

Tissue-Specific Gene Expression Identifies a Gene in the Lysogenic Phage Gifsy-1 That Affects *Salmonella enterica* Serovar Typhimurium Survival in Peyer's Patches

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In vivo expression technology was used to identify *Salmonella enterica* serovar Typhimurium genes that are transcriptionally induced when the bacteria colonize the small intestines of mice. These genes were subsequently screened for those that are transcriptionally inactive during the systemic stages of disease. This procedure identified *gipA*, a gene that is specifically induced in the small intestine of the animal. The *gipA* gene is carried on the lambdoid phage Gifsy-1. Consistent with the expression profile, the sole defect conferred by a *gipA* null mutation is in growth or survival in a Peyer's patch. The *gipA* strain is wild type in its ability to initially colonize the small intestine and invade the intestinal epithelium. The mutant also survives and propagates at wild-type levels during the systemic stages of disease. The *gipA* open reading frame is homologous to a family of putative insertion sequence elements, although our evidence shows that transposition is not required for *gipA* function in the Peyer's patch. These results suggest that the bacteria sense and respond to the particular environment of the Peyer's patch, a critical site for the replication of *Salmonella* serovar Typhimurium.

Salmonellae are invasive pathogens that cause a range of human diseases. Nontyphoid salmonellae usually cause gastroenteritis. Although this is often a self-limiting disease marked by diarrhea and abdominal cramps, the infection can be more severe, resulting in bacteremia, fever, or even death (36). After oral infection in mice, *Salmonella* spp. initially colonize the small intestine. The bacteria preferentially attach to and invade the M cells of the Peyer's patches (9, 26), mucosal lymphoid follicles that constitute the major site of antigen sampling and immune response in the small intestine (28). The bacteria gain access to the underlying lymphoid tissue and survive and multiply within the follicles. From this site, salmonellae eventually disseminate throughout the host, resulting in systemic infection (7, 26).

Analysis of the early steps of colonization and invasion by *Salmonella enterica* serovar Typhimurium using the BALB/c mouse model of infection and in vitro tissue culture models has significantly increased understanding of these processes (11). Three fimbriae have been implicated in the initial attachment of *Salmonella* serovar Typhimurium to the intestinal mucosa or Peyer's patch M cells: plasmid-encoded fimbriae (*pef*) (17), long polar fimbriae (*lpf*) (3, 4), and thin aggregative fimbriae (*curl*; *agf*) (47). Mutations that block the production of either Agf (48) or Lpf (4) increase the oral 50% lethal dose (LD₅₀) three- to fivefold. These modest effects are apparently due to functional redundancy of the fimbriae; the oral LD₅₀ of strains containing mutations in *pef*, *lpf*, *agf*, and *fim*, encoding type I fimbriae, is increased approximately 26-fold (48). After initial attachment, serovar Typhimurium uses the type III secretion system encoded on *Salmonella* pathogenicity island 1 (SPI1) to facilitate uptake by M cells (11, 19). The expression of the

secretion system is controlled in response to a specific combination of environmental signals that presumably acts as a cue that the bacteria are in the appropriate anatomic location (2, 41, 44).

Although much is known about the mechanism by which *Salmonella* gains entry through M cells to the Peyer's patches (19), very little is understood about the growth of *Salmonella* in Peyer's patch tissue. The SipB protein, injected into the host cell via the SPI1 type III secretion system, can induce apoptosis in macrophages (22, 30, 37), presumably in the macrophages that initially engulf serovar Typhimurium in the Peyer's patches. Whether the SPI1 system is important throughout the growth of serovar Typhimurium in the Peyer's patches is not clear. Recent work suggests that diarrhea caused by *Salmonella* is an inflammatory response mediated by invasion of intestinal epithelial cells and subsequent infiltration of neutrophils (11). However, even in, for example, calves, where diarrhea is the major symptom of *Salmonella* infection, Peyer's patches are a primary site of invasion that may lead to systemic disease (18). Therefore, understanding the growth of the bacteria in this particular environment is vital to understanding *Salmonella* pathogenesis.

In this study, we used in vivo expression technology (IVET) to identify operons that are transcriptionally induced in the animal during the infection process (32, 45). IVET is based on the premise that many genes whose products are specifically required for the infection process will be regulated such that they are expressed only at the proper time and place in the host. Thus, this system was designed to identify promoters that are transcriptionally active in the mouse (in vivo) but not significantly transcribed on normal laboratory media (in vitro). In the pIVET1 system, random bacterial promoters are fused to a promoterless copy of the *purA* gene in a $\Delta purA$ background. Serovar Typhimurium strains containing *purA* mutations are extremely attenuated at all stages of infection (13, 42). Therefore, only bacteria containing a *purA* fusion to a sufficiently active promoter can survive within host tissues. These bacteria are recovered from various host organs and

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Source or reference ^a
Strains		
<i>Salmonella</i> serovar Typhimurium		
14028	Wild type	ATCC ^b
JS120	14028 Δ purA3141::kan	25
JS130	14028 zjg8103::pir	25
JS131	JS120 constitutive pIVET1 fusion	
JS132	JS120 pIVET1::gipA'	
JS133	14028 zjg8101::kan pIVET1::gipA'	
JS134	14028 gipA1::MudCm	
JS135	14028 zii8104::Tn10dTc	
JS136	JS135 gipA2::MudJ	
JS137	14028 hilA339::kan	
JS200	JS135 Δ Gifsy-1::kan	
JS201	JS135 gfoJ::MudJ	
JS202	JS135 Δ gfoA::kan	
<i>E. coli</i> BW19851	RP4-2-Tc::Mu-1 kan::Tn7 integrant/creB510 hsdR17 endA1 zbf-5 uid(Δ MluI)::pir recA1 thi	35
Plasmids		
pWKS30	pSC101 Ap ^r	50
pJS302	pWKS30 gipA	
pCtsPara-GBE	pSC101 ts ori gam bet exo	12
pKD4	R6K ori FRT-kan-FRT	12

^aThis study, unless otherwise noted.

^bATCC, American Type Culture Collection.

screened for those that have low transcriptional activity in vitro by monitoring the expression of the *lac* operon located downstream of *purA*. Using this system, we have identified a locus that is specifically active during growth in the small intestine but is not significantly transcribed during the later, systemic stages of infection. We show here that this locus is carried on the Gifsy-1 bacteriophage and is important for the survival of serovar Typhimurium in the Peyer's patches of BALB/c mice.

MATERIALS AND METHODS

Bacterial strains and genetic manipulations. The bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, bacteria were cultured at 37°C in Luria-Bertani (LB) medium (43) containing, when appropriate, ampicillin at 50 μ g ml⁻¹, kanamycin at 50 μ g ml⁻¹, chloramphenicol at 20 μ g ml⁻¹, or tetracycline at 25 μ g ml⁻¹. Strains containing Δ purA were grown in medium supplemented with 27 μ g of adenine ml⁻¹ and 16 μ g of thiamine ml⁻¹ (AdB₁). Lactose MacConkey (43) agar containing kanamycin, ampicillin, and AdB₁ (lactose MacConkey/A/K/AdB₁) was used to monitor *lac* expression.

Kanamycin cassette insertion/deletions were constructed using a method developed by Datsenko and Wanner (12). PCR primers 60 nucleotides (nt) long were synthesized with 40 nt on the 5' ends corresponding to the ends of the desired deletions. The 3' 20 nt of each primer was annealed to the 5' or 3' end of an FRT-kan-FRT cassette in plasmid pKD4, where FRT is the site of Flp-mediated recombination. PCRs were carried out according to the Taq manufacturer's instructions (Life Technologies, Inc.). Plasmid pCtsPara-GBE was introduced into serovar Typhimurium strain 14028. This plasmid synthesizes the lambda recombination proteins Gam, Bet, and Exo when induced with arabinose. The plasmid-bearing strain was grown at 30°C in LB medium containing chloramphenicol and 1 mM arabinose and made electrocompetent (33). Approximately 800 ng of PCR product was transformed, and the cells were plated on LB medium containing kanamycin at 37°C. This procedure generally resulted in 30 to 50 Kn^r colonies. Each insertion/deletion was characterized by genetic mapping

and PCR. The insertion/deletions were transduced via P22 to construct isogenic strains for subsequent analysis.

IVET library. Chromosomal DNA was isolated from JS120 (Δ purA) as described previously (43) and partially digested with *Sau*3AI to give 4- to 6-kb fragments, which were subsequently separated by size using a 10 to 40% sucrose density gradient (1, 43). These fragments were cloned into a *Bgl*II site directly 5' of the promoterless *purA-lacZY* operon of pIVET1 (32, 45) and electroporated into *Escherichia coli* BW19851 (Pi⁺ Tra⁺). The Pi protein is required for replication of pIVET1 derivatives. Over 42,000 independent clones were obtained, sufficient to represent, with 99% confidence, a fusion to every kilobase of the chromosome in both orientations (10). This library was then mated with JS120 (Δ purA), where the plasmid must integrate into the chromosome via homologous recombination with the cloned fragment in order to be stably maintained. The resulting exconjugants were pooled, and this library was used for the initial selection.

IVET selection. The integrated pIVET1 library in JS120 was grown overnight in LB medium with ampicillin, kanamycin, and AdB₁ (LB/A/K/AdB₁). The culture was washed and resuspended in an equal volume of sterile 0.1 M sodium phosphate buffer (pH 8.0). Four 6-week-old female BALB/c mice were anesthetized with CO₂ and inoculated orally with 0.2 ml of the culture (approximately 10⁹ CFU). After 5 days, the mice were sacrificed by CO₂ asphyxiation; their small intestines were harvested and homogenized in 2.0 ml of sterile 150 mM NaCl. The homogenate was used to inoculate a separate, fresh LB/A/K/AdB₁ culture, which was grown overnight. This culture was used to orally inoculate naive mice for a second passage (four mice total) as described above. Five days after inoculation, the mice were sacrificed; their small intestines were harvested and homogenized as before. Serial dilutions were plated on lactose MacConkey/A/K/AdB₁ and incubated overnight. Ninety colonies that displayed a Lac⁻ phenotype were subsequently chosen for future analysis.

Cloning and sequencing of gipA. The integrated pIVET1::gipA' plasmid was recovered by P22 transduction (31) into the Pi-producing strain, JS130. Sequencing of the chromosomal region at the fusion joint was carried out at the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois using a primer for the 5' end of *purA*. Further sequence data were obtained by cloning, via partial *Sau*3AI digestion, a Cm^r insertional mutation located near the *gipA* open reading frame (ORF) into pWKS30. Restriction mapping was performed, and a suitable *Cla*I subclone of 1.8 kb that contained the entire *gipA* ORF (pJS302) was constructed. This clone was sequenced with primers specific for the plasmid and by primer walking. Sequence analyses were performed using the Wisconsin Package (Genetics Computer Group, Inc.).

Virulence studies and competition assays. Six- to 8-week-old female BALB/c mice were inoculated either orally or intraperitoneally (i.p.) with 0.2 ml of a bacterial suspension diluted in sterile 0.1 M sodium phosphate buffer (oral) or sterile 150 mM NaCl (i.p.). After 4 to 7 days, the mice were sacrificed by CO₂ asphyxiation; the ceca, small intestines, and/or spleens were recovered. These organs were homogenized in 2.0 ml of sterile 150 mM NaCl. Serial dilutions of the homogenates were plated on appropriate media to determine CFU per organ. Subsequent replica plating allowed us to determine the percentages of wild-type and mutant cells.

For Peyer's patch quantitation, mice were inoculated orally and sacrificed after 6 days. The intestines were removed, and the lumen was washed twice with 10 ml of sterile 150 mM NaCl using a syringe. The Peyer's patches were individually dissected and homogenized in 2.0 ml of 150 mM NaCl. Appropriate dilutions were plated on selective medium. Subsequent replica plating allowed us to determine the percentages of wild-type and mutant cells.

The LD₅₀ was calculated according to the protocol of Reed and Muench (40) using groups of six mice for each dose. The competitive index (CI) was defined as the output ratio (mutant/wild type) divided by the input ratio (mutant/wild type). Student's *t* test was used for statistical analysis.

IOC. Intestinal organ culturing (IOC) was performed essentially as described previously (4). Briefly, the small intestine was removed from a mouse and incubated in tissue culture medium in a petri dish. The intestine was washed with 10 ml of 150 mM NaCl, the ends were tied, and the intestine was filled with 10⁷ CFU of an approximately 1:1 mixture of mutant and wild-type bacteria. The intestine was incubated for 1.0 h at 37°C in 5% CO₂ to allow for attachment and invasion. The intestine was then washed extensively, and the most distal Peyer's patch was dissected. This Peyer's patch and the remaining small intestinal tissue were separately homogenized in 2.0 ml 150 mM NaCl, and dilutions were plated for CFU determinations. Subsequent replica plating allowed us to determine the ratio of wild-type to mutant recovered bacteria. The mean and standard deviation of the CI for five assays are reported.

Invasion of Henle-407 cultured epithelial cells. Henle-407 epithelial cells were obtained from the American Type Culture Collection and propagated according to supplier instructions. Invasion assays were performed as described previously (39). Bacterial cultures were grown under invasion conditions, and a 1:1 mixture of wild-type serovar Typhimurium and either the *gipA* mutant or a noninvasive *hilA* mutant was used as an inoculum. After lysis of the eukaryotic cells, dilutions were plated on the appropriate selective media. Subsequent replica plating allowed us to determine the percentages of wild-type and mutant cells. The mean and standard deviation of the CI for three assays are reported.

Isolation of chromosomal DNA and Southern blot analysis. Chromosomal DNA from colonies of 14028 isolated from various tissues of an infected animal

was isolated using the Qiagen chromosomal preparation protocol (Qiagen Inc.). Samples were digested with *Cla*I and run on a 1% agarose gel. The DNA was subsequently transferred to a nitrocellulose membrane as previously described (1). The probe, plasmid pJS302, was labeled (along with the parent plasmid containing no chromosomal insert), and hybridization was performed using a nonradioactive labeling and detection kit from DuPont NEN. Hybrids were detected using SuperSignal detection reagents (Pierce).

In vitro analysis of the regulation of *gipA*. Expression of the IVET fusion in vitro was determined following overnight growth under various environmental conditions. Previous experiments had shown that growth phase has no effect on the expression of *gipA*. Iron starvation assays were performed with LB medium in the presence of 100 mM diethylenetriamine-pentaacetic acid, an extracellular iron chelator. This concentration was determined empirically to cause a reasonable amount of growth inhibition in strain 14028. Microtiter β -galactosidase assays were performed as previously described (46).

Nucleotide sequence accession number. The *gipA* sequence has been entered into GenBank under accession number AF246666.

RESULTS

Rationale and identification of *gipA*. We have used the IVET system (32) to identify serovar Typhimurium genes that are induced while the bacterium is in the mouse (45; data not shown). For IVET selection, a strain must contain a fusion of *purA* to a gene that is transcriptionally active in the animal. For the purposes of this study, we concentrated on genes that were identified as being transcriptionally induced in the small intestine. We reasoned that a subset of these genes would include those whose products are specifically required for some aspect of the early infection process. This subset of genes would be expressed during the early stages but not during the systemic stages of disease. Accordingly, we screened for fusions that were induced only in the small intestine and not in the spleen. To accomplish this, we performed a competition assay with a bacterial strain containing an IVET fusion to an in vivo-induced gene of interest and JS131, a strain containing a pIVET1 fusion to a promoter that is sufficiently active to allow for the survival of the strain in any host tissue (data not shown). Mice were inoculated either orally or i.p. with an equal mixture of the two strains. After 3 to 5 days, the mice were sacrificed; the small intestines were removed from the orally infected animals, while the spleens were removed from the i.p. infected animals. We screened for fusion strains that were recovered from the small intestines after oral infection in numbers equal to or larger than those of the constitutive fusion strain but that were recovered in relatively small numbers from the spleen after i.p. infection. Of the 10 orally induced fusions that we tested, one clearly met the screening requirements. We named the corresponding gene *gipA* (for growth in Peyer's patches).

In order to confirm the induction of *gipA* in the small intestine, we repeated the competition assay. After oral inoculation, the *gipA* fusion strain invariably outcompeted JS131 in the small intestine, with a median CI of 3.27 (Table 2). In contrast, after i.p. inoculation, the *gipA* fusion strain was significantly outcompeted by JS131 (median CI, 0.07) (Table 2). These results confirm that the *gipA* gene is regulated such that it is specifically induced only in the early stages of infection.

Molecular characterization of *gipA*. The pIVET1::*gipA'* plasmid was recovered from the fusion strain via P22 transduction into a Pi-producing strain of serovar Typhimurium, where the plasmid can recircularize and replicate autonomously (31). The portion of the chromosomal insert closest to the fusion joint was sequenced using a primer that hybridized to the 5' end of the *purA* gene in pIVET1. The sequence data indicated that the fusion joint was 143 bp from the 5' end of an ORF. In order to clone the entire *gipA* locus, an MudCm insertion mutation was isolated in the chromosomal insert of the pIVET1::*gipA'* plasmid and recombined into the chromosome by P22 transduction (34) to yield JS134. Chromosomal DNA

TABLE 2. Competition assay with pIVET1 fusion-containing strains JS131 (constitutive) and JS132 (*gipA*)

Inoculum	Mouse	Organ	Total CFU recovered	CI ^a
Oral	A	Small intestine	3.7×10^3	1.44
	B		2.7×10^3	4.51
	C		2.4×10^3	1.66
	D		2.2×10^3	3.27
	E		8.8×10^2	4.15
i.p.	F	Spleen	4.8×10^6	0.09
	G		3.8×10^7	0.03
	H		9.6×10^6	0.07
	I		6.2×10^6	0.07
	J		1.8×10^6	0.13

^a Defined as the output ratio (JS131/JS132) divided by the input ratio (JS131/JS132). Results for oral and i.p. inocula are statistically different ($P < 0.0005$).

fragments containing the Cm^r marker were cloned into the low-copy-number pSC101 derivative pWKS30 (50). Restriction analysis and subcloning allowed the construction of pJS302, which contains a *Cla*I fragment with approximately 400 bp 5' and 200 bp 3' of the *gipA* ORF.

The complete DNA sequence of the *gipA* ORF and flanking regions was obtained. Analysis of this sequence suggested that *gipA* consists of a single ORF that shares significant homology with a group of IS891-like insertion elements found in a diverse group of organisms. Near the carboxy terminus of the predicted 411-amino-acid protein lies a cluster of highly conserved amino acids that include four completely conserved Cys residues (Fig. 1). The *gipA* ORF is located in the late operon of the Gifsy-1 bacteriophage between *gfoZ* (named *gfo* for Gifsy one) and *gfoU*, the orthologs of the phage lambda *Z* and *U* genes. However, the *gipA* ORF is in the opposite orientation with respect to these potential phage genes, both of which are intact.

Phenotypes of a *gipA* null mutation. The data presented above suggest that the *gipA* locus is specifically induced in the small intestine. Accordingly, we predicted that a strain containing a null mutation in *gipA* would be attenuated for survival after oral inoculation but would be unaffected for survival during a strictly systemic infection. To test this hypothesis, an MudJ insertion mutation was isolated within the ORF of *gipA*. The location of the insertion in the *gipA* ORF was confirmed by PCR.

To test the role of *gipA* in pathogenesis, we performed oral and i.p. competition assays between the *gipA*2::MudJ insertion mutant and an isogenic wild-type strain. The results, shown in Table 3, indicate that the *gipA* mutant was outcompeted more than twofold in the small intestine. Although this phenotype was subtle, it was very reproducible and statistically significant. In contrast, no defect was conferred by the *gipA* insertion mutation when the bacteria were injected i.p. Indeed, the *gipA* mutant outcompeted the wild-type strain, although this result was not statistically significant. Thus, consistent with the expression profile, the *gipA* insertion mutation confers a virulence defect in an oral but not an i.p. infection.

Mutations in Gifsy-1 structural genes do not confer a virulence phenotype. To ensure that the defect was not due to some effect on Gifsy-1 phage production, we isolated a MudJ insertion in the putative tail fiber gene *gfoJ*. An isogenic strain containing the *gfoJ*::MudJ insertion competed equally with the wild type after oral inoculation (Table 3). In addition, we constructed a deletion/kanamycin insertion in *gfoA*. Again, the

	296				354	
C01019	YQLEKY.GKK LILVNPAY.T	SKTCAKCGYV	KEDLS.LSDR	VFVCPNCGWI	ADRDYNASLNI	<i>Sulfolobus solfataricus</i>
C02018	YQLEKY.GKK LILVNPAY.T	SKTCARCQYV	KNDL.SLSR	VFVCPNCGWI	ADRDYNASLNI	<i>Sulfolobus solfataricus</i>
Orf406	YQLEKY.GKK LVLVNPY.T	SKTCARCQYV	NYDLT.LGDR	IFVCSRQCGWT	VDRDYNASLNI	<i>Sulfolobus sp.</i>
IS1136CF	YKCKFI.GIE LVVADRFPYS	SKTCSQCGEI	KKDLK.LKDR	VYNC.SCGLS	IDRDLNASINL	<i>Clostridium perfringens</i>
IS607	YKAKYS.NKE LLRADTYYP	SKTCSNCGQV	KQDLK.LKDR	IYQCLECGFE	LDRDINAAILN	<i>Helicobacter pylori</i>
Rv2885c	YKTGWY.GSS LVVADRWFPS	SKTCHACRHV	QDIGW...DE	KWQCDGCSIT	HQRDDNAAINL	<i>Mycobacterium tuberculosis</i>
Rv2978c	YKTVWY.GSA LVVADRWFPS	SKTCHACRHV	QDIGW...DE	QWQCDRCSV	HQRDDCAAINL	<i>Mycobacterium tuberculosis</i>
Rv2791c	YKTGWY.GSQ LVVADRWFPS	SKTCHVCGHV	QEIOW...AE	HWQCDSCSAS	HQRDDCAAINL	<i>Mycobacterium tuberculosis</i>
Tra	YKSEWY.GSS LYKVDRDFPS	SKLCSHC .HI	KNTTLRLSDA	KWTCSSCNTL	HDKDINAAILN	<i>Borrelia burgdorferi</i>
IS605	YKAQRK.AKT LLGIDRFFPS	SQLCSYCGFN	TGK.KHENIT	KFTCPHCNIT	HHRDYNASVNI	<i>Helicobacter pylori</i>
IS605b	YKAQRK.GKT LLSIDRFFPS	SQLCSYCGVN	TGK.KHESIT	KFTCPHCQT	HHRDYNASVNI	<i>Helicobacter pylori</i>
SCH35-10	YKAERY.GRT LVVIGRFEP	SQTCSTCGVK	DGP.KPLQVR	EWTCFACGTV	HDRDHNAAINV	<i>Streptomyces coelicolor</i>
Slr2062	AKAVMY.GRE FRTISRWEPT	SQRCSCCGEI	GGK.KELSVR	EWTCFLFCGAN	HDRDINAATNI	<i>Synechocystis sp.</i>
IS8301	YKAAWY.GRL VSKVSPYFPS	SQLCHDCGFK	NPEVKNLAVR	TWTCPNCGET	HDRDENAALNI	<i>Deinococcus radiodurans</i>
TM1044	YKAKRC.GIE VIKANRYFYS	SQMCSECGYI	NKEVKDLSVR	EWTCFVCGAH	HDRDVNAAKNL	<i>Thermotoga maritima</i>
YdcA	YKAATK.GVH LVKLDQWFAS	SKTCHCCGYK	MPEMP.LHKR	IWRCPFCGAE	HDRDINAALNI	Plasmid ColIb-P9
IS1341	YKLVVQ.GKK LLIKDKWFPS	SKTCSCCGRV	KESLS.LSER	TFRC.ECGFE	SDRDVNAAINI	Thermophilic bacterium PS3
GipA	YKGEWA.GRS VVAIDQFFPS	SKRCSCCGFI	MKKMP.LDVR	KWQCPFCGTD	HDRDVNAARNI	Bacteriophage Gifsy-1
YsnA	YKCAWY.GRE LVVIDRWFP	SKLCSGTCGTV	RGKLP.LNVR	EWTC.DCGAV	HDRDVNAARNI	<i>Streptomyces pristinaespiralis</i>
IS1136	YKTRA.GKT LVVIDRWYPS	SKTCSACGHL	LEKLS.LSTR	AWTCPGCRTR	HDRDINAAKNI	<i>Saccharopolyspora erythraea</i>
IS1253	YKATWY.GKN ILKVNRFAS	SKICSHCHHK	LDNLP.LSVR	NWTCPSQCOTH	HDRDTNAASNI	<i>Dichelobacter nodosus</i>
TM0135	YKAKLYGQV SEKVDPSY.T	SQTCPRCGHV	SKENRPDHGE	RFVCSQCGYE	EHADIVGAINI	<i>Thermotoga maritima</i>
O402	YK.QLWRGGQ VLAVPPAY.T	SQRCAVCGHT	AKENRLSQ.S	KFRQVQCGYT	ANADVNGARNI	<i>Escherichia coli</i>
Orf348	YK.QLWRGGQ VLAVPPAY.T	SQRCAVCGHT	AKENRLSQ.S	QFRQVQCGYT	ANADVNGARNI	Bacteriophage If1
IS606	YKQQ.HNGKL LVKVPPQY.T	SKTCHCCGNI	NHKLKLNH.R	QYWCLECGYR	EHRDINAANNI	<i>Helicobacter pylori</i>
TM0776	YKABQF.GIE VKLISEAN.T	SKTCEVCG..	.AKNPNNGRR	.YHCKACGFE	YHRDVGVAINI	<i>Thermotoga maritima</i>
TM1026	YKABQF.GIE VKLISEAN.T	SKTCEVCG..	.AKNPNNGRR	.YHCKTCGFE	YHRDVGVAINI	<i>Thermotoga maritima</i>
TM0047	YKAQIF.GIE VVRISEAH.T	SKTCEVCG..	.TQNRPSGRN	.YKACAGCGFE	YHRDVGVAINI	<i>Thermotoga maritima</i>
MJ0751	YKAKWD.GLD VEYVNPST	SKLCPICG..	.CKLDPNGR	LLKCNINCNLV	FDRDVVATFNL	<i>Methanococcus jannaschii</i>
Pab1802	YKAKWF.GVP VVYVNPKN.S	SKTCEPACG..	.GHLIPQEGR	LMKCLKCGLV	EDRDFIAVLNL	<i>Pyrococcus abyssi</i>
TnpA	YKAPLY.GSF VKEVNPRL.T	SRSCEPRCG..	.WVSRKVG.R	TFHCERCIGFT	MDRQLNASLNI	<i>Acidianus ambivalens</i>
IS891	YFGKVF.GVV TVAVPPHH.T	SQNCSCNCGEV	VKLSL..STR	THACPHCGHI	QDRDWNAAARNI	<i>Anabaena sp.</i>
	YKAEWY-GKE LVKVDP-FPS	SKTCSCCGHV	-KKLKPLS-R	KWTCPC-CGFE	HDRDINAAINI	Consensus

FIG. 1. Alignment of the carboxy-terminal region of GipA and homologs. Amino acids 296 to 354 of the 411-amino-acid GipA protein are shown. This region corresponds to the most highly conserved region of the protein. Amino acids that are identical in at least 30 of 32 proteins are shown in bold. Underlining indicates four completely conserved Cys residues. The designation of each putative protein is shown on the left, and the source is shown on the right. The accession numbers for the protein sequences, in order of appearance, are as follows: C01019, CAA69469; C02018, CAA69450; Orf406, CAA09118; IS1136CF, CAA60219; IS607, AF189015; Rv2885c, Q10809; Rv2978c, CAB05434; Rv2791c, CAA15586; Tra, AAC97568; IS605, AAC44690; IS605b, AAD08043; SCH35-10, CAB44417; Slr2062, BAA17870; IS8301, BAA32390; TM1044, AAD36121; YdcA, BAA75131; IS1341, JC4292; GipA, AF246666; YsnA, P54992; IS1136, JN0563; IS1253, AAB16749; TM0135, AE001699; O402, AAC74514; Orf348, AAC62159; IS606, AAD06403; TM0776, AAD35858; TM1026, AAD36103; TM0047, AAD35141; MJ0751, Q58161; Pab1802, CAB49750; TnpA, CAB56750; and IS891, AAA90935.

isogenic mutant competed equally well with the wild type after oral inoculation (Table 3). Thus, insertions or deletions in the late tail operon of Gifsy-1 do not confer a virulence defect. Thus, the insertion in *gipA* confers a specific defect.

Finally, we constructed a deletion/kanamycin insertion of the entire Gifsy-1 phage. In oral competition experiments, the Gifsy-1 deletion strain showed a sevenfold-decreased ability to grow or survive in the small intestine (Table 3). As has previously been reported (14), we found no defect conferred by the loss of Gifsy-1 after i.p. infection (data not shown). Thus, the loss of Gifsy-1 confers a phenotype essentially identical to that of the *gipA2::MudJ* insertion.

The *gipA* null mutation decreases growth or survival in the Peyer's patches. Previous studies (23) and observations from our own work indicated that, several days after an oral infection, most of the serovar Typhimurium found in the small intestine of mice is associated with the Peyer's patches. We reasoned that IVET selection would target genes that were transcriptionally active in the Peyer's patches because significant growth is required for selection. In order to compare the survival of the mutant and the wild type in the Peyer's patches, an oral competition assay was performed by inoculating a 1:1

TABLE 3. In vivo competition assays with wild-type serovar Typhimurium and isogenic mutants

Mutant	Route of inoculation ^a	Median CI ^b	No. of mice	P ^c
<i>gipA2::MudJ</i>	Oral	0.45	16	<0.05
	i.p.	2.32	9	NS
<i>gipA2::MudJ/pJS203</i>	Oral (Peyer's patch) ^d	0.10	5	<0.0005
	Oral (Peyer's patch) ^d	0.59	5	NS
<i>gfoI::MudJ</i>	Oral	1.5	5	NS
Δ <i>gfoA::kan</i>	Oral	1.3	5	NS
Δ Gifsy-1::kan	Oral	0.28	12	<0.00005

^a All inocula contained a 1:1 ratio of mutant to isogenic wild-type serovar Typhimurium (JS135). Doses given to BALB/c mice were 10² CFU i.p. and 10⁸ CFU orally. Bacteria were recovered from the small intestine after oral infection or the spleen after i.p. infection unless otherwise indicated.

^b Defined as output (mutant/wild type) divided by input (mutant/wild type).

^c Student's *t* test was used to compare output versus inoculum. NS, not significant.

^d Bacteria were recovered from individual dissected Peyer's patches after oral infection.

TABLE 4. Competition between wild type and the *gipA* mutant in individual Peyer's patches at an inoculum of 10^7 CFU^a

Peyer's patch	Total CFU recovered	% <i>gipA</i> mutant recovered
A	8.4×10^4	0.1
B	1.4×10^4	0.8
C	3.4×10^4	5
D	1.5×10^3	72
E	2.2×10^3	84
F	3.2×10^3	88
G	8.1×10^3	100

^a The strains used were JS135 and JS136.

mixture of the *gipA2::MudJ* mutant (JS136) and an isogenic wild-type strain (JS135). At the dose used for this analysis ($\sim 10^7$ CFU), any given Peyer's patch is likely to be colonized by only a single bacterium, resulting in a largely clonal population within a single patch. This phenomenon can be exploited to compare the growth of different bacterial strains within the same mouse. Three to five days after infection, individual Peyer's patches were excised, homogenized, and plated for total CFU determinations. Subsequent replica plating allowed us to determine the percentages of wild-type and *gipA2::MudJ* strains. The data indicated that the wild-type and mutant strains invaded and colonized Peyer's patches equally well (Table 4). However, the total number of CFU was consistently lower in Peyer's patches colonized by the mutant than in patches predominantly colonized by the wild type (Table 4). On average, the CFU of the mutant in a single colonized Peyer's patch was 5- to 10-fold lower than that in a Peyer's patch colonized by the wild type. These data suggested that the *gipA* product participates in some function required for survival and/or replication in the Peyer's patch.

To test the above hypothesis further, we inoculated a second group of mice using a 10-fold-larger inoculum, thus increasing the probability that each Peyer's patch would be colonized by both strains. In this manner, we could more easily compare the growth rates of the strains within a single Peyer's patch. We explicitly isolated the most distal Peyer's patch (7) in each of five mice 6 days postinfection and determined that the wild-type strain consistently out-competed the *gipA* mutant strain by approximately 10-fold (Table 3). Thus, the *gipA* mutation confers a defect in growth or survival in the Peyer's patches.

We performed an identical experiment with a *gipA2::MudJ* strain containing the *gipA*⁺ plasmid pJS302. The results (Table 3) show that the plasmid did complement the Peyer's patch defect, but perhaps not to wild-type virulence. However, the remaining defect in the plasmid-bearing strain was not statistically significant. These data suggest that the Peyer's patch defect conferred by the *gipA2::MudJ* mutation is due to a functional loss of GipA. This conclusion is further supported by several data presented above. First, *gipA* is a single ORF that is transcribed in the orientation opposite that of all the surrounding genes. Hence, a polar effect of the MudJ insertion is unlikely. Second, other mutations in the late operon, including an MudJ insertion, do not confer a virulence defect. Third, the Gifsy-1 deletion confers the same phenotype as the insertion mutation. Our data suggest that *gipA* is tightly regulated such that it is expressed only at the appropriate time. The fact that the plasmid-bearing strain did not behave exactly like the wild-type strain is perhaps due to inappropriate expression of *gipA* from the plasmid.

Mutations in *gipA* do not affect attachment to or invasion of the small intestine or growth in the cecum. The data presented in Table 4 show that equal numbers of randomly chosen Peyer's

patches were colonized with the *gipA* mutant and the wild type, suggesting that mutations in *gipA* do not confer an attachment or invasion defect. To further examine this possibility, we quantified attachment and invasion of the *gipA* mutant with respect to the wild type using the IOC technique developed by Bäumlner et al. (4). Ex vivo small intestines were inoculated with an equal mixture of the wild type (JS135) and the *gipA2::MudJ* mutant (JS136). After a 1-h incubation period, the *gipA* mutant and the wild type were recovered equally from both the distal Peyer's patch (CI = 1.02 ± 0.09 ; $n = 5$) and the small intestine (CI = 1.11 ± 0.08 ; $n = 5$). This result suggests that the *gipA* mutant is unaffected in its ability to initially attach to and invade the Peyer's patches and small intestine.

To confirm that *gipA* does not play a role in attachment to or invasion of intestinal epithelial cells, we performed in vitro invasion assays with Henle-407 cultured epithelial cells (39) using a *hilA* mutant as a negative control (2). After incubation of eukaryotic cells with a 1:1 mixture of wild-type and mutant bacteria, the *gipA2::MudJ* strain was equal to the wild-type strain in its ability to invade the eukaryotic cells (CI = 0.88 ± 0.26 ; $n = 3$). In contrast, the *hilA* strain was significantly out-competed in control experiments (CI = 0.08 ± 0.07 ; $n = 3$). Thus, the *gipA* mutation does not affect attachment or invasion of intestinal epithelial cells in tissue cultures.

Finally, we tested if the *gipA* insertion mutation had any effect on the ability of serovar Typhimurium to colonize or propagate in the cecum, which is devoid of Peyer's patches. A 1:1 mixture of the *gipA2::MudJ* mutant strain (JS136) and an isogenic wild-type strain (JS135) was used to orally infect a group of six mice. The mutant was recovered from the ceca in numbers equal to those of the wild type (CI = 0.95 ± 0.70 ; $n = 6$). These same mice did have a significantly lower number of mutant bacteria in the small intestine, and these data are included in those for the group of orally infected mice in Table 3. Taken together, the above results suggest that the *gipA* gene product is specifically involved in growth or survival in Peyer's patches and does not play a role in attachment to or invasion of the small intestine or growth in other areas of the intestine.

The *gipA* mutation does not confer a general growth defect. In order to test if the Peyer's patch phenotype could be accounted for by a simple growth defect, we performed competition assays with the *gipA2::MudJ* mutant (JS136) and the wild type (JS135) during growth in both rich and minimal media starting with an inoculum of approximately 200 cells and growing to saturation. The *gipA* strain showed no growth defect under these conditions (CI = 1.06 ± 0.19 for rich and 1.08 ± 0.08 for minimal; $n = 10$), suggesting that the defect in the Peyer's patch is not the result of a simple growth defect in the mutant.

A *gipA* mutation increases the oral LD₅₀. To further quantify the effects of the *gipA* insertion mutation, we determined the oral and i.p. LD₅₀s of our wild-type strain and the isogenic mutant strain. The oral LD₅₀ of the wild type in this study was similar to previously reported values (1.3×10^4 organisms). The oral LD₅₀ of the *gipA2::MudJ* mutant was 6.6×10^4 organisms. Thus, the *gipA* insertion resulted in an approximately fivefold attenuation after oral inoculation. In contrast, the LD₅₀ did not differ between the mutant and wild-type strains following i.p. inoculation. All mice inoculated with either the *gipA* mutant or the wild type died following i.p. inoculation of fewer than five organisms. These results are consistent with the expression profile and the previous data suggesting that *gipA* is specifically involved in an early stage of infection.

Transposition is not required for *gipA* function in the Peyer's patches. The sequence of the *gipA* ORF shows homology to those of a family of putative transposases. To determine whether this locus actively transposes during an infection, we isolated wild-type serovar Typhimurium from various sites of infection 6 days after oral inoculation. Bacteria were recovered from three independent Peyer's patches, the remaining small intestine, and the spleen by homogenizing the organs and plating for single colonies. Two or three individual colonies were chosen to represent each tissue, and chromosomal DNA was extracted from the isolates. This chromosomal DNA, as well as chromosomal DNA from the original inoculum, was digested with the restriction enzyme *ClaI*, which cuts outside of the *gipA* ORF. Southern blotting was performed using the intact *gipA* *ClaI* fragment as a probe. All samples produced an identical pattern representing the wild-type conformation of this locus (data not shown). The results indicated that a single copy of the *gipA* locus was present in the strains before and after growth in the Peyer's patches. The results suggest that a simple transposition event is not required for *gipA* function in the Peyer's patches.

Transcriptional regulation of *gipA*. The expression profile for *gipA* suggests that *gipA* is induced only in the small intestine. The constitutive fusion strain (JS131) used in the competition assay (Table 2) produces approximately 120 U of β -galactosidase in vitro. The pIVET1::*gipA'* fusion strain produces approximately 10 U of β -galactosidase activity. However, in animals, the *gipA* fusion outcompetes JS131 in the small intestine (Table 2). Since growth is dependent on expression of the fusion, this result suggests that *gipA* is induced at least 12-fold in the early stages of disease. We attempted to find laboratory conditions that would induce the *gipA* fusion in vitro. The pIVET1::*gipA'* fusion was moved into a *purA*⁺ background to ensure that there was no selection for expression of the fusion (JS133). We then measured the β -galactosidase activity produced by the fusion in response to a variety of in vitro conditions, including low iron, changes in pH, anaerobiosis, and PhoPQ-activating conditions (20). We also tested conditions known to induce other genes in the small intestine, such as the serovar Typhimurium invasion locus (2), and cholera toxin in both classical (24) and El Tor (21) *Vibrio cholerae* strains. None of the in vitro conditions that we used caused a significant induction of *gipA*, giving β -galactosidase activities ranging from 7.8 to 19 U, with a mean of 12.4 U. It is possible that *gipA* is induced in response to a signal that is specific to the small intestine. However, we cannot rule out the possibility that this gene is regulated in response to some environmental condition or combination of conditions that we have not tested.

DISCUSSION

We have used the IVET system (32, 45) to identify a serovar Typhimurium gene, *gipA*, that is specifically induced when the bacteria colonize the small intestine. By identifying genes that are expressed in a tissue-specific fashion, we expect to focus on gene products that are required for particular aspects of the infection process. We anticipated that oral selection would target genes induced in a Peyer's patch, since this is the site of significant bacterial growth in the small intestine (23) and growth is required for IVET selection. Indeed, we believe that *gipA* is specifically induced in the Peyer's patches and are performing experiments to explicitly address this issue. If true, then the *gipA* promoter is ideal for use in vaccine constructs, where the synthesis of antigens could be targeted to the Peyer's patches to induce mucosal immunity (16).

Our evidence suggests that a *gipA* null mutation confers a

growth or survival defect only in the Peyer's patches. The *gipA* mutant is unaffected in its ability to initially colonize the small intestine and invade Peyer's patches. Several lines of evidence support this conclusion. First, equal numbers of Peyer's patches were infected with primarily mutant or wild-type bacteria in a mixed inoculation. Second, the mutant showed no defect in colonization in an IOC model. Third, the mutant showed no defect in invasion of epithelial cells in tissue cultures, an assay that is well correlated with defects in entry into M cells of Peyer's patches (19). Moreover, no defect was conferred when the bacteria were grown systemically in animals or in laboratory cultures. Thus, the *gipA* defect is specific to the Peyer's patches, consistent with the expression profile. These data suggest that the bacteria sense the particular environment of the Peyer's patch and respond by producing products required at this site of replication.

The results presented here help to elucidate the early steps in serovar Typhimurium pathogenesis. In mice, it appears that a limited number of bacteria enter a Peyer's patch and propagate in this site (7, 23). The number of organisms in a Peyer's patch normally reaches approximately 10^4 (Table 4). However, the numbers of bacteria need only increase to 10^2 to 10^3 before the bacteria are able to initiate a systemic infection. Our mutant had an approximately 10-fold-decreased ability to propagate in the Peyer's patches, but because it could still grow to some extent, there was only a fivefold increase in the LD₅₀. Once past this bottleneck, the mutant is fully capable of causing disease.

The fact that the *gipA* mutant shows a defect in the Peyer's patches and not in the spleen emphasizes the inherent differences between these two lymphoid tissues. Distinct B-cell and T-cell subsets comprise a Peyer's patch (6). Indeed, the particular B cells apparently initiate differentiation of the lymphoid follicle (27). Although it is presumed that growth in macrophages is important for serovar Typhimurium after M-cell invasion, it is not clear in what cell types, if any, the bacteria grow in Peyer's patch tissue. For example, serovar Typhimurium is capable of invading both B cells and T cells in tissue cultures (49). There is apparently something unique about growth in the Peyer's patches that requires a specific set of bacterial gene products. Our results suggest that serovar Typhimurium senses this particular environment and responds accordingly.

The *gipA* ORF shows homology at the protein level to a family of putative insertion sequence element proteins from a wide array of prokaryotic species (Fig. 1). The elements present in *Dichelobacter nodosus*, a pathogen responsible for ovine foot rot (5), and *Helicobacter pylori*, the causative agent of duodenal and gastric ulcers (8), are both associated with clinically relevant pathogenicity determinants. Our evidence suggests that transposition is not required for *gipA* function in the Peyer's patches. Indeed, it is difficult to imagine how a transposition event would specifically enhance growth in a Peyer's patch. Alternatively, GipA could be a regulatory protein that is involved in the control of one or more virulence factors. An example is the Piv protein in *Moraxella lacunata*, which is homologous to a family of insertion sequence element "transposases" but acts via site-specific recombination to control pilin phase variation (29).

The putative protein sequences from the various GipA homologs are approximately 50% identical over their entire lengths. There is a particularly well-conserved motif near the C terminus of the protein that contains a number of invariant residues, including the amino acid sequence CXXC-15X-CXXC (Fig. 1). This motif is similar to motifs involved in metal ion binding in several other protein families. It most closely

resembles putative zinc finger motifs found in certain ribosomal proteins (51) but could also be involved in binding other metals (38). Since it is reasonable to believe that this protein may be involved in DNA binding, this highly conserved sequence could function as a zinc finger DNA binding domain.

The *gipA* ORF is carried in the late operon of the lambdoid phage Gifsy-1. This phage is fully functional and is capable of moving from wild-type serovar Typhimurium and lysogenizing cured strains (14). It is found in all serovar Typhimurium strains that have been examined (14). Our results (unpublished) suggest that Gifsy-1 is not present in other serovars of *Salmonella*, including enteritidis, typhi, dublin, and paratyphi. Although we cannot rule out the possibilities that the bacteriophage moves throughout the salmonellae and we just happened to examine serovar Typhimurium strains that were lysogenic and other isolates that were not, the simplest explanation for the results is that serovar Typhimurium strains particularly benefit from maintaining Gifsy-1. There is a complicated and poorly understood relationship between Gifsy-1 and Gifsy-2, another lambdoid phage that is lysogenic in strains of serovar Typhimurium (14, 15). This relationship pertains to phage production as well as virulence. Although, as in our studies, these authors showed that Gifsy-1-cured strains were fully virulent in an i.p. infection, they noted that Gifsy-1 can apparently complement certain virulence defects conferred by the loss of Gifsy-2 (14). Whether any genes carried on Gifsy-2 can specifically contribute to growth in the Peyer's patches is difficult to answer given the other virulence defects conferred by the loss of the Gifsy-2 phage (14).

Virulence in serovar Typhimurium is the sum action of a large number of virulence factors, each of which may contribute only slightly to overall pathogenesis. In order to completely understand the molecular mechanisms of pathogenesis, we must investigate all of these factors. By using tissue-specific gene expression as a guide, we have identified a gene whose product is involved in survival in the Peyer's patches. Although the defect conferred by a *gipA* null mutation is subtle and the molecular explanation for the defect is not immediately obvious, this information gives us a starting point for understanding the genes and gene products required for this critical aspect of serovar Typhimurium pathogenesis.

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