Phase variation of the lpf operon is a mechanism to evade cross-immunity between Salmonella serotypes

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Conventional wisdom holds that phase variation is a mechanism for immune evasion. However, despite fimbrial phase variation, mice previously exposed to Salmonella typhimurium are protected against a subsequent challenge. We evaluated whether lpf phase variation instead may be a mechanism to evade cross-immunity between Salmonella serotypes. Mice were immunized orally with S. typhimurium aroA mutants either that expressed the lpf operon (phase-on variant) or in which the entire lpf operon had been removed by deletion. During a subsequent challenge with virulent Salmonella enteritidis a selection against lpf phase-on variants was observed in mice previously exposed to S. typhimurium long polar fimbriae. Vaccination with S. typhimurium did not confer protection against challenge with S. enteritidis, presumably because lpf phase-off variants were able to evade cross-immunity. We propose that lpf phase variation is a mechanism to evade cross-immunity between Salmonella serotypes, thereby allowing their coexistence in a host population.

Bacteria are able to change the antigenic properties of their cell surface by two mechanisms: (i) antigenic variation, which leads to changes in the composition (e.g., the amino acid sequence) of surface structures, and (ii) phase variation, which involves a reversible gain or loss of an antigen. The hypothesis that antigenic variation is a mechanism to evade the host immune response, thereby allowing recurrent or persistent infection, is supported by elegant animal experiments and human volunteer studies performed with Borrelia hermsii and Neisseria gonorrhoeae (1-6). By analogy, it has been suggested that phase variation is a mechanism for immune evasion; however, there is little experimental evidence supporting this postulate.

Salmonella enterica serotype Typhimurium (S. typhimurium) expresses several surface antigens whose expression is regulated by phase variation. These include flagellar subunits (7-10), type 1 fimbriae (11-13), and long polar (LP) fimbriae (14). Immunization with flagellar antigens does not protect mice against infection with S. typhimurium (15, 16). It therefore has been speculated that the oscillation between “on” and “off” expression states of fimbrial or flagellar biosynthesis genes of S. typhimurium may be a mechanism for immune evasion (10). However, in addition to these variable antigens, the surface of S. typhimurium is decorated with conserved determinants such as lipopolysaccharide (LPS) (17). Immunization of mice with a S. typhimurium aroA mutant elicits high titers of antibodies directed against a single immunodominant LPS epitope, the O4-antigen (17, 18). Anti-O4 antibodies elicited by vaccination confer protection against a subsequent infection with virulent S. typhimurium. Flagellar or fimbrial phase variation does not allow S. typhimurium to evade protective immunity conferred by anti-O4 antibodies. Phase variation therefore is not a mechanism that allows S. typhimurium to cause recurrent infections. These considerations raise the question of what biological function the control of surface antigen expression by phase variation might serve during the interaction of S. typhimurium with its host.

Previous studies on fimbrial phase variation have focused on the recovery of Escherichia coli or Proteus mirabilis phase variants from organs, urine, or feces of infected hosts (19-25). It has become clear from this work that selection for fimbriate and nonfimbriate bacteria in the organ under investigation provides information regarding the function of an individual adhesin in the host. For instance, selection for type 1 fimbriate E. coli and P. mirabilis in the bladder is evidence for a role of type 1 fimbriae during colonization of this organ. Furthermore, bacteria isolated from the urine lack type 1 fimbriae, suggesting that an adherence defect of nonfimbriate phase variants leads to their elimination from the bladder (23-25). Finally, selection against type 1 fimbriated E. coli cells in the peritoneal cavity of mice may result from increased phagocytosis because of attachment of type 1 fimbriae to receptors of neutrophils (21, 22).

Although these data are useful for understanding the role of an individual adhesin during infection, they shed little light on the biological function of fimbrial phase variation. If the only selective pressure for or against fimbriated cells is imposed by the binding specificity of an adhesin in a particular organ, the advantage of controlling expression by a random, heritable on/off switch rather than by environmental cues alone is not apparent. Conventional wisdom regarding phase variation holds that it allows a fraction of a bacterial population to survive very sudden changes in their environment, but it is not clear what these sudden changes might be in the case of phase variation systems of S. typhimurium or related organisms.

Because the role of phase variation is not apparent when studying a single host pathogen combination, we explored the possibility that alterations of surface antigens may allow related pathogens, which circulate in the same host population, to avoid cross-immunity. To test this hypothesis, we have studied the role of lpf phase variation in S. typhimurium and Salmonella enterica serotype Enteritidis (S. enteritidis) during infection of mice. S. typhimurium and S. enteritidis are natural pathogens of mice, and both serotypes coexist in a rodent animal reservoir (26). The lpf operon encodes a fimbrial adhesin, which mediates attachment of S. typhimurium to murine Peyer’s patches (27-29). We have demonstrated recently that lpf phase variants can be identified by using a S. typhimurium strain, which carries a transcriptional fusion between the lpfABCD operon and the lacZYA genes of E. coli. Expression of this transcriptional fusion correlates with lpf-mediated attachment to Peyer’s patches, as shown by adhesion assays performed in a murine intestinal organ culture model (14). In this report, we have used this transcriptional fusion to study the effect of an immune response on the recovery of S. typhimurium and S. enteritidis lpf phase variants from mice.

Materials and Methods

Bacterial Strains and Growth Conditions. The S. typhimurium strains IR715 (nalidixic acid-resistant wild type) (30), AJB3...
Sequence Analysis of S. enteritidis LpfA and Construction of a Glutathione S-Transferase (GST)-LpfAse Fusion Protein. The lpfA gene was amplified from S. enteritidis strain CDC SSU7998 by using a primer pair described previously (14). To ensure that no mutations had been introduced by PCR amplification, products from two independent reactions were cloned into the vector pCR II (TA-cloning kit; Invitrogen) to give rise to plasmids pTN54 and pTN55, respectively. The nucleotide sequences of both plasmid inserts were determined and analyzed by using the MACVECTOR software package (Oxford). A translational fusion to GST was constructed by cloning the EcoRI–SalI-restricted PCR product into EcoRI–SalI-restricted pGEX-4T-2 (Amersham Pharmacia). Purification of the GST-LpfAse fusion protein was performed as described for a GST-LpfAs fusion protein (14).

Construction of Mutants. For construction of a S. typhimurium mutant carrying a deletion of the lpf operon, a 1.5-kb DNA region located upstream of lpfA was PCR-amplified by using the primers 5′-AATGGAGTGTATAGAGGTGGG-3′ and 5′-TCTAGACGTCTCACCGATAATGAAAAC-3′. The PCR product was cloned into plasmid pCRII (Invitrogen) to give rise to plasmid pMS1200. A 1.2-kb DNA region located downstream of lpfA was PCR-amplified by using the primers 5′-CCCGGGCTGTTGACCTTCAAGACAGATC-3′ and 5′-TCTACGGCCTTGGTTCACCTAACGAGC-3′. The PCR product was cloned into plasmid pCR II (Invitrogen) to give rise to plasmid pMS1201. Plasmid pMS1201 was restricted with XbaI–EcoRI, and the insert was cloned into XbaI–EcoRI-restricted plasmid pBlueScript SK(+) (37) to give rise to plasmid pMS1202. The insert of plasmid pMS1200 was excised by XhoI–KpnI restriction and cloned into XhoI–KpnI-restricted plasmid pMS1202 to give rise to plasmid pMS1203. Plasmid pMS1203 was linearized by HindIII restriction and ligated with a HindIII–DNA fragment carrying a kanamycin resistance cassette (KIXX; Amersham Pharmacia) to give rise to plasmid pMS1205. The insert of plasmid pMS1205 was excised by XbaI–KpnI restriction and cloned into XbaI–KpnI-restricted suicide vector pGP704 (38) to give rise to suicide plasmid pMS1208. E. coli strain S17–1 apr (pMS1208) was conjugated with S. typhimurium strain IR715 and exconjugants were selected on LB + Nal + Km plates. Exconjugants were patched onto LB + Cm plates, and a mutant, which was resistant to kanamycin but sensitive to carbenicillin, was selected and termed AQB103. Deletion of the lpf operon in AQB103 was confirmed by Southern hybridization (39) by using a DNA probe specific for lpf/CD (the insert of plasmid pMS1039) (29) and PstI-restricted chromosomal DNA of strains AQB103 and IR715 (35). Hybrids were detected by using the labeling and detection kit (nonradioactive) from Boehringer Mannheim.

Strains TN3 and TN4 were constructed by P22 transduction of the araA::Tn10 allele from S. typhimurium strain CL1509 into strains AQB33 and AQB103, respectively. TN3 and TN4 were unable to grow on minimal medium and did not form a halo around colonies on Chrome azurol S agar plates, thus confirming auxotrophy and loss of siderophore production caused by the araA mutation (36, 40).

A fusion between the lpfABCDE operon of S. enteritidis and the lacZYA genes of E. coli was constructed as described for S. typhimurium AJB33 (14). A spontaneous nalidixic acid-resistant derivative of S. enteritidis strain CDC SSU7998 was isolated on LB + Nal plates and termed TN2. Suicide plasmid pMS1096 (14) was introduced into TN2 by conjugation. An exconjugant selected on LB + Nal + Cm + X-gal plates was termed TN5. In strain TN5, the insertion of pMS1096 into the lpf operon was confirmed by Southern hybridization of MluI-restricted genomic DNA with a lpfCD-specific DNA probe (the labeled insert of plasmid pMS1039).

Animal Experiments. Before infection of 5- to 6-week-old female BALB/c mice, all bacteria were cultured as static overnight cultures in LB broth. In all experiments the bacterial titer of the inoculum was determined by spreading serial 10-fold dilutions on LB + X-gal + Te + Cm plates to quantify bacteria containing the lpf operon in the “on” or “off” expression state, respectively. Fecal pellets taken from naive mice on the day of immunization were suspended in PBS and spread on LB + X-gal + Te + Cm plates to ensure that the indigenous microflora is sensitive to these antibiotics. After inoculation, fecal pellets were collected 7 hr postinfection and at daily intervals and suspended in PBS, and serial 10-fold dilutions were spread onto LB + X-gal + Te + Cm plates. During challenge experiments, animals developing lethal signs of disease (anorexia) were euthanized, and the spleen was collected and homogenized in 5 ml of PBS by using a Stomacher (Tekmar, Cincinnati). Dilutions were plated to determine on/off ratios.

To determine whether lpf phase on/off ratios determined in the feces correlated with on/off ratios present in the cecum, a group of four mice was infected with 1 × 10⁹ colony-forming units (cfu)/animal of TN3. Mice were sacrificed after fecal pellets had been collected. The cecum was collected and homogenized in 5 ml of PBS by using a Stomacher (Tekmar), and serial 10-fold dilutions were spread onto LB + X-gal + Nal plates.

For immunization, groups of 10 mice were inoculated intra-gastrically with S. typhimurium araO4 mutants at a dose of 1 × 10⁶ cfu/animal contained in a 0.2-ml volume. Additional immunization experiments were performed with groups of five mice. Immunized mice were either challenged with a virulent S. typhimurium or S. enteritidis strain at day 70 postimmunization or were boosted with the same vaccine strain at 14 days postimmunization. Serum was collected from mice at day 14 after the booster immunization, and samples from each immunization group were pooled. Before challenge experiments, fecal pellets were collected from immunized mice to identify animals that had developed long-term carriage. Because phase switching occurred randomly, the on-to-off ratio determined for the inoculum varied between different strains during challenge experiments. Statistical analysis was performed after logarithmic conversion of phase on/off ratios. A Student’s t test was used to test whether the ratio present in the inoculum was significantly different from those recovered from animals. A t test for independent samples was used to calculate the significance of differences observed between ratios recovered from different immunization groups on a particular day postinfection.

A female New Zealand White rabbit was injected s.c. with approximately 1 mg of purified GST-LpfAs fusion protein. At approximately 1 mg per kg, the rabbit was boosted s.c. with the same dose. Serum was collected 15 days after the booster immunization. To remove antibodies that were not directed against LpfA, an E. coli strain [DH5α(pGEX-4T-2)] expressing the GST protein was used for absorption of the immune serum by using a protocol described previously (41). ELISA was performed by using a protocol described previ-
ously (42). Corning 96-well ELISA plates that were coated either with total S. typhimurium antigen (12.5 μg/well) or with purified GST, GST-LpfASt, or GST-LpfASe fusion protein (0.5 μg/well). Protein concentrations of purified proteins or the S. typhimurium whole-cell lysate were determined by using a Bradford assay kit (Bio-Rad). Samples (0.05 ml/well) were added to antigen-coated plates in triplicate 2-fold serial dilutions with 3% Blotto (3% powdered skim milk/0.04% anti-foam A/0.05% Tween 20/0.1% sodium azide in PBS) as the diluent. Mouse serum was detected by using goat anti-mouse IgG alkaline phosphatase (AP) conjugate, goat anti-mouse IgM-AP, and goat anti-mouse IgA-AP (Sigma). Rabbit serum was detected by using goat anti-rabbit Ig-AP (Sigma). Titers were expressed as the inverse of the highest dilution that gave an absorbance value greater than 0.1 when measuring $A_{405}$ on an ELISA plate reader (MR700 microplate reader; Dynatech).

Results

Fimbrial Phase Variants Are Recovered at Similar Ratios from Fecal Pellets and the Cecum of Immunized Mice. S. typhimurium strain AJB33 carries a transcriptional fusion between lpfABCDE and the reporter gene lacZYA, which alternates between transcriptionally active (on) and inactive (off) states of expression (14). On LB+X-gal plates, lpf phase-on and phase-off variants of AJB33 form colonies with a Lac− and Lac+ phenotype, respectively. Thus, the recovery of phase variants from infected animals can be monitored on LB+X-gal plates. To attenuate strain AJB33 for its use as a vaccine, an aroA::Tn10 allele was introduced by P22 transduction and the resulting strain was termed TN3.

A convenient method for monitoring the presence of lpf phase variants in immunized animals over time is to recover bacteria from fecal pellets at daily intervals. However, it is not known whether the ratio of fimbrial phase variants shed with the feces is similar to that present in the cecum, which contains the bulk of luminal S. typhimurium (43). A group of four mice was vaccinated with TN3 at a dose of $1 \times 10^9$/animal. The phase on phase-off ratios for organisms recovered from feces and the cecum were determined (Fig. 1). Although the phase on phase off ratios differed between mice, ratios recovered from feces and cecum of an individual mouse were, in each case, similar. These data showed that the ratio of lpf phase variants present in the cecum can be inferred by monitoring ratios recovered from fecal pellets of immunized mice.

The lpf Phase On/Phase Off Ratios Recovered from Fecal Pellets Collected During Immunization Closely Resemble That of the Inoculum. We next investigated whether the lpf phase on phase off ratio changes over time during oral immunization of mice with S. typhimurium strain TN3 (aroA lpfABCDE::lacZYA). Colonies with Lac+ or Lac− phenotypes were picked from a LB+X-gal+Cm+Tc plate and used to inoculate phase-on and phase-off cultures of TN3. Two groups of 10 mice were immunized with phase-on and phase-off cultures of TN3, respectively, and fecal pellets were collected after 7 hr and subsequently at daily intervals until day 10 postvaccination. At later time points, bacterial numbers recovered from fecal pellets were too low to determine lpf phase on phase off ratios. Mice immunized with a phase-off culture of TN3 shed predominantly phase-off variants during the 10-day period (Fig. 2A). Similarly, the lpf phase on phase off ratios recovered from mice immunized with a phase-on culture were similar to that of the inoculum (Fig. 2B). The finding that lpf phase on phase off ratios recovered from

![Fig. 1. Correlation of lpf phase on/phase off ratios shed in the feces with those present in the cecum of mice.](image1)

![Fig. 2. Recovery of lpf phase variants during immunization with a phase-off culture (A) or a phase-on culture (B) of strain TN3. The lpf phase on/phase off ratio present in the inoculum (solid bar) and the lpf phase on/phase off ratios recovered from fecal pellets collected after immunization are indicated (open bars represent mean \( \pm \) SE).](image2)
fetal pellets closely resembled that of the inoculum suggested that there was no overt selection for or against phase-on variants during immunization. We have demonstrated previously that in lpf phase-on cells of S. typhimurium strain AJB33, the LpfA fimbrial protein is present and exposed to the host immune system (14). Our data therefore suggested that mice inoculated with a phase-on culture of TN3 were exposed to LP fimbrial antigen for a period of at least 10 days postimmunization (Fig. 2B).

Construction of a S. typhimurium Strain Carrying a Deletion of the lpf Operon. We reasoned that the contribution of anti-LP fimbrial antibodies to an immune response could be inferred by comparing the responses elicited by vaccination with TN3 (aroA lpfABCDE::lacZYA) with that induced by immunization with a strain expressing all S. typhimurium antigens except LP fimbriae. For this purpose, a strain was constructed in which the entire lpf operon was removed by deletion. DNA regions upstream and downstream of the lpf operon were PCR-amplified and cloned on opposite sites of a kanamycin resistance cassette (KIXX; Amersham Pharmacia). The kanamycin resistance marker flanked by the lpf upstream and downstream DNA sequences was cloned into suicide vector pGP704, and the resulting construct (pMS1208) was introduced into S. typhimurium strain IR715 (nalidixic acid resistant wild type) by conjugation. A lpf deletion mutant arising by allelic exchange was termed AJB103. Deletion of the lpf operon in AJB103 was confirmed by Southern hybridization by using a 2-kb DNA fragment specific for lpfCD. No hybridization signal was detected with genomic DNA of S. enteritidis IR715 (nalidixic acid resistant wild type) by conjugation. A single copy fusion between S. typhimurium strain TN3 (aroA lpfABCDE::lacZYA) or a culture of TN4 (aroA Δlpf). Two mice in the group immunized with TN4 developed chronic carriage and were sacrificed. To allow nonspecific defense mechanisms, such as macrophage activation, to return to their normal level before infection, mice were challenged at 70 days postimmunization. At this time, both immunized groups and a group of five naive mice of the same age were infected with a phase-on culture of S. enteritidis strain TN5 (lpfABCDE::lacZYA) at a dose of 1.1 x 10^7 cfu/mouse. No selection against phase-on variants of S. enteritidis was detected in mice immunized with a S. typhimurium lpf deletion mutant (TN4) when compared with naive mice (P > 0.05) (Fig. 3). In contrast, the phase on/phase off ratios of TN5 recovered at days 2–5 postinfection of mice immunized with a lpf phase-on variant of S. typhimurium were significantly lower than those recovered at corresponding days from the naive group (P < 0.05) or the group immunized with TN4 (P < 0.05). Thus, immunization with a S. typhimurium lpf phase-on variant resulted in selection against lpf phase-on variants of S. enteritidis during a subsequent challenge.

We next determined whether immunization with S. typhimurium elicits protection against a homologous or heterologous challenge. Challenge with S. enteritidis at a dose of 1.1 x 10^7 cfu/mouse caused lethal morbidity in naive mice as well as in mice immunized with a S. typhimurium vaccine (Table 2). To assess whether immunization with S. typhimurium would confer a low level of protective immunity, the experiment was repeated with a lower S. enteritidis challenge dose (1.5 x 10^6 cfu/mouse). The mortality in the group of naive mice was not higher than that recorded for the two groups of mice immunized with S. typhimurium. Thus, oral immunization with a S. typhimurium vaccine did not confer protection against a subsequent challenge with virulent S. enteritidis. In a control experiment, mice immunized with a S. typhimurium vaccine were challenged orally after 70 days with 1.9 x 10^7 cfu of a virulent S. typhimurium strain (AJB33). Whereas mice immunized with a S. typhimurium vaccine were protected against challenge with virulent S. typhimurium, naive mice developed lethal signs of disease and were euthanized.

Table 1. Murine anti-Salmonella antibody titers elicited by S. typhimurium vaccine strains

<table>
<thead>
<tr>
<th>Vaccination with strain</th>
<th>Serum IgG titer</th>
<th>Serum IgA titer</th>
<th>Serum IgM titer</th>
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</thead>
<tbody>
<tr>
<td>TN4</td>
<td>4,096</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>TN3</td>
<td>4,096</td>
<td>128</td>
<td>64</td>
</tr>
<tr>
<td>Naïve control</td>
<td>&lt;16</td>
<td>&lt;16</td>
<td>&lt;16</td>
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Table 2. Protection against challenge with Salmonella serotypes 70 days postimmunization with a S. typhimurium vaccine

<table>
<thead>
<tr>
<th>Immunization with S. typhimurium vaccine strain</th>
<th>Death/total after challenge with strain (dose)</th>
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<tbody>
<tr>
<td>S. enteritidis</td>
<td>S. enteritidis</td>
</tr>
<tr>
<td>TN5 (1.5 x 10^6 cfu/mouse)</td>
<td>TN5 (1.1 x 10^7 cfu/mouse)</td>
</tr>
<tr>
<td>3/5</td>
<td>4/5</td>
</tr>
<tr>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>None</td>
<td>5/5</td>
</tr>
</tbody>
</table>

phology was detectable when incubation of plates was allowed to continue at room temperature for a period of a week. Alternation between Lac+ (phase on) and Lac− (phase off) phenotypes occurred through a heritable phase variation mechanism because inoculation of broth cultures with bacteria picked from a Lac+ colony gave rise to a considerably higher proportion of Lac− colonies than inoculation with bacteria picked from a Lac− colony. These data indicated that, like the lpf operon of S. typhimurium, the lpf operon of S. enteritidis oscillates between phase-on and phase-off expression states at the transcriptional level.

Immunization with S. typhimurium Results in Selection Against S. enteritidis lpf Phase-On Variants During a Subsequent Challenge. We next addressed the question of whether immunization with a S. typhimurium vaccine causes selection against S. enteritidis expressing the lpf operon. Groups of five mice were immunized with a phase-on culture of S. typhimurium strain TN3 (aroA lpfABCDE::lacZYA) or a culture of TN4 (aroA Δlpf). Two mice in the group immunized with TN4 developed chronic carriage and were sacrificed. To allow nonspecific defense mechanisms, such as macrophage activation, to return to their normal level before infection, mice were challenged at 70 days postimmunization. At this time, both immunized groups and a group of five naive mice of the same age were infected with a phase-on culture of S. enteritidis strain TN5 (lpfABCDE::lacZYA) at a dose of 1.1 x 10^7 cfu/mouse. No selection against phase-on variants of S. enteritidis was detected in mice immunized with a S. typhimurium lpf deletion mutant (TN4) when compared with naive mice (P > 0.05) (Fig. 3). In contrast, the phase on/phase off ratios of TN5 recovered at days 2–5 postinfection of mice immunized with a lpf phase-on variant of S. typhimurium were significantly lower than those recovered at corresponding days from the naive group (P < 0.05) or the group immunized with TN4 (P < 0.05). Thus, immunization with a S. typhimurium lpf phase-on variant resulted in selection against lpf phase-on variants of S. enteritidis during a subsequent challenge.

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**LPfA of *S. enteritidis* and *S. typhimurium* Are Cross-Reactive.** Anti-LPfA antibodies have been shown to select against *S. typhimurium* Lpf phase-on variants during infection of mice (14). Our finding that vaccination with LP fimbriate *S. typhimurium* causes selection against *S. enteritidis* Lpf phase-on variants therefore suggested that LpfA of both serotypes may be conserved antigenically. To test this hypothesis, a DNA fragment containing base pairs 49–534 of the *lpfA* ORF, corresponding to the coding region of the mature LpfA protein after removal of its signal sequence, was PCR-amplified from *S. enteritidis*. Comparison of the nucleotide sequence of the *S. enteritidis* PCR product with the *S. typhimurium* sequence revealed two C-to-T transversions located at positions 147 and 332 of the *lpfA* ORF, respectively. The divergence at position 147 was a synonymous change, which did not alter the amino acid sequence of LpfA. In contrast, the nucleotide substitution at position 332 resulted in a replacement of threonine present in *S. typhimurium* LpfA (LpfA St) by isoleucine in the *S. enteritidis* orthologue (LpfA Se).

To provide direct evidence for cross-reactivity of fimbrial proteins from different *Salmonella* serotypes, anti-GST-LpfA St immune serum of a rabbit was preabsorbed with an *E. coli* strain expressing GST. Subsequently, the antibody titers against GST, GST-LpfA St, and GST-LpfA Se were determined by ELISA (Table 3). The antibody titers against LpfA St and LpfA Se differed by only one 2-fold dilution, thus confirming that these proteins are antigenically cross-reactive.

**Discussion**

Phase variation is thought to be a mechanism that allows bacteria to evade an antibody response (45). However, because protection against *S. typhimurium* is conferred by antibodies directed against LPS, phase variation of fimbrial and flagellar antigens does not allow this pathogen to evade an immune response of a previously exposed host. To understand how *S. typhimurium* may benefit from phase variation, we extended the analysis of a single host/pathogen combination to include related pathogens, which coexist in the same host population. *S. typhimurium* and *S. enteritidis* both circulate in a rodent animal reservoir and are the *Salmonella* serotypes most frequently isolated from mice or rats (26). To persist in rodent host populations, these serotypes must be able to generate, on average, at least one secondary case of infection from a primary case. The average number of animals that become infected from a single infected rodent can be defined as the case reproductive number. Theory indicates that the case reproductive number is directly proportional to the density of susceptible hosts (46). Immunization of a proportion of animals in a colony will reduce the density of susceptible hosts and may lead to herd immunity if the case reproductive number becomes less than 1. These considerations suggest that infection with one *Salmonella* serotype can reduce the case reproductive number of a second serotype sharing an immunodominant antigen. Thus, cross-reactive antibodies produced by a mammalian host during infection can induce between-serotype competition by influencing the transmission dynamics of pathogens (47). Evasion of between-serotype competition therefore is important for coexistence of different *Salmonella* serotypes in the rodent host population. Consistent with this idea, vaccination

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**Table 3. Immune serum raised against LpfA St is cross-reactive with LpfA Se**

<table>
<thead>
<tr>
<th>Wells coated with</th>
<th>Serum antibody titer</th>
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<tbody>
<tr>
<td>GST-LpfA St</td>
<td>512</td>
</tr>
<tr>
<td>GST-LpfA Se</td>
<td>256</td>
</tr>
<tr>
<td>GST</td>
<td>~8</td>
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of mice with *S. typhimurium* does not elicit cross-immunity against subsequent challenge with *S. enteritidis* or vice versa (Table 2) (17, 18). Thus, *S. typhimurium* and *S. enteritidis* evade between-serotype competition while circulating in murine host populations.

Both cellular and humoral responses can confer immunity to infection with *Salmonella* serotypes (48). Lack of cross-protection between *S. enteritidis* and *S. typhimurium* is, at least in part, caused by differences in their O-antigen. The majority of antibodies elicited by immunization with heat-killed bacteria or with a live attenuated *S. typhimurium*aroA vaccine is directed against the O-antigen (17, 49, 50). The O-antigen of *S. typhimurium* (O-antigen formula O4,5,12) consists of three epitopes: the O12-antigen (a trisaccharide backbone consisting of mannose → rhamnose → galactose →), the O4-antigen (an abequose branch linked to the O5-antigen (acylation of the abequose branch). The O4-antigen is the dominant determinant, and immunization of mice with a *S. typhimurium*aroA vaccine results in anti-O4 titers that are 10-fold higher than antibody titers directed against other O-antigen epitopes (17). Furthermore, the anti-O4 titers generated during immunization with an arO4 vaccine confer immunity to subsequent challenge with *S. typhimurium* (17, 18).

*S. enteritidis* has the O-antigen formula O9,12. The O12-antigen, which is present in both *S. typhimurium* and *S. enteritidis*, is a minor determinant, and anti-O12 titers elicited by immunization of mice are too low to cause protective immunity (16, 49). Instead, immunization of mice with *S. enteritidis* LPS results in a protective antibody response directed against the O9-antigen, a tyvelose branch of the O-antigen trisaccharide backbone. Because immunization of mice with a *S. typhimurium* vaccine results in a protective response against the O4-antigen, which is not present in *S. enteritidis*, between-serotype competition is evaded (16–18).

Immunization with a GST-LpfA protein of *S. typhimurium* elicited cross-reactive antibodies against *S. enteritidis* LpfA (Table 3). Thus, both serotypes share a surface antigen that previously has been shown to be exposed to the immune system during infection (14). No selection against *S. enteritidis* lpf phase-on variants was observed in mice vaccinated with the *S. typhimurium* lpf deletion mutant. In contrast, immunization of mice with a *S. typhimurium*aroA vaccine expressing the *lpf* operon resulted in selection against phase-on variants of *S. enteritidis* during a subsequent oral challenge (Fig. 3). Although the relative numbers of *S. enteritidis* lpf phase-on variants were reduced, the *lpf* phase-off variants were able to evade immunity against LP fimbrial antigens of *S. typhimurium*, as suggested by the lack of protection against a heterologous challenge (Table 2). These data support the idea that evasion of cross-immunity between *S. typhimurium* and *S. enteritidis* is, in part, mediated by phase variation of the *lpf* operon. Hence, evasion of cross-immunity by fimbrial phase variation may be among the mechanisms that allow *S. typhimurium* and *S. enteritidis* to evade between-serotype competition, thereby facilitating their coexistence in a rodent animal reservoir. This selective advantage is not apparent when studying an isolated host–pathogen interaction. The biological function of phase variation thus may be similar to the role of O-antigen polymorphism among *Salmonella* serotypes, because both mechanisms confer an advantage at the pathogen population level. To validate this hypothesis, experiments are under way in our laboratory to assess the degree of protection conferred by an immune response against LP fimbriae during challenge experiments with locked-on phase mutants.

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