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Selective Capture of *Salmonella enterica* Serovar Typhi Genes Expressed in Macrophages That Are Absent from the *Salmonella enterica* Serovar Typhimurium Genome

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Thirty-six *Salmonella enterica* serovar Typhi-specific genes, absent from the *Salmonella enterica* serovar Typhimurium genome, that were expressed in human macrophages were identified by selective capture of transcribed sequences. These genes are located on 15 unique loci of the serovar Typhi genome, including *Salmonella* pathogenicity islands (SPI-7, SPI-8, and SPI-10) and bacteriophages (ST15, ST18, and ST35).

Salmonella enterica is composed of more than 2,400 serovars that can infect humans and a great diversity of mammals, birds, and reptiles (17). Some *Salmonella* serovars are closely related genetically but differ in their host range. For example, *Salmonella enterica* serovar Typhi is the etiologic agent of typhoid fever and is a host-adapted serovar that is specific for humans, whereas *S. enterica* serovar Typhimurium is a broad-host-range pathogen. Since there is no animal model to study serovar Typhi pathogenicity, little is known about the specific factors contributing to its ability to cause typhoid fever and its adaptation to the human host. Consequently, what is known about serovar Typhi pathogenicity has been largely extrapolated from studies of serovar Typhimurium infections in mice. Although serovar Typhi and serovar Typhimurium share many virulence properties, each serovar causes a distinct type of disease in humans. Serovar Typhi is associated with systemic infections, whereas serovar Typhimurium is usually associated with localized gastroenteritis. The complete genome sequences of serovar Typhi strains CT18 and Ty2 and serovar Typhimurium strain LT2 are now available (7, 21, 23). There are significant differences between serovar Typhi and serovar Typhimurium, and unique regions of serovar Typhi DNA are scattered along the chromosome. Such unique regions can be single genes or groups of gene blocks (up to 100 genes). In a comparison with serovar Typhimurium LT2, 601 genes (13.1% of the serovar Typhi genome) in 82 regions were found to be unique to serovar Typhi CT18, and in a comparison with serovar Typhi, 479 genes (10.9% of the serovar Typhimurium genome) were found to be unique to serovar Typhimurium (21). Gene clusters unique to particular bacteria are likely to contribute to adaptation to particular environments or host niches and may contribute to pathogenicity. Thus, we can consider the possibility that serovar Typhi possesses unique genetic information that may be important for systemic spreading and survival in the human host.

As survival within macrophages is an essential step for *Salmonella* pathogenesis (10), we have used selective capture of transcribed sequences (SCOTS) (6, 14) to identify genes expressed by serovar Typhi within macrophages. In order to obtain sequences unique to serovar Typhi (i.e., absent from the serovar Typhimurium genome), we have used an additional strain-specific enrichment step, previously described to identify in vivo-expressed pathogen-specific genes from avian-pathogenic *Escherichia coli* (8). The human macrophage-like cells, derived from the monocyte cell line THP-1 (ATCC TIB-202), were infected with serovar Typhi ISP1820 as described previously (5, 6). The infected monolayers were lysed 2 h postinfection by the addition of TRIzol (Invitrogen), and RNA was prepared according to the manufacturer's instructions. A 5-μg sample of RNA was isolated from infected macrophages or under other growth conditions, treated with RNase-free DNase, and reverse transcribed by random priming (6, 11). Three rounds of bacterial cDNA capture were done as described previously (5, 6, 14). The cDNA mixtures obtained following SCOTS were used as probe templates or for competitive hybridization enrichment. The cDNA enrichment for serovar Typhi-specific sequences was obtained by using an excess (10 μg) of genomic DNA from serovar Typhimurium strain SL1344 as described previously (8). cDNAs were then cloned after three rounds of this competitive hybridization enrichment using the original TA cloning kit (Invitrogen) according to the manufacturer's instructions. Cloned inserts were sequenced at our sequencing facility. Database searches and DNA and protein similarity comparisons were carried out with the BLAST algorithms (1) available from the National Center for Biotechnology Information. We sequenced 60 clones, corresponding to 45 different genes, and 36 of these clones corresponded to distinct genes that are specific to serovar Typhi.

Serovar Typhi-specific genes. We identified 36 genes expressed intracellularly by serovar Typhi that are absent from serovar Typhimurium (Table 1). The genomic locations of the 36 genes were analyzed, and 15 distinct regions ranging in

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TABLE 1. Unique ORFs identified

Class	ORF	Gene and/or possible function	Locus (size in kb)	Presence[f]	
				BLAST[a]	Array[b]
SPI-7	STY4526	Hypothetical protein	STY4521–STY4680 (134)	Ty	Ty,[e]Du,[e]Pa[e]
	STY4530	*topB*, topoisomerase B			
	STY4534	Hypothetical protein			
	STY4536	*ssb*, single-strand binding protein			
	STY4539	*pilL*, putative exported protein			
	STY4573	Hypothetical protein			
	STY4574	Hypothetical protein			
	STY4589	Hypothetical protein			
	STY4643	*apl*, phage regulatory protein	Prophage $SopE_{ST}$		
	STY4650	*yjhP*, hypothetical protein			
	STY4654	*vexB*, Vi polysaccharide export			
	STY4656	*tviE*, Vi polysaccharide biosynthesis			
	STY4664	Putative DNA helicase			
	STY4669	Putative membrane protein			
SPI-8	STY3280	Bacteriocin fragment	STY3273–STY3292 (7)	Ty, Pa	Ty, Ag, Pa, Sf
SPI-10	STY4848	Putative transposase	STY4821–STY4853 (33)	Ty, Pa	Ty, Pa, Ja[e]
Prophages					
ST15	STY1635	Putative bacteriophage protein	STY1591–STY1643 (34)	Ty	Ty[c]
	STY1643	*min* DNA invertase			
ST18	STY2005	Conserved hypothetical protein	STY2003–STY2077 (49.3)[g]	Ty	Ty,[c]Cs,[e]Pa, Pc[e]
	STY2016	Putative bacteriophage protein			
	STY2020	Putative bacteriophage protein			
	STY2022	Putative exported protein			
	STY2026	Putative bacteriophage protein			
ST35	STY3663	Hypothetical protein	STY3658–STY3706 (35)	Ty	Ty
	STY3695	*pin*, DNA invertase			Du,[c,e] Mo[c,e]
Fimbriae	STY0206	*staB*, chaperone protein	STY0201–STY0207 (4.7)	Ty	Ty, Se,[d]Mo, Or
	STY0207	*staA*, fimbrial protein			
	STY3920	*stgC*, usher	STY3918–STY3922 (5)	Ty	Ty,[d]Se,[d]Du,[d]En[d]
Regulators	STY3845	*yiiF*, CopG family	STY3844–STY3845 (0.8)	Ty, Pa	Ty, Pa, Ja
	STY4412	DeoR family	STY4412–STY4415 (4)	Ty, Pa, En, Du	Ty, Pa, Du, En, Ga, Ja, Me, Mo, Mu, Or
Other	STY0115	Putative insertion sequence transposase	STY0114–STY0115 (1.6)	Ty	Ty, Pa, Ag, Mu
	STY2732	Conserved hypothetical protein	STY2731–STY2732 (2.5)	Ty, Pa, Bo, En, Du	NA
	STY3068	Hypothetical protein	STY3064–STY3071 (9)	Ty, Pa	Ty, Pa, Se, Sf
	STY3948	Hypothetical protein	STY3948–STY3950 (3)	Ty, Bo	Ty, Bo, Ag, Se, Sf
	STY3950	Hypothetical protein			
	STY4221	Putative aminotransferase	STY4216–STY4222 (6)	Ty, Pa	Ty, Pa

[a] Includes unfinished genome (see text).
[b] Microarray data are from reference 25. Listed ORF(s) were present in all tested strains of the same serovar.
[c] Microarray data are from reference 30.
[d] Southern blot hybridization data are from reference 31.
[e] Full-length region was not present in all isolates tested.
[f] Ag, serovar Agona; Bo, *S. bongori*; Cs, serovar Choleraesuis; Du, serovar Dublin; En, serovar Enteritidis; Ga, serovar Gallinarum; Ja, serovar Java; Me, serovar Muenster; Mo, serovar Montevideo; Mu, serovar Muenchen; Or, serovar Oranienburg; Pa, serovar Paratyphi A; Pc, serovar Paratyphi C; Se, serovar Sendai; Sf, serovar Senftenberg; Ty, serovar Typhi; NA, not available.
[g] Genes STY2038 to STY2077 are missing in strain ISP1820.

length from 0.8 kb to 134 kb and containing at least two genes per locus were identified (Table 1). SCOTS identified serovar Typhi-specific regions as small as 0.8 kb in length. BLAST comparisons with microbial genomes (including unfinished genomes) at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/sutils /genom_table.cgi) were also performed to verify the presence of the identified regions in 10 *Salmonella* strains from different serovars that have been or are currently being sequenced. Strains in the genome databases included serovar Typhi strains CT18 (7) and Ty2 (21), serovar Typhimurium strains LT2 (23), DT104 (Sanger Institute), and SL1344 (Sanger Institute), serovar Dublin (University of Illinois at Urbana-Champaign), serovar Enteritidis LK5 (University of Illinois at Urbana-Champaign), *Salmonella bongori* 12419 (Sanger Institute), serovar Paratyphi A strain ATCC 9150 (Washington University), and serovar Paratyphi B strain SPB7 (Washington University). Seven regions were present only in the two serovar Typhi strains, and five regions were present in both serovar Typhi and serovar Paratyphi (Table 1). An extensive data set of gene distributions among a diversity of 79 *Salmonella* strains (25) was also used to determine the distribution of the genes identified by SCOTS in the *Salmonella* species (Table 1).

Hybridization profiles. Expression of the identified genes under other growth conditions was verified. Serovar Typhi cDNAs were obtained by three rounds of SCOTS from bacteria grown in vitro in Luria-Bertani (LB) broth (log phase), overnight in tissue culture medium (complete RPMI medium),

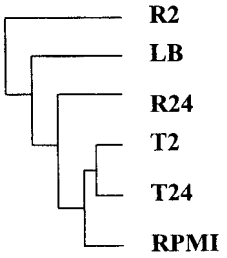

FIG. 1. Dendrogram presenting the hierarchical clustering of serovar Typhi-specific gene expression under different growth conditions, as determined by hybridization with SCOTS cDNA probes. Signal intensities were background subtracted and normalized to the IS*200* control. The log (base 2) of this ratio was used for hierarchical clustering and graphical representation.

or following infection of THP-1 human macrophages for 2 h and 24 h (T2 and T24) or RAW 264.7 murine macrophages for 2 h and 24 h (R2 and R24). These cDNAs were then used as probes against the 36 serovar Typhi-specific genes by Southern blotting, using the Dig High Prime DNA labeling and detection starter kit II (Roche Diagnostics). The hybridization signals for each gene product were quantified by densitometry. SCOTS cDNA was previously used as a probe hybridized to a membrane array to investigate global gene transcription by *Helicobacter pylori* (15). The hybridization results from the different experiments were clustered together using default parameters of the hierarchical clustering (HCL) function of the TIGR MeV (27) (Fig. 1). The results of the hybridization profile experiments using infection of murine macrophages (R2 and R24) were closer to those of the in vitro experiments (with LB broth and RPMI medium) than to those of the experiments using human macrophages (T2 and T24) (Fig. 1). This could be because the phenotype of serovar Typhi is host restricted, because only serovar Typhi-specific genes were analyzed, or because many of the serovar Typhi genes expressed in human macrophages are repressed in both LB broth and murine macrophages. Three major hybridization profiles for the serovar Typhi-specific genes were obtained as follows: (i) 14 genes exhibited weaker hybridization signals when the cDNA probes from infected murine macrophages were used (Fig. 2A); (ii) 11 genes exhibited equal signal intensities in both macrophages (Fig. 2B); and (iii) 6 genes exhibited weaker hybridization signals when the T2 and T24 cDNA probes from infected human THP-1 macrophages were used (Fig. 2C). The hybridization patterns of the five remaining genes (STY0207, STY1635, STY2026, STY2732, and STY3695) did not correspond to the three main hybridization profiles.

SPIs and prophages. Among the serovar Typhi-specific sequences, 25 genes were located on *Salmonella* pathogenicity islands (SPIs) or prophage-like elements (Table 1). It is clear that pathogenicity islands such as SPIs (20), as well as phage (2), contribute to strain- or serovar-specific genomic differences and the evolution and virulence of bacterial pathogens. Sixteen of the serovar Typhi-specific genes identified are located on SPI-7, -8, or -10. SPI-7 is a 134-kb region of mosaic structure (22, 24), and 14 identified genes are located on SPI-7. Seven of these genes belong to a putative DNA transfer system (Table 1), and two genes, STY4654 (*vexB*) and STY4656 (*tviE*), are part of the *viaB* locus, which encodes the Vi capsule. The

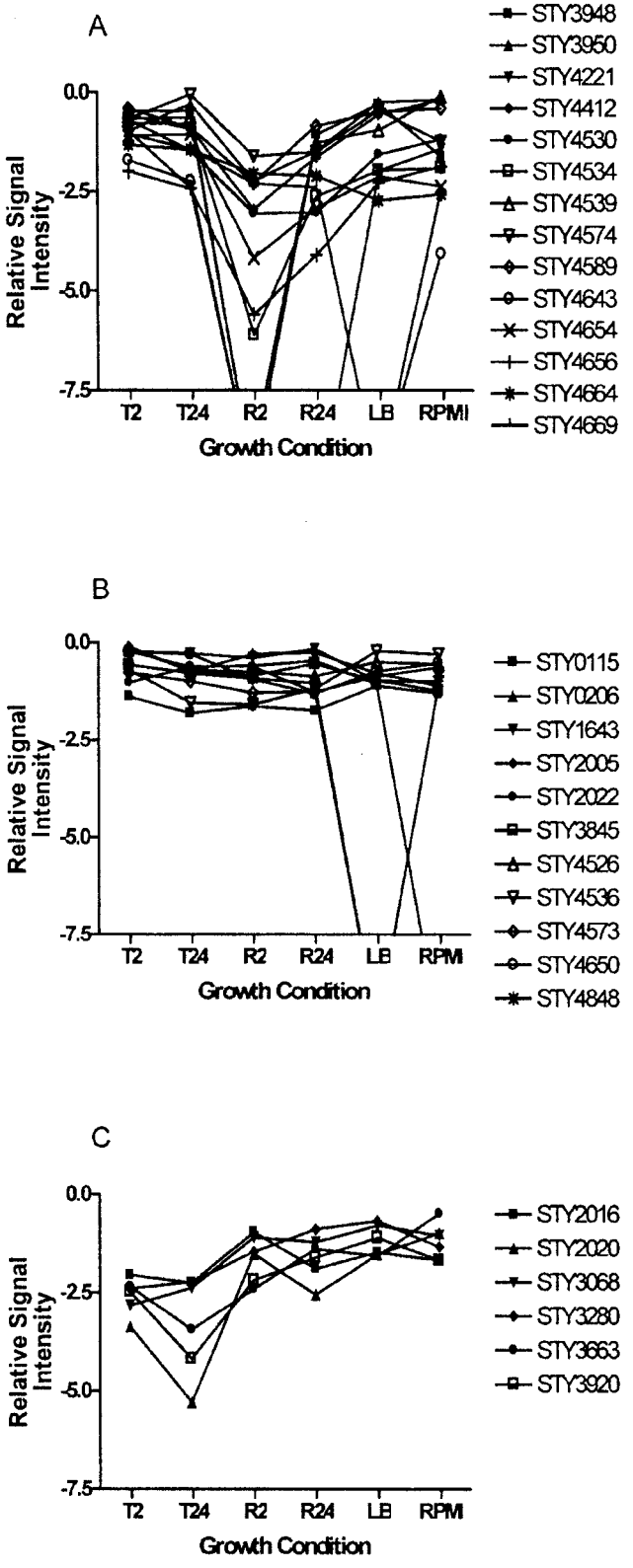

FIG. 2. Relative expression levels of serovar Typhi-specific genes under different growth conditions (see the legend to Fig. 1 for details). (A) genes with signal intensities greater in THP-1 macrophages than in RAW 264.7 macrophages; (B) genes with equal signal intensities in both macrophages; (C) genes with signal intensities lower in THP-1 macrophages than in RAW 264.7 macrophages.

Vi capsule seems to play a role in reducing early inflammatory responses from intestinal epithelial cells during infection with serovar Typhi (28). Similarly, the *viaB* locus may also influence the macrophage host response, as Vi contributes to the survival of serovar Typhi within human macrophages (16). We observed a weaker hybridization signal for the *vexB* and *tviE* genes from cDNA probes derived from serovar Typhi-infected murine macrophages than from those obtained from infected human macrophages (Fig. 2A). Thus, the Vi capsule may play a role in host specificity. In our previous studies, we also identified genes *tviB* and *vexA* of the *viaB* locus to be expressed in human macrophages (5). Hybridization signals for SPI-7 genes were always detected in human macrophages (Fig. 2A and B). We identified 10 genes carried by four different prophage-like elements: ST15, ST18, ST35, and the bacteriophage $SopE_{ST}$. Five of these genes were located on ST18, including STY2005, which codes for a hypothetical protein with a conserved GG DEF domain. The GGDEF domain is involved in signal transduction of virulence genes (12) and represents a protein family involved in the regulation of the production of cellulose and biofilms in serovar Typhimurium (13). The bacteriophage $SopE_{ST}$ is located within SPI-7 (see above), and one gene, *apl*, was identified by SCOTS. In bacteriophage 186 of *E. coli*, *apl* encodes a stress response protein that is both a repressor of *c*I and an excisionase (26). This may reflect that inside the macrophages, bacterial as well as lysogenic phage or phage-related genes respond to many stress conditions. It was previously demonstrated that phage induction in vivo was necessary for expression of virulence factors encoded by phage, such as the Shiga toxin (32, 33).

Fimbrial and pilus-encoding genes. *Salmonella* contains a number of putative fimbrial and pilus-encoding systems (19). However, overall, little is known about their function, the conditions under which these putative structures are expressed, their role in virulence, and their possible relationship to bacterial host adaptation. By using SCOTS, we have identified genes encoding two putative fimbriae, Sta and Stg, and a type IV pilus system expressed inside human macrophages. STY3920 (*stgC*) contains a premature stop codon that disrupts the expected open reading frame (ORF) encoding the usher and is therefore considered a pseudogene in serovar Typhi strain ISP1820 (data not shown) and other serovar Typhi strains (31). The Sta and Stg fimbriae were present in all serovar Typhi strains tested and demonstrated a scattered distribution within *Salmonella* serovars (Table 1). The type IV pili encoded by the serovar Typhi *pil* operon are located on SPI-7 (see above). Type IV pili of serovar Typhi facilitate bacterial entry into human intestinal epithelial cells and mediate binding to the host cell cystic fibrosis transmembrane conductance regulator. The STY4539 (*pilL*) gene did not hybridize with cDNA probes from inside murine macrophages (Fig. 2A). Some adhesins, such as thin aggregative fimbriae encoded by the *agf* (*csg*) genes and plasmid-encoded fimbriae, were previously shown to be expressed during serovar Typhimurium infection of macrophages (9). It is currently unknown whether production of either fimbriae or pili by an intracellular pathogen such as serovar Typhi when inside host cells confers a specific advantage for virulence or host specificity.

Other unique regions. Two putative transposase genes, STY0115 and STY4848 (SPI-10), were identified by SCOTS. These two transposase genes were detected constitutively (Fig. 2B). In serovar Typhimurium, some transposase genes, such as *tnpA*, STM1860, and STM2904, are also expressed intracellularly (9). Regulation of transposases is not well understood to date, and their expression may reflect the bacterial growth state (4). However, a putative transposase gene, *gipA*, carried on phage Gifsy-1 of serovar Typhimurium, enhances growth or survival of serovar Typhimurium in the Peyer's patches of the murine small intestine (29). Putative regulatory proteins encoded by STY3845 and STY4412, putative regulators related to CopG and DeoR, respectively, were identified. STY4221 is located on a 6-kb region and encodes a putative aminotransferase that shows identity with a hemolysin of *Treponema denticola* (3). We have identified two out of three ORFs (STY3948 and STY3950) in a 3-kb region that encodes hypothetical proteins that are conserved in some *Vibrio* spp. In serovar Typhi, this locus is inserted in the region corresponding to the galactonate *dgo* operon in serovar Typhimurium, which is absent from the serovar Typhi genome.

Despite the powerful genomic and bioinformatic tools available to us, we currently have a limited understanding of the molecular basis of infectious diseases. The availability of complete sequences of numerous bacterial pathogens or serovars, combined with functional genomics, will be invaluable in efforts to further understand the in vivo expression of bacterial genes and mechanisms of bacterial pathogenesis. A genomic comparison of *Salmonella* serovars Typhi and Typhimurium identified 601 serovar Typhi-specific genes (23). In this report, we were able to determine that 36 serovar Typhi-specific genes were expressed during macrophage infection. The majority of the serovar Typhi-specific genes were located on SPIs and phages, which is not surprising, as these elements are horizontally acquired or transferred (2, 20). Some of the putative proteins identified in this study that are products of genes located on SPIs, phages, or chromosomes may represent new effector proteins or fitness factors. In serovar Typhimurium, several type III effector proteins, such as SopE, are encoded by prophages; some, such as SipABCD (SPI-1) or SseABCDEFG (SPI-2), are encoded by SPIs; and others, such as SopA, SopD, and SifA, are encoded by genes located on small regions dispersed throughout the chromosome (18, 34, 35). Further characterization is needed to determine the contribution of these serovar Typhi-specific genes to pathogenesis. By using SCOTS, we have been able to identify in vivo-expressed genes unique to a specific pathogen. These kinds of data cannot be obtained solely by a comparison of genomes, and this is why functional genomic approaches such as SCOTS represent a very powerful tool to better understand bacterial pathogenesis.

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