

# *Salmonella enterica* Highly Expressed Genes Are Disease Specific

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**During in vitro broth culture, bacterial gene expression is typically dominated by highly expressed factors involved in protein biosynthesis, maturation, and folding, but it is unclear if this also applies to conditions in natural environments. Here, we used a promoter trap strategy with an unstable green fluorescent protein reporter that can be detected in infected mouse tissues to identify 21 *Salmonella enterica* promoters with high levels of activity in a mouse enteritis model. We then measured the activities of these and 31 previously identified *Salmonella* promoters in both the enteritis and a murine typhoid fever model. Surprisingly, the data reveal that instead of protein biosynthesis genes, disease-specific genes such as *Salmonella* pathogenicity island 1 (SPI-1)-associated genes and genes involved in anaerobic respiration (enteritis) or SPI-2-associated genes and genes of the PhoP regulon (typhoid fever), respectively, dominate *Salmonella* in vivo gene expression. The overall functional profile of highly expressed genes suggests a marked shift in major transcriptional activities to nutrient utilization during enteritis or to fighting against the host during typhoid fever. The large proportion of known and novel essential virulence factors among the identified genes suggests that high expression levels during infection may correlate with functional relevance.**

Gene expression in microorganisms during in vitro culture is dominated by a small number of highly expressed genes (31, 45, 59) that are predominantly involved in protein biosynthesis, maturation, and folding (transcription/translation factors, ribosomal proteins, proteases, and chaperones). The essential functions of many of the gene products, the strongly biased codon usage that corresponds to abundant tRNAs, and the biased amino acid composition that might minimize the substantial biosynthesis energy costs (1) indicate the high biological significance of these genes.

Some of these genes could also dominate gene expression in pathogenic bacteria during infection (45). Numerous studies have investigated pathogenic in vivo gene expression by diverse techniques such as in vivo expression technology (IVET), differential fluorescence induction, in vivo-induced antigen technology, and microarray transcriptome analysis (9, 38, 54, 56, 58, 60, 62, 66, 72, 74), but these studies have mostly focused on genes that are specifically induced during infection, whereas genes with high levels of in vitro activity have been mostly disregarded. The in vivo significance of such “constitutive” genes thus remains largely unclear.

We have recently developed a modified differential fluorescence induction (72) technique that allowed identification of a large number of *Salmonella enterica* promoters with very high activity in infected spleen in a murine typhoid fever model, regardless of their in vitro activity (14, 61, 72). In this strategy, a *Salmonella* green fluorescent protein (GFP) promoter trap library is sorted for high fluorescence in infected mice by two-color flow cytometry, which suppresses interfering host autofluo-

rescence (14). Utilization of unstable GFP variants such as GFP\_OVA decreases sensitivity and thus allows selective identification of very strong promoters (61, 76).

In the present study, 21 *Salmonella* promoters with high levels of activity in a different disease model, the recently described murine enteritis model (3), were identified. The combination of these promoters and previously identified promoters with high activity in the typhoid fever model enabled us to analyze highly expressed *Salmonella* genes under two distinct diseases conditions in the same host species. The results indicate an unexpected dominance of disease-specific genes and a marked shift in the overall functional profiles during enteritis and systemic disease.

## MATERIALS AND METHODS

**Bacterial strains, *Salmonella* promoter trap library, and construction of mutants.** Streptomycin-resistant virulent *Salmonella enterica* serovar Typhimurium SL1344 (41) was used for all infection experiments. *Salmonella* organisms were cultured in LB medium containing 0.4% NaCl to minimize detrimental effects of high GFP levels (D. Bumann, unpublished observations). Appropriate antibiotics (streptomycin, 90  $\mu\text{g ml}^{-1}$ ; ampicillin, 100  $\mu\text{g ml}^{-1}$ ; and kanamycin, 50  $\mu\text{g ml}^{-1}$ ) were added.

We used an episomal GFP\_OVA promoter trap library in SL1344 with about 20-fold genome coverage that was previously described (61). In brief, sheared *Salmonella enterica* serovar Typhimurium SL1344 genomic DNA fragments (each, 500 to 700 bp) were inserted upstream of a promoterless *gfp\_ova* gene on a medium-copy-number plasmid. The resulting plasmid library containing about  $1.1 \times 10^6$  independent transcriptional fusions was transformed into strain SL1344, yielding  $10^6$  independent transformants.

Fluorescence-activated cell sorter (FACS)-sorted fluorescent clones were characterized by PCR amplification of the inserted DNA fragment with flanking primers up2 (5'-GTGATGTCGGCGATATAG) and do2 (5'-GAATTGGGA CAACTCCAG), followed by restriction fragment fingerprinting using frequently cutting endonucleases Tsp509I, AluI, and HpaII. Nonredundant clones were sequenced using primer do (5'-TACTCATATGTATATCTCCTTCTTA). Associated *Salmonella* genes were identified by comparison with the genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Both the primary annotation (53) and the somewhat different annotation of The Institute for Genomic Research (available at <http://www.tigr.org>) were used. Three transcrip-

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tional fusions were located within regions of the *Salmonella* genome sequence that lack annotated genes in the right orientation (fragment I, nucleotides 1656707 to 1656128 [nucleotides in the genome sequence]; fragment II, nucleotides 2807962 to 2807445; fragment III, nucleotides 1334351 to 1333800), and these were excluded from further analysis (see Results).

Strains with a chromosomal *gfp* insertion were constructed as previously described (61). In brief, *gfp.mut2* was fused to a kanamycin resistance cassette by PCR, and products were integrated into the *Salmonella* chromosome at the predicted start codon of the respective gene using the temperature-sensitive helper plasmid pKD46 carrying a Lambda Red recombinase expression cassette (22). This recombinase protects the ends of linear DNA fragments and mediates homologous recombination with extraordinary efficiency, thus enabling directed chromosomal insertion of PCR-generated fragments with very short (40-bp) terminal homologous regions. Correct transformants were identified using appropriate flanking and internal primers, and pKD46 was cured during growth at 37°C, as verified by loss of resistance to ampicillin. For constructing deletion strains, target genes were replaced by the kanamycin-resistance cassette by Lambda Red recombinase-mediated homologous recombination (22) with the PCR primers described at <http://falkow.stanford.edu/whatwedo/wanner/stmA-primers>.

**Mouse *Salmonella* infection models.** A recently described enteritis model has been developed for C57BL/6 mice (3). To allow direct comparison to our previous data set (61), we used BALB/c mice instead. For the enteritis model, mice were pretreated with 20 mg streptomycin; 1 day later, mice were intragastrically infected with 10<sup>9</sup> CFU *Salmonella* cells as previously described (3). At 1 day or 2 days postinfection, mice were killed, and ceca were prepared and homogenized. Each cecum generally contained 10<sup>7</sup> to 10<sup>8</sup> CFU *Salmonella* cells and showed typical signs of inflammation that are dependent on functional *hilA* (data not shown), as observed previously for C57BL/6 mice (3).

For the typhoid fever model, mice were systemically infected with 100 to 200 CFU by tail vein injection. After four days mice were killed and the spleen was removed for analysis. At this stage, spleen contained 10,000 to 40,000 CFU.

To determine virulence defects, groups of five streptomycin-pretreated mice were infected intragastrically (enteritis) or groups of five untreated mice were infected systemically (typhoid fever) with a mixture of wild-type and mutant *Salmonella* strains. Two days (enteritis) or 5 days (typhoid fever) postinfection, mice were killed and the CFU numbers in the cecum or spleen were determined for both wild-type and mutant strains by plating on selective media. The competitive index (CI) was calculated as (output mutant/wild-type ratio)/(input mutant/wild-type ratio). The significance of growth defects of the mutants compared to wild-type *Salmonella* was evaluated using the *t* test on logarithmically transformed CI data (expectation value CI = 1; log CI = 0).

**Sorting of promoter trap library and single-clone analysis.** To identify clones carrying transcriptional fusions with high levels of activity in a murine enteritis model, three streptomycin-treated mice were intragastrically infected with the *Salmonella* promoter trap library (10<sup>9</sup> CFU). One day later, ceca were removed, homogenized in phosphate-buffered saline (PBS), and treated with 0.1% (final concentration) Triton X-100. After 100-fold dilution into PBS, GFP\_OVA-expressing *Salmonella* cells were sorted by two-color flow cytometry using a biosafety level 2-equipped high-speed sorter (FacsDIVA; BD Biosciences) as previously described (14, 61). In brief, background fluorescence from other gut particles interfering with detection of GFP-expressing *Salmonella* was suppressed based on ratiometric comparison of green and orange emission intensities. Compared to background fluorescence, GFP-expressing *Salmonella* strains have a substantially higher green/orange emission ratio (14). In addition, forward and sideward scatter properties were used to further distinguish *Salmonella* cells from larger particles. An appropriate scatter gate was set using cecal contents of mice infected with a brightly fluorescent *Salmonella* strain that could be easily identified by green and orange emissions alone. The flow cytometer was calibrated each time with previously established calibration standards (76).

From the pooled cecal contents of three *Salmonella* library-infected mice, 40,000 *Salmonella* cells containing >20,000 GFP\_OVA molecules were sorted and cultivated on agar plates. The clones were pooled, and a total of 10<sup>9</sup> CFU was administered to three streptomycin-treated mice. One day later, cecal contents were prepared as described above and pooled. A total of 4,000 *Salmonella* cells containing >100,000 GFP molecules were sorted and cultivated on agar plates.

To compare *Salmonella* gene expression during enteritis and typhoid fever, we also included some of our previously identified typhoid fever-associated promoters (61). In that study, many overlapping promoter-carrying inserts had been obtained in different sort cycles. For each promoter, only the activity of the fusion with the smallest distance between insert end and start codon of the respective gene was measured. In the present study, we used only those fusions that were obtained from a single sort cycle for high activity in vivo, while the

other original sort cycles selecting for certain in vitro activities in LB culture were disregarded. This was done to avoid any in vitro bias in comparing data from the two disease models. As a result, for some promoters we had to measure fusions other than those of the previous study. The resulting combined data set contained 50 different promoters (Tables 1 and 2).

GFP\_OVA expression of individual clones was analyzed in the enteritis and typhoid fever models in mouse infection experiments by two-color flow cytometry using a FACS SORT (BD Biosciences) flow cytometer. For several fusions (35 in the typhoid fever model and 13 in the enteritis model), two to eight independent replicate measurements were done, and a median coefficient of variance of 25% was obtained. The results of independent measurements (where applicable) are shown in Tables 1 and 2. The activity of 10 identified promoters was also determined using single-copy chromosomal *gfp* fusions (Table 3; two independent replicates each). The flow cytometer was calibrated each time with previously established calibration standards (76). In contrast to the sorting of the libraries (see above), samples for these experiments were fixed with 2% formalin. This treatment decreased fluorescence intensities by about 25%, in agreement with previous observations (35), and this was taken into account for calculating GFP contents. The fluorescence intensity of fixed samples was constant over several hours of incubation at 4°C, thus permitting comparative measurements of different samples.

**Determination of GFP and GFP\_OVA fluorophore formation and degradation.** Logarithmic- or stationary-phase in vitro cultures or freshly prepared cecal or splenic homogenates containing GFP- or GFP\_OVA-expressing *Salmonella* strains were diluted 10 fold in prewarmed (37°C) and aerated LB medium containing chloramphenicol (170 µg ml<sup>-1</sup>) to block de novo protein synthesis. The mixtures were vigorously agitated at 37°C; after different time intervals, aliquots were removed and fixed in 2% formaldehyde in PBS. GFP or GFP\_OVA fluorescence was then quantified by two-color flow cytometry.

## RESULTS

### GFP detection in the gut despite limited oxygen availability.

Our strategy to identify *Salmonella* promoters with high levels of activity during murine enteritis was based on GFP fluorescence detection. GFP fluorophore formation is oxygen dependent (37) and thus might be impaired in the anaerobic gut environment (36, 63). To evaluate this potential problem, streptomycin-treated mice (3) were infected with initially non-fluorescent stationary-phase *Salmonella* cells carrying a transcriptional *gfp\_ova* fusion to the ribosomal promoter *P*<sub>rpsA</sub> (Fig. 1A). Two-color flow cytometry of cecal contents obtained 8, 24, or 48 h postinfection revealed *Salmonella*-like particles with high green fluorescence (Fig. 1B; data not shown) and emission ratios typical for GFP (14), indicating that GFP synthesis and at least partial fluorophore formation occurred in the gut. Background fluorescence emission of other gut particles including bacteria and tissue fragments could be spectrally separated from GFP fluorescence (14) by two-color flow cytometry (data not shown).

Further incubation of freshly prepared cecal content in vigorously agitated LB broth containing 170-µg ml<sup>-1</sup> chloramphenicol to block GFP de novo synthesis enhanced *Salmonella* fluorescence by 33% ± 4% (Fig. 1D, solid triangles), followed by a decline with a half-life of 88 ± 5 min that is typical for degradation of unstable GFP\_OVA (61). *Salmonella* expressing stable GFP.mut2 (19) in the gut showed a similar initial fluorescence increase during in vitro incubation in LB broth containing 170 µg ml<sup>-1</sup> chloramphenicol, but no degradation as expected (Fig. 1D, open triangles). Fluorescence enhancement was also observed when GFP\_OVA- or GFP.mut2-expressing *Salmonella* cells grown under oxygen-limiting in vitro conditions were analyzed by this assay (Fig. 1E), suggesting that the early fluorescence increase might reflect oxygen-dependent fluorophore formation in a fraction of initially non-

TABLE 1. *Salmonella* operons with high expression during enteritis

First locus <sup>a</sup>	Gene	Operon <sup>b</sup>	Function (classification <sup>c</sup> )	Expression level <sup>d</sup>		Previous evidence for expression/attenuation <sup>e</sup> (reference)
				Enteritis	Typhoid fever	
STM3668	<i>yiaK</i>	<i>yiaK-O-lyxK-sgbHUE</i>	Uptake and utilization of lyxose ascorbate (P)	470	30	NA
STM4501		<i>STM4501-4500</i>	Putative cytoplasmic protein, putative SAM-dependent methyltransferase (U)	380	40	NA
STM4315	<i>rtsA</i>	<i>rtsAB</i>	Regulator of SPI-1 expression (V)	290	b.t.	NA
STM0974	<i>focA</i>	<i>focA-pflB</i>	Formate transporter, pyruvate formate lyase (P)	300, 210	b.t.	exp (78)
STM2283	<i>glpT</i>	<i>glpTQ</i>	Glycerol transporter (P)	240	30	exp (15)
STM2344		<i>STM2344-2340</i>	PTS system, utilization of unknown carbohydrates (P)	210	40	NA
STM0744	<i>ybgC</i>	<i>ybgC-tolQRAB-pal-ybgF</i>	Maintenance of cell envelope integrity (P)	210	35	att* (tolB) (8)
STM3881	<i>rbsD</i>	<i>rbsDACBK</i>	Ribose ABC transporter (P)	230, 170	40, 35, 30, 25	exp (15)
STM0608	<i>ahpC</i>	<i>ahpCF</i>	Alkylhydroperoxide reductase (P)	180	b.t.	NA
STM2876	<i>hilA</i>	<i>hilA-iagB</i>	Regulator of SPI-1 expression, cell invasion protein (V)	270, 120	b.t.	att (3, 69)
STM1328			Putative outer membrane protein (U)	170	b.t.	NA
STM2328	<i>nuoA</i>	<i>nuoA-N</i>	NADH dehydrogenase I (P)	140	50	NA
STM2875	<i>hilD</i>		Regulator of SPI-1 expression (V)	120, 100, 90, 85, 85, 80, 80	65, 45	NA
STM2330	<i>lrhA</i>		Regulation of flagella, motility, chemotaxis genes (P)	160, 130, 80, 70	15, b.t.	NA
STM2886	<i>sicA</i>	<i>sicA-sipBCDA-iacP</i>	SPI-1 effector proteins and chaperons (V)	120, 90	b.t.	att (3, 69)
STM1155	<i>htrB</i>		Lipid A biosynthesis (P)	105	b.t.	NA
STM0260	<i>dniR</i>		Regulator of nitrite reductase (P)	90	40	NA
STM0191	<i>fhuA</i>	<i>fhuACDB</i>	Ferrichrome uptake system (P)	130, 45	b.t.	exp (38)
STM2867	<i>hilC</i>		Regulator of SPI-1 expression (V)	40	b.t.	NA
STM4423			Putative AraC-type DNA-binding domain-containing protein (U)	35	b.t.	NA
STM2079	<i>wzzB</i>		LPS synthesis (P)	35, 20	15	NA

<sup>a</sup> First locus downstream of the identified promoter. Loci with the prefix STM were annotated by McClelland et al. (53).

<sup>b</sup> Predicted operons according to published data and operon prediction at TIGR.

<sup>c</sup> Classification into functional categories: P, physiological; V, virulence; U, unknown function.

<sup>d</sup> GFP\_OVA levels in ceca of infected streptomycin-pretreated mice or in spleens of intravenously infected mice in 1,000 copies per *Salmonella* cell (each value represents one infected mouse); b.t., below detection threshold of 10,000 GFP copies per cell in spleens of infected mice.

<sup>e</sup> exp, expression in gut; att, inactivation of the operon leads to loss of enteritis phenotype in mice or cattle; att\* (tolB), inactivation results in *tolB*-dependent attenuation in typhoid fever model; NA, no information available.

fluorescing GFP molecules. To account for this nonfluorescing GFP without laborious in vitro incubation steps, fluorescence intensities of immediately fixed cecal contents were multiplied by a normalization factor of 1.33. However, the major conclusions of this study would not be changed in the absence of applying this normalization factor (see below).

In contrast to this enteritis model, mice that had not received a streptomycin pretreatment had no fluorescent *Salmonella* in their ceca 1 day after intragastric infection with initially nonfluorescent *Salmonella* carrying the  $P_{\text{rpsA}}\text{-gfp\_ova}$  fusion, suggesting an absence of de novo GFP biosynthesis. Initially highly fluorescent *Salmonella* remained so in the cecum for 24 h, but aerobic incubation did not result in maturation of recently expressed GFP\_OVA; the degradation assay showed very little turnover activity (data not shown), suggesting that the detected GFP\_OVA may have been derived from the inoculum instead of being synthesized de novo in the gut. This apparent lack of ribosomal promoter activity is in agreement with the failure of *Salmonella* to colonize the gut of untreated mice or to cause intestinal pathology (3).

After systemic infection, initially nonfluorescent *Salmonella* carrying a transcriptional *gfp\_ova* fusion to the ribosomal promoter  $P_{\text{rpsA}}$  synthesized de novo GFP\_OVA in the spleen (Fig. 1C), and fluorophore formation was already complete in vivo (Fig. 1F). GFP\_OVA had a similar half-life ( $83 \pm 5$  min) in *Salmonella* in spleen (Fig. 1F) compared to *Salmonella* in gut lumen (Fig. 1D), suggesting that this degradable GFP variant allows detection of very strong *Salmonella* promoters with similar sensitivity (76) in both disease models.

**Identification of *Salmonella* promoters with high levels of activity during enteritis.** To identify *Salmonella* promoters

with high levels of activity in the gut, a diverse plasmid-based *Salmonella* GFP-promoter trap library was FACS sorted for high fluorescence in the cecal contents of streptomycin-pretreated, intragastrically infected mice. Ninety-six recovered clones were characterized by PCR restriction fragment length polymorphism, revealing 34 nonredundant clones. Sequencing of the corresponding inserts revealed 24 different promoters, 21 of which were associated with an appropriately oriented downstream open reading frame (Table 1). Three promoters were located within open reading frames or associated with open reading frames in the reverse direction (for sequence information, see Materials and Methods). Incomplete annotation by gene prediction programs, as well as transcription of noncoding genome sequences, is not uncommon (68). In particular, the identified promoters might drive expression of small RNAs or antisense RNAs, but we could not find clear evidence for that. Specific sequence properties of the insert-plasmid fusion might also have resulted in artificial promoter activities. We therefore excluded these promoters from further analysis.

High levels of in vivo activity in all identified promoters was confirmed by separate infection experiments (Table 1). To further validate the identified promoters, *gfp* was chromosomally integrated downstream of 10 selected promoters. For six of these constructs, GFP expression in the native genomic context and copy number was sufficiently high for detection in duplicate experiments (Table 3), confirming the successful identification of promoters with exceptionally high in vivo activity (35). The residual four promoters might be active but not strong enough to overcome the high detection threshold for

TABLE 2. *Salmonella* operons with high expression during typhoid fever

First locus <sup>a</sup>	Gene	Operon <sup>b</sup>	Function (classification) <sup>c</sup>	Expression level <sup>d</sup>		Previous evidence for expression/attenuation <sup>e</sup> (reference)
				Enteritis	Typhoid fever	
STM2781	<i>virK</i>		PhoP-regulated gene (V)	Part	720, 510, 480, 430, 370	att (24)
STM1602	<i>sifB</i>		SPI-2 effector protein (V)	10	300, 280, 380, 360	NA
STM1406	<i>ssaG</i>	<i>ssaG-L</i>	SPI-2 TTSS (V)	b.t.; b.t.; b.t. (+)	280, 270, 270, 220, 180, 120	exp. att (73)
STM2080	<i>ugd</i>		UDP-glucose/GDP-mannose dehydrogenase (V)	b.t.; b.t.; b.t.	160, 160, 150, 120, 65	exp (73)
STM1393	<i>ssaB</i>	<i>ssaBC</i>	SPI-2 TTSS (V)	b.t.; b.t.; b.t.	170, 150, 150, 90, 65	att (71)
STM4157	<i>sseK1</i>		SPI-2 effector protein (V)	15	150, 120, 75	NA
ST64B	<i>sb26 (sseK3)</i>		Putative SPI-2 effector protein (V*)	Part	120, 110, 75	NA
STM1397	<i>sseA</i>		SPI-2 effector protein (V)	b.t.	130, 70	exp. att (40, 79)
STM1231	<i>phoP</i>	<i>phoPQ</i>	Virulence gene regulator (V)	b.t., b.t. (+)	180, 65, 50	exp. att (38, 55, 79)
STM1330			Putative DNA/RNA nonspecific endonuclease (P)	Part	85, 70	NA
STM1672			Putative cytoplasmic protein (U)	5	90, 80, 60	NA
PSLT046			Putative carbonic anhydrase (V)	17	90, 60	exp. att (73)
STM1633	<i>mig-5</i>		Putative periplasmic binding protein (V**)	10	100, 45	NA
STM1088	<i>pipB</i>		Putative cytoplasmic protein (V)	b.t., b.t. (+)	110, 65, 55, 45	att* (47)
STM0617	<i>naa</i>		Putative effector protein (V)	10	70, 45	exp (73)
STM3195	<i>ribB</i>		RNase I (P)	190	60, 50, 45, 40	NA
STM1275	<i>yaof</i>		Riboflavin synthesis (P)	20, 10	55, 35	att (this study)
STM0701	<i>speF-potE</i>		Putative hemolysin (V)	40	45, 40	att (this study)
STM2666	<i>pheLA</i>		Polyamine metabolism (P)	230, 195, 75	60, 45, 30	NA
STM1224	<i>sifA</i>		Aromatic amino acid biosynthesis (P)	b.t., b.t. (+)	45, 40	att (6)
STM0972	<i>sopD2</i>		SPI-2 effector protein (V)	b.t., b.t. (+)	45, 40, 35	att (11)
STM1583			Putative cytoplasmic protein (U)	b.t., b.t.	50, 40, 30	NA
STM2875	<i>hilD</i>		Regulator of SPI-1 expression (V)	40, 25	50, 35, 30	NA
STM2328	<i>nuaA</i>		NADH dehydrogenase (P)	25, 20	40, 35	NA
STM1269	<i>aroQ</i>	<i>nuaA-N</i>	Putative chorismate mutase (P)	b.t., b.t.; b.t. (+)	35, 30	Not attenuated (this study)
STM2585A	<i>pagK2</i>		Homologue of <i>pagK</i> (V)	b.t. (+)	35, 30	NA
STM1333	<i>thrS</i>		Threonine tRNA synthetase (P)	125	40, 25	NA
STM0809			Putative inner membrane protein (U)	b.t., b.t.	30, 30	NA
STM2780	<i>pipB2</i>		SPI-2 TTSS effector protein (V)	Part	30, 25	att (47)
STM1444	<i>shyA</i>		Transcriptional regulator for hemolysin (MarR family) (V)	b.t., b.t. (+)	30, 25	att (50)
STM0474	<i>ybaJ</i>	<i>ybaJ-htha</i>	Putative cytoplasmic protein, negative regulator of SPI-1 expression (V)	30	25, 20	NA

<sup>a</sup> First locus downstream of the identified promoter. Loci with prefixes STM and PSLT were annotated by McClelland et al. (53).

<sup>b</sup> Predicted operons according to published data and operon prediction at TIGR.

<sup>c</sup> Classification into functional categories: P, physiological; V, virulence; V\*, virulence associated according to homology to known virulence factors or genomic context (see text); U, unknown function.

<sup>d</sup> GFP\_OVA levels in ceca of infected streptomycin-pretreated mice and in spleens of infected mice in 1,000 copies per *Salmonella* cell (each value represents one infected mouse); part, only a fraction of the total population has a GFP content higher than the detection limit of 4,000 copies per cell in ceca of infected streptomycin-pretreated mice; b.t., below detection threshold; (+), a minority of *Salmonella* cells (<10%) express detectable amounts of GFP\_OVA.

<sup>e</sup> exp. expression in infected mice; att, inactivation of the operon attenuates *Salmonella* for systemic virulence in mice; att\*, inactivation results in attenuation, but the responsible gene has not yet been identified; NA, no information available.



TABLE 3. *Salmonella* in vivo GFP expression from chromosomal promoter fusions in cecum of streptomycin-pretreated mice

Locus	Gene	Expression level in enteritis model <sup>a</sup>
STM0608	<i>ahpC</i>	12, 35
STM2283	<i>glpT</i>	10, 13
STM3881	<i>rbsD</i>	9, 15
STM0974	<i>focA</i>	8, 11
STM4315		8, 10
STM1328		7, 10
STM2330	<i>lrhA</i>	b.t., b.t.
STM2344		b.t., b.t.
STM2876	<i>hilA</i>	b.t., b.t.
STM4423		b.t., b.t.

<sup>a</sup> GFP.mut2 levels in 1,000 copies per *Salmonella* cell in ceca of infected streptomycin-pretreated mice. b.t., below detection threshold of 4,000 GFP copies per cell. Each value represents one infected mouse.

single-copy reporter constructs. In addition, autoregulatory effects might occur for promoters  $P_{hilA}$ ,  $P_{lrhA}$ , and  $P_{stm4423}$  that drive expression of regulatory genes. For example, *gfp* inserted directly upstream of the *hilA* gene might interfere

with *HilA* expression, which is required for full  $P_{hilA}$  activity (23).

Previous data qualitatively confirm the in vivo activity of six of the identified *Salmonella* promoters, which further supports our data set. In particular, *HilA* (expressed from  $P_{hilA}$ ) and *SipA* and *SipB* (expressed from  $P_{sicA}$ ) are required for full *Salmonella* virulence in murine and/or bovine colitis models, suggesting that they are expressed in the gut (33, 69, 80). In addition, an IVET study has revealed intestinal *fhuA* expression (38). Indirect evidence for bacterial expression of *rbsDACB*, *glpTQ*, and *pflB* has been provided by transcriptome analysis of *Escherichia coli* growing on intestinal mucus (15) and *Vibrio cholerae* in a rabbit ileal loop model (78).

We restricted our analysis to the small minority of exceptionally highly active promoters by using the insensitive, degradable GFP\_OVA reporter variant and very stringent sort criteria. The high redundancy of the sorted sublibrary (see above) might suggest substantial coverage of such promoters. On the other hand, the small set of 21 promoters obviously represents only a small part of all *Salmonella* activities during

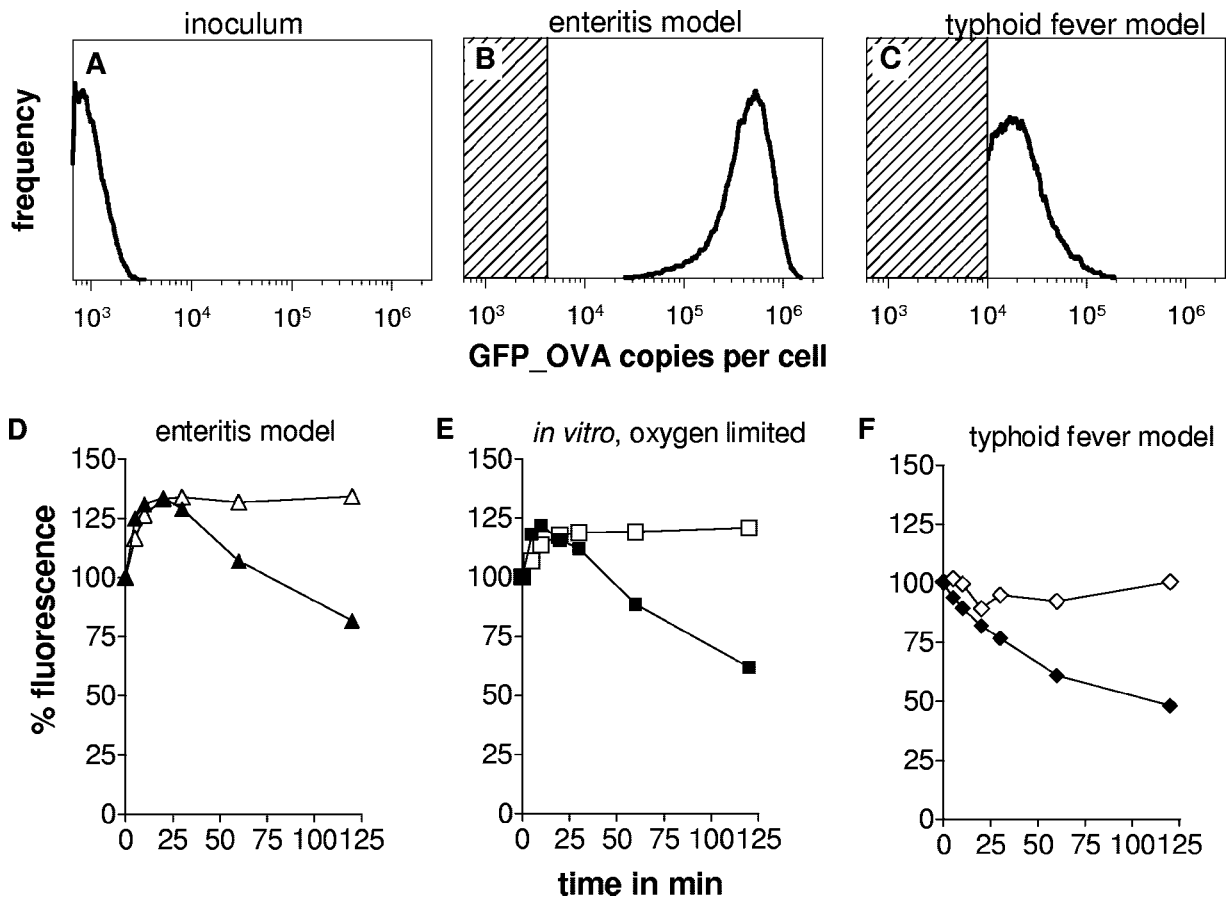


FIG. 1. GFP\_OVA content in *Salmonella* cells carrying a transcriptional  $P_{fpsA}$ -*gfp\_ova* fusion during enteritis and typhoid fever, as determined by flow cytometry. (A) Mice were infected with initially nonfluorescing *Salmonella* cells from a stationary in vitro culture. *Salmonella* were recovered 8 h after oral infection from infected ceca (B) or 4 days after systemic infection from spleen (C). The shaded areas represent GFP\_OVA levels below the detection threshold in the respective tissue homogenates. Similar data were obtained in three independent experiments. To assess GFP maturation and degradation, *Salmonella* cells expressing stable GFP (open symbols) or nonstable GFP\_OVA (solid symbols) were freshly prepared from infected ceca (D), in vitro cultures with limited aeration (E), or infected spleen (F) and incubated in fully aerated LB medium containing chloramphenicol to block de novo synthesis. At different time intervals, GFP fluorescence was determined by flow cytometry.

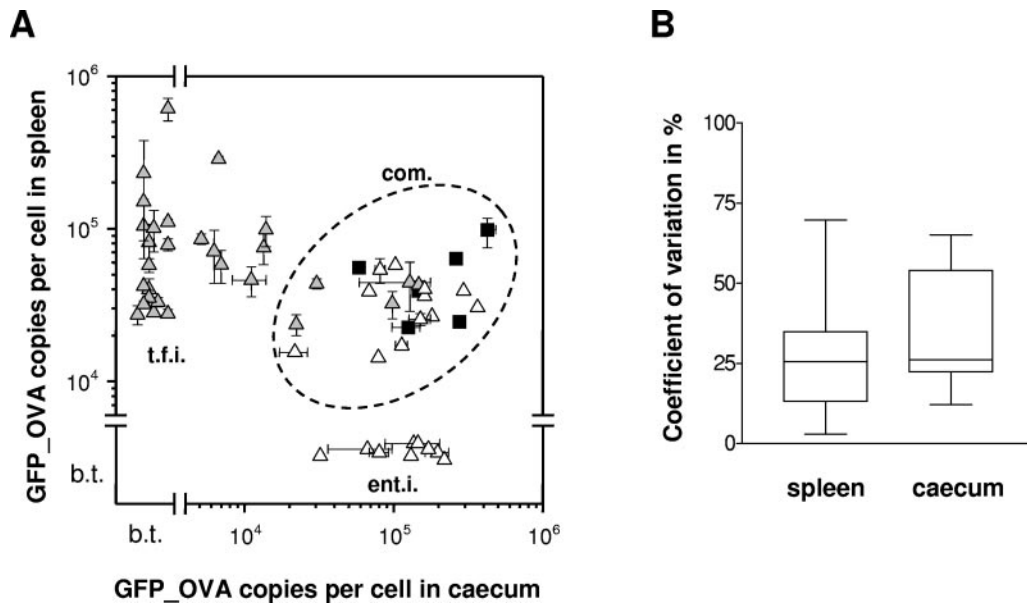


FIG. 2. (A) *Salmonella* promoter activities in murine colitis (cecum) and typhoid fever (spleen) models. Promoter fusions were originally obtained from flow cytometric sorts of infected cecal lumen (open triangles) or spleen (gray triangles). For comparison, activities of five ribosomal promoters are also shown (black squares). b.t., below detection threshold; com, commonly active promoters; ent.i., enteritis induced; t.f.i., typhoid fever induced. Error bars indicate the range of repeated measurements. (B) Coefficient of variation of results obtained from repeated measurements of individual promoter-*gfp\_ova* fusions in typhoid fever (spleen) and enteritis (cecum) models. The plots shows ranges (whiskers) and quartiles (the boxes extend from the 25th percentile to the 75th percentile, and the lines within boxes depict the medians).

enteritis. Interestingly, only 2 out of the 21 promoters identified ( $P_{hilD}$  and  $P_{nuoA-N}$ ) were previously found by our high-coverage analysis with the same *Salmonella* promoter trap library in a murine typhoid fever model (61).

**Disease-dependent *Salmonella* promoter activities.** To compare *Salmonella* gene expression during different disease conditions in the same host species, we combined the enteritis-associated promoters with 31 previously identified typhoid fever-associated promoters that were sorted for high in vivo activity (61), resulting in a set of 50 different *Salmonella* promoters with high in vivo activity (for promoter selection, see Materials and Methods; two promoters were isolated from both models). We measured the activity of each fusion in the typhoid fever and enteritis models by two-color flow cytometry (Tables 1 and 2; Fig. 2A). We carried out two to eight replicate measurements for 35 fusions in the typhoid fever model and for 13 fusions in the enteritis model. All results from individual infection experiments are shown in Tables 1 and 2, and the observed ranges are shown in Fig. 2A. We were unable to perform full triplicate measurements for all 100 settings (50 fusions in two disease models) because of restrictions on animal experimentation. However, the available replicate data revealed a median coefficient of variation of 25% for promoter activity measurements in different mice (or 7% for the log-transformed values) (Fig. 2B), and this moderate level of variance may allow semiquantitative comparisons. This comparison is further limited by analyzing only a single time point per disease model. Pathogen gene expression during infection can considerably vary over time (74), but a detailed kinetic study was beyond the scope of the present study.

Keeping these limitations in mind, an unexpected large proportion of the 50 characterized promoters showed activities

that differed substantially (up to several orders of magnitude) between the two disease models ("disease-specific promoters") (Fig. 2A). Some promoter fusions showed high levels of activity in both models, but most of them were still much more active during enteritis (median GFP\_OVA ratio of enteritis/typhoid fever, around 5). The same trend of higher activity during enteritis was also observed with *gfp\_ova* fusions to ribosomal promoters that we had previously identified in an unrelated project (Fig. 2A, black squares). We classified this group of promoters as "commonly active" (Fig. 2A).

For some promoter fusions with very high activity in the typhoid fever model, we also observed a small fraction of fluorescent *Salmonella* cells in the enteritis model (Table 2). From a quantitative comparison of volumetric FACS particle counts and plating results (14), we estimated that these subpopulations always represented <10% of the total *Salmonella* population in the gut lumen, while the large majority of *Salmonella* cells had an undetectably low GFP\_OVA content. Some of these promoters are known to be active only within host cells (see below), suggesting that these small fluorescent populations could represent a few *Salmonella* cells that had been phagocytosed by host cells infiltrating the gut lumen or lamina propria cells that were later released from damaged villi (17, 34). To simplify the interpretation, we excluded these minor populations from further analysis.

**Properties of disease-specific and commonly expressed genes.** Twenty-seven of 50 *Salmonella* promoters (56%) with very high levels of activity during infection drive expression of genes with no homologue in the closely related species *E. coli*, which was more than expected (32% of all annotated *Salmonella* genes lack *E. coli* homologues). The absence of homologues in *E. coli* was particularly evident for disease-specific promoters

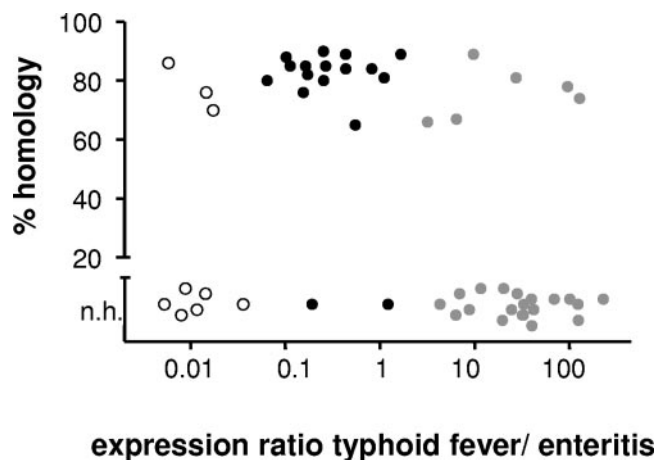


FIG. 3. Homology of highly in vivo-expressed *Salmonella* genes to *E. coli* K-12. A majority of *Salmonella* genes with disease-specific expression (enteritis, open circles; typhoid fever, gray circles; for classifications, see the legend to Fig. 2A) lack *E. coli* homologues, whereas most commonly expressed genes (black circles) are conserved in *E. coli*. Promoters that were only partially detected in one of the models were assumed to have median expression values close to the respective detection thresholds (6,000 molecules for typhoid fever and 4,000 molecules for enteritis) for calculating expression ratios. Promoters with undetectable expression in one of the disease models were assumed to have median expression values of about a quarter of the respective detection thresholds, based on the typical bandwidths of GFP measurements. n.h., homology below 5%.

(Fig. 3). *Salmonella* genes with no *E. coli* homologue also shared an atypically low GC content (data not shown), suggesting that they might have been acquired by horizontal gene transfer from an unknown microorganism. As a consequence of low GC content, these genes had a markedly different codon usage compared to “classical” highly expressed genes such as ribosomal proteins and chaperones, resulting in poor codon adaptation indices (reference 25 and data not shown). Moreover, the calculated metabolic costs for amino acid biosynthesis (1) of these genes were significantly higher than the genome average (data not shown), indicating that these genes were not

yet optimized for low energy costs. *Salmonella* thus appeared to adapt to different disease conditions, mainly by using genes that lack properties of “typical” highly expressed genes.

In contrast to the disease-specific genes, most of the commonly expressed genes had an *E. coli* homologue (Fig. 3), but both codon usage and metabolic costs were not significantly different from the genome average (data not shown). Some “typical” highly expressed genes such as ribosomal genes (high conservation, highly biased codon usage, and low estimated biosynthetic costs) were also strongly expressed during infection (Fig. 2), but these genes did not appear to dominate *Salmonella* in vivo gene expression.

**Functional profile of *Salmonella* gene expression in enteritis and typhoid fever.** The individual comparison of 50 different promoter activities revealed marked disease-specific *Salmonella* gene expression. To compare the overall functional distribution of in vivo highly expressed genes, promoters were classified as “virulence associated,” “physiological,” or “function unknown” (for detailed descriptions of individual genes, see Discussion), and the respective GFP-based promoter activities were calculated for both disease models (Fig. 4). These data represented only a first coarse estimate of the functional profile of highly in vivo-expressed *Salmonella* genes because of the limited number of promoter fusions and replicate measurements, as well as potentially distorting effects of copy number, topology, and position of the fusion junctions. With these limitations in mind, the overall analysis revealed a striking functional difference between the two different disease models. In the enteritis model, *Salmonella* expressed predominantly genes involved in general physiological functions such as nutrient utilization and energy conversion, while virulence genes that were mostly associated with SPI-1 accounted for only a minor fraction of gene expression (Fig. 4A). In marked contrast, *Salmonella* in the typhoid fever model predominantly expressed virulence genes that were mostly associated with *Salmonella* pathogenicity island 2 (SPI-2) or the PhoPQ regulon, whereas genes involved in general physiology were comparatively weakly expressed (Fig. 4B). These quantitative differ-

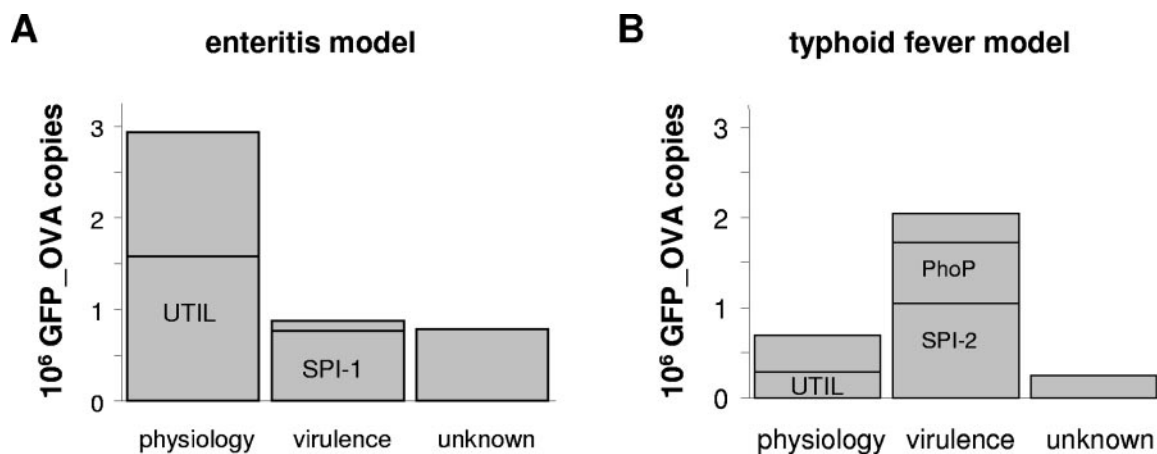


FIG. 4. Functional profile of highly expressed *Salmonella* genes during enteritis (A) or typhoid fever (B). Expression levels determined as the number of GFP<sub>OVA</sub> copies per cell of promoters associated with physiology, virulence, or unknown function were separately summed. The cumulative activity of promoters associated with nutrient utilization and energy conversion (UTIL), SPI-1, SPI-2, or the PhoPQ regulon (PhoP) is also shown.

TABLE 4. Phenotype of *Salmonella* mutants in different disease models

Mutation	Enteritis model			Typhoid fever model		
	CI <sup>a</sup>	log CI <sup>b</sup>	P value <sup>c</sup>	CI <sup>a</sup>	log CI <sup>b</sup>	P value <sup>c</sup>
<i>fnr</i>	0.025	-1.8 ± 0.4	0.0007	0.251	-0.7 ± 0.4	0.011
<i>yaoF</i>	0.47	-0.36 ± 0.2	NS	0.035	-1.69 ± 0.5	0.0003
<i>aroQ</i>	0.85	-0.09 ± 0.2	NS	1.278	-0.08 ± 0.2	NS
<i>ribB</i>	0.0030	-2.7 ± 0.6	0.0031	<0.0001	Less than -4	<0.0001

<sup>a</sup> CI = competitive index (geometric mean of data from five mice). For calculations see Materials and Methods.

<sup>b</sup> Arithmetic mean of log-transformed CI values ± standard deviation.

<sup>c</sup> P values from two-tailed *t* test of log-transformed CI values. NS, not significant.

ences in gene expression between the two disease models were compatible with the predominant identification of physiology-associated promoters in the enteritis sort (Table 1) and virulence-associated promoters in the typhoid fever sort (Table 2) and also reflected the weaker expression of physiology-associated and ribosomal promoters in the typhoid fever model (Fig. 2).

**Disease-specific expression correlates with functional requirements.** Complementary sets of *Salmonella* virulence genes are known to be involved in enteritis induction (SPI-1-associated genes) and systemic typhoid fever (SPI-2-associated genes; PhoPQ regulon). Our expression analysis revealed that for most of these genes, the specific expression pattern reflected the differential relevance (i.e., SPI-2 genes were highly expressed during typhoid fever but not during enteritis; for detailed description of individual genes, see Discussion), in good agreement with our initial analysis (14).

To further explore the relationship between disease-specific expression and function, we selected four previously uncharacterized *Salmonella* genes that were not associated with SPI-1, SPI-2, or PhoPQ. In particular, we generated a mutant lacking the global anaerobiosis regulator *fnr* to test the combined relevance of several identified Fnr-dependent genes associated with anaerobic respiration and mixed acid fermentation that were selectively expressed during enteritis. We also tested mutants lacking the putative hemolysin *yaoF* or the periplasmic chorismate mutase *aroQ*, which were both selectively expressed during typhoid fever, and a mutant that lacked the riboflavin biosynthesis gene *ribB* with high expression in both disease models. All four *Salmonella* mutants were compared to the isogenic wild-type strain SL1344 by competitive infection experiments in both disease models.

Three of the four mutants had strong in vivo phenotypes (Table 4). The global anaerobiosis regulator *fnr* was essential during enteritis and had a weak role in typhoid fever. Limited oxygen availability in the gut (36), which was also reflected by delayed GFP fluorophore formation (Fig. 1D), appeared to result in a requirement for anaerobic metabolism. The *yaoF* mutant was attenuated in the typhoid fever model, where it was specifically expressed, but not during enteritis. The riboflavin biosynthesis gene *ribB* was essential in both disease models, in agreement with its expression pattern. In contrast to these three mutants, the *aroQ* mutant had no detectable phenotype even during typhoid fever, where *aroQ* was selectively expressed. A chorismate mutase isozyme, which is a part of *pheA*, was highly expressed during typhoid fever (Table 2) and might compensate for deficient *aroQ*. Taken together, these data

confirmed and extended evidence for extensive *Salmonella* adaptation to various disease conditions by differential expression of specifically required genes.

## DISCUSSION

Highly in vivo-expressed pathogen genes could provide important targets for novel strategies for diagnosis, prevention, and treatment of infectious diseases. Moreover, major in vivo activities, as defined by gene expression patterns, could provide detailed insight into pathogen biology under relevant conditions. In vitro data for diverse bacterial species suggest that highly expressed genes encode predominantly highly conserved factors involved in protein biosynthesis and folding including transcription factors, ribosomal proteins, and chaperones. Many of these genes share a strongly biased codon usage, and this has been exploited to predict highly expressed genes in both environmental and pathogenic bacteria (44, 46). Extensive in vivo gene expression data have been obtained for various pathogens; these data could be used for testing the relevance of protein biosynthesis genes during infection, but most studies focused on genes specifically induced during infection, while constitutively expressed genes were mostly disregarded. To identify highly in vivo-expressed genes independently of whether they are expressed or not in vitro, we analyzed *Salmonella* promoter activities in murine models of enteritis and typhoid fever by a quantitative GFP reporter assay. As a critical precondition for this approach, we demonstrated that GFP fluorescence can be used to monitor *Salmonella* gene expression in the gut despite low oxygen availability.

In total, we identified 21 strong *Salmonella* promoters in the enteritis model and combined them with 31 previously identified strong promoters from the typhoid fever model (61). Interestingly, none of the associated *Salmonella* genes with high expression during infection belonged to the highly conserved core of transcription factors, ribosomal proteins, and chaperones that typically dominate gene expression in vitro. Moreover, most highly in vivo expressed genes did not share the "typical," strongly biased codon usage of ribosomal genes, suggesting a limited utility of theoretical predictions based on codon adaptation indices. While protein biosynthesis is essential for bacterial growth under all conditions, these data suggested that it might not be the dominating activity in *Salmonella* during infection.

It might be expected that instead an activity related to host-pathogen interactions might dominate *Salmonella* gene expression in both intestinal and systemic infections. However, only 2



out of 50 identified promoters were commonly isolated from the two diseases models, despite substantial coverage of strong promoters. Moreover, semiquantitative activity measurements revealed that a surprisingly large proportion of the identified strong promoters were differentially regulated between enteritis and typhoid fever. Many of these differences might simply reflect different microenvironments in the spleen and the cecum. Indeed, for several identified promoters, previous *in vitro* evidence suggested differential regulation under conditions that are thought to mimic the gut lumen or intracellular compartments in infected host cells (51). Our data provided some *in vivo* confirmation of these data (see below). More importantly, the present study revealed that these and other disease-specific genes might actually dominate overall *Salmonella* *in vivo* gene expression.

**Enteritis-specific genes.** Several genes with specific high expression during enteritis are known to be involved in virulence. In particular, four identified promoters were associated with the type III secretion system encoded on SPI-1 that is known to be important for enteritis induction and invasion. The  $P_{\text{sicA}}$ -associated *sicA-sipBCDA-iacP* operon drives expression of the chaperone SicA that also has regulatory functions, the SPI-1 translocon components (and effectors) SipB and SipC, and the effector SipA (21). The *rtsA*, *hilA*, and *hilC* genes encode members of a complex regulatory cascade that positively regulates expression of various SPI-1-associated genes (26, 64).

The SPI-1-associated *rtsA* gene is cotranscribed with *rtsB*, which encodes a negative regulator of flagellum synthesis (26). Interestingly, we found a second negative regulator of flagellum genes (*lrhA*) with specific expression during enteritis, which can also downregulate motility and chemotaxis genes (49). However, the functional relevance of these negative flagella gene regulators in the murine colitis model remained unclear, since we also found high levels of enteric expression of the *flhDC* operon encoding the master regulator of class II flagellar genes and expression of the major flagellin gene *fliC* (data not shown). Indeed, direct examination of freshly prepared cecal contents of intragastrically infected mice revealed highly motile green fluorescent *Salmonella* cells (data not shown), in agreement with recent functional evidence for *Salmonella* motility and chemotaxis in the murine enteritis model (65). Interestingly, transcriptome analysis of *Vibrio cholerae* from stool samples revealed repression of chemotaxis genes, although *Vibrio* was also directly observed to be highly motile in the same samples (54). These apparently contradictory results might be related to posttranscriptional regulation, which is not revealed in promoter or transcriptome studies.

Several other identified genes had specific physiological functions. A formate transporter (FocA) and the pyruvate formate lyase (PflB) are involved in mixed acid fermentation during anaerobic growth of *Salmonella*, which is consistent with oxygen limitation in the gut. *PflB* is also upregulated in *Vibrio cholerae* in a rabbit ileal loop model (78). The strong virulence defect of our regulator *fmr* mutant (Table 4) supported the high relevance of anaerobic *Salmonella* metabolism in the enteritis model.

The enterically expressed *fhuACDB* operon encodes a ferri-chrome uptake system that utilizes ferrichrome siderophores produced by fungi (20). The expression of this operon con-

firmed a previous IVET result (38) and might reflect limited iron availability (7) in the gut during enteritis.

The *ahpCF* operon that encodes an alkylhydroperoxide reductase involved in detoxification of reactive oxygen and nitrogen intermediates was found to be specifically expressed in the colitis model, potentially reflecting antibacterial host responses during cecum inflammation. The enteric expression was verified with a GFP insertion in the native chromosomal locus (Table 3). AhpC is induced during interaction with macrophages, and the gene product is recognized as an antigen during systemic infection (29, 67); but in our hands, neither an episomal nor the chromosomal promoter fusion was sufficiently active for detection in infected spleen.

Finally, two genes with unknown function were specifically expressed in the enteritis model. *STM4423* encodes an *araC*-type regulator and *STM1328* encodes a conserved bacterial protein (COG3528).

**Typhoid fever-specific genes.** The vast majority of genes that were found to be associated with typhoid fever-specific promoters are known or potential virulence factors and reside in clusters of presumably horizontally acquired genes ("genomic islands") (data not shown). In particular, we identified nine operons that are associated with the type III secretion system encoded on SPI-2. This system is essential for systemic virulence and has been shown to interfere with microtubuli and actin networks, vesicle trafficking, and phagoendosome maturation resulting in the exclusion of inducible nitric oxide synthase and NADPH oxidase from the *Salmonella*-containing vacuole (75). Among the operons identified in our data set were previously characterized structural components expressed from  $P_{\text{ssaB}}$  and  $P_{\text{ssaG}}$  and secreted effectors/translocon components and their associated chaperones expressed from  $P_{\text{sseA}}$ ,  $P_{\text{sifA}}$ ,  $P_{\text{sifB}}$ ,  $P_{\text{pipB}}$ ,  $P_{\text{pipB2}}$ ,  $P_{\text{sopD2}}$ , and  $P_{\text{sseK1}}$  (11, 12, 30, 40, 47, 48, 67). We also identified up to three novel candidate genes, *STM1583*, *STM1633*, and *sb26* (carried on phage ST64B, which is absent in the sequenced strain LT2) (70), that are potentially associated with SPI-2, as they bear serine-rich N-terminal domains potentially acting as a signal for type III secretion (32). Indeed, *sb26* is a close homologue to secreted *sseK1* (*STM4157*) and *sseK2* (*STM2137*) and has recently been renamed *sseK3* (48). The association of *STM1633* with virulence functions was suggested by its location in a pathogenicity islet that also includes the SPI-2 effector *sseJ*.

The key regulator *phoP* and five other genes of the PhoPQ regulon (*slyA*, *ugd*, *pagK2*, *virK*, and *mig-5*) were found to be selectively expressed in the typhoid fever model. SlyA itself is another transcriptional regulator (13). Members of the PhoPQ regulon regulate lipopolysaccharide structural modifications and changes in the composition of outer membrane proteins that render *Salmonella* more resistant to antimicrobial peptides (27). Finally, *yaoF*, which is weakly homologous to a *Shigella* hemolysin (57), represents a novel typhoid fever-specific virulence factor (Table 4).

In addition to the large number of known or putative virulence factors, three genes with putative housekeeping functions were found to be selectively expressed in the typhoid fever model, with products including a dispensable periplasmic chorismate mutase (expressed by *aroQ*) (Table 4), and the two endonucleases RNase I (*rna*) and the nonspecific DNA/RNA endonuclease *STM1330*, which is homologous to an extracel-

lular nuclease of *Serratia marcescens*. Interestingly, another RNA-degrading enzyme, polynucleotide phosphorylase, profoundly influences the infection biology of *Salmonella* (16). Possibly, these nucleases modulate mRNA turnover for regulation of virulence gene expression, degrade antibacterial nets produced by neutrophils (10), and/or are involved in scavenging nucleic acids for nucleoside supply (5).

Finally, we identified the typhoid fever-associated hypothetical operon, *STM0809-STM0810*, which encodes putative inner membrane proteins that are exclusively present in *S. enterica* serovar Typhimurium but absent in *S. enterica* serovar Typhi, and *STM1672*, which is present in both serovars but absent in nonpathogenic *E. coli*.

**Commonly expressed genes.** Most of the commonly highly expressed genes have a physiological function. In particular, we identified the two biosynthetic genes *ribB* and *pheA*. RibB is involved in the biosynthesis of riboflavin, an essential precursor of flavin adenine dinucleotide and flavin mononucleotide cofactors, and is required in both disease models (Table 4). *pheA* encodes the bifunctional chorismate mutase/prephenate dehydratase involved in phenylalanine biosynthesis. Four commonly expressed promoters drive the expression of operons involved in carbohydrate uptake and utilization. RbsDACBK and GlpTQ mediate the uptake of ribose and glycerol-3-phosphate, respectively. The *yiaK* operon and the putative operon *STM2344-2340* encode carbohydrate uptake systems and utilization enzymes. The expression of *glpTQ* and the *yiaK* operons can be induced in vitro by anaerobiosis (42, 77), and their somewhat higher expression in the gut may reflect the oxygen limitation in this compartment compared to that in the spleen. Two identified promoters drive expression of proteins involved in respiration. NADH dehydrogenase I (*nuoA-N*) is expressed under both aerobic and anaerobic conditions (2), and DniR is a positive regulator of the formate-dependent respiratory nitrite reductase, which uses formate as electron donor to reduce nitrite to ammonia in anaerobic respiration (43). In addition, genes of lipopolysaccharide synthesis (*htrB* and *wzzB*) and maintenance of cell envelope integrity (*ybgC-tolQRAB-palybgF* operon), the *speF-potE* operon whose gene products are involved in polyamine metabolism, and the gene of the threonine tRNA synthetase (*thrS*) were found to be commonly expressed. The commonly expressed putative operon *STM4501-STM4500* encodes a protein broadly conserved in bacteria (COG3811) and a putative *S*-adenosylmethionine-dependent methyltransferase.

Surprisingly, two virulence-associated promoters were found to be active in both disease models, and one of these ( $P_{\text{hilD}}$ ) was independently identified in nonredundant clones in both disease models. HilD is a positive regulator in a regulatory cascade that leads to expression of SPI-1-associated genes, which are required for induction of enteritis but not during systemic infection. HilD expression is only modestly regulated under different in vitro conditions (52), and HilD activity is diminished by direct interaction with Hile (4) suggesting that intracellular *hilD* transcription might not correlate with SPI-1 activity. The second commonly expressed virulence promoter drives expression of the *ybaJ-hha* operon. While the function of YbaJ is unknown, Hha negatively regulates expression of Hila, which itself is a positive regulator of SPI-1-associated genes (28). The complex regulation of SPI-1 is still incompletely

understood, and the functional relevance of enteric expression of the *ybaJ-hha* operon is presently unclear.

**Global functional adaptations.** In addition to the differential regulation of individual operons, the data also suggested marked differences in the major functions of highly expressed genes during enteritis and typhoid fever. While nutrient utilization and energy conversion appeared to dominate *Salmonella* transcription during enteritis, virulence factor transcription seemed to dominate during typhoid fever. This reallocation of transcriptional activities to complementary functions closely corresponded to differential microenvironmental constraints, including competition with the host and other gut microorganisms for nutrients and low oxygen availability during enteritis and potent antimicrobial immune responses that impair intracellular *Salmonella* growth during typhoid fever. Indeed, the highly expressed genes play an outstanding role for *Salmonella* virulence, since at least 16 (32%) of the identified 50 operons with very high levels of expression during infection encoded genes with essential functions in one or both disease models (Tables 1, 2, and 4). This high proportion was remarkable, compared to the low frequency of essential genes among in vivo-expressed genes that had been previously identified with the nonquantitative IVET technology (18, 39) and an estimated 4% essential virulence genes in the entire *Salmonella* genome (8). Exceptionally high in vivo expression levels thus appeared to correlate with functional importance; this could reflect economical use of the substantial biosynthesis costs that are associated with highly expressed genes.

However, it is not yet clear whether these strong promoter activities correlate with high abundance of the corresponding proteins. Technical difficulties currently make it difficult to determine the protein composition of *Salmonella* in infected mouse tissues, but the atypically high AT content and the potentially suboptimal codon usage of many SPI-2-associated genes and genes of the PhoP regulon suggest that despite high levels of transcription of these virulence genes, the corresponding proteins might be less abundant than other well-translated proteins. Further studies are required to clarify this issue to more fully characterize *Salmonella* adaptation to different disease conditions on the proteome level.

Another issue that requires further study is the relevance of the mouse enteritis model for human disease. Several histological features show that some important aspects of human disease are reproduced by the mouse model. However, in mice this model leads eventually to lethal systemic infection, while in immunocompetent patients, gastroenteritis is not associated with detectable systemic dissemination. We utilized the mouse model, since it allowed comparison of systemic disease and enteritis in the same animal host species, but further studies are needed to validate our findings in more realistic model systems.

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