OmpC Is the Receptor for Gifsy-1 and Gifsy-2 Bacteriophages of Salmonella

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Mutations in the Salmonella enterica serovar Typhimurium ompC gene conferred resistance to Gifsy-1 and Gifsy-2 bacteriophages. Selection for complementing plasmids yielded clones of ompC. Introduction of an ompC clone into Escherichia coli conferred the ability to adsorb Gifsy phage. These data show that OmpC is the receptor for Gifsy-1 and Gifsy-2 phages.

Two inducible prophages, Gifsy-1 and Gifsy-2, contribute to virulence in Salmonella enterica serovar Typhimurium. The Gifsy-1 and Gifsy-2 bacteriophages are partially homologous to each other but are heterogeneous and integrate at different sites in the chromosome, 57 and 23.8 centisomes, respectively (7). Serovar Typhimurium strains cured of Gifsy-2 are significantly attenuated in mice. Virulence factors encoded on Gifsy-2 include SodCl, a periplasmic superoxide dismutase (3, 6), and SseI/SrfH (12, 21), a protein secreted by a host-contact-dependent secretion system (12). The Gifsy-2 phage also harbors an antivirulence gene, grvA, that decreases the virulence of serovar Typhimurium (8). The Gifsy-1 phage carries the gtpA gene, which affects the ability of serovar Typhimurium to grow within Peyer’s patches in the small intestine of mice (19).

Isolation of Gifsy-2-resistant mutants. Previously, we found that the use of a galE derivative of a doubly cured (Gifsy-1- and Gifsy-2-cured) strain as a recipient resulted in a higher plating efficiency and larger plaques for both Gifsy-1 and Gifsy-2 phages (8). In adsorption assays (see below), a galE mutant adsorbed Gifsy-2 phage 14-fold more efficiently than the isogenic galE" strain. The UDP-galactose permease encoded by galE is required for synthesis of the outer core and the O antigen of lipopolysaccharide (11). These results suggested that the receptor(s) for the Gifsy phage was an outer membrane protein that, in the wild-type background, is partially blocked by the O antigen.

To identify the outer membrane receptor for Gifsy-2, we isolated serovar Typhimurium mutants that were resistant to Gifsy-2 phage. Because it is difficult to generate high-titer lysates of Gifsy-1 or Gifsy-2 phage, we have been unable to isolate clear-plaque mutants. Therefore, we performed an enrichment procedure for phage-resistant mutants. Four independent pools of greater than 10,000 MudCm insertions in the galE Gifsy-1- and Gifsy-2-cured strain were generated (5) and subcultured (1:100) in 2 ml of Luria-Bertani (LB) medium containing 20 μg of chloramphenicol ml\(^{-1}\). After 3 to 4 h at 37°C, approximately \(5 \times 10^5\) sodC1::aph Gifsy-2 phage were added to each pool. The sodC1::aph insertion confers kanamycin resistance but does not have an effect on Gifsy-2 phage production (8). A significant fraction of the phage-infected cells should have lysed in the overnight culture. Phage-resistant mutants and lysogens of Gifsy-2, which would be kanamycin resistant, should have survived. After overnight incubation at 37°C, the pools were subcultured (1:100) in LB medium for 3.5 h. To induce lysis of strains lysogenized by Gifsy-2, a 35-μl aliquot of 0.3% \(\mathrm{H}_2\mathrm{O}_2\) was added to each pool, which was then incubated overnight at 37°C. This procedure was repeated six times.

Following this enrichment procedure for phage-resistant mutants, each pool was diluted and plated on LB-chloramphenicol agar. Eight colonies from each independent pool were isolated and scored for kanamycin resistance to ensure that they were not Gifsy-2 lysogens. All tested colonies were chloramphenicol resistant and kanamycin sensitive. There were no detectable plaques when \(5 \times 10^5\) Gifsy-2 phage were spotted on top agar lawns of these strains. Spotting phage onto a lawn of the galE Gifsy-1- and Gifsy-2-cured parent strain resulted in a confluent zone of clearing.

P22 lysates were grown on one representative strain from each pool and used to transduce the MudCm insertions into the galE Gifsy-1- and Gifsy-2-cured parent strain. Characterization of the resulting transductants showed that all four independent MudCm insertions conferred resistance to both Gifsy-2 and Gifsy-1 phages, as determined by a plaque assay. The data suggested that Gifsy-2 and Gifsy-1 phages use the same receptor.

Next, we demonstrated that the MudCm insertion mutations did not affect Gifsy phage replication or assembly. Each MudCm mutation was moved into a wild-type Gifsy-1- and Gifsy-2-containing background and a Gifsy-1-cured and Gifsy-2-containing background. The titers of at least six independent overnight cultures of each strain were determined for Gifsy-1 and Gifsy-2 phages. All of the strains containing the MudCm insertions produced the same titer of Gifsy-1 or Gifsy-2 phage as the isogenic parent strain (\(~10^6\) Gifsy-1 phage PFU ml\(^{-1}\); \(~10^7\) Gifsy-2 phage PFU ml\(^{-1}\)). These results suggested that the MudCm insertions did not block phage production but rather caused loss of the Gifsy phage receptor.

Gifsy-resistant mutations affect ompC. To determine the locations of the MudCm insertions, we used semirandom PCR (1) to generate DNA templates and determined the DNA sequence from the left end of each MudCm insertion. The MudCm markers were inserted in sopH2, napC, yojI, and resC. We
FIG. 1. Outer membrane proteins from the Gifsy-resistant mutants and the ompC" plasmid-complemented strains. The region of the gel corresponding to 30- to 40-kDa proteins is shown. All Salmonella strains were galeE96 Gifsy-1- and Gifsy-2-cured derivatives of serovar Typhimurium strain 14028 (8). Plasmid-bearing strains also contained the zgg-8103::pir" allele (9). Lanes: A, E. coli (DH5αpir"); B, pompC" in E. coli ("p" before the gene indicates that the gene was carried on a plasmid); C, ompC"; D, pompC" in ompC"; E, ompC96::Tn10; F, pompC" in ompC96::Tn10; G, Δ[(sspH2)*MudCm" (ompC)]; H, pompC" in Δ[(sspH2)*MudCm" (ompC)].

noted that all of these insertions were near ompC, which encodes a major outer membrane porin protein. We subsequently determined that the MudCm insertions were 100% linked by P22 transduction to the known ompC96::Tn10 insertion (4). The simplest explanation for these results was that each of the MudCm insertions was associated with a deletion that extended through ompC, and it was the loss of the OmpC porin that resulted in phage resistance.

Selection for plasmids that complement Gifsy phage resistance. To further identify the defect conferred by the MudCm insertions, we selected plasmids that would complement the mutations and restore phage sensitivity. Each of the MudCm insertions was transduced into a galeE Gifsy-1- and Gifsy-2-cured strain containing the zgg-8103::pir" allele (9), which allows for the replication of Pi-dependent plasmids. The resulting strains were transformed with a library of random 5-kb serovar Typhimurium chromosomal fragments in plasmid pGP704 (13). Each transformation mixture was divided into four pools and grown overnight in LB medium containing 50 μg of ampicillin ml⁻¹. These pools were then subcultured 1:100 in the same medium for 4 h. Approximately 5 × 10⁵ sodC1:aph Gifsy-2 phage were added to each culture, which was then incubated for 3 to 4 h at room temperature without agitation. The mixture was then plated on LB agar containing 50 μg of kanamycin ml⁻¹ to select for Gifsy-2 lysogens. For each MudCm mutant, we obtained 1 to 5 kanamycin-resistant colonies. The kanamycin-resistant colonies simultaneously became Gifsy-1 phage sensitive, as determined by a plaque assay. Nontransformed MudCm insertion strains never yielded kanamycin-resistant colonies in control experiments.

Restriction analysis suggested that the complementing plasmids isolated in the four independent MudCm insertion mutants all contained the same 3-kb chromosomal insert. Therefore, one of these plasmids, pTH73, was characterized further. DNA sequences of the ends of the chromosomal insert were obtained using plasmid primers. The data showed that plasmid pTH73 contained the entire ompC gene and partial fragments of the yojN and aphE genes. To show more clearly that the ompC gene was responsible for complementing the Gifsy phage resistance phenotype, we deleted a fragment (372 bp) of the aphE gene on plasmid pTH73 to create plasmid pTH77, which contains the entire ompC gene, less than 400 bp of the aphE gene, and less than 100 bp of the yojN gene. Plasmid pTH77 was moved into each pir" MudCm insertion mutant. In all instances, the ompC" plasmid was able to restore sensitivity to both Gifsy-1 and Gifsy-2 phages to wild-type levels, as determined by plaque assays. These data confirmed that the loss of the OmpC protein in the MudCm insertion mutants conferred resistance to Gifsy phage.

Phage resistance phenotype of an ompC" insertion mutant. The data presented above suggested that OmpC is the receptor for Gifsy-1 and Gifsy-2 phages. However, the MudCm insertions are apparently associated with deletions that remove genes surrounding ompC. Therefore, we characterized the phenotypes conferred by a known ompC mutation, ompC96::Tn10 (4). This insertion was moved into the galeE Gifsy-1- and Gifsy-2-cured strain by P22 transduction. First, we examined OmpC production in the ompC96::Tn10 strain and the MudCm insertion strains. Outer membrane proteins from the Gifsy-resistant mutants and the plasmid-complemented strains were isolated as previously described (14) and displayed on an 11% polyacrylamide gel containing 4 M urea (10). All of the MudCm mutants (one representative is shown) and the ompC96::Tn10 mutant lacked the 36-kDa OmpC protein; strains containing the ompC" plasmid isolated from the Gifsy sensitivity selection procedure overexpressed this outer membrane protein (Fig. 1).

No detectable Gifsy-1 or Gifsy-2 plaques were observed when the ompC96::Tn10 mutant was used as a recipient in plaque assays (Table 1). This defect was complemented to

### TABLE 1. Effects of ompC alleles on Gifsy phage interactions

<table>
<thead>
<tr>
<th>Strain*</th>
<th>ompC&quot;</th>
<th>pompC&quot; in ompC&quot;</th>
<th>ompC96::Tn10</th>
<th>pompC&quot; in ompC96::Tn10</th>
<th>E. coli</th>
<th>pompC&quot; in E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Efficiency of plating</td>
<td>100 ± 15</td>
<td>107 ± 11</td>
<td>&lt;0.004</td>
<td>105 ± 19</td>
<td>&lt;0.004</td>
<td>&lt;0.004</td>
</tr>
<tr>
<td>Phage unadsorbed</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&gt;99.5 ± 6</td>
<td>&gt;99.5 ± 33</td>
<td>&gt;99.5 ± 3</td>
<td>&gt;99.5 ± 3</td>
</tr>
<tr>
<td>Frequency of lysogeny</td>
<td>100 ± 31</td>
<td>88 ± 26</td>
<td>&lt;9</td>
<td>109 ± 3</td>
<td>&lt;9</td>
<td>&lt;9</td>
</tr>
</tbody>
</table>

| Efficieny of plating | 100 ± 13 | 89 ± 19 | <0.006 | 82 ± 16 | <0.006 | <0.006 |
| Phage unadsorbed | 0.3 ± 0.3 | 0.2 ± 0.1 | >99.5 ± 22 | 0 ± 0.02 | 81 ± 31 | 0.02 ± 0.46 |
| Frequency of lysogeny | 100 ± 19 | 99 ± 29 | <0.1 | 83 ± 14 | <0.1 | 0.8 ± 0.46 |

*All Salmonella strains were galeE96 Gifsy-1- and Gifsy-2-cured derivatives of strain 14028 (8). Plasmid-bearing strains contained the zgg-8103::pir" marker (9), which has no effect on Gifsy biology. E. coli strains were DH5αpir".

* Results are given as means and standard deviations and are relative to the results of an identical assay with no adsorbing cells added. Four samples were tested.

* Results are given as means and standard deviations and are relative to the results of an identical assay with no adsorbing cells added. Four samples were tested.
approximately wild-type levels by the ompC+ plasmid pTH77. We performed adsorption assays to test directly if the ompC mutation affected phage binding. A 100-μl aliquot of an overnight bacterial culture was centrifuged, and the cells were resuspended in 0.1 ml of LB medium containing approximately 10⁶ Gifsy-1 or Gifsy-2 phage. The mixture was allowed to stand at room temperature for 1 h. Approximately 50 μl of chloroform was added, and the sample was vortexed and immediately centrifuged to pellet cells and adsorbed phage. The number of phage remaining in suspension was determined by plating dilutions on lawns of phage-sensitive bacteria. The ompC+ parent strain adsorbed greater than 99% of both Gifsy-1 and Gifsy-2 phages. In contrast, the ompC mutant adsorbed less than 0.5% of Gifsy-1 or Gifsy-2 phage. The ompC+ plasmid restored adsorption to wild-type levels (Table 1). Finally, we determined the frequency of lysogeny as described above using the soilC::aph Gifsy-2 phage or a ΔgmrB::kan Gifsy-1 phage (T. D. Ho, C. D. Ellermeier, and J. M. Slauch, unpublished data), both of which confer resistance to kanamycin. There were no detectable lysogens of either Gifsy-1 or Gifsy-2 when the ompC gale E. coli Gifsy-1- and Gifsy-2-cured mutant was used as a recipient. As expected, the ompC+ plasmid restored the ability of the recipient to become lysogenized. Thus, mutations in ompC confer a Gifsy-1- and Gifsy-2-resistant phenotype. An ompC mutant does not adsorb phage and cannot be lysogenized. A gale+ ompC Gifsy-1- and Gifsy-2-cured mutant also could not be lysogenized by either Gifsy-1 or Gifsy-2 (data not shown), showing that the phage resistance phenotype is due solely to the loss of OmpC and is not dependent on the O-antigen defect.

Salmonella OmpC allows Gifsy-1 and Gifsy-2 phage to be adsorbed by Escherichia coli. We tested Gifsy phage sensitivity in a restriction-negative E. coli strain with and without the Salmonella ompC+ plasmid. Neither Gifsy phage was able to cause plaque formation or lysogenize E. coli strain DH5αλpir+ (Table 1). This strain is sensitive to the OmpC-specific E. coli phage SS4 (16) and therefore produces OmpC. Although there was some variability in the assay, the level of adsorption of Gifsy-1 or Gifsy-2 by DH5αλpir+ was not significantly different from that observed with the serovar Typhimurium ompC strain. However, when plasmid pTH77 was brought into DH5αλpir+ (Fig. 1), the resulting strain was able to adsorb greater than 99% of both Gifsy-1 and Gifsy-2 phages (Table 1).

When Gifsy-1 and Gifsy-2 phages were spotted on lawns of DH5αλpir+/pTH77, no plaques were observed. We also did not detect any lysogens of the Gifsy-1 phage. However, the DH5αλpir+/pTH77 recipient could be lysogenized by the kanamycin-resistant Gifsy-2 phage at a low but detectable frequency (Table 1). Thus, Gifsy-2 was able to enter E. coli cells bearing the serovar Typhimurium ompC+ plasmid. Because the DH5αλpir+ strain is recA and Gifsy phage induction is RecA dependent (T. D. Ho, L. Shaughnessy, and J. M. Slauch, unpublished data), the Gifsy-2 prophage was moved by P1 transduction into a recA+ E. coli strain (MC4100) (17). The resulting recA+ E. coli lysogen (JS198) of Gifsy-2 was not able to produce Gifsy-2 phage, as detected by plague plaque or lysogeny assays with sensitive E. coli (DH5αλpir+/pTH77) or serovar Typhimurium (JS178) as the recipient. Thus, E. coli lysogens of Gifsy-2 were incapable of producing Gifsy-2 phage. These results are consistent with our observation that Gifsy-2 was incapable of causing the formation of plaques on a lawn of E. coli expressing serovar Typhimurium OmpC. Taken together, these results further showed that serovar Typhimurium OmpC is the receptor for both Gifsy-1 and Gifsy-2 phages. Clearly, both phages are adsorbed by E. coli cells that express Salmonella OmpC. However, the production of phage requires both the presence of and an appropriate interaction with certain host factors. Lysogeny also requires host functions and an appropriate site in the genome. It is not surprising that E. coli does not meet all of these requirements for Gifsy-1 and Gifsy-2.

Although osmoregulation of E. coli ompC has been well documented (18), Salmonella does not appear to regulate porin expression in response to osmolarity. In S. enterica serovar Typhi, ompC is expressed at high levels in the presence of both low osmolarity and high osmolarity (15). We have also found that ompC-hacZ fusions are not osmoregulated in serovar Typhimurium (unpublished data). Thus, the receptor for Gifsy-1 and Gifsy-2 phages is most likely available in the presence of different osmolarity conditions.

The fact that E. coli OmpC does not bind Gifsy phage suggests that the phage binding sites are located in regions that differ between the two OmpC proteins. Serovar Typhimurium OmpC and E. coli OmpC are 83% identical and 86% similar at the protein level. The structure of the related protein OmpF from E. coli is known (2), and the OmpC structure can be inferred by aligning the two sequences. The porin proteins are trimeric structures in which each monomer forms a β barrel in the outer membrane, with certain loops that connect β sheets being exposed to the external medium (2). Most of the differences in sequence between the serovar Typhimurium and E. coli OmpC proteins are in surface-exposed loops, which correspond approximately to amino acids 25 to 29, 63 to 69, 154 to 168, 248 to 258, and 332 to 344 in the mature serovar Typhimurium protein. Differences between the serovar Typhimurium and E. coli proteins are particularly evident in the loop from amino acids 154 to 168, in which E. coli OmpC has an extra six amino acids, and the loop from amino acids 248 to 258, in which the E. coli protein is missing nine amino acids. Vakharia and Misra (20) used three OmpC-specific phages to isolate mutations affecting E. coli OmpC. Interestingly, 23 of 24 of these phage-resistant mutations altered amino acids in these two loops of the protein. These differences in surface-exposed regions are likely to account for the inability of E. coli OmpC to adsorb the Gifsy phages.

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