a set of enteric pathogens, including four *Salmonella* serovars of differing host range, tissue tropism, and disease manifestation.

Table 1 shows that DNAs prepared from seven of these *ivi* gene fragments hybridized strongly to genomic DNA prepared from one or more *Salmonella* serovars and not to any other pathogens tested (with the exception of *iviXVII*). The *Salmonella*-specific regions fell into three distinct classes based on their respective hybridization patterns to the four *Salmonella* serovars tested. These serovars include the broad host range *S. typhimurium* and *Salmonella newport* (warm- and cold-blooded animals), limited host range *Salmonella choleraesuis* (humans, pigs), and the host-adapted *Salmonella typhi* (humans). Class I sequences hybridized to all *Salmonella* serovars tested. In contrast, Class II and III sequences were serovar specific: Class II hybridized to all serovars except *S. typhi*, whereas Class III hybridized only to broad host-range serovars. Control hybridizations show that a typical core sequence marker that is shared between many species, e.g., triose- phosphate isomerase (*tpi*; $\approx$80% DNA homology between *Salmonella* spp. and *Escherichia coli*), hybridized to all species tested, and the *Salmonella* plasmid virulence gene, *spvB*, hybridized only to *Salmonella* serovars tested that contain the *Salmonella* virulence plasmid, pSLT (all serovars but *S. typhi*).

***Salmonella*-Specific Regions.** To determine the extent of the chromosomal regions that are distinctive to *Salmonella*, the *ivi* gene fragments from these regions were used as hybridization probes against a library of λ clones containing *S. typhimurium* genomic DNA. Positively hybridizing λ clones were selected and fragments of these (typically 1–3 kb) were used as probes against genomic DNA prepared from the collection of pathogens shown in Table 1. Each line in Fig. 1 represents an assemblage of overlapping λ and/or plasmid clones in a *Salmonella*-specific region.

Fig. 1 shows that three of the *Salmonella*-specific regions are present in all *Salmonella* serovars tested (lines 1–3) and are

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Abbreviations: IVET, *in vivo* expression technology; CI, competitive index.

*To whom reprint requests should be addressed.

Table 1. *ivi* sequences that are distinct within and between *Salmonella* serovars

	ivi gene probes*								
	Class I				Class II	Class III			
Organism	*iviVI-A*	*iviXVII*	*iviXVIII*	*iviXIX*	*iviXXI*	*iviXXII*	*iviXXIV*	*tpi*	*spvB*
Salmonella									
S. typhimurium 14028	++++	++++	++++	++++	++++	++++	++++	++++	++++
S. newport	++++	++++	++++	++++	++++	++++	++++	++++	++++
S. choleraesuis	++++	++++	++++	++++	++++	−	−	++++	++++
S. typhi Ty2	++++	++++	++++	++++	+	−	−	++++	−
E. coli†									
EPEC	−	+	−	−	−	−	−	+++	−
UPEC	−	−	−	−	−	−	−	+++	−
APEC	−	−	−	−	−	−	−	+++	−
RDEC	−	−	−	−	−	−	−	+++	−
XL1	−	−	−	−	−	−	−	+++	−
Other pathogens									
Shigella flexneri 2a‡	−	−	−	−	−	−	−	+++	−
Shigella dysenteriae SHA-1	−	−	−	−	−	−	−	+++	−
Klebsiella pneumoniae‡	−	+++	−	−	−	−	−	+++	−
Serratia marcescens‡	−	++	−	−	−	−	−	+++	−
Citrobacter freundii‡	−	−	−	−	−	−	−	+++	−
Pseudomonas aeruginosa 103	−	++	−	−	−	−	−	++	−
Aeromonas hydrophila SSU	−	−	++	−	−	−	−	++	−
Enterobacter aerogenes‡	−	−	−	−	−	−	−	++	−
Enterobacter cloacae‡	−	−	−	−	−	−	−	++	−
Plant pathogens									
Pantoea agglomerans‡	−	−	−	−	−	−	−	+	−
Erwinia carotovora‡	−	−	−	−	−	−	−	+	−

*DNA prepared from *ivi* genes of atypical base composition (≤49% or ≥57%) were used as molecular probes to hybridize against genomic DNA isolated from a series of gram-negative pathogens. G + C content of the *ivi* probes was determined by sequencing 233 bp to 326 bp from the *ivi* join: *iviVI-A*, 42%; *iviXVII*, 63%; *iviXVIII*, 46%; *iviXIX*, 60%; *iviXXI*, 38%; *iviXXII*, 26%; *iviXXIV*, 57%; *tpi* (51% over 172 bp); *spvB* (49% over 127 bp).

†Enteropathogenic *E. coli* (EPEC O127:K63), uropathogenic *E. coli* (UPEC O1:K1:H7), avian pathogenic *E. coli* (APEC O1:K1), rabbit diarrheal *E. coli* (RDEC-1).

‡ATCC type strain.

++++, a signal equal to that seen with parental *S. typhimurium* DNA; −, a signal less than or equal to a 10-fold dilution of parental *S. typhimurium* DNA; +, ++, and +++ represent signals intermediate to that seen with 10-fold diluted and full-strength parental *S. typhimurium* DNA.

designated Class I. The *iviVI-A* region (line 1) corresponds to a chromosomal segment of atypical base composition (>35 kb), part of which was known to be present in *Salmonella* spp., but not in *E. coli* (10, 21, 22). A second Class I region encodes two *ivi* fusions, *iviXVII* and *iviXXV* (line 2), that reside in a region involved in vitamin B_{12} biosynthesis (23) and propanediol utilization (*pdu*) (ref. 24; T. A. Bobik, personal communication). Last, the *iviXVIII* region (line 3) is comprised of Class I sequences that are separated by a short intervening segment of *E. coli*-like core sequence.

The remaining three regions have proven to be mosaics of *Salmonella* serovar-specific sequences (lines 4–6). The *iviXIX* and *iviXX* regions (line 4) comprise two distinct blocks of different hybridization classes, Class I and Class III, respectively, separated by a small core sequence. Similarly, the *iviXXI* region (line 5) comprises two serovar-specific hybridization classes, Class II and Class III. Notably, a portion of the *iviXXI* region does not hybridize to DNA prepared from the laboratory strain of *S. typhimurium*, LT2, thus distinguishing not only one serovar from another but also individual strains within a serovar. Last, *iviXXII* and *iviXXIII* define an ≈34-kb Class III region that is flanked on either side by Class I sequences (line 6). One of the two flanking Class I regions was previously known to be present in *Salmonella* spp., but not present in *E. coli*, containing the flagellar-switch region (*hin*), and may extend as far as the *Salmonella*-specific *tct* locus (25, 26).

Association of Mobile Elements with *Salmonella*-Specific Acquired Sequences. Fig. 1, line 1 shows a component of an *rhs* (recombination hot spot) repetitive element, termed an H-repeat (H-rpt), flanks the *iviVI-A* region on one end (27). This H-rpt insertion-like sequence is present in all *Salmonella* serovars tested (Class I) and such elements have been associated with the acquisition of virulence functions in several pathogens (27–30). Another *rhs* is also present in this region, defined by *iviXXIV*. In contrast to the H-rpt, this *rhs* is only present in broad host-range *Salmonella* serovars (Class III) and encodes a predicted peptide motif involved in carbohydrate binding (31, 32), suggesting a possible role in adhesion. The portion of the *iviVI-A* region flanked on both ends by insertion sequences may be inherited as an independent unit.

Several mobile elements reside within the Class III *iviXXII* region (Fig. 1, line 6). Examples include insertion elements IS3 and IS1351, and homologues to functions encoded on the cryptic prophage, CP4–57. Both Class III junction points of this region are associated with homologues to the CP4–57 integrase, *slpA*, a homologue that defines the junction of a ToxR-regulated gene cluster in *Vibrio cholerae* (33). Moreover, one junction point lies near *ssrA* (encoding 10Sa RNA), a tRNA-like structure that is part of the insertion site for CP4–57 in *E. coli* (34) and the insertion site of a pathogenicity island in *Dichelobacter nodosus* (35).

***Salmonella*-Specific Sequences Contribute to Virulence.** Directed deletions of the *Salmonella*-specific regions were constructed to determine the overall contribution of these regions to pathogenesis (36). Table 2 shows that deletions in 3/6 *Salmonella*-specific regions confer a defect in virulence as determined in a CI assay. A CI < 1.0 indicates that the mutant is less fit than the wild type. Strains MT2040, MT2037, and MT2055 exhibit a CI that is significantly reduced from 1.0 as determined by the *t* test ($P < 0.05$). Removal of the Class III

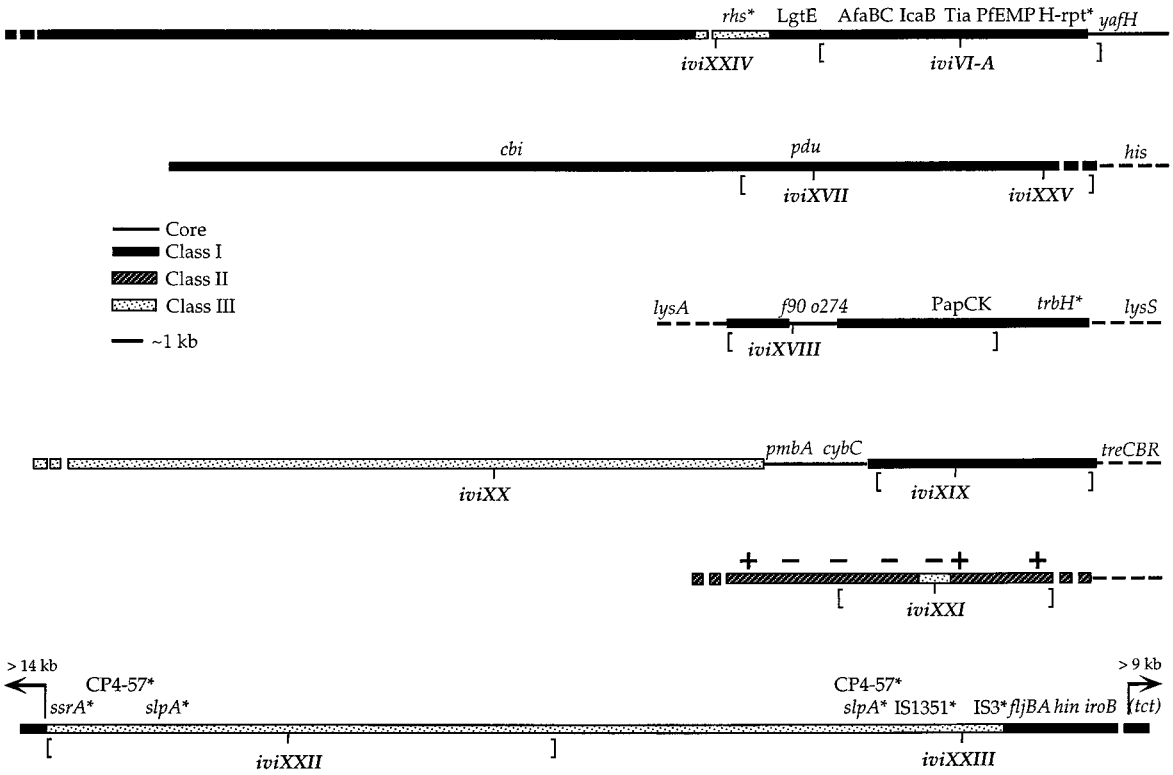

FIG. 1. *Salmonella*-specific regions identified by IVET. Each line represents an assemblage of overlapping λ and/or plasmid clones in a *Salmonella*-specific region. The boxed sequences represent *Salmonella*-specific regions that are distinct to *Salmonella* spp. and are not present in other enteric species tested. Class I sequences hybridized to all *Salmonella* serovars. Class II sequences hybridized to all *Salmonella* serovars tested with the exception of *S. typhi*. Class III sequences hybridized only to broad host-range serovars (*S. typhimurium* and *S. newport*). Core sequences are those shared between *Salmonella* spp. and *E. coli*. Each region was identified by hybridization to a probe from an *ivi* gene fragment of atypical base composition as determined by sequencing >200 bp starting from the *ivi* junction. Protein designations indicate predicted similarities ranging from 30–81% identity at the amino acid level over a minimum of 50 deduced residues. Protein similarities include enterotoxigenic *E. coli* (ETEC) Tia (10, 12) and AfaBC, involved in afimbrial adhesin in uropathogenic and diarrheal-associated *E. coli* strains (13), PfEMP-1, involved in attachment of *Plasmodium falciparum*-infected red blood cells to peripheral epithelium (10, 14) and IcaB, an intercellular adhesin molecule of *Staphylococcus epidermidis* that functions in biofilm formation (15). LgtE of *Neisseria meningitidis* is involved in modification of surface lipopolysaccharide (16). PapC and PapK are adherence-related functions of uropathogenic *E. coli* (UPEC) (17, 18). Gene designations denote similarity ranging from 61–96% at the DNA level over a minimum of 129 nucleotides. The *S. typhimurium* open reading frames *f90* and *o274* are homologues of *E. coli* genes that do not map to the corresponding region of *E. coli* (minute 47 in *E. coli* vs. minute 65 in *S. typhimurium*). Similarly, *Salmonella pap*-like sequences map to minute 65, which is near, but distinct from, the corresponding *papCK*-containing region of uropathogenic *E. coli* at minute 67 (19). Brackets designate the endpoints of deletions tested in the CI assay. The "+" designation over the *iviXXI* region indicates that the corresponding chromosomal segment hybridizes to DNA prepared from the laboratory strain of *S. typhimurium*, LT2; a "−" designation indicates that it does not hybridize to DNA prepared from LT2. For all other regions, probes hybridized equally well to DNA prepared from *S. typhimurium* strains 14028 and LT2. The * designates homologies that are associated with mobile genetic elements that are discussed in the text with the exception of *trbH*, which is associated with a pathogenicity island of *Dichelobacter nodosus* (20).

iviXXII region (17 kb) conferred the largest virulence defect in BALB/c mice (>300-fold) and showed reduced survival (3.2-fold) in RAW 264.7 cultured murine macrophages. Removal of other *ivi* regions conferred smaller defects in mice. The additive effect of such small contributions might have a significant impact on fitness within the host and, over time, lead to the formation of distinct serovars and species with differing host range, tissue tropism, and disease manifestation.

DISCUSSION

We have employed IVET to identify six *Salmonella* regions of atypical base composition that are not present in many other species tested, including several pathogenic strains of *E. coli*. These *Salmonella*-specific regions fall into three classes with respect to their distribution within four *Salmonella* serovars of differing host range: Class I sequences are present in broad host-range and host-adapted *Salmonella* serovars; Class II are present in all but the host-adapted serovar, *S. typhi*; Class III are present only in broad host-range serovars and not *S. typhi* nor the limited host-range serovar, *S. choleraesuis*. Many of these *Salmonella*-specific regions are required for full virulence and are associated with mobile elements, such as phage and insertion sequences that may have contributed to their acquisition by horizontal transfer. The incremental acquisition of functions encoded within these regions may contribute to evolution of species, host range, and disease manifestation.

Recent studies on microbial speciation have estimated that up to 30% of the genome sequences may differ between the closely related species *S. typhimurium* and *E. coli* (1, 3, 25). These differences have been attributed to genomic flux; the loss and acquisition of genes after divergence from a common ancestor. Indeed, ≈25% of the more than 100 *ivi* genes recovered from the IVET selection in *S. typhimurium* have no homologue in the DNA database (4, 10). Moreover, we have shown that at least 10 of these *ivi* genes reside in 6 distinct regions of atypical base composition and removal of a portion of 3 of these regions conferred a virulence defect in a murine typhoid fever model.

It is expected that at least some of the *in vivo*-induced *Salmonella*-specific sequences encode functions responsible for the speciation and subsequent evolution of distinct *Salmonella* serovars. Sequences present in all *Salmonella* spp. (Class I) are likely to have been inherited early in the divergence of

Table 2. Deletions of *Salmonella*-specific regions confer virulence defects

Strain	*ivi* gene deleted	Map position, min*	CI, *n*†
MT2040	*iviVI-A*	7	0.36 ± 0.13 (6)
MT2037	*iviXVII; iviXXV*	44	0.24 ± 0.16 (4)
MT2080	*iviXXI*	57	1.7 ± 1.6 (9)
MT2055	*iviXXII*	60	0.003 ± 0.002 (4)‡
MT2060	*iviXVIII*	65	2.0 ± 1.2 (4)
MT2058	*iviXIX*	96	0.99 ± 0.70 (5)

Salmonella-specific sequences were deleted according to methods described in ref. 36; deletions range in size from 6 to 25 kb.

*Map positions of *ivi* genes were determined by Mu*d*P22 mapping (37) and refined by linkage to known markers in the *S. typhimurium* chromosome (38).

†All deletion mutants used in the CI assay were derivatives of IVET-selected parental *ivi* fusion strains recovered from spleens after intraperitoneal (i.p.) infection of BALB/c mice or from infected RAW 264.7 cultured macrophages. BALB/c mice were i.p. infected with a 1:1 ratio of deletion mutant: wild type (MT2057). Five days after infection, the bacterial cells were recovered from the spleen; the CI is the ratio of mutant/wild-type bacteria recovered. *n*, Number of mice tested. All deletion mutants exhibit wild-type levels of growth on Luria broth medium as evidence by colony size and number after overnight incubation with the exception of MT2055, which shows a mild growth defect. The extent of material removed in the deletion mutants is shown by brackets in Fig. 1.

‡MT2055 exhibits reduced survival (3.2-fold) compared to the wild type 27 hr after infection of RAW 264.7 cultured murine macrophages.

Salmonella, encoding functions that allow it to occupy niches that are unavailable to its nearest relatives, similar to the proposed inheritance of *S. typhimurium* pathogenicity island 1 (SPI-1) (39). Sequences not found in all serovars (Classes II and III) represent later acquisitions or losses of functions that reflect the lifestyle of each serovar, as is proposed for unique virulence regions within avian pathogenic *E. coli*, which are not present in *E. coli* K-12 (40). In our studies, all probes were derived from *ivi* genes in the broad host range *S. typhimurium* and thus, by definition, *S. typhimurium* is a member of all hybridization classes described here. Therefore, it should not be interpreted from our results that *S. typhimurium* contains every sequence that is specific to the salmonellae. Rather, it is expected that probes derived from *ivi* genes of other serovars would identify sequences not present in *S. typhimurium*; indeed, *S. typhi* contains 118 kb of a sequence that is not present in *S. typhimurium* (41).

Many of the *Salmonella*-specific regions encode predicted proteins with sequence similarity to virulence proteins from a wide variety of pathogens and removal of these regions confers a virulence defect. For example, Fig. 1 (line 1 and legend) shows that the *iviVI-A* region encodes predicted products that show similarity to several adhesin-like molecules (10, 12–15) and a lipopolysaccharide modification enzyme (16). The *iviXVII* region (Fig. 1, line 2) allows growth on 1,2-propanediol (24) and the virulence defect observed after its removal may reflect the significance of using alternative carbon sources *in vivo*. Moreover, the acquisition of this region in *Salmonella* spp. is thought to have contributed to its divergence from the closely related *E. coli* (1, 3). The *iviXXII* region (Fig. 1, line 6) contains phage-like functions, including *ssrA*, a tRNA-like structure that is part of the insertion site for the *E. coli* cryptic prophage CP4–57 (34) and the insertion site of a pathogenicity island in *D. nodosus* (35). Removal of a portion of this region conferred the greatest defect in virulence. Deletions of some *Salmonella*-specific regions did not show an effect in the murine infection model, but may encode functions that are either redundant or important for virulence in other hosts.

The association of pathogenicity islands with phage-like functions that integrate into the chromosome at phage attachment sites is well documented (5). These attachment sites are often within the well-conserved, omnipresent tRNA genes that may facilitate phage-mediated horizontal transfer of virulence functions by providing compatible integration sites within many recipient species. Moreover, the importance of functions encoded within integrated phages themselves to virulence has been demonstrated by the increased serum resistance in λ lysogens of *E. coli* (42). Along these lines, the recently sequenced *E. coli* genome reveals many cryptic phage functions that may enhance fitness, although the ability to produce lytic phages apparently has been lost (43).

From what organisms were these sequences acquired? The composition of acquired sequences will tend to drift over time (ameliorate) to the G + C content of the recipient (44), thus masking the identity of the donor organism. In cases where there is no DNA sequence homology, protein similarity may provide clues to the functions encoded by *Salmonella*-specific DNA, but not the source of the acquired sequence. For example, many *Salmonella*-specific DNA regions such as *iviVI-A* (Fig. 1, line 1) exhibit no homology to related species at the DNA level. However, the predicted IviVI-A gene product shows significant similarity over its entire length to enterotoxigenic *E. coli* Tia (10). This lack of DNA homology to other species suggests that the source of these acquired sequences remains to be identified. Perhaps they have been acquired from other pathogenic, commensal, or environmental organisms that shared similar animal or environmental reservoirs at some time during their evolutionary history.

The *Salmonella*-specific *in vivo*-induced regions comprise a fossil record of events that have lead to the evolution of distinct species and serovars. These acquired *in vivo*-selected sequences not only distinguish one pathogen from another but may also point to the functions involved in host/pathogen interactions, which lead to the host specificity and tissue tropism of each species, i.e., the functions that contribute to specific disease or carrier state caused by a given serovar in a given host. The elucidation of species-specific genes in clinically relevant bacteria may provide probes for the rapid and sensitive identification of infectious organisms.

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