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Molecular, functional, and evolutionary analysis of sequences specific to *Salmonella*

(genome organization/enteric bacteria/virulence/transcriptional regulators)

EDUARDO A. GROISMAN*†, MARK A. STURMOSKI*, FELIX R. SOLOMON*, ROBERT LIN*, AND HOWARD OCHMAN‡

*Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63110; and ‡Department of Biology, University of Rochester, Rochester, NY 14627

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ABSTRACT **In that salmonellae have been implicated in an unprecedented array of diseases, sequences found to be specific to this species are often thought to be involved in the virulence attributes not seen in other enteric bacteria. To identify the molecular, genetic, and phenotypic characteristics that differentiate bacterial species, we analyzed five cloned DNA fragments that were originally described as being confined to *Salmonella*. Most of these segments mapped to unique positions on the *Salmonella typhimurium* chromosome indicative of independent evolutionary events, and three had G+C contents considerably lower than that of the *Salmonella* genome, suggesting that they arose through horizontal transfer. The nucleotide sequence was determined for one of the clones exhibiting an atypical base composition. This 4.9-kb fragment contained an open reading frame with structural similarity to the LysR family of transcriptional regulators. Strains harboring deletions in this region were tested for >120 phenotypic characteristics including the effects on a collection of environmentally regulated *lac* gene fusions. In addition, all deletion strains behaved like the wild-type parent when tested for virulence in mice.**

The enteric bacteria classified as *Salmonella* have been recovered from a wide variety of mammalian and nonmammalian species (1) and are the principal etiological agents of gastroenteritis and enteric fever in humans and domesticated animals (2). Several virulence properties, including toxin production, the invasion of mammalian epithelial cells (3, 4), survival within macrophages (5), and resistance to the antibiotic effects of small peptides (6, 7), set salmonellae apart from other species of enteric bacteria. *Salmonella typhimurium*, in its capacity to cause a lethal infection in mice, is commonly used as a model for typhoid fever, and this has led to the extensive characterization of factors associated with its pathogenicity (8). Genes specific to *Salmonella* could be responsible for its virulence properties, as observed for loci required in the invasion of intestinal epithelium (9).

Aside from the considerable diversity in virulence characteristics, species of enteric bacteria differ with respect to metabolic and biochemical attributes, such as the ability to ferment lactose and utilize citrate (10). Variation in these factors as well as several molecular criteria, including linkage maps, genome sizes, and G+C contents, suggest that large-scale changes have occurred within the genomes of enteric bacteria since they diverged (10, 11). The most comprehensive information concerning the genetic structure and organization of bacterial genomes has come from comparative analyses of *Escherichia coli* and *S. typhimurium* (12). The chromosomes of these species are well conserved with respect to size and the order, orientation, and spacing of genes. However, when the linkage maps of *E. coli* and *S. typhimurium* are aligned, there is a large inversion encompassing >10% of the chromosome and some 30 loop structures, with regions ranging from 20 to 80 kb restricted to one of the species (11, 12). Although some of these regions harbor genes encoding the metabolic properties used to differentiate *E. coli* and *S. typhimurium*, the ancestry and function of most sequences residing on loops remains unknown.

Through the systematic screening of an *S. typhimurium* DNA library, Fitts (13) recovered numerous clones containing DNA sequences that were apparently unique to salmonellae. Hybridization studies revealed that five of these (with the prefix RF) clones contained sequences that were present in all or in a majority of strains of *Salmonella* (13). "*Salmonella*-specific" clones are useful for diagnostic procedures (13, 14) and are also likely to contain genes that encode products that distinguish *Salmonella* from other enteric species. Such products may have a role in pathogenicity, host range, vitamin B_{12} biosynthesis—which occupies some 1% of the genome (15, 16)—or the survival of *Salmonella* outside of the animal host environment. These *Salmonella*-specific DNA sequences may also provide insights on the origin and ancestry of the *Salmonella* genome.

In this paper, we examined the DNA fragments described by Fitts (13) as being confined to salmonellae. These clones were analyzed with respect to map location, nucleotide content, and phylogenetic distribution to discover the factors contributing to their presence in the *Salmonella* genome. The entire nucleotide sequence of one of these clones was determined to identify features that account for its origin in *Salmonella* and potential similarities to known genes.¶ We established that a portion of this clone accommodates an open reading frame whose sequence and structure are related to the LysR family of transcriptional regulators. In addition, we have performed scanning-deletion analysis of this segment of the chromosome to establish the functional role of this region.

MATERIALS AND METHODS

LB, MacConkey, and M63 minimal media have been described (17). Antibiotics were used at the following concentrations: kanamycin, 40 μg/ml; ampicillin, 50 μg/ml; tetracycline, 10 μg/ml; chloramphenicol, 40 μg/ml. Protamine sulfate was incorporated into LB agar plates at a final concentration of 1 mg/ml. Phage P22-mediated transduction was performed as described (18). Plasmid DNA was introduced into *S. typhimurium* by either P22 transduction or by electroporation. Restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories, Boehringer Mannheim, or New England Biolabs.

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†To whom reprint requests should be addressed.

¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. L03407).

Plasmids RF305, RF319, RF321, RF333, and RF356 were obtained from the Agricultural Research Culture Collection (Peoria, IL). The following *Salmonella* strains were used. Strain X3761, the virulent wild-type strain used in our studies, was obtained from R. Curtiss III (Washington University, St. Louis, MO). Strains EG5227, EG5229, EG5233, EG5235, and EG5237 were derivatives of X3761 containing insertion/deletions in *Salmonella*-specific regions (see *Results*). Strains JF511, JF512, JF514, JF515, JF570, and JF663 harboring *lac* gene fusions to phosphate starvation inducible genes (19) and strains JF556, JF738, JF888, JF895, JF896, JF897, JF900, JF926, JF927, JF928, JF1101, JF1102, JF1103, JF1104, JF1105, JF1107, and JF2389 containing *lac* gene fusions to oxygen-regulated loci (20) were obtained from J. Foster (University of South Alabama, Mobile, AL). Further information about these environmentally responsive *lac* gene fusions can be found in refs. 19 and 20. Isogenic *sinR* derivatives of these strains were constructed by transducing the *kan* Δ*sinR* marker from strain EG5229.

To map the position of the five RF clones in the *S. typhimurium* genome, we hybridized vector-free DNA fragments from each clone to filters containing an ordered array of DNA prepared after induction of strains harboring Mud–P22 prophages scattered around the *Salmonella* chromosome (21). The RF clones were originally generated by insertion of *Bam*HI fragments into the *Bam*HI site of the plasmid vector YEp13 (13). To determine the base ratios of each of the five clones, the inserts were released from the vector by digestion with *Bam*HI, separated by agarose gel electrophoresis, recovered by electrophoresis onto DEAE membranes (Schleicher & Schuell), and processed as described (22).

To construct strains harboring chromosomal insertion/deletions, we used P22-mediated transduction to introduce plasmids harboring the mutated DNA into *polA* recipients. We selected for the kanamycin-resistance marker present in the mutated plasmid insert and screened for loss of the vector-encoded ampicillin-resistance marker. The kanamycin-resistance gene introduced into the mutated plasmids was from either pUC4-K (Pharmacia) or pEG5086 (23). Metabolic capabilities of mutant strains were assayed on the Biolog ES MicroPlate (Biolog, Hayward, CA). Virulence properties of the mutant strains were examined by inoculating BALB/c mice by either the intragastric or the intraperitoneal routes, using doses ranging from 5 to 50 times the LD_{50} of the wild-type isogenic parent strain.

For Southern blot hybridization experiments, 1 μg of DNA from the bacterial species listed in Table 2 was digested with *Eco*RI and electrophoresed through an 0.8% agarose gel at 8 V/cm in 0.5× TBE (1× TBE = 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3). After electrophoresis, the DNA was transferred to a nylon support membrane [Photogene (BRL)], and hybridizations to each radiolabeled *Bam*HI insert proceeded for 18 h at 65°C in high phosphate buffer (0.5 M NaCl/0.1 M sodium phosphate, pH 7.0/5 mM EDTA) supplemented with 0.2% Sarkosyl. Prior to autoradiography, filters were washed for two 30-min periods at 65°C in 0.1× standard saline citrate/0.1% SDS. Hybridizations to filters containing DNAs from natural isolates of *E. coli* followed identical procedures. For nucleotide sequencing, CsCl-purified plasmid RF321 as well as subclones of the *Bam*HI insert were treated as described (24) using Sequenase (United States Biochemical). The initial round of sequencing employed oligonucleotide primers complementary to the region adjacent the *Bam*HI site of the YEp13 vector (5′-TATCGACTACGCGATCA-3′ and 5′-ATGCGTCCGGCGTAGA-3′). To obtain sequence information beyond the range of individual primers, we constructed additional internal primers as needed to sequence both strands of the DNA template. Analyses of nucleotide sequence data were performed using the GCG program package (25). Other molecular biological protocols were performed as described (26).

RESULTS

Characterization of RF Clones. The *Salmonella*-specific fragments were heterogeneous with respect to base composition, map location, and phylogenetic distribution (Table 1). The sizes of the cloned inserts were identical to those determined by Fitts (13), except that RF305 was supplied as an 8.8-kb fragment containing two internal *Bam*HI sites. As reported by Fitts (13), the five clones had different restriction maps, suggesting that each fragment originated from distinct regions of the chromosome. The map position of each clone was determined by hybridization to an ordered library of *Salmonella* DNA and two of the five clones—RF319 and RF356—were situated between 57 and 60 minutes. Since the level of resolution of this mapping procedure is ≈3 minutes or 150 kb, it is not possible to establish the exact location, order, or proximity of these two clones. However, based on the restriction maps and G+C contents, clones RF319 and RF356 do not represent duplicate isolates. Clone RF321—originally mapped between 4 and 7 minutes—was found to be 6% linked to *proBA* by phage P22 transduction in experiments using strains harboring insertion/deletions in this region.

In that the G+C content of bacterial genomes is relatively homogeneous over the entire chromosome and atypical base compositions are often taken as evidence of horizontal transfer (27), we determined the base ratios of each insert to establish the ancestry of these clones. Three of the RF inserts—RF319, RF321, and RF333—had base compositions of ≈45% G+C, much lower than the overall G+C content of 52–54% estimated for *Salmonella*. The RF clones with low G+C contents resided at distinct positions on the chromosome, demonstrating that each arose by an independent event.

Phylogenetic Distribution of RF Clones. The five RF clones were originally designed as probes for the selective detection of *Salmonella* because they were present in a majority of salmonellae and absent from other enteric species (13). To delineate the phylogenetic distribution and potential origin of each insert, we performed a series of hybridizations to filters containing genomic DNAs from several species of bacteria (Table 2). Only one of the clones—RF333—was confined to *Salmonella*; others varied in their distributions among the tested organisms. Aside from its presence in *Salmonella*, RF321 was also detected in an isolate of *Klebsiella pneumoniae* and subsequent analysis revealed that the hybridization signal produced by RF321 corresponded to a plasmid present in the tested strain of *Klebsiella*.

Interestingly, RF319 was present in *Shigella flexneri* but not in the single isolate of *E. coli* K-12 included in the survey of representative bacterial species (Table 2). Since all species of *Shigella* are more closely related to *E. coli* than to *Salmonella* (28), we examined a genetically diverse collection of 72 isolates of *E. coli* (29) for sequences that hybridize to RF319. Twenty of these strains contained sequences homologous to RF319, and in more than one-third of the cases (7/20), the hybridizing sequences were associated with plasmids, which

Table 1. Physical properties and map positions of *Salmonella*-specific clones

Name	Size, kb	G+C content, %	Map position, minutes
RF305	8.6	53.4	54–57
RF319	6.0	45.2	57–60
RF321	4.9	44.9	4–7*
RF333	5.7	44.5	28.5–31.5
RF356	4.2	53.1	57–60

*There is 6% linkage of RF321 to *proBA* indicating that its map position is adjacent to 7 minutes at ≈20 kb counterclockwise from *proBA*.

Table 2. Distribution of *Salmonella*-specific clones among other bacteria

Bacteria	DNA sequence				
	RF321	RF333	RF305	RF319	RF356
S. typhimurium	+	+	+	+	+
E. coli			+		+
Shigella flexneri			+	+	+
Citrobacter freundii			+		
Klebsiella pneumoniae	+		+		+
Enterobacter aerogenes			+		
Enterobacter cloacae					
Proteus vulgaris					
Providencia stuartii			+		
Erwinia herbicola			+		
Erwinia carotovora			+		
Serratia marsescens					
Zymomonas mobilis					+
Pseudomonas aeruginosa					
Bacillus subtilis					

Presence or absence of homologs to DNA sequences from RF clones was determined by Southern blot hybridization using vector-free fragments. + indicates the presence of a hybridization signal in the designated species.

could account for its dissemination among species. Natural isolates more closely related to *E. coli* K-12 did not contain sequences that hybridized to RF319, whereas those strains genetically similar to *Shigella* spp. produced a positive signal.

As expected, the two clones with G+C contents typical of *Salmonella* DNA hybridized to the largest number of species (Table 2). Although these clones, RF305 and RF356, mapped to adjacent regions and had very similar base compositions, the differences in their phylogenetic distributions further indicate that these fragments did not share a common evolutionary history.

Nucleotide Sequence Analysis of RF321. Based on its anomalous G+C content and its phylogenetic distribution, RF321 was selected for further evaluation. To identify features that may relate to its origin in the *Salmonella* genome and to uncover open reading frames that may elucidate its function, we determined the nucleotide sequence of the 4.9-kb insert in clone RF321. The base composition of the insert was not uniform: there were stretches with G+C contents as low as 30% and others with contents >52% (Fig. 1). We identified 12 open reading frames with the capacity to encode proteins of at least 67 amino acids and only the largest showed significant similarity to known proteins in the GenBank data base (Release 67.0).

The large open reading frame in the RF321 insert spanned ≈1 kb—the typical length for a bacterial coding region—and encoded a potential protein with a predicted molecular weight of 36,322. Analysis of this open reading frame revealed a G+C content of <40%. Consistent with the possibility that the RF321 segment was acquired horizontally, this open reading frame showed a base composition at the third positions of codons of only 35% G+C and utilized codons rarely observed in *Salmonella* genes. The protein encoded by this gene exhibited a high degree of similarity to the LysR family of transcriptional regulators (Fig. 2) and was named SinR for *Salmonella*-insert regulator. This family of proteins includes OxyR, whose activity is directly affected by oxidation (30), *Rhizobium* NodD (31), and *Citrobacter* AmpR (32), which are modified by small effector molecules. SinR did not show a bias in the Lys/Arg ratio, usually detected in this family of regulators (33). Several *sinR* homologues are transcribed divergently from operons that fall under their transcriptional control; however, inspection of the regions adjacent to *sinR* failed to reveal other long open reading frames.

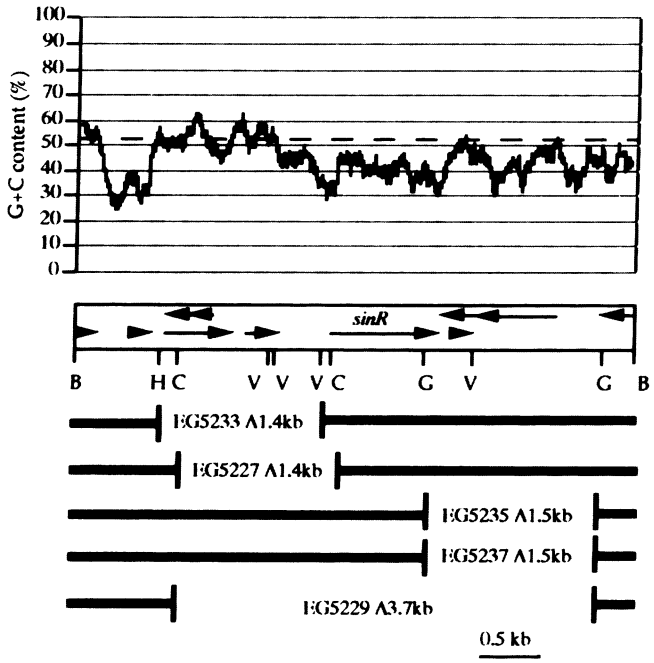

FIG. 1. (*Upper*) G+C content of the *Bam*HI insert of clone RF321. The graph was created using the program DELTA GRAPH PRO on a Macintosh computer with data generated by the program WINDOWS (GCG) to determine the percentage G+C (window, 100 bases; sliding increment, 1). The *y* axis indicates percentage G+C content, and the *x* axis corresponds to the nucleotide sequence plotted in the middle position for that window [i.e., the first data point corresponds to position 50 (window 1–100), the second data point corresponds to position 51 (window 2–101), etc., up to position 4830]. Broken line at 52% indicates the reported G+C content of *Salmonella* DNA. (*Lower*) Restriction map of *Bam*HI insert of clone RF321. Position and orientation of open reading frames >67 amino acids long are represented by arrows. Restriction enzyme sites are as follows: B, *Bam*HI; C, *Bcl* I; G, *Bgl* II; H, *Hin*dIII; V, *Eco*RV. Size and position of chromosomal segments absent from five strains harboring insertion/deletions in the *sinR* region. Sizes of deleted DNA are indicated in kilobase pairs next to the name of each construct. Strains EG5235 and EG5237 lack the identical 1.5-kb region but contain the same *kan* insert in opposite orientations.

Mutational Analysis of the RF321 Chromosomal Region: *sinR* Is Not an Essential Gene. To evaluate the potential functions of SinR, we constructed five strains harboring insertion/deletions ranging from 1.4 to 3.7 kb in this region of the chromosome (Fig. 1). Strains harboring these mutations were viable, indicating that the deleted regions did not encode genes essential for growth in LB agar. The mutants were not auxotrophs and were able to grow on minimal medium containing glycerol, glucose, succinate, malate, acetate, or galactose as sole carbon and energy sources. Deletion strains showed patterns identical to those produced by the wild-type parent strain in their biochemical properties tested with Biolog plates, which assay the ability to utilize 95 carbon sources. We attempted to identify potential targets of SinR regulation by screening a collection of 23 *lac* gene fusions previously described as being regulated by various environmental conditions including phosphate concentration (19) and oxygen tension (20). As shown by the Lac phenotype of isogenic constructs, SinR did not appear to be involved in the control of any of these loci.

To investigate the role of SinR in the control of virulence functions, we tested the mutants for their ability to grow on plates containing protamine and also to cause a lethal infection in mice. All mutants behaved like the wild-type strain in protamine agar plates, an ability that has been shown to be necessary for mouse virulence (E.A.G., unpublished data).

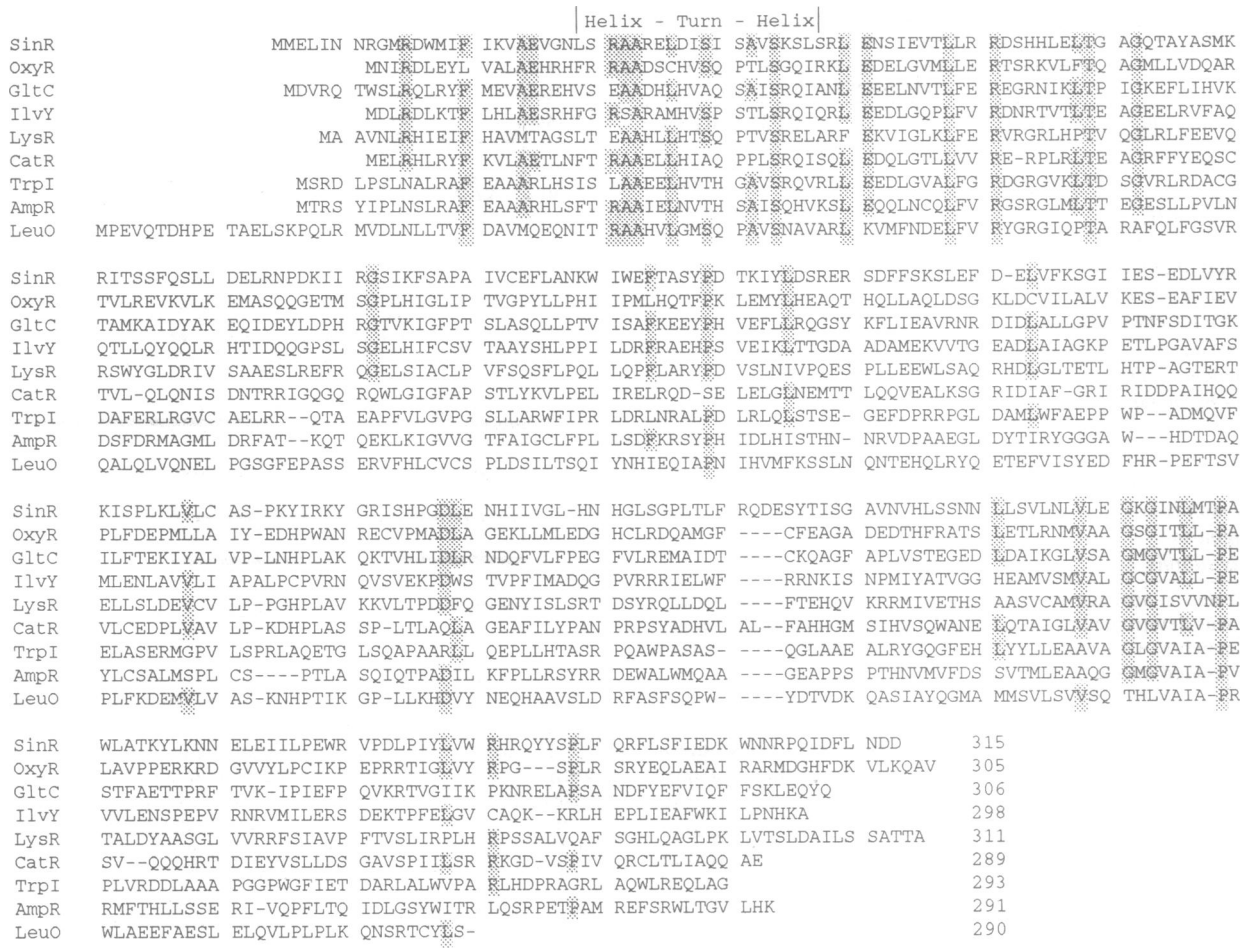

FIG. 2. Alignment of SinR and eight additional members of the LysR family of transcriptional regulators. Sequences were obtained from GenBank, EMBL, Swiss-Prot, and the National Biomedical Research Foundation Protein Information Resource data bases. Positions of residues shared by SinR and at least four other proteins are shaded.

We then tested their virulence properties directly by inoculating BALB/c mice with doses equivalent to 5 and 50 times the LD_{50} by both the oral and intraperitoneal routes of inoculation and found that the strains were still virulent. This indicated that the mutated sequences do not contribute to a virulence phenotype associated with disease for mice.

DISCUSSION

Ancestry of *Salmonella*-Specific Sequences. The genomes of the enteric bacteria *E. coli* and *S. typhimurium* show conservation in size and genetic organization, but differ with respect to ≈300 kb (10–12). In this paper, we have analyzed five clones that had been previously described as being confined to the salmonellae (13). The two clones with G+C contents similar to that of *Salmonella* harbored DNA sequences that hybridized to a variety of enteric species (Table 2). The remaining three clones had base compositions much lower than that of the *S. typhimurium* chromosome, suggesting acquisition via a horizontal transfer event from a distantly related organism. These data reinforce the view of a mosaic structure of the *Salmonella* chromosome recently postulated after analysis of the *phoN* (34) and *rfb* (35, 36) loci. The most likely mechanism of genetic acquisition in *Salmonella* is conjugation and, in support of this view, we have established that one of the clones of low G+C contents—RF321—is associated with plasmid sequences in *Klebsiella pneumoniae*. Moreover, 400 bp upstream of the *sinR* locus is a 72-bp region with 78% identity to a portion of the 145-kb plasmid harboring the gene for the heat-stable toxin I (*estA1*) from *E. coli* 18D (37). The *estA1* gene is generally flanked by insertion elements (37, 38) that could promote its transfer between species. We detected no *estA1* homologues in the nucleotide sequence for RF321 (Fig. 1) or in hybridization studies to *S. typhimurium* DNA.

Two of the RF clones mapped to the 57–60 minutes, a region known to encode characters that are *Salmonella*-specific: *hin*, which is necessary for phase variation of flagellar genes (39), the *tct* operon required for transport of tricarboxylates (40), and loci required for invasion of epithelial cells (4, 41, 42). Based on the restriction enzyme profiles of RF319 and RF356, these clones do not correspond to genes already mapped to this region. The clone containing *sinR* mapped approximately a half minute counterclockwise of *proBA*. No loci have been mapped to this region of the *S. typhimurium* chromosome (43), suggesting that genes corresponding to this segment are involved in unexplored metabolic activities. This clone, as well as RF333 (localized at 28.5 to 31.5 minutes), mapped to regions that are devoid of recognized loops indicating that the portion of the chromosome confined to *Salmonella* in these two regions must be <20 kb.

***Salmonella* Regulators and the Control of Virulence Functions.** Analysis of RF321 revealed the presence of an open reading frame, SinR, with a high degree of similarity to the LysR family of transcriptional regulators. SinR harbored a helix–turn–helix motif characteristic of this family of proteins and is most likely involved in binding target DNA molecules. Despite the screening of >120 phenotypes and the production of β-galactosidase for a collection of environmentally-

regulated *lac* gene fusions accounting for ≈1% of the genome (19, 20), we were unable to define the particular physiological or environmental conditions in which SinR is required. Our experiments did indicate that *sinR* is not an essential gene and is not required for virulence in mice.

Transcriptional regulators, such as PhoP, Crp, and OmpR, are known to control virulence functions in *Salmonella* (44–47). Mutations in the genes encoding these regulators attenuate the virulence of *S. typhimurium* in mice. However, *phoP*, *crp*, and *ompR* are present in numerous nonpathogenic enteric species and their roles cannot be limited to virulence in the mammalian host. For example, mutations in *phoP* result in one of the highest attenuating effects in *S. typhimurium* by preventing growth within macrophages and rendering *Salmonella* hypersusceptible to host microbicidal peptides (5, 6). While most enterics harbor *phoP* homologues (44), they lack *phoN* (34), a *Salmonella*-specific gene regulated by PhoP with no role in pathogenesis (6).

There are three hypotheses to explain the origin of pathogenic characters in microorganisms: (*i*) the differences observed with *Salmonella* could be due to the accumulation of allelic variants in essentially the same set of genes; (*ii*) the presence of yet undescribed *Salmonella*-specific loci that, if introduced into avirulent species such as *E. coli*, would render it pathogenic; or (*iii*) the differential regulation of essentially the same complement of genes could account for the observed differences in a manner similar to that postulated by King and Wilson (48) to account for morphological differences among primates. To date, the majority of loci found to be unique to *Salmonella* are not involved in pathogenesis, whereas several loci involved in virulence have a wide phylogenetic distribution.

Function of *Salmonella*-Specific Sequences. Because the ecology of *Salmonella* is characterized by short periods in animal hosts, one might not expect the majority of genes to be involved in virulence. Consistent with this postulate, it has recently been shown that only 5% of the transposon insertion mutants of a wild-type *S. typhimurium* strain affect virulence patterns in mice (E.A.G. and F. Heffron, unpublished data). It is likely that sequences confined to salmonellae are required for survival within an ecological niche specific for this organism outside the animal host. A reflection of a response to environmental variables is exemplified by the systems involved in phosphate scavenging in enteric bacteria. The *Salmonella*-specific *phoN* encodes a nonspecific acid phosphatase with no apparent role in virulence (6, 34) and *E. coli* harbors an alkaline phosphatase with no counterpart in the *S. typhimurium* genome.

SinR may regulate other *Salmonella*-specific loci or control expression of genes also present in other enterics but in response to different types of environmental cues. Identification of the targets of SinR regulation may shed light on the time and circumstances in the life cycle of *Salmonella* where this protein is active and provide information on the habitats of this organism.

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