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

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Edited by Stanley Falkow, Stanford University, Stanford, CA, and approved September 14, 1999 (received for review March 26, 1999)

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*. Fimbrial or flagellar 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 *lpfABCDE* 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), AJB33

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: LP, long polar; LPS, lipopolysaccharide; cfu, colony-forming units; X-gal, 5-bromo-4-chloro-3-indoyl β-D-galactoside; GST, glutathione *S*-transferase.

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(*lpfABCDE*::*lacZYA*) (14), and CL1509 (*aroA*) (31) are derivatives of *S. typhimurium* strain ATCC14028 and have been described. The *S. enteritidis* wild-type isolate CDC SSU7998 has been described (32). The *E. coli* strain TA One Shot was purchased from Invitrogen. *E. coli* strains S17–1 λ*pir* (33) and DH5α (34) have been described. All bacteria were routinely cultured in LB broth or on plates (35). Minimal medium agar plates (35) and Chrome azurol S agar plates (36) were prepared as described elsewhere. If appropriate, antibiotics were included at the following concentrations: 50 μg/ml nalidixic acid (LB+Nal), 30 μg/ml chloramphenicol (LB+Cm), 100 μg/ml kanamycin (LB+Km), 100 μg/ml carbenicillin (LB+Cb), and 20 μg/ml tetracyclin (LB+Tc). When required, the Lac indicator 5-bromo-4-chloro-3-indoyl β-D-galactoside (X-gal) was added to LB plates at a final concentration of 40 mg/liter (LB+X-gal).

Sequence Analysis of *S. enteritidis lpfA* and Construction of a Glutathione *S*-Transferase (GST)-LpfA$_{Se}$ 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 *Eco*RI–*Sal*I-restricted PCR product into *Eco*RI–*Sal*I-restricted pGEX-4T-2 (Amersham Pharmacia). Purification of the GST-LpfA$_{Se}$ fusion protein was performed as described for a GST-LpfA$_{St}$ 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′-CCCGGGCTGTTGACCTTCAAGACAGATC-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 *lpfE* was PCR-amplified by using the primers 5′-CTCGCTTTTTGCCCTG-GATAG-3′ and 5′-TCTAGACGTCTCACCGATAATGAAAAC-GAC-3′. The PCR product was cloned into plasmid pCRII to give rise to plasmid pMS1201. Plasmid pMS1201 was restricted with *Xba*I–*Eco*RI, and the insert was cloned into *Xba*I–*Eco*RI-restricted plasmid pBluescript SK(+) (37) to give rise to plasmid pMS1202. The insert of plasmid pMS1200 was excised by *Xho*I–*Kpn*I restriction and cloned into *Xho*I–*Kpn*I-restricted plasmid pMS1202 to give rise to plasmid pMS1203. Plasmid pMS1203 was linearized by *Hin*dIII restriction and ligated with a *Hin*dIII-DNA fragment carrying a kanamycin resistance cassette (KIXX; Amersham Pharmacia) to give rise to plasmid pMS1205. The insert of plasmid pMS1205 was excised by *Xba*I–*Kpn*I restriction and cloned into *Xba*I–*Kpn*I-restricted suicide vector pGP704 (38) to give rise to suicide plasmid pMS1208. *E. coli* strain S17–1 λ*pir* (pMS1208) was conjugated with *S. typhimurium* strain IR715 and exconjugants were selected on LB+Nal+Km plates. Exconjugants were patched onto LB+Cb plates, and a mutant, which was resistant to kanamycin but sensitive to carbenicillin, was selected and termed AJB103. Deletion of the *lpf* operon in AJB103 was confirmed by Southern hybridization (39) by using a DNA probe specific for *lpfCD* (the insert of plasmid pMS1039) (29) and *Pst*I-restricted chromosomal DNA of strains AJB103 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 *aroA*::Tn*10* allele from *S. typhimurium* strain CL1509 into strains AJB33 and AJB103, 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 *aroA* 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 *Mlu*I-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+Tc+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+Tc+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+Tc+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/phase off ratios determined in the feces correlated with on/off ratios present in the cecum, a group of four mice was infected with 1×10^9 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 intragastrically with *S. typhimurium aroA* mutants at a dose of 1×10^9 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-LpfA$_{St}$ fusion protein. At day 16 postimmunization, 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-

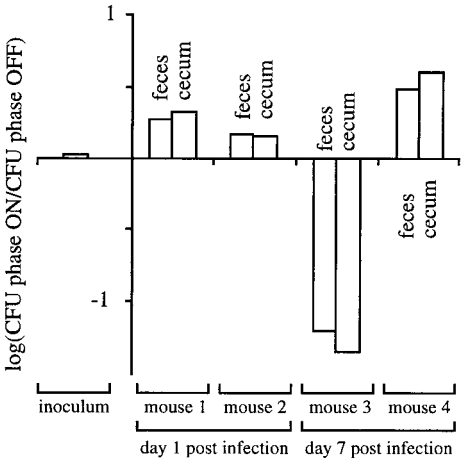

Fig. 1. Correlation of *lpf* phase on/phase off ratios shed in the feces with those present in the cecum of mice.

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-LpfA$_{St}$, or GST-LpfA$_{Se}$ 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*::Tn*10* 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 1×10^9/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. 2*A*). 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. 2*B*). The finding that *lpf* phase on/phase off ratios recovered from

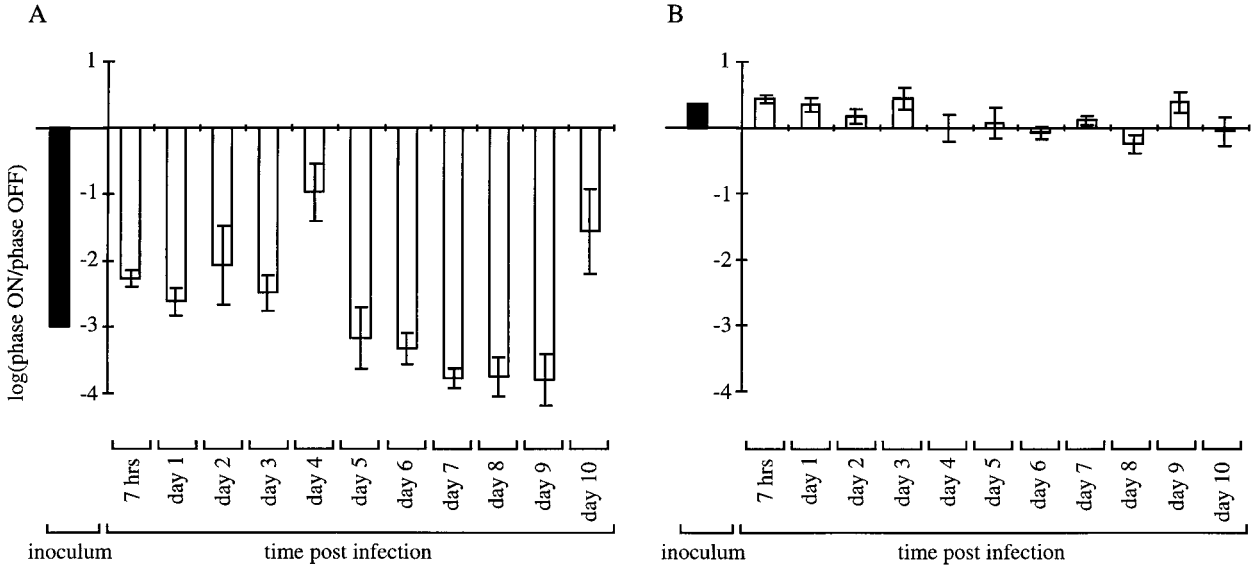

Fig. 2. Recovery of *lpf* phase variants during immunization with a phase-off culture (*A*) or a phase-on culture (*B*) of strain TN3. The *lpf* phase-on/phase-off ratio present in the inoculum (solid bar) and the *lpf* phase-on/phase-off ratios recovered from fecal pellets collected after immunization are indicated (open bars represent mean ± SE).

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

Vaccination with strain	Serum IgG titer	Serum IgA titer	Serum IgM titer
TN4	4,096	64	64
TN3	4,096	128	64
Naïve control	<16	<16	<16

fecal 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. 2*B*).

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. typhimurium* AJB103, thus confirming deletion of *lpf* biosynthesis genes (data not shown). To attenuate strain AJB103 for its use as a vaccine for mice, an *aroA*::Tn*10* allele was introduced by P22 transduction, and the resulting strain was termed TN4.

To ensure that the presence or absence of the *lpf* operon does not alter immunogenicity of a vaccine strain, groups of five mice were immunized and boosted by using either strain TN4 (*aroA* Δ*lpf*) or a phase-on culture of TN3 (*aroA lpfABCDE*::*lacZYA*). The titer of anti-*Salmonella* antibodies in serum collected 14 days after the booster immunization was determined by ELISA (Table 1). Similar or identical titers were elicited in both treatment groups.

Construction of a *S. enteritidis* Strain Carrying a Fusion Between the *lpf* Operon and the *lacZYA* Genes. The *lpf* operon is present in *S. enteritidis* strain CDC SSU7998 as shown previously by Southern hybridization with DNA probes specific for *lpfA*, *lpfCD*, and *lpfE* (44). A single copy fusion between *lpfABCDE* and the *lacZYA* operon of *E. coli* was constructed in *S. enteritidis* as described previously for *S. typhimurium* strain AJB33. A spontaneous nalidixic acid-resistant derivative of *S. enteritidis* strain CDC SSU7998 was isolated and termed TN2. Suicide plasmid pMS1096, which contains the *lpfE* ORF cloned in front of the promoterless *lacZYA* genes (14), was introduced into TN2 by conjugation. An exconjugant, in which pMS1096 was integrated into the chromosome to form a transcriptional fusion between *lpfABCDE* and *lacZYA*, was isolated and termed TN5. When *S. enteritidis* strain TN5 was spread on LB+X-gal+Cm plates, Lac^+ and Lac^- colony phenotypes were observed. A sectored morphology 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 by 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.

Table 2. Protection against challenge with *Salmonella* serotypes 70 days postimmunization with a *S. typhimurium* vaccine

Immunization with *S. typhimurium* vaccine strain	Death/total after challenge with strain (dose)		
	S. enteritidis TN5 (1.5×10^6 cfu/mouse)	*S. enteritidis* TN5 (1.1×10^7 cfu/mouse)	*S. typhimurium* AJB33 (1.9×10^7 cfu/mouse)
TN3	3/5	4/5	0/3
TN4	3/3	3/3	0/3
None	3/5	5/5	5/5

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* Δ*lpf*). 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 naïve mice of the same age were infected with a phase-on culture of *S. enteritidis* strain TN5 (*lpfABCDE*::*lacZYA*) at a dose of 1.1×10^7 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 naïve 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 naïve 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 1.1×10^7 cfu/mouse caused lethal morbidity in naïve 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 (1.5×10^6 cfu/mouse). The mortality in the group of naïve 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 1.9×10^7 cfu of a virulent *S. typhimurium* strain (AJB33). Whereas mice immunized with a *S. typhimurium* vaccine were protected against challenge with virulent *S. typhimurium*, naïve mice developed lethal signs of disease and were euthanized.

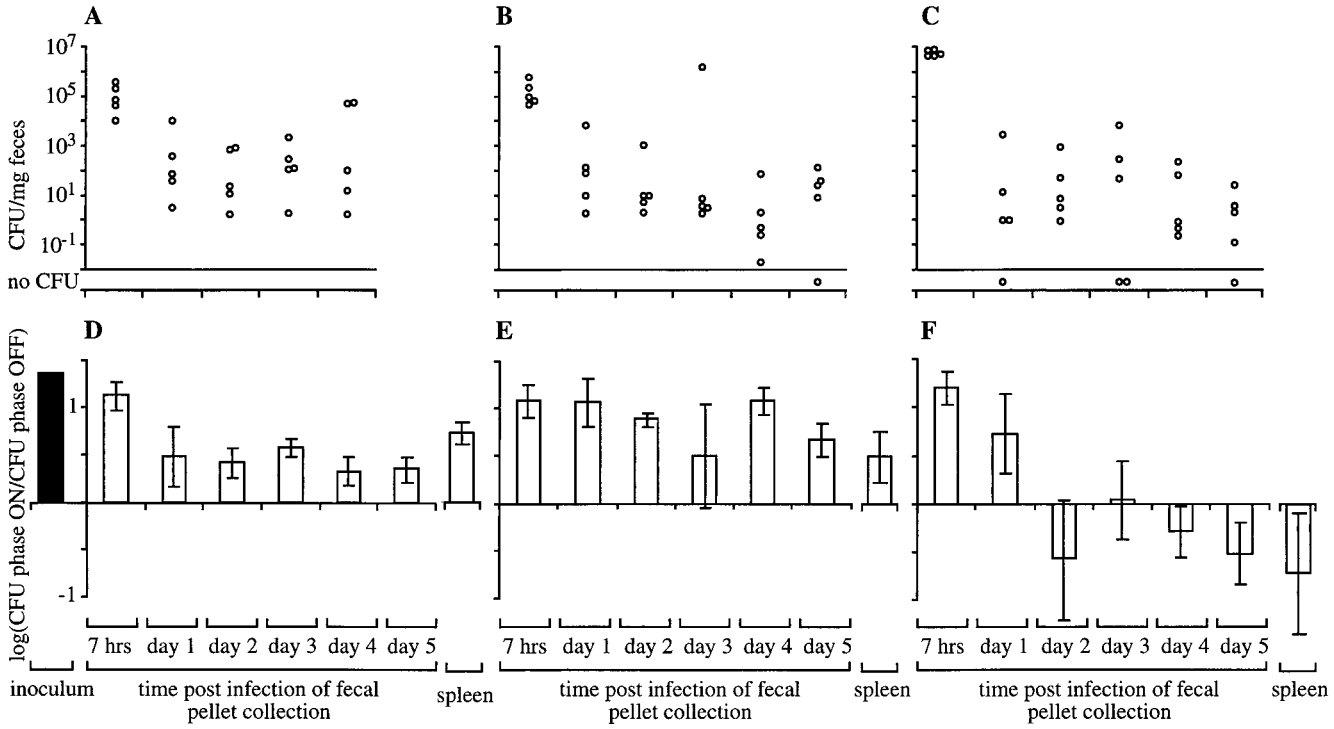

Fig. 3. Recovery of *S. enteritidis lpf* phase variants from infected mice. Mice were either naïve (*A* and *D*) or had been immunized before infection either with a *S. typhimurium lpf* deletion mutant (TN4) (*B* and *E*) or a *lpf* phase-on culture of *S. typhimurium* strain TN3 (*C* and *F*). Total numbers of bacteria recovered from feces (*A–C*) are given for individual animals (○). The limit of detection was approximately 0.02 cfu/mg feces. The *S. enteritidis lpf* phase on/phase off ratios present in the challenge inoculum (solid bar) or recovered from fecal pellets or the spleens (open bars represent mean ± SE) are shown (*D–F*). In *D*, bacteria were recovered from the cecum at day 5. The spleen was collected from moribund animals between 5 and 7 days postinfection.

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.

Table 3. Immune serum raised against $LpfA_{St}$ is cross-reactive with $LpfA_{Se}$

Wells coated with	Serum antibody titer
GST-$LpfA_{St}$	512
GST-$LpfA_{Se}$	256
GST	<8

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

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), and the O5-antigen (acetylation 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 *aroA* 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, 17, 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, 49).

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.

We thank R. Kingsley for absorption of the immune serum and R. Tsolis for critical comments on the manuscript. Work in A.B.'s laboratory is supported by Public Health Service Grants AI40124 and AI44170.

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