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Identification of GtgE, a Novel Virulence Factor Encoded on the Gifsy-2 Bacteriophage of *Salmonella enterica* Serovar Typhimurium

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The Gifsy-2 temperate bacteriophage of *Salmonella enterica* serovar Typhimurium contributes significantly to the pathogenicity of strains that carry it as a prophage. Previous studies have shown that Gifsy-2 encodes SodCI, a periplasmic Cu/Zn superoxide dismutase, and at least one additional virulence factor. Gifsy-2 encodes a *Salmonella* pathogenicity island 2 type III secreted effector protein. Sequence analysis of the Gifsy-2 genome also identifies several open reading frames with homology to those of known virulence genes. However, we found that null mutations in these genes did not individually have a significant effect on the ability of *S. enterica* serovar Typhimurium to establish a systemic infection in mice. Using deletion analysis, we have identified a gene, *gtgE*, which is necessary for the full virulence of *S. enterica* serovar Typhimurium Gifsy-2 lysogens. Together, GtgE and SodCI account for the contribution of Gifsy-2 to *S. enterica* serovar Typhimurium virulence in the murine model.

Salmonella enterica serovar Typhimurium infects millions of people each year, resulting in diseases ranging from mild self-limiting gastroenteritis to enteric fever and death. *S. enterica* serovar Typhimurium infection requires colonization and growth of the organism in a variety of host tissues. The multiple steps in the infection process involve an array of virulence factors, each of which might make only a small contribution to overall pathogenesis. Many of these virulence genes are found on horizontally acquired segments of DNA, such as pathogenicity islands and islets and bacteriophages (12, 17, 19).

Most, if not all, isolates of *S. enterica* serovar Typhimurium are lysogens of bacteriophage Gifsy-2 (9), which is a lambdoid bacteriophage having the same relative gene order as the prototype phage λ. *S. enterica* serovar Typhimurium strains cured of Gifsy-2 are significantly attenuated in the mouse (8), indicating that Gifsy-2 contributes virulence factors to its host. One of these factors is SodCI, a periplasmic Cu/Zn superoxide dismutase (5, 6) that presumably protects the bacteria against superoxide produced by macrophages (6). *S. enterica* serovar Typhimurium strains with mutant *sodCI* are attenuated in macrophages as well as in mice (5). Gifsy-2 also carries the unusual gene *grvA*. Mutations in *grvA* increase virulence as measured in a competition assay. This so-called antivirulence phenotype is absolutely dependent on *sodCI*, suggesting that GrvA is also involved in SodCI-mediated resistance to phagocytic superoxide (15).

It is clear that Gifsy-2 contributes more than *sodCI* to virulence in *S. enterica* serovar Typhimurium lysogens (8, 15). Based on sequence analysis and other evidence, there are several candidates for the additional virulence determinant(s) carried by Gifsy-2 (Fig. 1). The putative product of *gtgA* is 75% identical to PipA, encoded on *Salmonella* pathogenicity island 5 (SPI-5) (18). *S. enterica* serovar Dublin strains with mutant *pipA* showed reduced fluid secretion and intestinal inflammation in a bovine-ileal-loop model (28). These mutants were unaffected in the mouse model of systemic infection (28). Interestingly, the Gifsy-1 lambdoid phage carries a gene, *gogA*, which is essentially identical in sequence and relative position to Gifsy-2 *gtgA* (10). Upstream of *sodCI* is the *ailT* gene, whose product has homology to the Lom/Ail family of proteins (10). In *S. enterica* serovar Typhimurium, other members of this family include PagC, involved in macrophage survival (1, 23) (39% identical to AilT) and Rck, which is encoded by the virulence plasmid and which confers serum resistance (14) (35% identical to AilT). The *sseI*/*srfH* gene is under the control of SsrAB, a two-component regulatory system encoded on SPI-2, and is transcriptionally induced in macrophages (29). The SseI protein is secreted into the eukaryotic cell cytoplasm by the SPI-2 type III secretion system (20). The role of *sseI*/*srfH* in an animal infection has not been previously reported. The *gtgF* gene, proximal to *attR*, is predicted to encode a small 63-amino-acid protein that is 76% identical to that encoded by *msgA*. A mutation in the *msgA* gene increased the intraperitoneal (i.p.) 50% lethal dose of *S. enterica* serovar Typhimurium 300-fold and attenuated the strain in macrophage survival assays (11). A second MsgA homolog, SrfE (48% identical to GtgF) was identified as the product of an SsrAB-regulated gene (29).

The purpose of this study was to identify the Gifsy-2 determinants that have a quantifiable effect on *S. enterica* serovar Typhimurium virulence. Here we show that the contribution of the Gifsy-2 phage to virulence in mice is largely dependent on *sodCI* and *gtgE*, a unique gene with no significant homologs in the sequence databases. Thus, although other gene products

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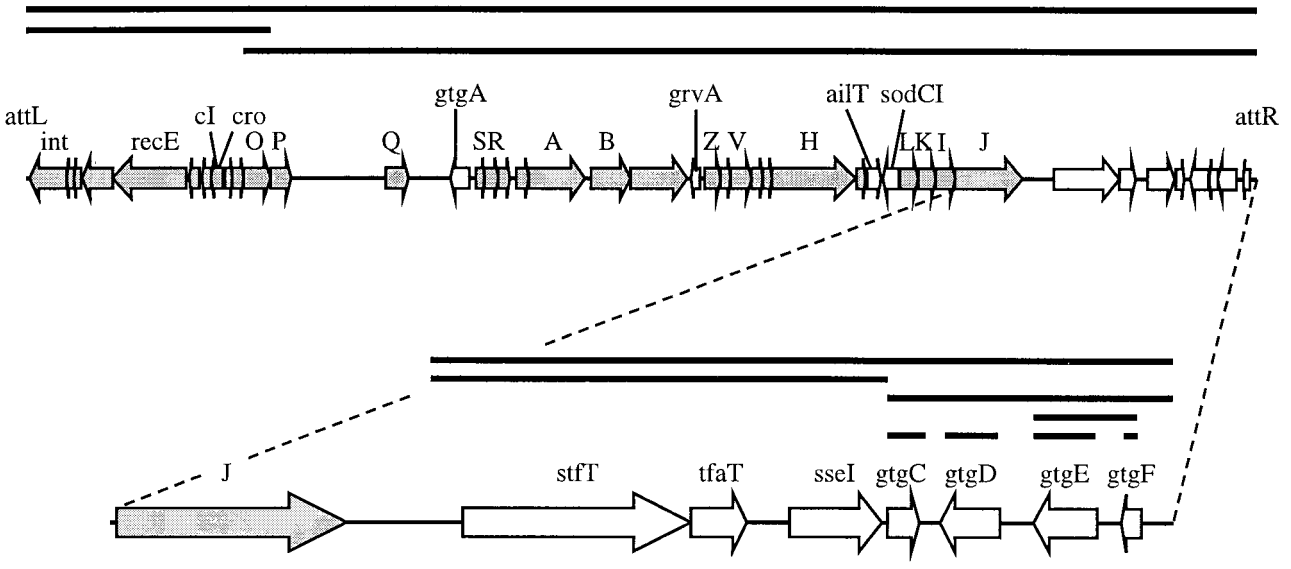

FIG. 1. Gene organization of the Gifsy-2 bacteriophage. Data were from reference 18 and our laboratories. (Top) Open reading frames with identifiable orthologs in phage lambda are labeled. Most of these genes are termed *gft* (Gifsy-2), e.g., *gftO*. White arrows, putative virulence genes; gray arrows, putative phage genes; lines, deletion intervals. (Bottom) Gene organization of the B region. Genes with no obvious role in phage production are termed *gtg* (Gifsy-2 gene) (10).

encoded on Gifsy-2 might interact with the host, they do not have a measurable role in the mouse model of infection.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains used are described in Table 1. All strains generated for this study are isogenic derivatives of *S. enterica* serovar Typhimurium strain 14028 (7). Plasmid pNFB9 is a derivative of *pir*-dependent plasmid pGP704 (22) and carries the *int* gene and the *attP* site of bacteriophage Gifsy-1. This plasmid can integrate at Gifsy-1 attachment site *attG1* (N. Figueroa-Bossi and L. Bossi, unpublished results). Plasmids pNFB13 and pNFB14, constructed in the course of this work, are derivatives of pNFB9 carrying the *gtgE* gene and neighboring DNA (coordinates 1142327 to 1143385 of the *S. enterica* serovar Typhimurium chromosome) (18) in opposite orientations.

TABLE 1. *S. enterica* serovar Typhimurium strains

Strain[a]	Genotype	Deletion end points[b]	Source or reference[c]
14028	Wild type		ATCC[d]
JS159	Δ(G-2 B)::Km	1136168–1144030	15
JS192	*sodCI*::*aph*		15
JS221	Δ*gtgEF*::Km	1142583–1143681	
JS222	Δ*gtgEF sodCI*::*aph*		
JS223	ΔGifsy-2::Km	1098189–1144030	
JS224	Δ(*attL-gftO*)::Km	1098189–1107180	
JS225	Δ(*gftO-attR*)::Km	1106211–1144030	
JS226	ΔGifsy-2::Cm	1098189–1144030	
JS227	Δ*ailT*::Km	1129388–1129938	
JS228	Δ*gtgC*::Km	1141042–1141428	
JS229	Δ(*stfT-sseI*)::Km	1136168–1141011	
JS230	Δ(*gtgC-attR*)::Km	1141042–1144030	
JS231	attλ::pRA102		
JS232	ΔGifsy-2 *att*λ::pRA102::pGP704		
JS233	ΔGifsy-2 *att*λ::pRA102::p*sodCI*$^+$		
JS234	ΔGifsy-2 *att*λ::pRA102::p*gtgE*$^+$		
JS235	ΔGifsy-2 *att*λ::pRA102::p*gtgE*$^+$ *sodCI*$^+$		
MA6054	*ara-907 araD901*::MudJ		
MA6938	Δ*gtgD*::Km	1141637–1142195	
MA7073	Δ*gtgE*::Km	1142583–1143216	
MA7075	Δ*gtgF*::Km	1143531–1143681	
MA7137	Δ*gtgE attG1*::pNFB13 *ara-907 araD901*::MudJ		
MA7156	Δ*gtgE*::Km *attG1*::pNFB14		
MA7164	Δ*gtgE*::Km *attG1*::pNFB9		
MA7165	Δ*gtgE attG1*::pNFB9 *ara-907 araD901*::MudJ		

[a] All strains are derived from *S. anterica* serovar Typhimurium strain 14028 (7).
[b] Numbers indicate the base pairs that are deleted (inclusive) as defined in the *S. enterica* serovar Typhimurium LT2 genome sequence (National Center for Biotechnology Information). The DNA insert in pNFB13 and pNFB14 corresponds to interval 1142327 to 1143385.
[c] This study unless otherwise noted.
[d] ATCC, American Type Culture Collection.

Strains were grown in or on Luria-Bertani (LB) or glucose NCE media (16). Ampicillin (AP; 100 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (20 μg/ml) were used as selective antibiotics. The color indicator 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at a concentration of 80 μg/ml. P22 transductions were performed as previously described by using P22 HT 105/*int-201* (16).

Generation of insertion/deletion mutations using λ Red recombinase. Insertion/deletion mutations in Gifsy-2 were constructed via the λ Red recombinase method (4, 30) using constructs developed by Datsenko and Wanner (4). PCR primers of 50 to 60 nucleotides (nt) were synthesized with 30 to 40 nt on the 5′ ends corresponding to the ends of the desired deletion. The 3′ 20 nt of each primer anneal to plasmids pKD3 and pKD4 or to pKD13 at the 5′ or 3′ end of an antibiotic resistance cassette flanked by FRT sites for Flp-mediated recombination (4). PCRs were carried out according to the manufacturer's instructions (Invitrogen, Carlsbad, Calif.). Plasmid pKD46 was introduced into *S. enterica* serovar Typhimurium strain 14028. This plasmid synthesizes the λ recombination proteins Gam, Bet, and Exo when induced with arabinose (1 mM). The plasmid-bearing strain was grown at 30°C in LB medium–AP–1 mM arabinose and was made electrocompetent (16). Approximately 800 ng of PCR product was transformed, and the cells were plated on LB medium containing the appropriate antibiotic and incubated at 37°C. This generally resulted in 30 to 50 antibiotic-resistant colonies, representing Red-mediated integration of the cassette by homologous recombination with the 30- to 40-bp ends of the PCR fragment. Each insertion/deletion mutation was characterized by genetic mapping and PCR. The insertions/deletions were transduced via P22 to construct isogenic strains for subsequent analysis. The precise endpoints of the deletions are indicated in Table 1.

Removal of antibiotic cassettes using Flp recombinase. A temperature-sensitive plasmid carrying the Flp recombinase (pCP20) (2) was transformed into strains containing the insertion/deletion mutations. The resulting transformed colonies were restreaked twice in the presence of AP at 30°C to select for the plasmid. Flp mediates site-specific recombination between the FRT sites that flank the antibiotic resistance cassette, resulting in loss of the marker. The strains were then restreaked on LB agar at 37 to 42°C twice. The resulting strains were checked for loss of the temperature-sensitive plasmid and the antibiotic resistance cassette.

Virulence assays. Strains were grown overnight in LB broth at 37°C with aeration and washed once in an equal volume of 0.15 M NaCl. For each experiment, the two strains of interest were mixed 1:1 and the mixture was diluted in 0.15 M NaCl. Female BALB/c mice (Harlan Sprague-Dawley, Inc., Indianapolis, Ind., or Iffa-Credo, Lyon, France) approximately 6 weeks old were inoculated with the mixture, containing 200 to 2,000 bacterial cells. Inocula were plated on LB plates and then replica plated onto appropriate selective media to determine the total number and percentage of bacteria from each inoculated strain. In some cases, one of the two strains was marked with a chromosomal *ara-lac* operon fusion (*araD901*::MudJ) to simplify screening. This fusion was verified not to have any effect on the ability of *S. enterica* serovar Typhimurium to infect mice (see Table 3).

Mice inoculated i.p. were sacrificed after 4 to 5 days, and their spleens were removed, homogenized, diluted, and plated on LB plates. We routinely recovered 10^7 to 10^8 bacteria per spleen. Replica plating on selective media or on medium supplemented with X-Gal and arabinose allowed us to determine the percentage of each strain. The competitive index (CI) was calculated as (percentage of strain A recovered/percentage of strain B recovered)/(percentage of strain A inoculated/percentage of strain B inoculated). The CI of each set of assays was analyzed statistically by using Student's *t* test. In instances where a virulence defect was observed, the mutant strain was reconstructed and the competition assay was repeated to ensure that the phenotype was the result of the deletion mutation.

In vitro growth assays. Equal volumes of overnight LB cultures of mutant and wild-type bacteria were mixed, washed, diluted, and inoculated into glucose NCE medium (200 to 400 bacteria into 5 ml). Each inoculum was plated on laboratory media to precisely determine the number of bacteria and the percentage of the mutant in the mixture. After 24 h growth at 37°C with aeration, cultures were diluted in saline and plated on laboratory media. The percentages of mutant bacteria recovered were analyzed as in the in vivo competition assays.

RESULTS

Gifsy-2 phage encodes multiple virulence determinants. Previous studies had shown that, while *sodCI* was a major contributor to virulence, it is not the only virulence factor carried on the Gifsy-2 phage (8, 15). To identify these virulence determinants, we constructed insertions/deletions in Gifsy-2 using the λ Red recombinase method (4, 30) and determined the virulence of these deletion strains in competition assays after i.p. injection. First, a strain with the entire Gifsy-2 phage and its proposed attachment sites deleted was competed against the isogenic wild-type strain. The virulence of the ΔGifsy-2::Km (JS223) strain was attenuated >100-fold with respect to the wild-type strain (Table 2), consistent with previous data (8, 15). This decrease in virulence was much greater than the sixfold attenuation conferred by a *sodCI* insertion (Table 2). Therefore, Gifsy-2 must contribute some other virulence determinant(s) in addition to *sodCI*.

TABLE 2. Competition assays with Gifsy-2 deletion strains

Relevant genotype[a]	Strain	Median CI[b]	No. of mice[c]	P[d]
Δ(Gifsy-2)::Km	JS223	0.0068	4	<0.0005
sodCI::*aph*	JS192	0.15	9	<0.0005
Δ(*attL-gftO*)::Km	JS224	0.70	3	NS
Δ(*gftO-attR*)::Km	JS225	0.0082	2	0.001
Δ*ailT*::Cm	JS227	0.94	4	NS
Δ(G-2 B)::Km	JS159	0.15	9	<0.0005
Δ(*stfT-sseI*)::Km	JS229	0.87	6	NS
Δ(*gtgC-attR*)::Km	JS230	0.18	3	0.001
Δ*gtgC*::Km	JS228	0.72	5	NS
Δ*gtgD*::Km	MA6938	0.75	6	NS
Δ*gtgF*::Km	MA7075	1.36	5	NS
Δ*gtgEF*::Km	JS221	0.16	10	<0.0005
Δ*gtgE*::Km	MA7073	0.14	5	0.0006

[a] All strains competed against wild-type 14028 except for strain MA7073, which competed against strain MA6054 (*ara-907 araD901*::MudJ).
[b] CI was calculated according to the formula in Materials and Methods, with the mutant as strain A and the wild type as strain B.
[c] All assays were performed i.p. using BALB/c mice.
[d] Student's *t* test was used to compare output versus inoculum. NS, not significant.

An additional virulence factor(s) is located in the Gifsy-2 B region. To narrow down the region of Gifsy-2 that carried the additional virulence gene(s), we generated large deletions of the Gifsy-2 phage and determined the effects on virulence. We found that, in an otherwise wild-type background, a deletion from *attL* through *gftO*, which includes the immunity region of Gifsy-2, had no effect on virulence (Fig. 1; Table 2). However, a deletion from *gftO* through *attR*, which includes *sodCI*, attenuates to approximately the same degree as a deletion of the entire Gifsy-2 phage (Fig. 1; Table 2). This indicated that both *sodCI* and the additional virulence determinant(s) are located within this deletion interval.

Based on sequence analysis, one possible candidate for the additional Gifsy-2 virulence factor was *ailT*, a gene whose presumed product is homologous to several serum resistance proteins (10). To determine if *ailT* contributes to virulence, we constructed a null mutation in the gene. The *ailT* insertion/deletion mutant competed evenly against an isogenic wild-type strain in an i.p. competition assay (Table 2). This showed that the *ailT* gene did not have a significant effect on virulence during i.p. infection of BALB/c mice.

In phage λ, genes located between phage gene J and *attR*, termed the B region, are not necessary for the production of viable phage (3). To further delineate the location of additional virulence genes on the Gifsy-2 phage, we deleted the

TABLE 3. Competition assays with *gtgE* and *gtgE*$^+$ complemented strains[a]

Strain A		Strain B		Median CI[b]	No. of mice[c]	P^d
Relevant genotype	Name	Relevant genotype	Name			
Δ*gtgE*::Km	MA7073	Wild type	MA6054	0.14	5	0.0006
Δ*gtgE attG1*::pNFB9 (vector)	MA7164	Δ*gtgE attG1*::pNFB 13(*gtgE*$^+$)	MA7137	0.15	4	0.001
Δ*gtgE attG1*::pNFB9 (vector)	MA7165	Δ*gtgE attG1*::pNFB 14(*gtgE*$^+$)	MA7156	0.10	4	0.002
Δ*gtgEF sodCI*::*aph*	JS222	ΔGifsy-2::Cm	JS226	2.1	9	0.01

[a] All strains used in these assays were isogenic with the wild type (14028). In the assays in the first three rows from the top, competing strains were screened by plating on medium supplemented with X-Gal and arabinose (see full genotypes in Table 1).
[b] CI was calculated according to the formula in Materials and Methods.
[c] All assays were performed i.p. using BALB/c mice.
[d] Student's *t* test was used to compare output versus inoculum. NS, not significant.

analogous B region of the Gifsy-2 phage (Fig. 1) and inserted a kanamycin resistance cassette, designating the mutation Δ(G-2 B). The Gifsy-2 B region deletion mutant (JS159) was ninefold attenuated compared to the wild type (Table 2). This suggested that the Gifsy-2 B region contained a virulence gene(s) that contributes to the attenuation of a strain cured of the Gifsy-2 phage.

Previously identified genes in the B region have no effect on virulence in the whole animal. Recently, it has been shown that *sseI*/*sfrH*, located in the B region, is transcriptionally induced in macrophages (29) and encodes a protein that is secreted by the SPI-2 type III secretion system (20). We tested whether this gene was responsible for the contribution of the B region to virulence. A deletion of *stfT* through *sseI* (Fig. 1) was constructed, and the mutant strain (JS229) was tested for virulence in competition assays against the wild type (14028). We found that the deletion mutant competed evenly with the wild type (Table 2). Thus, none of the genes in this interval were responsible for the contribution of the B region to virulence. Although *sseI* is induced within a macrophage and the resulting protein is secreted into the cytoplasm of eukaryotic cells, loss of this product does not significantly affect *S. enterica* serovar Typhimurium virulence in BALB/c mice after i.p. inoculation.

Identification of the *gtgE* virulence gene. The above results indicated that the virulence determinant in the B region was located downstream of *sseI*. This was confirmed by deleting genes *gtgC* through *attR* (Fig. 1). The resulting strain (JS230) was attenuated to approximately the same degree as the Δ(G-2 B) strain (Table 2). We then constructed deletions in the four major open reading frames in this region, *gtgC*, *gtgD*, *gtgE*, and *gtgF*. Strain JS221, with the *gtgE* and *gtgF* open reading frames deleted, was attenuated to the same extent as the Δ(G-2 B) strain (Table 2). Deletion of *gtgC*, *gtgD*, or *gtgF* had no significant effect on virulence (Table 2). These results indicate that *gtgE* is the primary virulence determinant in this region. This was confirmed by assaying a strain with a deletion of only *gtgE*; MA7073 is attenuated to the same extent as the Δ*gtgEF* mutant. The fact that insertions/deletions in either the upstream or downstream open reading frames have no effect suggests that *gtgE* is transcribed independently of *gtgF* and *gtgD*.

To determine whether the virulence phenotypes observed in vivo were due to general growth defects, an assay involving competition between the Δ*gtgEF* mutant and the wild type in minimal medium was performed. There was no significant difference between the mutant and wild type after overnight growth (median CI = 0.83; $n = 6$). Indeed, deletion of the entire Gifsy-2 phage did not affect growth of *S. enterica* serovar Typhimurium in minimal medium (median CI = 0.94; $n = 6$).

Complementation of the *gtgE* virulence gene. To ensure that the observed phenotype is due to loss of *gtgE* function, a wild-type copy of the *gtgE* gene was cloned in either orientation onto plasmid pNFB9, which contains the attachment site and the *int* gene of bacteriophage Gifsy-1. Expression of Gifsy-1 integrase causes the plasmid to integrate via site-specific recombination at the Gifsy-1 attachment site (*attG1*) in *S. enterica* serovar Typhimurium. (Loss of the Gifsy-1 phage does not affect virulence in i.p. infection assays [8, 25].) Strains with *gtgE* in Gifsy-2 deleted and containing either of the *gtgE*$^+$ plasmids integrated at *attG1* competed against the isogenic Δ*gtgE* strain containing the integrated vector. As shown in Table 3, the strains containing the single-copy *gtgE*$^+$ plasmids behaved like the wild type in these assays. Taken together, these data indicate that the virulence defect conferred by the Δ(G-2 B) mutation is due to loss of *gtgE* function.

The *gtgE* and *sodCI* genes are the major virulence determinants carried by Gifsy-2. The data above indicate that *gtgE* and *sodCI* are responsible for the contribution of Gifsy-2 to virulence. To explicitly test this, we constructed a *sodCI gtgE* double mutant and tested its virulence phenotype. Because *gtgE* and *sodCI* with deletion/insertion mutations both encoded kanamycin resistance, we used the Flp recombinase method to delete the marker cassette inserted into the *gtgE* deletion mutant (see Materials and Methods). The absence of the *gtgE* gene was confirmed by PCR. In competition assays, this Δ*gtgEF* mutant competed evenly with the original Δ*gtgEF*::Km mutant (data not shown). The double mutant was tested in competition assays against a strain with the Gifsy-2 phage deleted (JS226). The *sodCI gtgE* double-mutant strain outcompeted the strain with Gifsy-2 only twofold (Table 3). Given that the Gifsy-2 deletion strain is approximately 150-fold attenuated, this demonstrated that SodCI and GtgE are the major contributors to virulence encoded by Gifsy-2 in *S. enterica* serovar Typhimurium. However, deletion of the entire phage apparently confers an additional, but subtle, virulence defect. This could be due to an additive effect of losing several independent Gifsy-2 genes, which may include putative virulence factors, but also phage functions such as *recE* and the immunity region (Δ[*attL-gftO*]::Km; Table 2).

Having shown that the *gtgE* and *sodCI* genes are necessary virulence determinants carried by Gifsy-2, we tested if *gtgE* and *sodCI* genes are sufficient to complement the virulence defect due to a Gifsy-2 deletion. We cloned wild-type copies of the

TABLE 4. Competition assays with complemented strains

Mutant strain[a]		Median CI[b]	No. of mice[c]	P^d
Relevant genotype	Name			
Δ(Gifsy-2) pGP704 (vector)	JS232	0.014	12	< 0.0005
Δ(Gifsy-2) p*sodCI*$^+$	JS233	0.28	11	< 0.0005
Δ(Gifsy-2) p*gtgE*$^+$	JS234	0.67	12	NS
Δ(Gifsy-2) p*gtgE*$^+$ *sodCI*$^+$	JS235	0.62	12	NS

[a] All strains competed against an isogenic wild-type strain (JS231).
[b] CI was calculated according to the formula in Materials and Methods, with the mutant as strain A and the wild type as strain B.
[c] All assays were performed i.p. using BALB/c mice.
[d] Student's *t* test was used to compare output versus inoculum. NS, not significant.

gtgE and the *sodCI* genes, separately and together, on *pir*-dependent plasmid pGP704 (22). The vector and resulting plasmids were each integrated at the λ attachment site (as in reference 15) in strains with Gifsy-2 deleted, and the resulting strains competed against an isogenic Gifsy-2$^+$ strain. As shown in Table 4, the ΔGifsy-2 strain containing the *sodCI*$^+$ plasmid resembles a Δ*gtgE* strain. Surprisingly, introduction of the *gtgE*$^+$ plasmid restores virulence essentially to a wild-type level. Indeed, introduction of *sodCI*$^+$ in this background does not result in any significant virulence increase. We presume that altered expression of the genes from the plasmids affects the level of complementation. However, it is clear from this data that both SodCI and GtgE contribute significantly to the virulence of *S. enterica* serovar Typhimurium strain 14028.

DISCUSSION

The pathogenic potential of *Salmonella* strains is dependent on the contribution of a wide array of virulence factors. Many of these have been acquired on pathogenicity islands and islets over evolutionary time. Bacteriophages also contribute significantly to virulence in *Salmonella* and many other pathogenic bacteria, allowing the acquisition and exchange of virulence factors on a much more rapid time scale. Lambdoid phage Gifsy-2 is required for full virulence of lysogenic *S. enterica* serovar Typhimurium strains. Deletion of Gifsy-2 attenuates virulence 100-fold as measured in an i.p. competition assay. Gifsy-2 carries a number of potential virulence factors. However, we have shown that the virulence defect conferred by loss of Gifsy-2 is largely dependent on the loss of only two Gifsy-2 genes, *sodCI* and *gtgE*.

Consistent with results for previous mutations that genetically separated Gifsy-2 phage production and virulence (15), two results presented here confirm that SodCI and GtgE function independently of the Gifsy-2 phage life cycle. First, the Δ(*attL-gftO*)::Km strain is fully virulent. This deletion should block Gifsy-2 excision, late gene transcription, and replication. Second, wild-type *gtgE* and *sodCI*, when separated from the phage genome, complement the virulence defect conferred by a Gifsy-2 deletion. Thus, expression and function of *gtgE* and *sodCI* are independent of Gifsy-2 phage biology. This is in contrast to what is found for some phage-produced virulence factors such as Shiga toxin 2, carried on lambdoid phages in lysogenic Shiga toxin-producing *Escherichia coli* strains. In these cases, toxin production is under the transcriptional control of the late operon, such that toxin is produced only when the phage is induced, killing the bacterial cell (27).

SodCI is a periplasmic Cu/Zn superoxide dismutase, important for protection of the bacterium against phagocytic superoxide in the animal (5, 6). The *gtgE* gene encodes a putative protein of 228 amino acids with no significant homologs in other bacterial species found in the National Center for Biotechnology Information database. The GtgE protein is acidic (pI 4.6) with no apparent N-terminal signal sequence or transmembrane domains, suggesting a cytoplasmic protein. Epitope-tagging experiments indicate that GtgE is produced by *S. enterica* serovar Typhimurium cells growing in vitro as well as intracellularly in HEp-2 epithelial cells (26). Thus, GtgE represents a novel bacterial virulence factor. It is formally possible that the protein is secreted, for example, by the SPI-2 type III secretion system, given that the signals for type III secretion are not easily recognized. It is also possible that GtgE is a type III chaperone. If this is true, then its target protein is not carried on Gifsy-2 or Gifsy-1. Indeed, the phenotype conferred by loss of GtgE is apparently more severe than that conferred by loss of any previously identified SPI-2 effector except SifA (24). Information regarding the biochemical function of GtgE will require further analyses.

Gifsy-2 genes with homology to known virulence factors individually had no significant effect on the virulence of *S. enterica* serovar Typhimurium in i.p competition assays. There are several possible explanations for these results. Perhaps some of the genes have been acquired only recently by the Gifsy-2 phage and have not evolved or adapted to the appropriate regulatory circuitry. Alternatively, the products of these genes might perform redundant functions. For example, a second, nearly identical copy of the *gtgA* gene of Gifsy-2 is found in the genome of the Gifsy-1 prophage. Another likely possibility is that these loci specify activities involved in aspects of infection that are not reflected in the mouse model or that are too subtle to measure in whole-animal experiments. SseI is clearly secreted into the eukaryotic cell by the SPI-2 type III secretion system (20). However, loss of SseI produced no discernible phenotype. Indeed, the lack of a significant phenotype in a mouse model is not uncommon with effector proteins (see references 13 and 21 for examples). This does not mean that these proteins do not have some role in the host, and it will be important to understand their biochemical function in order to fully understand *Salmonella* pathogenesis.

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