Applied and Environmental Microbiology, Dec. 2007, p. 7522–7530 Vol. 73, No. 23
0099-2240/07/$08.00+0 doi:10.1128/AEM.01481-07

Genome-Wide Screen of *Salmonella* Genes Expressed during Infection in Pigs, Using In Vivo Expression Technology▽

Yanyan Huang,[1,2] Christopher L. Leming,[2] Mitsu Suyemoto,[2] and Craig Altier[1]*

Department of Population Medicine and Diagnostic Sciences, Cornell University, Ithaca, New York 14853,[1] and College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina 27606[2]

Received 2 July 2007/Accepted 26 September 2007

Pigs are a food-producing species that readily carry *Salmonella* but, in the great majority of cases, do not show clinical signs of disease. Little is known about the functions required by *Salmonella* to be maintained in pigs. We have devised a recombinase-based promoter-trapping strategy to identify genes with elevated expression during pig infection with *Salmonella enterica* serovar Typhimurium. A total of 55 clones with in vivo-induced promoters were selected from a genomic library of ~10,000 random *Salmonella* DNA fragments fused to the recombinase *cre*, and the cloned DNA fragments were analyzed by sequencing. Thirty-one genes encoding proteins involved in bacterial adhesion and colonization (including *bcfA*, *hscA*, *rffG*, and *yciR*), virulence (*metL*), heat shock (*hscA*), and a sensor of a two-component regulator (*hydH*) were identified. Among the 55 clones, 19 were isolated from both the tonsils and the intestine, while 23 were identified only in the intestine and 13 only in tonsils. High temperature and increased osmolarity were identified as environmental signals that induced in vivo-expressed genes, suggesting possible signals for expression.

Serovars of *Salmonella enterica* infect a variety of hosts, from domestic livestock to humans. The outcomes of *Salmonella* infection can range from asymptomatic carriage to severe disease. The two common disease syndromes caused by *Salmonella*, septicemia and enteritis, have been actively studied, the former through the use of a mouse model and the latter primarily with calves (23, 38, 57, 85). After decades of effort, the genetic factors utilized by *Salmonella* to cause both enteric and systemic infection are becoming clearer. *Salmonella* pathogenicity islands (SPI) 1 through 5 have been shown to be required for functions essential to *Salmonella* virulence, including the penetration of epithelial cells and survival in macrophages (6, 11, 23, 30, 45, 58). Thus, these SPIs are essential to producing overt disease in a wide range of animal species.

Another important aspect of *Salmonella* infection is its persistence and asymptomatic carriage in animals that serve as reservoirs for contamination of human food. Salmonellosis remains the leading cause of death and is second only to campylobacterosis in illness numbers in the United States among bacterial food-borne diseases (54). Pigs are a food-producing species that readily carry *Salmonella* but, in the great majority of cases, do not show clinical signs of diseases. Surveys have shown that up to six serotypes can be isolated from clinically normal pigs on a single farm (16). Pork products were implicated in 2.9% of all *Salmonella* outbreaks during the years 1983 to 1987 (82). Between 1988 and 1992, 18% of the outbreaks caused by consumption of contaminated meat that were reported to the CDC were due to ham and pork (5). Swine also shed antimicrobial-resistant *Salmonella* that pose a threat to food safety. With increasing frequency, *Salmonella* isolates obtained from pigs are resistant to one or multiple antimicrobials. Recent studies have shown that at least half, and in some cases over 90%, of *Salmonella* isolates obtained from commercially raised swine in the United States are multiresistant (20, 27). Therefore, the high rate of unapparent infections makes pigs potential incubators of *Salmonella*, allowing the expansion of bacterial populations and threatening human health. It also makes pigs an important species for the study of mechanisms by which *Salmonella* is maintained in animal species that fail to show overt disease.

To effectively survive and persist in animals, *Salmonella* must coordinate gene expression in response to varied environments during the process of infection. Little is known about genetic factors required for *Salmonella* carriage in clinically healthy animal hosts with persistent shedding of bacteria in feces. A characteristic that all *Salmonella* infections share is colonization of the gastrointestinal tract, and so genetic factors important for colonization may be required. In chickens, another important species carrying *Salmonella* without causing overt disease, mutants of lipopolysaccharide biosynthesis have shown reduced intestinal colonization (13, 14, 87). Fimbrial adhesions are also thought to be potential factors for mediating attachment to intestinal surfaces by *S. enterica* serovar Typhimurium (1, 10, 35), and there is evidence that nonmotile mutants of *Salmonella* are deficient in colonization (4). However, the colonization factors of *Salmonella* are thought to be host specific (10, 59, 74, 86). It is thus unknown whether the genetic factors required for *Salmonella* persistence in pigs differ from those of other animal species.

Besides the genetic factors, a variety of environmental conditions present within animal hosts have been shown to provide signals that control *Salmonella* gene expression. An early step in the pathogenesis of *Salmonella* infection is bacterial penetration of the intestinal epithelium. Many of the genes required for epithelial penetration are found within SPI1. It has been demonstrated that the regulation of invasion genes requires a

* Corresponding author. Mailing address: Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853. Phone: (607) 253-3926. Fax: (607) 253-3907. E-mail: ca223@cornell.edu.

▽ Published ahead of print on 5 October 2007.

coordinated response to varied environmental signals. Low oxygen tension and high osmolarity, conditions of the ileum, have been implicated in the induction of SPI1 invasion genes (3). Transcription of invasion genes has also been shown to be repressed by bile (65), and we have previously shown that acetate can induce invasion gene expression in *Salmonella* (41). The environmental conditions present in animals thus might also provide plausible signals for other *Salmonella* functions required for life in animal hosts.

The goal of the current study was to identify *Salmonella* genes induced during infection of the pig and the environmental signals plausibly inducing the expression of those in vivo-induced genes. In the past decade, many techniques have been developed to study bacterial genes that are expressed during infection of animal hosts, such as signature-tagged mutagenesis (87), differential fluorescence induction (88), in vivo expression technology (49), and microarray analysis (42). Here, we have used a recombinase-based in vivo expression technology in combination with a signature-tagging approach to identify genes expressed during infection of pigs with *Salmonella enterica* serovar Typhimurium as a means to identify genes that may be important for *Salmonella* carriage in pigs. The results indicate that *S. enterica* serovar Typhimurium induces a variety of genes in this animal host, including those involved in adhesion, two-component regulation, survival in macrophages, and anaerobic metabolism, as well as unknown functions. Furthermore, we demonstrate that environmental conditions present in pigs, elevated temperature and increased osmolarity, induce the expression of some of these in vivo-induced genes.

MATERIALS AND METHODS

Library screening for in vivo-induced genes. For the screening approach, we used a recombinase-based system previously developed to identify differentially expressed *Salmonella* genes (2). This system consists of a cassette integrated into the *Salmonella* chromosome that harbors *npt*, encoding kanamycin resistance, and *sacB*, for sucrose susceptibility, flanked by a pair of *loxP* sites. On a plasmid is a promoterless derivative of *cre* encoding the Cre recombinase of phage P1 that recognizes the *loxP* sites as its targets. The fusion of an active promoter to *cre* induces recombination of the two *loxP* sites and deletion of the intervening DNA, allowing selection on sucrose. Fusion of promoters active only when exposed to a specific environment, in this case after infection of pigs, induces bacterial conversion to sucrose resistance only after bacteria have been exposed to that environment, thus selecting for differentially expressed bacterial genes. We created a library of *Salmonella* genomic DNA fragments by partial digestion of total genomic DNA from *S. enterica* serovar Typhimurium strain 798, originally isolated from a pig (93), with HaeIII, AluI, or RsaI and then size fractionated the DNA to isolate fragments of 1 to 2 kb (Fig. 1). These three libraries were pooled and fragments were cloned into the PmlI site of the ampicillin-marked plasmid pCA19, placing them upstream of a promoterless derivative of the phage P1 recombinase *cre.* A derivative of strain ATCC 14028s carrying a chloramphenicol resistance marker and a pair of chromosomal *loxP* sites flanking *npt* (kanamycin resistance) and *sacB* (sucrose susceptibility) was transformed with this library, with an initial selection on morpholinepropanesulfonic acid (MOPS) minimal agar with ampicillin (100 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (25 μg/ml). (Strain ATCC 14028s was used in these experiments because strain 798 carrying the *sacB* cassette proved not to be sucrose susceptible; ATCC 14028s also infects pigs in high numbers, similar to strain 798.) Selection on kanamycin removed constitutively active promoters from the library, thus leaving DNA fragments with no promoter activity and regulated promoters not expressed on laboratory media.

Approximately 10^4 independent library transformants were pooled for administration to pigs. Approximately 1×10^{10} bacteria, representing this pool of 10^4 clones, were administered orally to two 7-week-old pigs. After 48 h, the pigs were sacrificed and the contents of the entire intestinal tract were harvested. Cecal and colonic contents were diluted in phosphate-buffered saline, passed through a gauze filter, and then centrifuged to concentrate bacteria. Ileal contents were in a small volume and of liquid consistency and so were used directly. All samples were plated onto salmonella-shigella agar with added chloramphenicol, ampicillin, and 5% sucrose. On this medium, only bacteria that had lost the *loxP* cassette, along with the intervening *sacB*, should have been present due to the differential expression of *cre*. The loss of the *loxP* cassette was verified by susceptibility to kanamycin. We further confirmed the size of all in vivo-induced fragments to be in the range of 1 to 2 kb by PCR amplification using primers homologous to sequence that flanks the inserted fragment.

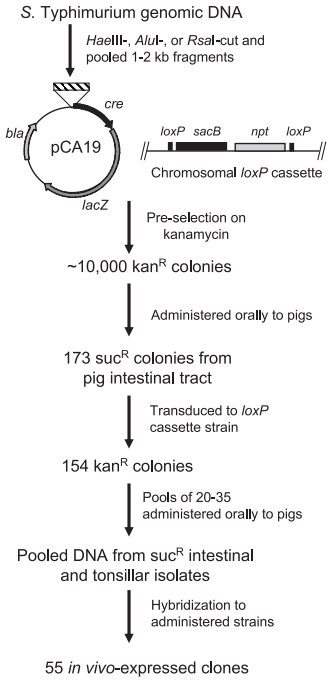

FIG. 1. Identification of in vivo-induced genes. A genomic library of approximately 10,000 random *Salmonella* DNA fragments was fused to *cre* and preselected on kanamycin (Kan^r) to eliminate constitutive promoters from the population. The library was then administered to two pigs, and the intestinal contents were cultured on selective medium containing 5% sucrose (Suc^r), selecting for bacteria that lost the *loxP* cassette, along with the intervening *sacB*, due to the differential expression of *cre*. Each plasmid was reintroduced into the strain carrying the intact *loxP* cassette by P22 transduction; of 173 transductants, 154 remained kanamycin resistant. These were divided into groups of 20 to 35 each, and each group was used to infect two pigs. Sucrose-resistant *Salmonella* isolates were isolated from the ileum and the tonsils, and these were used to make pooled probes by PCR amplifying the cloned fragments of each. Probes were used in colony blots to determine which members of the input pool had reproducibly converted to sucrose resistance after animal infection, producing 55 promoter fragments that were expressed again in both of the pigs that had received the bacterial pool.

Verification of in vivo induction. Plasmids carrying in vivo-induced genes were individually reintroduced into the strain with the intact chromosomal *loxP* gene cassette by transduction with phage P22. Only the transductants remaining kanamycin resistant when grown on MOPS minimal medium were retained for further study. We next tested the gene expression of those transductants in pigs again, using a modified signature-tagging method. A sample of an overnight culture of each strain was fixed to a nylon membrane using a dot blot apparatus according to the manufacturer's directions (Millipore, Billerica, MA). The membranes were incubated with the colony side up in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 minutes twice and in neutralizing solution (0.5 M Tris pH 7.5, 1.5 M NaCl) for 10 minutes twice. Membranes were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and were then fixed by UV cross-linking at 1,200 W. Each strain was applied to five membranes; each membrane contained 20 to 35 strains. Equal volumes of these 20 to 35 strains were pooled to create an inoculum for two 7-week-old pigs, with each pig receiving approximately 1×10^{10} bacteria. After 48 h, the pigs were sacrificed.

The contents of 10 to 15 cm of the distal ileum were harvested, diluted in MOPS minimal base, passed through a filter stomacher bag, and then centrifuged to concentrate bacteria. For tonsil samples, both sides of the tonsils of the soft palate were swabbed using two cotton swabs, which were washed with MOPS minimal base and centrifuged to concentrate bacteria. All samples were plated onto salmonella-shigella agar with added chloramphenicol (for the chromosomal marker), ampicillin (for the plasmid marker), and 5% sucrose. Sucrose-resistant bacteria were recovered as four output pools, one each for the ileum and the tonsils for each pig. Plasmid DNA was prepared from the inoculated and output pools using a Qiagen (Valencia, CA) plasmid midi kit. The DNA inserts present in the pools were PCR amplified using the single primer 5′-GCGGCCGCACG TGCGGCCGC, homologous to sequence that flanks the inserted DNA fragment on both its ends in pCA19. PCR products were purified using a Qiagen PCR purification kit. The purified PCR products were labeled using a second PCR amplification with a digoxigenin (DIG) probe synthesis kit (Roche Applied Science, Indianapolis, IN), and these PCR products were used as probes to hybridize to the membrane containing the corresponding inoculated bacteria for each pig. We hybridized membranes with probes from five sources: one made from the input pool, two made from the output pools for the tonsils (one from each pig), and two for the intestinal contents (one from each pig). Hybridizations were performed using DIG Easy Hyb (Roche Applied Science, Indianapolis, IN) overnight at 42°C, and a DIG wash and block buffer set and biotin luminescence detection kit were used according to the manufacturer's directions for washing and detection. Blots were detected using a luminescence imager (Lumi-Imager; Boehringer Mannheim).

Sequencing. Sequences were obtained using an Applied Biosystems (Foster City, CA) 3130xl genetic analyzer or by the MClab DNA Sequencing Service (San Francisco, CA). The sequencing primers were 5′-CATTTTCCAGGTATG CTCAG, which is located in *cre*, and 5′-AGTAGGTTGAGGCCGTTG, located upstream from the inserted DNA fragment in pCA19. The location of each of the cloned fragments was determined by comparison to the sequenced genome of *S. enterica* serovar Typhimurium strain LT2 (GenBank accession number AE006468).

β-Galactosidase assays. β-Galactosidase assays were performed using an enhanced β-galactosidase assay kit with chlorophenol red-β-D-galactopyranoside (CRPG) following the manufacturer's directions (Genlantis, San Diego, CA) for a 96-well microtiter plate assay, with minor alterations. We used one drop of chloroform and one drop of 0.1% sodium dodecyl sulfate to lyse cells. Levels of β-galactosidase expression were measured by the catalytic hydrolysis of the CRPG substrate to a dark red product. The β-galactosidase levels were calculated using the following equation: β-galactosidase = (1,000 × absorbance at 595 nm)/[(sample volume) × (duration of reaction) × (absorbance at 600 nm)]. Absorbance was read using a Power Wave Xs 96-well plate reader (Bio-Tek Instruments Inc., Winooski, VT). To assess the effect of temperature on gene expression, strains were grown in 96-well sealed plates as standing overnight cultures in LB broth at 30°C, 37°C, and 42°C. To test the effects of osmolarity, strains were grown aerobically in 96-well plates as standing overnight cultures in MOPS minimal medium at 37°C with or without the addition of 0.4 M NaCl. Triplicate cultures of each strain were assayed for *lacZ* expression by CRPG-enhanced β-galactosidase assays.

Statistical analysis. For β-galactosidase assays, two-sample *t* tests were performed to determine which means differed at a P level of ≤ 0.05, using the SAS System for Windows 8 and MINITAB release 14.

RESULTS AND DISCUSSION

Recombinase-based screening for in vivo-induced genes in the pig. The high rate of unapparent infections makes pigs potential incubators of *Salmonella*, allowing the expansion of bacterial populations in the animal host. Therefore, we sought to establish an experimental model using pigs to study mechanisms of *Salmonella* infection in clinically healthy animals. We reasoned that there might be bacterial genes required for survival that are expressed specifically in pigs. We selected 55 cloned fragments from a genomic library of 10,000 fragments by using a recombinase-based system that we had previously developed to identify *Salmonella* genes that are differently expressed when bacteria are exposed to specific environmental conditions (2) (Fig. 1). Of these, 19 were isolated from both the tonsils and the intestine, while 23 were identified only in the intestine and 13 only in tonsils. To characterize these in vivo-expressed genes, we sequenced the cloned fragments and compared them to the *Salmonella* genome database (53). Thirty-one cloned fragments of the 55 clones corresponded to 32 unique genes with known or putative functions (Table 1), as some clones carried more than one gene and some clones were found more than once. Six of the cloned fragments, corresponding to *hydH*, *hpaB*/*hpaR*, *wecC*/*rffG*, *yciR*, STM1731, and STM0611/0612/0613, were found twice, and one cloned fragment containing three genes (STM2755/2756/2757) was found three times. In addition, 21 clones found in this screen, representing 18 chromosomal loci, were in an orientation relative to *cre* opposite to that of the annotated gene predicted to be carried on the fragment (see details below). Among those reverse-oriented fusions, *ygiK* was recovered four times independently, as it occurred on fragments of different sizes, while *rpoN* and STM1368 were similarly independently identified twice. Besides the 31 clones with known functions and 21 clones with reverse-oriented fusions, we failed to obtain readable sequences for 3 cloned fragments. These results therefore show that by using a promoter trap strategy in combination with a signature-tagging approach, large *Salmonella* genomic libraries can be produced and screened to identify in vivo-induced genes.

Genes for synthesis of fimbriae and lipopolysaccharide. Three genes identified by this screen have previously been implicated in the colonization of animals (33, 84, 87). One cloned fragment included *bcfA*, encoding a fimbrial subunit. Fimbriae have been shown to function as intestinal colonization factors in *Salmonella* serovars (35, 79, 80). The expression of serotype Typhimurium fimbrial antigens is induced during the infection of mice (33). *bcfA* is specifically expressed during the infection of bovine ligated ileal loops, but not in vitro (34). One other cloned locus found in this screen included two adjacent genes, *wecC* and *rffG*, involved in the production of enterobacterial common antigen (55), a cell surface glycolipid present in all gram-negative enteric bacteria (39). We found this fragment twice, on different-sized fragments, from the tonsils of one pig and from both the tonsils and the intestine of another pig. This gene and its homologues have been shown to be important for colonization for a number of bacterial pathogens. Mutation of *rffG* produces reduced virulence in the plant pathogen *Erwinia carotovora* subsp. *atroseptica* when inoculated into potato plant stems (84). It has been shown that *rffG* in *Escherichia coli* is a functional homologue of *rfbB*, with both of these genes encoding dTDP-glucose hydratases (50). Serotype Typhimurium has both *rfbB* and *rffG*, which also encode dTDP-glucose hydratases (84), and a mutant of *rfbB* in serotype Typhimurium has been shown to exhibit reduced intestinal colonization of chicks (87). Thus, the importance of these genes identified by our screen is supported by studies in other animal and plant species and suggests that the method is sufficient to identify genes important to the existence of *Salmonella* in pigs.

Sensor of a zinc tolerance two-component system. Two clones encoding a sensor of the HydH/HydG two-component regulator were found in this screen, with both carrying *hydH* but on fragments of different sizes. HydH/HydG has also been designated ZraS/ZraR (*z*inc *r*esistance-*a*ssociated *s*ensor/regu-

TABLE 1. *Salmonella* genes induced during infection of pigs

Category	Gene	Function	Tissue(s)
Adherence	*wecC*/*rffG*	UDP-*N*-acetyl-D-mannosaminuronic acid dehydrogenase/dTDP-glucose 4,6-dehydratase	Intestine and tonsil
	bcfA	Fimbrial subunit	Intestine and tonsil
	fdx-hscA	Involved in assembly of Fe-S clusters	Intestine
	yciR	Putative diguanylate cyclase/phosphodiesterase	Intestine and tonsil
Two-component regulator	*hydH*	Sensory kinase in two-component regulatory system with HydG	Intestine and tonsil
Aromatic hydroxylase	*hpaB*/*hpaR*	4-Hydroxyphenylacetate catabolism	Intestine and tonsil
Virulence	*metL*	Aspartokinase II-homoserine dehydrogenase II	Intestine
Metabolism	*cysQ*	Sulfite biosynthetic protein	Intestine and tonsil
	sppA	Protease IV	Intestine and tonsil
	cbiF/*cbiG*	Synthesis of vitamin B_{12} adenosyl cobalamide precursor	Tonsil
Putative function	STM0611/0612/0613	Putative hydrogenase protein	Intestine
	oadB	Putative sodium ion pump	Tonsil
	rlgA	Putative integrase	Tonsil
	STM2689	Pseudogene	Intestine
	STM1731	Putative catalase	Intestine and tonsil
	STM2755/2756/2757	Putative sugar phosphate aminotransferase/putative hexulose 6-phosphate synthase	Tonsil
	yiiG	Putative cytoplasmic protein	Intestine
	STM0325	Putative IS*3* transposase	Intestine
	STM4489	Putative superfamily I DNA helicase	Intestine
	STM4320/*pheR*	Putative *merR* family bacterial regulatory protein	Intestine
	ybbP	Putative inner membrane protein	Intestine and tonsil
	STM1634/1635	Putative amino acid ABC transporter permease component	Intestine and tonsil
	ybgH	Putative POT family transport protein	Intestine
Reverse-oriented fusions	*ygiK*	Putative inner membrane protein	Intestine and tonsil
	STM1368	Putative Na^+-dicaboxylate symporter	Intestine and tonsil
	yciA/*yciB*	Intracellular septation protein A/B	Intestine
	sitC	Fur-regulated *Salmonella* iron transporter	Intestine
	kduD	2-Deoxy-D-gluconate 3-dehydrogenase	Intestine
	parA	Plasmid partition protein A	Intestine
	galP	Galactose/proton symporter	Intestine
	shdA	Fibronectin binding protein	Intestine
	Pslt068	Putative ParB-like nuclease	Intestine
	napA	Periplasmic nitrate reductase	Intestine and tonsil
	hflK	FtsH modulator	Intestine and tonsil
	stbD	Putative fimbrial usher	Intestine and tonsil
	rpoN	DNA-directed RNA polymerase subunit N	Intestine and tonsil
	slt-trpR	Soluble lytic murein transglycosylase	Intestine and tonsil
	Pslt026	Putative periplasmic protein	Intestine and tonsil
	wcaL	Putative glycosyl transferase	Tonsil

lator), as it is involved in zinc tolerance (47). It has been proposed that ZraS/ZraR senses high zinc concentrations and activates the expression of *zraP* to contribute to zinc tolerance (47). The level of *hydG* mRNA has also been shown to be increased threefold in *E. coli* cultures after the addition of $ZnSO_4$ (46). The dietary zinc requirement for swine is 50 to 100 ppm, which is more than that for other tested livestock (7). Therefore, it is possible that *hydH* was expressed in both the tonsils and the intestine of pigs during *Salmonella* infection in response to the high zinc concentration present in pigs due to zinc supplementation of feed. Alternatively, it has also been shown that *hydH* of *E. coli* is expressed during infection of the human gut (36). Thus, the two-component zinc tolerance system HydH/HydG may be important for bacteria during life within animal hosts.

***yciR*, encoding GGDEF and EAL domains.** Another gene identified in this screen was *yciR*, also designated *gcpE* (*G*GDEF domain-*c*ontaining *p*rotein E) (26). We cloned *yciR* twice independently, on different-sized fragments, once from tonsils and once from the intestine. *yciR* encodes a protein containing GGDEF and EAL domains, representing a class of proteins found in many bacterial species. Serotype Typhimurium has 12 proteins containing GGDEF domains and 14 proteins with EAL domains (26, 81). Such proteins control the intracellular concentration of the global second messenger c-di-GMP, with the GGDEF domain stimulating c-di-GMP production and the EAL domain c-di-GMP degradation (77). c-di-GMP has been identified as a global regulator responsible for motility, adhesion, biofilm formation, and virulence (56). Deletion of *yciR* of serotype Typhimurium affects cellulose

production and biofilm formation (26), while a different EAL domain protein has been indicated to control bacterial survival in mice (31). Together, these findings suggest that *yciR* and the modulation of c-di-GMP levels may be involved in *Salmonella* colonization or survival in pigs.

Genes for assembly of Fe-S clusters and the heat shock response. Another cloned fragment plausibly affecting *Salmonella* persistence in pigs contained the *hscBA-fdx* operon, which is involved in the assembly of Fe-S clusters (83) and probably cotranscribed with the Fe-S cluster *iscSUA* genes (95). HscA, a chaperone, has been shown to be regulated by the Fe-S cluster assembly protein IscU and the cochaperone HscB (75). The ferredoxin (Fdx) is proposed to be involved in electron transfer (28). Therefore, this *hscBA-fdx* operon plays a central role in the assembly machinery of Fe-S clusters, which function in a number of cellular processes, including gene regulation (37). It has been shown that the bacterial species *Xenorhabdus nematophila* requires an intact *iscRSUA-hscBA-fdx* operon to colonize *Steinernema carpocapsae* nematodes (51). Thus, this operon might also be important for serotype Typhimurium colonization or carriage in animals. In particular, within this operon *hscA* encodes a 66-kDa heat shock protein which is a homologue of the heat shock protein DnaK (32). It has been shown that *Salmonella* heat shock proteins are induced upon infection of macrophages (8), and a heat shock protein of the size of HscA has been shown to be responsible for binding of serotype Typhimurium to intestinal mucus (18). These findings could thus implicate *hscA* as a gene affecting *Salmonella* colonization and persistence in pigs.

Genes involved in the degradation of aromatic compounds. A clone carrying two adjacent genes, *hpaB* and *hpaR*, was found twice on different cloned fragments. *hpaB* and *hpaR* are both components of the 4-hydroxyphenylacetate (4-HPA) degradative pathway in *E. coli* (22, 52). *hpaB* and the gene adjacent to it, *hpaC*, form a single transcription unit and encode the large and small components of a two-component 4-HPA 3-monooxygenase. *hpaB* encodes the flavoprotein, whereas *hpaC* codes for a coupling oxidoreductase (21, 64) which increases the hydroxylase activity of HpaB (63). In *E. coli*, the *hpa* catabolic genes are organized in two transcribed operons in the same orientation: the upper operon (*hpaBC*) and the meta operon (*hpaGEDFHI*) (62, 70). The *hpa* pathway of *E. coli* is regulated by two proteins, HpaA as an activator and HpaR as a repressor, reverse oriented to the two operons (22, 62). In serotype Typhimurium, the gene arrangement of the *hpa* operons is different from that in *E. coli*; the upper operon (*hpaBC*) and the meta operon (*hpaGEDFHI*) are divergently transcribed. *hpaR* of *Salmonella* is transcribed in the same orientation as *hpaBC* but opposite to that of *hpaGEDFHI*. Therefore, it is not yet clear whether the promoter activity identified from this clone originates from the promoter of *hpaB* or that of *hpaR*. The regulatory circuits of these aromatic catabolic pathways have also not been well established in *Salmonella*. Although aromatic compounds are highly abundant in soil and water, it has also been suggested that there are sources of aromatic compounds in the gastrointestinal tract, a majority of them being derived from the fermentation of aromatic amino acids and with some provided by plant materials (29). Thus, the fact that *hpaB* or *hpaR* of *Salmonella* was induced in pigs suggests that *Salmonella enterica* serotype Typhimurium is able to degrade certain aromatic compounds when living in an animal host.

Virulence functions. Only one gene identified in our screen, *metL*, has been previously shown to be involved in *Salmonella* virulence. This gene encodes the bifunctional enzyme aspartokinase II-homoserine dehydrogenase II (AKII-HDII) and was carried on a clone isolated from the intestine. AKII-HDII catalyzes two independent proximal steps in the prokaryotic biosynthetic pathways that convert aspartate to lysine, threonine, and methionine (94), and a serotype Typhimurium mutant of *metL* exhibits reduced virulence in mice (17). *metL* might therefore be important for *Salmonella* maintenance in pigs as well. No genes in any of the *Salmonella* pathogenicity islands were found in this study. This was not unexpected, as mutants of SPI1 or SPI2 genes have been shown to maintain their ability to colonize the chick intestine (59), suggesting that serotype Typhimurium is much less reliant upon SPI1 and SPI2 to establish and maintain infection in animals that fail to show overt disease, such as pigs and chickens.

Genes required for vitamin B_{12} synthesis. One cloned fragment carried the two adjacent genes *cbiF* and *cbiG*, which regulate vitamin B_{12} synthesis (71). Serotype Typhimurium synthesizes B_{12} only during anaerobic growth and can use B_{12} as a cofactor in at least three reactions (71). B_{12} synthesis requires the expression of a single 20-gene operon, named *cob*, which maps to centisome 44 of the serotype Typhimurium chromosome and includes 3 *cob* and 17 *cbi* genes (72). Propanediol, a by-product of food digestion, induces the transcription of the *cob* operon dependent upon PocR, a regulatory protein of the AraC family (12). Vitamin B_{12} is required for degradation of ethanolamine and propanediol, both of which are carbon sources present in the gastrointestinal tract (43, 69). It is therefore possible that *Salmonella* induces the *cob* operon to utilize these nutrient sources while living in the intestinal tract. The *cbiF*/*cibG* clone identified in this screen, however, was found only in tonsils. It remains possible that *cbiF*/*cbiG* was also induced in the intestinal tract but that we failed to find it there. For this fusion, and all other fusions described in this work, the specific location of induction could not be made with complete certainty, as the design of the screen does not ensure the complete recovery of all isolates from both body sites tested. Alternatively, it is possible that environmental signals exist specifically in the tonsils that induce the *cob* operon, either as a requirement for life in the tonsil itself or as a prelude to passage into the intestinal tract. Our previous work has shown that the invasion of epithelial cells by *Salmonella* is coregulated with propanediol and ethanolamine catabolism, including *cob* expression (40, 41). It is therefore possible that environmental cues encountered by *Salmonella* coordinately regulate functions important to life in an animal host, including both metabolic and virulence functions.

Genes of other functions. Other genes found in this screen included a protease (*sppA*), an integrase (*rlgA*), a gene used for cysteine synthesis (*cysQ*) (60), and those with the putative functions shown in Table 1. Therefore, our screen has identified diverse classes of *Salmonella* genes that appear to be induced in vivo.

Reverse-oriented fusions. We also found that 21 of the fragments identified in this screen were cloned in the direction that placed the annotated gene contained on the fragment in an

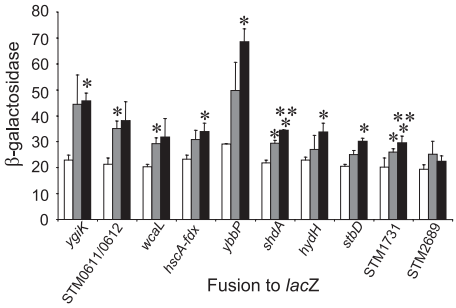

FIG. 2. Effects of temperature on in vivo-induced gene fusions. Strains carrying a fusion of the indicated gene to *lacZ* were grown as standing overnight cultures in LB at 30°C (white bars), 37°C (gray bars), and 42°C (black bars). Triplicate cultures of each strain were assayed for *lacZ* expression using CRPG-enhanced β-galactosidase assays. Single asterisks show a significant increase ($P \leq 0.05$) when the strain was grown at 42°C or 37°C compared to 30°C. Double asterisks show a significant increase ($P \leq 0.05$) when the strain was grown at 42°C compared to 37°C. The β-galactosidase concentration was calculated as defined in Materials and Methods. Error bars show standard deviations. STM2689 is shown as a representative of fusions that were not induced under these conditions.

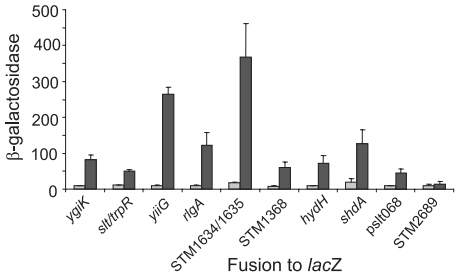

FIG. 3. Effects of osmolarity on in vivo-induced gene fusions. Strains carrying a fusion of the indicated gene to *lacZ* were grown as standing overnight cultures in MOPS minimal medium (gray bars) or with the addition of 0.4 M NaCl (black bars). Triplicate cultures of each strain were assayed for *lacZ* expression in CRPG-enhanced β-galactosidase assays. All fusions shown, except for STM2689, produced a significant increase ($P \leq 0.05$) in expression due to the addition of the NaCl. STM2689 is shown as representative of fusions that were not induced under these conditions. The β-galactosidase concentration was calculated as defined in Materials and Methods. Error bars show standard deviations.

orientation opposite to that of *cre*, thus producing no obvious promoter fusion. Examination of the sequence of these clones showed that they did not carry portions of adjacent genes in the opposite orientation, nor were they composed of concatenated DNA fragments from disparate regions of the genome. Misannotaion of the genome is unlikely as an explanation, because most of the cloned fragments carried genes with recognized functions, such as *shdA*, *rpoN*, and *wcaL* (Table 1), while three cloned fragments with putative functions in *Salmonella* (STM1368, *ygiK*, and *yciA*/*yciB*) have homologues with recognized functions in another organism. Similar identification of reverse-oriented fusions has been repeatedly observed by others but without complete explanation (9, 49, 66, 67, 89). Although some of these reverse-oriented fusions may contain no genuine promoter element, it is possible that others of them do. Three of the 21 were found independently twice or more in our screen, while 4 of these were induced by elevation of temperature and 5 were induced by osmolarity, as described below. One proposed explanation is that these fusions represent promoters that act to control gene expression by an antisense regulation mechanism (61, 76). Further investigation of these clones will be required to elucidate their functions.

Identification of environmental factors that induce gene expression. A number of environmental conditions likely to be present within animal hosts have been studied as possible stimuli for *Salmonella* gene expression. Specifically, for *Salmonella* invasion genes, oxygen tension, osmolarity, growth phase, pH, and the presence of bile have all been implicated in the control of gene expression (3, 19, 24, 44, 48, 65). We therefore next determined whether any of our in vivo-induced genes responded to similar conditions. The reporter plasmid used in this study carries a promoterless *lacZ* immediately downstream from *cre* (2), and so we used β-galactosidase assays to assess changes in gene expression in response to environmental signals. One plausible means to induce expression in pigs is via a change in temperature. Pigs have a normal body temperature of ~39 to 40°C, higher than that of humans. To test the effects of temperature, we tested the expression of clones when strains were grown at 30°C, 37°C, and 42°C. We found that nine of the cloned fragments induced *lacZ* expression with statistical significance at 42°C and/or 37°C compared to growth at 30°C (Fig. 2). Two of the reverse-oriented fusions, *shdA* and *stbD*, were also significantly increased at 42°C versus 37°C. *fdx-hscA* was induced by high temperature, as expected, as *hscA* is a heat shock protein. *hydH*, the sensor kinase of the zinc tolerance two-component system, was also induced by high temperature. Other induced genes included those encoding a putative hydrogenase (STM0611/0612), an inner membrane protein (*ybbP*), and a putative catalase (STM1731). Four of nine clones induced by high temperature were reverse-oriented fusions, those that contained *ygiK*, *wcaL*, *shdA*, and *stbD*. As described above, these fragments carried no identified promoter elements that would induce the conditional expression of the *lacZ* fusion. The fact that they were significantly induced by changes in temperature, however, suggests that transcription at these loci might indeed occur in response to altered environmental conditions.

Previous studies have demonstrated that an increase in osmolarity has a global effect on gene expression in *E. coli* (90–92). In the gastrointestinal tracts of mammals, bacteria are faced with hyperosmolarity (73). High osmolarity has been implicated in the induction of *Salmonella* invasion genes (24), and so it might induce other genes required for infection and survival. Therefore, we tested osmolar stress by growing our strains with the addition of 0.4 M sodium chloride to the medium (91). Nine of the clones were induced by increased osmolarity (Fig. 3). These included genes encoding the sensor kinase *hydH*, an integrase (*rlgA*), and a predicted amino acid ABC transporter (STM1634). Increased osmolarity also induced the reverse-oriented fusions carrying *shdA* and Pslt068. Thus, osmolarity is likely a signal for the induction of *Salmonella* genes in pigs and induces some transporters and membrane proteins. We also tested anaerobiosis, increased zinc by addition of $ZnSO_4$, iron limitation by addition of the iron-chelating agent 2,2′-dipyridyl, rich versus minimal medium, and cold shock as possible inducers of gene expression, since all these conditions

have previously been shown to induce one or several in vivo-induced genes. However, none of these conditions induced the expression of any in vivo-induced genes identified in this study.

Conclusions. In this work, we performed a comprehensive *Salmonella* genomic library screen to identify genes differentially expressed upon infection of pigs using a recombinase-based in vivo expression technology. As the first such screen in this animal species to be reported, we identified some common colonization factors that likely verify the utility of the approach. These genes included *bcfA*, *wecC*, *rffG*, and *yciR*, which are involved in surface adherence and were isolated from both tonsils and the intestinal tract. An early step for *Salmonella* colonization is adherence to cell surfaces. Thus, regardless of the location, *Salmonella* likely uses these colonization factors to establish itself in the body. We also found novel factors not previously known to be induced upon bacterial interaction with an animal host. These included *hydH*, which may indicate that zinc acts as a signal during *Salmonella* infection, and *hpaB*, the product of which catalyzes the degradation of 4-HPA, suggesting the use of aromatic acids as energy sources. Interestingly, we also identified a gene, *yciR*, with putative diguanylate cyclase and phosphodiesterase functions used for the production and degradation of c-di-GMP. As *yciR* has been shown to be used for biofilm formation in *Salmonella* (26), and as c-di-GTP as a second messenger has recently been identified as important in other pathogenic bacteria (15, 25, 68, 78), the induction of this gene may play an important role for *Salmonella* after animal infection. Although no screen of this type can provide an exhaustive account of all genes induced in vivo, this study provides information for further research on *Salmonella* survival and colonization in animals that carry the pathogen without clinical signs, which may lead to new therapies or prevention strategies to reduce the contamination of the human food supply.

ACKNOWLEDGMENTS

We thank Morgan Raley for invaluable assistance with sequencing.

This project was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, award number 2005-35201-16270.

REFERENCES

1. **Althouse, C., S. Patterson, P. Fedorka-Cray, and R. E. Isaacson.** 2003. Type 1 fimbriae of *Salmonella enterica* serovar Typhimurium bind to enterocytes and contribute to colonization of swine in vivo. Infect. Immun. **71:**6446–6452.
2. **Altier, C., and M. Suyemoto.** 1999. A recombinase-based selection of differentially expressed bacterial genes. Gene **240:**99–106.
3. **Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee.** 1996. Co-ordinate regulation of Salmonella typhimurium invasion genes by environmental and regulatory factors is mediated by control of hilA expression. Mol. Microbiol. **22:**703–714.
4. **Barrow, P. A., J. M. Simpson, and M. A. Lovell.** 1988. Intestinal colonisation in the chicken by food-poisoning Salmonella serotypes; microbial characteristics associated with faecal excretion. Avian Pathol. **17:**571–588.
5. **Bean, N. H., J. S. Goulding, M. T. Daniels, and F. J. Angulo.** 1997. Surveillance for foodborne disease outbreaks—United States, 1988–1992. J. Food Prot. **60:**1265–1286.
6. **Behlau, I., and S. I. Miller.** 1993. A PhoP-repressed gene promotes *Salmonella typhimurium* invasion of epithelial cells. J. Bacteriol. **175:**4475–4484.
7. **Berger, L. L.** 1987. Salt and trace minerals for livestock, poultry and other animals. Salt Institute, Alexandria, VA.
8. **Buchmeier, N. A., and F. Heffron.** 1990. Induction of Salmonella stress proteins upon infection of macrophages. Science **248:**730–732.
9. **Camilli, A., and J. J. Mekalanos.** 1995. Use of recombinase gene fusions to identify Vibrio cholerae genes induced during infection. Mol. Microbiol. **18:**671–683.
10. **Carnell, S. C., A. Bowen, E. Morgan, D. J. Maskell, T. S. Wallis, and M. P. Stevens.** 2007. Role in virulence and protective efficacy in pigs of Salmonella enterica serovar Typhimurium secreted components identified by signature-tagged mutagenesis. Microbiology **153:**1940–1952.
11. **Chan, K., C. C. Kim, and S. Falkow.** 2005. Microarray-based detection of *Salmonella enterica* serovar Typhimurium transposon mutants that cannot survive in macrophages and mice. Infect. Immun. **73:**5438–5449.
12. **Chen, P., D. I. Andersson, and J. R. Roth.** 1994. The control region of the *pdu/cob* regulon in *Salmonella typhimurium*. J. Bacteriol. **176:**5474–5482.
13. **Craven, S. E.** 1994. Altered colonizing ability for the ceca of broiler chicks by lipopolysaccharide-deficient mutants of Salmonella typhimurium. Avian Dis. **38:**401–408.
14. **Craven, S. E., N. A. Cox, J. S. Bailey, N. J. Stern, R. J. Meinersmann, and L. C. Blankenship.** 1993. Characterization of Salmonella california and S. typhimurium strains with reduced ability to colonize the intestinal tract of broiler chicks. Avian Dis. **37:**339–348.
15. **D'Argenio, D. A., M. W. Calfee, P. B. Rainey, and E. C. Pesci.** 2002. Autolysis and autoaggregation in *Pseudomonas aeruginosa* colony morphology mutants. J. Bacteriol. **184:**6481–6489.
16. **Davies, P. R., F. Geradus, E. M. Bovee, J. A. Funk, W. E. M. Morrow, F. T. Jones, and J. Deen.** 1998. Isolation of Salmonella serotypes from feces of pigs raised in a multiple-site production system. J. Am. Vet. Med. Assoc. **212:**1925–1929.
17. **De Groote, M. A., T. Testerman, Y. Xu, G. Stauffer, and F. C. Fang.** 1996. Homocysteine antagonism of nitric oxide-related cytostasis in Salmonella typhimurium. Science **272:**414–417.
18. **Ensgraber, M., and M. Loos.** 1992. A 66-kilodalton heat shock protein of *Salmonella typhimurium* is responsible for binding of the bacterium to intestinal mucus. Infect. Immun. **60:**3072–3078.
19. **Ernst, R. K., D. M. Dombroski, and J. M. Merrick.** 1990. Anaerobiosis, type 1 fimbriae, and growth phase are factors that affect invasion of HEp-2 cells by *Salmonella typhimurium*. Infect. Immun. **58:**2014–2016.
20. **Farrington, L. A., R. B. Harvey, S. A. Buckley, R. E. Droleskey, D. J. Nisbet, and P. D. Inskip.** 2001. Prevalence of antimicrobial resistance in salmonellae isolated from market-age swine. J. Food Prot. **64:**1496–1502.
21. **Galan, B., E. Diaz, M. A. Prieto, and J. L. Garcia.** 2000. Functional analysis of the small component of the 4-hydroxyphenylacetate 3-monooxygenase of *Escherichia coli* W: a prototype of a new flavin:NAD(P)H reductase subfamily. J. Bacteriol. **182:**627–636.
22. **Galan, B., A. Kolb, J. M. Sanz, J. L. Garcia, and M. A. Prieto.** 2003. Molecular determinants of the hpa regulatory system of Escherichia coli: the HpaR repressor. Nucleic Acids Res. **31:**6598–6609.
23. **Galan, J. E., and R. Curtiss III.** 1989. Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells. Proc. Natl. Acad. Sci. USA **86:**6383–6387.
24. **Galan, J. E., and R. Curtiss III.** 1990. Expression of *Salmonella typhimurium* genes required for invasion is regulated by changes in DNA supercoiling. Infect. Immun. **58:**1879–1885.
25. **Galperin, M. Y.** 2004. Bacterial signal transduction network in a genomic perspective. Environ. Microbiol. **6:**552–567.
26. **Garcia, B., C. Latasa, C. Solano, F. Garcia-del Portillo, C. Gamazo, and I. Lasa.** 2004. Role of the GGDEF protein family in Salmonella cellulose biosynthesis and biofilm formation. Mol. Microbiol. **54:**264–277.
27. **Gebreyes, W. A., P. R. Davies, W. E. Morrow, J. A. Funk, and C. Altier.** 2000. Antimicrobial resistance of *Salmonella* isolates from swine. J. Clin. Microbiol. **38:**4633–4636.
28. **Giel, J. L., D. Rodionov, M. Liu, F. R. Blattner, and P. J. Kiley.** 2006. IscR-dependent gene expression links iron-sulphur cluster assembly to the control of O_2-regulated genes in Escherichia coli. Mol. Microbiol. **60:**1058–1075.
29. **Goldman, P.** 1983. Biochemical pharmacology and toxicology involving the intestinal flora. Academic Press, Inc., New York, NY.
30. **Groisman, E. A., and H. Ochman.** 1993. Cognate gene clusters govern invasion of host epithelial cells by Salmonella typhimurium and Shigella flexneri. EMBO J. **12:**3779–3787.
31. **Hisert, K. B., M. MacCoss, M. U. Shiloh, K. H. Darwin, S. Singh, R. A. Jones, S. Ehrt, Z. Zhang, B. L. Gaffney, S. Gandotra, D. W. Holden, D. Murray, and C. Nathan.** 2005. A glutamate-alanine-leucine (EAL) domain protein of Salmonella controls bacterial survival in mice, antioxidant defence and killing of macrophages: role of cyclic diGMP. Mol. Microbiol. **56:**1234–1245.
32. **Hoff, K. G., D. T. Ta, T. L. Tapley, J. J. Silberg, and L. E. Vickery.** 2002. Hsc66 substrate specificity is directed toward a discrete region of the iron-sulfur cluster template protein IscU. J. Biol. Chem. **277:**27353–27359.
33. **Humphries, A., S. Deridder, and A. J. Baumler.** 2005. *Salmonella enterica* serotype Typhimurium fimbrial proteins serve as antigens during infection of mice. Infect. Immun. **73:**5329–5338.
34. **Humphries, A. D., M. Raffatellu, S. Winter, E. H. Weening, R. A. Kingsley, R. Droleskey, S. Zhang, J. Figueiredo, S. Khare, J. Nunes, L. G. Adams, R. M. Tsolis, and A. J. Baumler.** 2003. The use of flow cytometry to detect expression of subunits encoded by 11 Salmonella enterica serotype Typhimurium fimbrial operons. Mol. Microbiol. **48:**1357–1376.
35. **Humphries, A. D., S. M. Townsend, R. A. Kingsley, T. L. Nicholson, R. M.**

Tsolis, and A. J. Baumler. 2001. Role of fimbriae as antigens and intestinal colonization factors of Salmonella serovars. FEMS Microbiol. Lett. **201:**121–125.
36. **John, M., I. T. Kudva, R. W. Griffin, A. W. Dodson, B. McManus, B. Krastins, D. Sarracino, A. Progulske-Fox, J. D. Hillman, M. Handfield, P. I. Tarr, and S. B. Calderwood.** 2005. Use of in vivo-induced antigen technology for identification of *Escherichia coli* O157:H7 proteins expressed during human infection. Infect. Immun. **73:**2665–2679.
37. **Johnson, D. C., P. C. Dos Santos, and D. R. Dean.** 2005. NifU and NifS are required for the maturation of nitrogenase and cannot replace the function of isc-gene products in Azotobacter vinelandii. Biochem. Soc Trans. **33:** 90–93.
38. **Jones, B. D., and S. Falkow.** 1994. Identification and characterization of a *Salmonella typhimurium* oxygen-regulated gene required for bacterial internalization. Infect. Immun. **62:**3745–3752.
39. **Kuhn, H. M., U. Meier-Dieter, and H. Mayer.** 1988. ECA, the enterobacterial common antigen. FEMS Microbiol. Rev. **4:**195–222.
40. **Lawhon, S. D., J. G. Frye, M. Suyemoto, S. Porwollik, M. McClelland, and C. Altier.** 2003. Global regulation by CsrA in Salmonella typhimurium. Mol. Microbiol. **48:**1633–1645.
41. **Lawhon, S. D., R. Maurer, M. Suyemoto, and C. Altier.** 2002. Intestinal short-chain fatty acids alter Salmonella typhimurium invasion gene expression and virulence through BarA/SirA. Mol. Microbiol. **46:**1451–1464.
42. **Lawley, T. D., K. Chan, L. J. Thompson, C. C. Kim, G. R. Govoni, and D. M. Monack.** 2006. Genome-wide screen for Salmonella genes required for long-term systemic infection of the mouse. PLoS Pathog. **2:**e11.
43. **Lawrence, J. G., and J. R. Roth.** 1995. The cobalamin (coenzyme B_{12}) biosynthetic genes of *Escherichia coli*. J. Bacteriol. **177:**6371–6380.
44. **Lee, C. A., and S. Falkow.** 1990. The ability of Salmonella to enter mammalian cells is affected by bacterial growth state. Proc. Natl. Acad. Sci. USA **87:**4304–4308.
45. **Lee, C. A., B. D. Jones, and S. Falkow.** 1992. Identification of a Salmonella typhimurium invasion locus by selection for hyperinvasive mutants. Proc. Natl. Acad. Sci. USA **89:**1847–1851.
46. **Lee, L. J., J. A. Barrett, and R. K. Poole.** 2005. Genome-wide transcriptional response of chemostat-cultured *Escherichia coli* to zinc. J. Bacteriol. **187:** 1124–1134.
47. **Leonhartsberger, S., A. Huber, F. Lottspeich, and A. Bock.** 2001. The hydH/G genes from Escherichia coli code for a zinc and lead responsive two-component regulatory system. J. Mol. Biol. **307:**93–105.
48. **Lundberg, U., U. Vinatzer, D. Berdnik, A. von Gabain, and M. Baccarini.** 1999. Growth phase-regulated induction of *Salmonella*-induced macrophage apoptosis correlates with transient expression of SPI-1 genes. J. Bacteriol. **181:**3433–3437.
49. **Mahan, M. J., J. M. Slauch, and J. J. Mekalanos.** 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259:**686–688.
50. **Marolda, C. L., and M. A. Valvano.** 1995. Genetic analysis of the dTDP-rhamnose biosynthesis region of the *Escherichia coli* VW187 (O7:K1) *rfb* gene cluster: identification of functional homologs of *rfbB* and *rfbA* in the *rff* cluster and correct location of the *rffE* gene. J. Bacteriol. **177:**5539–5546.
51. **Martens, E. C., J. Gawronski-Salerno, D. L. Vokal, M. C. Pellitteri, M. L. Menard, and H. Goodrich-Blair.** 2003. *Xenorhabdus nematophila* requires an intact *iscRSUA-hscBA-fdx* operon to colonize *Steinernema carpocapsae* nematodes. J. Bacteriol. **185:**3678–3682.
52. **Martin, M., A. Gibello, J. Fernandez, E. Ferrer, and A. Garrido-Pertierra.** 1991. Catabolism of 3- and 4-hydroxyphenylacetic acid by Klebsiella pneumoniae. J. Gen. Microbiol. **137:**621–628.
53. **McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson.** 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature **413:**852–856.
54. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. **5:**607–625.
55. **Meier-Dieter, U., R. Starman, K. Barr, H. Mayer, and P. D. Rick.** 1990. Biosynthesis of enterobacterial common antigen in Escherichia coli. Biochemical characterization of Tn10 insertion mutants defective in enterobacterial common antigen synthesis. J. Biol. Chem. **265:**13490–132497.
56. **Mendez-Ortiz, M. M., and J. Membrillo-Hernandez.** 2005. Proteins with GGDEF and EAL domains: their role in bacterial metabolism. Rev. Latinoam. Microbiol. **47:**130–139. (In Spanish.)
57. **Miller, S. I., and J. J. Mekalanos.** 1990. Constitutive expression of the *phoP* regulon attenuates *Salmonella* virulence and survival within macrophages. J. Bacteriol. **172:**2485–2490.
58. **Mills, D. M., V. Bajaj, and C. A. Lee.** 1995. A 40 kb chromosomal fragment encoding Salmonella typhimurium invasion genes is absent from the corresponding region of the Escherichia coli K-12 chromosome. Mol. Microbiol. **15:**749–759.
59. **Morgan, E., J. D. Campbell, S. C. Rowe, J. Bispham, M. P. Stevens, A. J. Bowen, P. A. Barrow, D. J. Maskell, and T. S. Wallis.** 2004. Identification of host-specific colonization factors of Salmonella enterica serovar Typhimurium. Mol. Microbiol. **54:**994–1010.
60. **Neuwald, A. F., B. R. Krishnan, I. Brikun, S. Kulakauskas, K. Suziedelis, T. Tomcsanyi, T. S. Leyh, and D. E. Berg.** 1992. *cysQ*, a gene needed for cysteine synthesis in *Escherichia coli* K-12 only during aerobic growth. J. Bacteriol. **174:**415–425.
61. **Osorio, G. C., A.** 2003. Hidden dimensions of *Vibrio cholerae* pathogenesis. ASM News **69:**396–401.
62. **Prieto, M. A., E. Diaz, and J. L. Garcia.** 1996. Molecular characterization of the 4-hydroxyphenylacetate catabolic pathway of *Escherichia coli* W: engineering a mobile aromatic degradative cluster. J. Bacteriol. **178:**111–120.
63. **Prieto, M. A., and J. L. Garcia.** 1994. Molecular characterization of 4-hydroxyphenylacetate 3-hydroxylase of Escherichia coli. A two-protein component enzyme. J. Biol. Chem. **269:**22823–22829.
64. **Prieto, M. A., A. Perez-Aranda, and J. L. Garcia.** 1993. Characterization of an *Escherichia coli* aromatic hydroxylase with a broad substrate range. J. Bacteriol. **175:**2162–2167.
65. **Prouty, A. M., and J. S. Gunn.** 2000. *Salmonella enterica* serovar Typhimurium invasion is repressed in the presence of bile. Infect. Immun. **68:** 6763–6769.
66. **Rainey, P. B.** 1999. Adaptation of Pseudomonas fluorescens to the plant rhizosphere. Environ. Microbiol. **1:**243–257.
67. **Rollenhagen, C., and D. Bumann.** 2006. *Salmonella enterica* highly expressed genes are disease specific. Infect. Immun. **74:**1649–1660.
68. **Romling, U., M. Gomelsky, and M. Y. Galperin.** 2005. c-di-GMP: the dawning of a novel bacterial signalling system. Mol. Microbiol. **57:**629–639.
69. **Rondon, M. R., A. R. Horswill, and J. C. Escalante-Semerena.** 1995. DNA polymerase I function is required for the utilization of ethanolamine, 1,2-propanediol, and propionate by *Salmonella typhimurium* LT2. J. Bacteriol. **177:**7119–7124.
70. **Roper, D. I., T. Fawcett, and R. A. Cooper.** 1993. The Escherichia coli C homoprotocatechuate degradative operon: hpc gene order, direction of transcription and control of expression. Mol. Gen. Genet. **237:**241–250.
71. **Roth, J. R., J. G. Lawrence, and T. A. Bobik.** 1996. Cobalamin (coenzyme B12): synthesis and biological significance. Annu. Rev. Microbiol. **50:**137–181.
72. **Roth, J. R., J. G. Lawrence, M. Rubenfield, S. Kieffer-Higgins, and G. M. Church.** 1993. Characterization of the cobalamin (vitamin B_{12}) biosynthetic genes of *Salmonella typhimurium*. J. Bacteriol. **175:**3303–3316.
73. **Russell, J. B., and G. N. Jarvis.** 2001. Practical mechanisms for interrupting the oral-fecal lifecycle of Escherichia coli. J. Mol. Microbiol. Biotechnol. **3:**265–272.
74. **Shah, D. H., M. J. Lee, J. H. Park, J. H. Lee, S. K. Eo, J. T. Kwon, and J. S. Chae.** 2005. Identification of Salmonella gallinarum virulence genes in a chicken infection model using PCR-based signature-tagged mutagenesis. Microbiology **151:**3957–3968.
75. **Silberg, J. J., T. L. Tapley, K. G. Hoff, and L. E. Vickery.** 2004. Regulation of the HscA ATPase reaction cycle by the co-chaperone HscB and the iron-sulfur cluster assembly protein IscU. J. Biol. Chem. **279:**53924–53931.
76. **Silby, M. W., P. B. Rainey, and S. B. Levy.** 2004. IVET experiments in Pseudomonas fluorescens reveal cryptic promoters at loci associated with recognizable overlapping genes. Microbiology **150:**518–520.
77. **Simm, R., M. Morr, A. Kader, M. Nimtz, and U. Romling.** 2004. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. Mol. Microbiol. **53:**1123–1134.
78. **Tamayo, R., J. T. Pratt, and A. Camilli.** 2007. Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. Annu. Rev. Microbiol. **61:**131–148.
79. **Tanaka, Y., and Y. Katsube.** 1978. Infectivity of Salmonella typhimurium for mice in relation to fimbriae. Nippon Juigaku Zasshi **40:**671–681.
80. **Tanaka, Y., Y. Katsube, T. Mutoh, and K. Imaizumi.** 1981. Fimbriae of Salmonella typhimurium and their role in mouse intestinal colonization of the organism. Nippon Juigaku Zasshi **43:**51–62.
81. **Tatusov, R. L., M. Y. Galperin, D. A. Natale, and E. V. Koonin.** 2000. The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Res. **28:**33–36.
82. **Tauxe, R. V.** 1991. Salmonella: a postmodern pathogen. J. Food Prot. **54:** 563–568.
83. **Tokumoto, U., and Y. Takahashi.** 2001. Genetic analysis of the isc operon in Escherichia coli involved in the biogenesis of cellular iron-sulfur proteins. J. Biochem. (Tokyo) **130:**63–71.
84. **Toth, I. K., C. J. Thorpe, S. D. Bentley, V. Mulholland, L. J. Hyman, M. C. Perombelon, and G. P. Salmond.** 1999. Mutation in a gene required for lipopolysaccharide and enterobacterial common antigen biosynthesis affects virulence in the plant pathogen Erwinia carotovora subsp. atroseptica. Mol. Plant-Microbe Interact. **12:**499–507.
85. **Tsolis, R. M., L. G. Adams, T. A. Ficht, and A. J. Baumler.** 1999. Contribution of *Salmonella typhimurium* virulence factors to diarrheal disease in calves. Infect. Immun. **67:**4879–4885.
86. **Tsolis, R. M., S. M. Townsend, E. A. Miao, S. I. Miller, T. A. Ficht, L. G.**

Adams, and A. J. Baumler. 1999. Identification of a putative *Salmonella enterica* serotype Typhimurium host range factor with homology to IpaH and YopM by signature-tagged mutagenesis. Infect. Immun. **67:**6385–6393.

87. **Turner, A. K., M. A. Lovell, S. D. Hulme, L. Zhang-Barber, and P. A. Barrow.** 1998. Identification of *Salmonella typhimurium* genes required for colonization of the chicken alimentary tract and for virulence in newly hatched chicks. Infect. Immun. **66:**2099–2106.
88. **Valdivia, R. H., and S. Falkow.** 1997. Fluorescence-based isolation of bacterial genes expressed within host cells. Science **277:**2007–2011.
89. **Wang, J., A. Mushegian, S. Lory, and S. Jin.** 1996. Large-scale isolation of candidate virulence genes of Pseudomonas aeruginosa by in vivo selection. Proc. Natl. Acad. Sci. USA **93:**10434–10439.
90. **Weber, A., and K. Jung.** 2002. Profiling early osmostress-dependent gene expression in *Escherichia coli* using DNA macroarrays. J. Bacteriol. **184:** 5502–5507.
91. **Weber, A., S. A. Kogl, and K. Jung.** 2006. Time-dependent proteome alterations under osmotic stress during aerobic and anaerobic growth in *Escherichia coli*. J. Bacteriol. **188:**7165–7175.
92. **Weber, H., T. Polen, J. Heuveling, V. F. Wendisch, and R. Hengge.** 2005. Genome-wide analysis of the general stress response network in *Escherichia coli*: σ^S-dependent genes, promoters, and σ factor selectivity. J. Bacteriol. **187:**1591–1603.
93. **Wood, R. L., A. Pospischil, and R. Rose.** 1989. Distribution of persistent Salmonella typhimurium infection in internal organs of swine. Am. J. Vet. Res. **50:**1015–1021.
94. **Zakin, M. M., N. Duchange, P. Ferrara, and G. N. Cohen.** 1983. Nucleotide sequence of the metL gene of Escherichia coli. Its product, the bifunctional aspartokinase II-homoserine dehydrogenase II, and the bifunctional product of the thrA gene, aspartokinase I-homoserine dehydrogenase I, derive from a common ancestor. J. Biol. Chem. **258:**3028–3031.
95. **Zheng, L., V. L. Cash, D. H. Flint, and D. R. Dean.** 1998. Assembly of iron-sulfur clusters. Identification of an iscSUA-hscBA-fdx gene cluster from Azotobacter vinelandii. J. Biol. Chem. **273:**13264–13272.