Population Heterogeneity of *Salmonella enterica* Serotype Typhimurium Resulting from Phase Variation of the *lpf* Operon In Vitro and In Vivo

Robert A. Kingsley, Eric H. Weening, A. Marijke Keestra, and Andreas J. Bäumlér*

Department of Medical Microbiology and Immunology, College of Medicine, Texas A&M University
System Health Science Center, College Station, Texas 77843-1114

Received 17 December 2001/Accepted 5 February 2002

The *lpf* fimbrial operon oscillates between phase ON and phase OFF expression states, thereby generating heterogeneity within *S. enterica* serotype Typhimurium populations with regard to expression of long polar fimbrial antigens. To determine whether the proportion of *lpf* phase variants changes with growth conditions, the *lpf* phase ON content of cultures was determined after in vitro and in vivo passage. After passage in Luria-Bertani (LB) broth for 120 generations, 96% of cells in a serotype Typhimurium culture carried the *lpf* operon in the phase ON expression state, regardless of the phase ON/OFF ratio in the inoculum. In contrast, a culture passaged on LB agar plates for 500 generations contained approximately 2% *lpf* phase ON cells. Differences in the *lpf* phase ON content of cultures passaged in broth and on plates were not caused by an outgrowth of *lpf* phase ON or *lpf* phase OFF cells, since deletion of *lpf* biosynthesis genes did not alter the phase ON/OFF ratio attained after passage. Instead, growth in LB broth resulted in a eightfold increase in the phase OFF-to-ON transition frequency and a decrease of the *lpf* phase ON-to-OFF transition frequency by a factor of 150 compared to growth on LB agar plates. After infection of naïve CBA/J mice with an *lpf* phase ON culture of serotype Typhimurium, the proportion of *lpf* phase ON cells continuously decreased over time, regardless of whether the strain carried intact fimbrial biosynthesis genes. These data suggest that elaboration of fimbriae does not have a major influence on the population heterogeneity produced by phase variation of the *lpf* operon in naïve mice.

The *lpfABCDE* operon is a cluster of fimbrial biosynthesis genes involved in colonization of the murine intestine by *Salmonella enterica* serotype Typhimurium (3–5). Expression of *lpf* has been studied by fusing the promoterless *lacZYA* operon of *Escherichia coli* directly downstream of the *lpfE* stop codon, thereby generating a single-copy transcriptional fusion to the intact fimbrial operon (17). A serotype Typhimurium strain carrying the *lpfABCDE::lacZYA* fusion gives rise to Lac⁺ (phase ON) and Lac[−] (phase OFF) colony phenotypes, suggesting that the *lpf* operon oscillates between phase ON and phase OFF expression states at the transcriptional level (17). Colonies with the Lac[−] and the Lac⁺ phenotypes are composed predominantly of *lpf* phase OFF variants (approximately 99.8% of cells) and of *lpf* phase ON variants (approximately 85% of cells), respectively. Thus, *lpf* phase OFF and *lpf* phase ON cells give rise to progeny that are predominantly of the same phase, indicative of a heritable switching mechanism. However, *lpf* phase variants arise at a frequency that is considerably higher than the natural mutation rate. Phase OFF cells give rise to *lpf* phase ON variants at a frequency (f_{ON}) of 2.4×10^{-4} /generation, while *lpf* phase ON cells give rise to *lpf* phase OFF variants at a frequency (f_{OFF}) of 6.8×10^{-3} /generation (17). The mechanism of *lpf* phase variation is currently unknown. Phase variation of the *pef* fimbrial operon of serotype Typhimurium has recently been shown to involve Dam

methylation (14). This mechanism is not likely to be involved in *lpf* phase variation, since Dam methylation sites are not present in its promoter region (3). Phase variation of the *lpf* operon at the transcriptional level results in population heterogeneity with regard to expression of the LpfA fimbrial protein. This conclusion is supported by the finding that a selection against *lpf* phase ON cells is observed in mice previously immunized with a purified glutathione *S*-transferase–LpfA fusion protein (17).

The phenomenon of phase variation raises the question of what proportion of cells present in natural serotype Typhimurium populations are *lpf* phase ON. However, it is difficult to predict the *lpf* phase ON content of serotype Typhimurium populations circulating in natural host reservoirs because two of the relevant parameters are unknown. First, it is an open question as to what proportion of *lpf* phase ON cells may be shed from an infected animal. Secondly, it is not known how growth of serotype Typhimurium outside the host, which may occur during fecal-oral transmission, may affect the proportion of *lpf* phase ON cells in a population. A number of factors may contribute to the population heterogeneity of serotype Typhimurium with respect to expression of the *lpf* operon in vitro and in vivo. One factor influencing the proportion of *lpf* phase ON cells present in a serotype Typhimurium culture are the *lpf* phase transition frequencies, f_{OFF} and f_{ON} , which have previously been determined during growth on LB agar plates (17). This study demonstrates that f_{OFF} is greater than f_{ON} , suggesting that prolonged culture on LB agar plates will give rise to a serotype Typhimurium population in which the majority of cells carry the *lpf* operon in the phase OFF expression state. A

* Corresponding author. Mailing address: Department of Medical Microbiology and Immunology, College of Medicine, Texas A&M University System Health Science Center, Reynolds Medical Building, College Station, TX 77843-1114. Phone: (979) 862-7756. Fax: (979) 845-3479. E-mail: abaumlér@tamu.edu.

FIG. 1. Construction of a $\Delta lpfABCDE::lacZYA$ transcriptional fusion in *S. enterica* serotype Typhimurium. A schematic drawing of the homologous recombination event occurring between the flanking regions of the *lpf* operon carried on plasmid pRA168 and the serotype Typhimurium chromosome (IR715) is shown on top. The predicted product of this recombination event (RAK48 chromosome) is shown below. A subsequent homologous recombination event occurring between the *lpf* promoter carried on plasmid pRA161 and the serotype Typhimurium chromosome (RAK48) is shown next. The predicted product of this event (RAK49) is shown at the bottom. *bla*, β -lactamase gene, carbenicillin resistance marker on pRA168; *cat*, chloramphenicol acetyltransferase gene, chloramphenicol resistance marker on pRA161; *Km^r*, kanamycin resistance marker inserted between the *lpf* flanking regions on pRA168.

second factor altering the culture composition with respect to expression of the *lpf* operon may be the selection for or against the expression of fimbrial proteins encountered under different in vitro or in vivo growth conditions. For instance, selection for the expression of type 1 fimbriae of serotype Typhimurium, which are encoded by the *fim* operon and whose expression is regulated by phase variation, has been observed during growth in static broth culture (19, 20). It is therefore possible that certain in vitro growth conditions may favor outgrowth of *lpf* phase ON or phase OFF cells. Similarly, *lpf*-mediated colonization of intestinal surfaces may provide a selective advantage during growth in vivo, since *lpf* phase ON variants possess an increased ability to colonize murine Peyer's patches in an intestinal organ culture model (17). On the other hand, previous exposure of mice to LpfA results in selection against *lpf* phase ON cells during a challenge (15–17). These data suggest that naïve mice infected with serotype Typhimurium may over time develop an immune response against LpfA, thereby generating a selective advantage for *lpf* phase OFF cells during persistence in an animal. Thus, the ratio of *lpf* phase ON to phase OFF cells encountered in natural populations may be affected both by *lpf* phase transition frequencies and by selection for or against expression of fimbriae.

In this study we investigated which of these factors influence the proportion of *lpf* phase ON cells in a serotype Typhi-

murium culture grown in vitro under standard laboratory conditions and in vivo in a mouse model.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. enterica* serotype Typhimurium strain ATCC 14028 was obtained from the American Type Culture Collection. Strain IR715 is a virulent nalidixic acid-resistant derivative of strain ATCC 14028 described previously (23). AJB33 is a derivative of IR715 which carries a single-copy chromosomal *lpfABCDE::lacZYA* transcriptional fusion (17). Strain AR3675, a derivative of ATCC 14028 containing a *lac* reporter fusion to *fluB*, was described previously (24). *E. coli* strains S17-1 λ pir and DH5 α are described elsewhere (10, 21).

Bacteria were cultured aerobically at 37°C in Luria-Bertani (LB) broth (tryptone, 10 g/liter; yeast extract, 5 g/liter; NaCl, 10 g/liter) in a roller or on LB agar (16 g/liter) plates. If appropriate, antibiotics were added at the following concentrations: carbenicillin, 100 mg/liter; chloramphenicol (CM), 30 mg/liter; kanamycin (KM), 60 mg/liter; and nalidixic acid (NAL), 50 mg/liter. β -Galactosidase activity was detected by the addition of 60 mg of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) per liter to LB agar plates.

Construction of mutants. A strain containing a deletion from bp 64 of the *lpfA* open reading frame to 172 bp upstream of the stop codon of the *lpfE* open reading frame was constructed as follows (Fig. 1). Flanking regions of the desired deletion were amplified by PCR using the primers 5' GGAAATCAGGCGGGAACTG 3' and 5' CGGGATCCGCAGAAAGTGGAACTACAGCGAGAG 3' and the primers 5' CGGGATCCCATCTGGTGGGGAGCAACAATAC 3' and 5' GCCAAACAGTGAAAGAAGACGAAG 3'. The PCR products were digested with *Bam*HI and ligated with a *Bam*HI-digested kanamycin resistance cassette in a three-component reaction. The product of this ligation reaction consisting of both PCR products flanking the kanamycin resistance cassette was

cloned into suicide vector pGP704 (11), and the resulting construct was designated pRA168. Suicide plasmid pRA168 was introduced into serotype Typhimurium strain IR715 by conjugal transfer from *E. coli* S17-1 λ pir, and exconjugants were selected on LB+NAL+KM plates. Mutants carrying the kanamycin resistance cassette and a deletion of the *lpf* operon were identified by their sensitivity to carbenicillin (loss of pGP704 vector sequences). A colony was selected and designated RAK48 (Fig. 1). Deletion of the *lpf* operon in RAK48 was confirmed by Southern hybridization using the cloned *lpfA* gene as a probe (data not shown).

A transcriptional fusion of the *lpf* promoter (P_{lpf}) with *lacZYA* was constructed in strain RAK48 by directional cloning of the PCR product amplified with the primers 5' GGAAATCAGGCGGGAACTG 3' and 5' GCAGAAGTGGA ACTACAGCGAGAG 3' into suicide vector pFUSE (6), yielding suicide plasmid pRA161 (Fig. 1). pRA161 was introduced into strain RAK48 by conjugal transfer from *E. coli* S17-1 λ pir and recombination of the plasmid into the chromosome selected on LB+NAL+CM agar plates. One such exconjugant was selected and designated strain RAK49 (Fig. 1). Integration of pRA161 into the serotype Typhimurium chromosome was confirmed by Southern hybridization using the labeled PCR product as a probe (data not shown).

Southern hybridization. Isolation of genomic DNA and Southern transfer of DNA onto a nylon membrane were performed as previously described (2). Hybridization was performed at 65°C in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% (wt/vol) sodium dodecyl sulfate, 5% (wt/vol) dextran sulfate, and 1:20 liquid block (Amersham Pharmacia). Two 15-min washes were performed under nonstringent conditions at room temperature in 2× SSC–0.1% sodium dodecyl sulfate. Labeling of DNA probes with fluorescein-11-dUTP and detection with anti fluorescein antiserum was performed with the Gene Images labeling and detection kit (Amersham Pharmacia).

Determination of phase equilibrium. For determination of phase equilibrium on LB agar plates, LB+CM+X-Gal plates were initially inoculated with a suspension containing approximately 10^4 CFU from either an *lpf* phase ON or an *lpf* phase OFF colony of AJB33 or RAK49. Bacteria were grown overnight at 37°C, and the number of CFU and the *lpf* phase ON/OFF ratio were determined by resuspending all colonies in 5 ml of phosphate-buffered saline (PBS) and plating serial 10-fold dilutions on LB+CM+X-Gal agar plates. A second passage was inoculated with approximately 10^4 CFU and incubated overnight. Serial passage on LB agar plates was continued until the *lpf* phase ON/OFF ratio in the culture originally inoculated with an *lpf* phase ON colony was similar to that of a culture originally inoculated with an *lpf* phase OFF colony. Each experiment was performed in triplicate. Phase equilibrium in broth was determined in essentially the same way, except that serial 10-fold dilutions of overnight cultures were directly plated on LB+CM+X-Gal agar plates to determine the number of each phase variant in the culture. Approximately 10^4 CFU were used to inoculate the subsequent culture.

Calculation of phase transition frequencies during growth on LB agar plates and in LB broth. Calculation of f_{ON} was based on theoretical predictions developed by Stocker (22). The number of phase ON CFU in the inoculum of the phase equilibrium experiments (see above) was used to calculate the number of phase ON CFU theoretically arising by cell doubling if the culture went through the number of generations experimentally determined for overnight growth. We assumed that the actual number of phase ON CFU in the culture following overnight growth (determined experimentally) was the sum of the number of phase ON CFU theoretically arising by cell doubling of phase ON cells present in the inoculum plus the number of phase ON CFU arising by phase variation from phase OFF cells (i.e., switching back to the original phase was ignored). Therefore, the number of phase ON variants arising by phase variation was calculated by subtracting the number of phase ON cells theoretically arising by cell doubling from the total number of phase ON CFU determined experimentally. The total number of CFU after overnight growth (N), the number of generations the culture went through (g , $\log_{10} N/\log_{10} 2$), and the number of phase ON variants (M) present in each culture was determined. The f_{ON} phase transition frequency was calculated by the formula $(M/N)/g$ (8). After logarithmic transformation of the data, a Student t test (two tailed) was used to determine whether differences between f_{ON} phase transition frequencies were statistically significant.

Animal experiments. Eight- to ten-week-old female CBA/J (Jackson Laboratory) mice were used throughout this study. Prior to inoculation, 100 mg of fresh fecal pellets was collected from each mouse, homogenized in 1 ml of sterile PBS (pH 7.4), and tested for the presence of nalidixic acid-resistant flora by plating on LB+NAL. No nalidixic acid-resistant coliforms were detectable. Bacteria were routinely cultured overnight at 37°C in a roller prior to inoculation. In all experiments the bacterial titer and, when appropriate, the *lpf* phase ON/OFF ratio of the inoculum were determined by spreading serial 10-fold dilutions on

LB agar plates containing the appropriate antibiotics and X-Gal. Mice were inoculated intragastrically with approximately 10^9 CFU in a volume of 200 μ l of saline (0.85%, wt/vol) by gavage. At various time points, approximately 100 mg of fresh fecal pellets was collected from each mouse and homogenized in 1 ml of PBS (pH 7.4). Serial 10-fold dilutions were plated on LB+NAL containing X-Gal, and the *lpf* phase ON/OFF ratio was determined. Mice were sacrificed at various time points, and Peyer's patches, mesenteric lymph nodes, and livers were aseptically removed. The *lpf* phase ON/OFF ratio recovered from the organs was determined by plating serial 10-fold dilutions of homogenized organs on LB+X-Gal agar containing the appropriate antibiotics.

RESULTS

Phase equilibrium during growth on agar or in liquid media. Theoretical considerations predict that phase variation will result in a stable proportion of phase ON cells after a bacterial culture went through a sufficient number of generations. For example, *S. enterica* serotype Typhimurium strain AJB33 (*lpfABCDE::lacZYA*) gives rise to Lac⁺ (phase ON) and Lac[−] (phase OFF) colony phenotypes on agar plates (17). A phase OFF colony of AJB33 presumably arises from a single bacterium which was in the phase OFF expression state. Therefore, the initial proportion of phase ON cells, M/N , in a phase OFF colony is 0. During growth of the phase OFF colony, phase ON variants will arise during each generation at the frequency f_{ON} . Therefore, the proportion of phase ON cells will increase with the number of generations the population went through. Since phase OFF cells also arise from phase ON cells, M/N will stabilize and oscillate around an equilibrium value after the population has gone through a sufficient number of generations on LB agar plates. This phase equilibrium is attained when the number of phase OFF variants arising from phase ON cells equals the number of phase ON variants arising from phase OFF cells. Given a fraction M/N of phase ON cells and a fraction $1 - M/N$ of phase OFF cells in equilibrium, this relationship can be described by $(M/N)f_{OFF} = (1 - M/N)f_{ON}$. After solving this equation for M/N in an equilibrium culture ($M/N = f_{ON}/[f_{OFF} + f_{ON}]$), the proportion of cells expressing fimbriae in equilibrium can be estimated from the transition frequencies. The phase transition frequencies determined for AJB33 during growth on LB agar plates ($f_{OFF} = 6.8 \times 10^{-3}$ /generation and $f_{ON} = 2.4 \times 10^{-4}$ /generation) (17) indicate that in a serotype Typhimurium culture grown to phase equilibrium on LB agar plates, the proportion of *lpf* phase ON cells is approximately 3.4%.

To test this prediction experimentally, we determined the phase equilibrium attained when bacterial growth is maintained by daily passage of LB broth cultures initially inoculated with approximately 10^4 CFU from a suspension of either an *lpf* phase ON or an *lpf* phase OFF colony of AJB33 (*lpfABCDE::lacZYA*). Daily passages were achieved by dilution of a stationary-phase overnight culture with sterile LB broth. Each dilution resulted in growth from approximately 10^4 CFU, thus maintaining the population heterogeneity of the previous culture. Cultures inoculated with a phase ON colony and those inoculated with a phase OFF colony contained identical proportions of phase ON cells after they had gone through approximately 120 generations (Fig. 2A). This proportion remained constant thereafter, indicating that phase equilibrium had been reached. Surprisingly, equilibrium LB broth cultures of AJB33 contained approximately 96% *lpf* phase ON cells rather than the 3.4% phase ON cells predicted by the phase

FIG. 2. Phase-variant composition of cultures passed in broth and solid medium. Each variant or strain type was enumerated at each passage; the means of three repeats ($\pm$ standard deviation) are plotted. (A) Proportion of phase variants of AJB33 (*lpfABCDE::lacZYA*) during planktonic growth in LB broth inoculated with a predominantly phase ON (open squares) or phase OFF (filled squares) inoculum. (B) Proportion of phase variants of AJB33 during growth on LB agar plates inoculated with a predominantly phase ON (open squares) or phase OFF (filled squares) inoculum. (C) Proportion of phase variants of AR3675 (*fhuB::lacZYA*) and IR715 (wild type) during growth on LB agar plates inoculated with an approximately 10:1 ratio (AR3675 to IR715; open squares) or during planktonic growth in LB broth inoculated with an approximately 1:100 ratio (AR3675 to IR715; open circles). (D) Proportion of phase variants of RAK49 (Δ *lpfABCDE::lacZYA*) during planktonic growth in LB broth inoculated with a predominantly phase ON (open squares) or phase OFF (filled squares) inoculum. (E) Proportion of phase variants of RAK49 during growth on LB agar plates inoculated with a predominantly phase ON (open squares) or phase OFF (filled squares) inoculum.

transition frequencies determined after growth on LB agar plates.

We reasoned that differences between the phase equilibrium determined experimentally and that predicted from the phase transition frequencies might result from the use of different growth conditions. That is to say, the *lpf* phase transition frequencies had previously been determined by analyzing AJB33 (*lpfABCDE::lacZYA*) phase ON and phase OFF colonies grown on solid medium (LB agar plates) (17). In contrast, the phase equilibrium shown in Fig. 2A was attained during passage in liquid culture (LB broth). In order to test this notion, we determined the phase equilibrium attained as a result of bacterial growth through daily passage of AJB33 on LB agar plates. Passages were initially inoculated with a suspension of a phase ON or a phase OFF colony containing approximately 10^4 CFU. When bacteria were passed on LB agar plates, the equilibrium point was reached after approximately 500 generations at approximately 2% phase ON cells (Fig. 2B). Thus, the phase equilibrium reached after passage on LB agar plates was similar to that predicted by the phase transition frequencies described previously (17).

The different equilibrium phase ON contents of AJB33 (*lpfABCDE::lacZYA*) cultures grown either in LB broth (96% phase ON cells) or on LB agar plates (2% phase ON cells) suggested that growth conditions may alter phase transition

frequencies or that phases may differ in their ability to grow on plates and in broth. Growth curves of LB broth cultures inoculated either with a phase ON or a phase OFF colony of AJB33 were not significantly different, suggesting similar growth rates of both phases in liquid culture (data not shown). Similarly, phase ON and phase OFF colonies of AJB33 contained similar numbers of CFU, suggesting that cells of both phases have similar growth rates on LB agar plates (data not shown). To directly test the possibility that outgrowth may occur as a result of expression of long polar (LP) fimbriae encoded by the *lpf* operon, a transcriptional fusion between the *lpf* promoter (P_{lpf}) and the *lacZYA* operon was constructed in a strain carrying a deletion of *lpf* fimbrial biosynthesis genes (RAK49) (Fig. 1). Phase equilibrium for RAK49 was attained at an *lpf* phase ON/OFF ratio similar to that observed for strain AJB33 after passage either in LB broth or on LB agar plates (Fig. 2D and E). Thus, expression of LP fimbrial proteins did not influence phase equilibrium points attained by passage in LB broth or on LB agar plates.

We next examined the possibility that outgrowth in LB broth may occur as a result of expression of the *lac* reporter genes. That is, although an LB broth culture inoculated with a phase OFF colony of AJB33 (*lpfABCDE::lacZYA*) initially contains only a small proportion of phase ON cells, subsequent passage in LB broth results in a culture containing 96% phase ON cells,

TABLE 1. Phase transition frequency of *lpfABCDE* on agar and in broth culture

Growth condition	f_{OFF}	f_{ON}	% Phase ON at equilibrium
LB agar plates	4.7×10^{-3}	9.5×10^{-5}	2
LB broth	3.2×10^{-5}	7.6×10^{-4}	96

thereby raising the possibility that expression of *lacZYA* mediates outgrowth in LB broth. To test this idea, we used a serotype Typhimurium strain (AR3675) containing a *lac* reporter fusion to *fhuB*, a gene that is expressed in vitro but is not required for growth in rich media (24). We then determined whether expression of the *lac* reporter genes in AR3675 would result in an outgrowth of this strain when grown in coculture with the serotype Typhimurium wild type (ATCC 14028). To this end, LB broth was inoculated with a 1:100 mixture of strain AR3675 and ATCC 14028 to mimic the ratio of Lac⁺ to Lac⁻ cells in a phase OFF colony of AJB33. Growth of this culture was then maintained by daily passage in LB broth. We expected that the fraction of AR3675 in the mixed culture would increase if expression of the *lac* reporter genes promoted outgrowth of serotype Typhimurium Lac⁺ cells in broth. On the contrary, we observed a small decrease in the proportion of AR3675 over the first 200 generations (Fig. 2C), suggesting that expression of the *lac* reporter genes does not promote outgrowth in LB broth. The alternate hypothesis, namely, that expression of the *lac* reporter genes would result in a growth defect of serotype Typhimurium on agar plates, was investigated by inoculating LB plates with a 10:1 mixture of AR3675 and ATCC 14028 to mimic the ratio of Lac⁺ to Lac⁻ cells present in a phase ON colony of AJB33. Growth of this culture was then maintained by daily passage in LB agar plates. We expected that the fraction of AR3675 in the mixed culture would decrease if expression of the *lac* reporter genes would result in a growth defect on agar plates. However, there was no apparent change in the proportion of AR3675 during the first 200 generations, suggesting that expression of the *lac* reporter genes in serotype Typhimurium does not result in a growth defect on LB agar plates (Fig. 2C).

Our results demonstrate that neither expression of the *lac* reporter genes nor expression of LP fimbrial proteins resulted in outgrowth of *lpf* phase ON or phase OFF cells during culture in LB broth or on LB agar plates. These data suggested that M/N in an equilibrium culture is predominantly a function of the *lpf* phase transition frequencies. We next calculated f_{ON} for serotype Typhimurium strain AJB33 during growth in LB broth and on LB agar plates (Table 1) based on the theoretical prediction that the proportion of phase variants arising in a culture increases linearly with the number of generations (g) through which the culture goes if the growth rates of both phases are equal (22). The f_{ON} for growth on LB agar plates was calculated to be 9.5×10^{-5} /generation, which is 2.5-fold lower than a previous estimate (17); however, this difference was not statistically significant ($P > 0.05$). The f_{ON} during growth in LB broth was calculated to be 7.6×10^{-4} /generation, which is significantly higher ($P < 0.05$) than f_{ON} for growth on LB agar plates. These estimates for f_{ON} and the equilibrium phase ON contents (M/N) determined for growth in LB broth or on LB agar plates (Fig. 2) were used to derive an estimate

for f_{OFF} from the following equation: $M/N = f_{ON}/(f_{OFF} + f_{ON})$. The f_{OFF} for growth on LB agar plates was calculated to be 4.7×10^{-3} /generation, which was similar to a previous estimate (17). The f_{OFF} during growth in LB broth was calculated to be 3.2×10^{-5} /generation, which is considerably lower than f_{OFF} determined for growth on LB agar plates. The approximately 8-fold increase of f_{ON} during growth on LB broth compared to growth on LB agar plates and the approximately 150-fold increase of f_{OFF} during growth on LB agar plates compared to growth on LB broth supported the idea that differences in the phase equilibrium content were due to changes in phase transition frequencies.

***lpf* phase ON content of serotype Typhimurium cultures during in vivo passage.** Passage of AJB33 (*lpfABCDE::lacZYA*) under different in vitro growth conditions gave rise to cultures that differed considerably, at equilibrium, with regard to their *lpf* phase ON content (Fig. 2). This observation raised the question of whether the proportion of *lpf* phase ON cells in a serotype Typhimurium population during growth in vivo is more similar to that attained after in vitro passage in LB broth or to that observed after in vitro passage on LB agar plates. To address this question, we determined the *lpf* phase ON/OFF ratio of AJB33 recovered from fecal pellets over 21 days after inoculation of eight CBA/J mice. CBA/J mice were chosen since they are naturally resistant to serotype Typhimurium but shed the pathogen with their feces for several weeks after oral inoculation, thereby providing an opportunity to monitor in vivo growth for prolonged periods. Following inoculation of mice with 10^9 CFU of an AJB33 *lpf* phase ON culture, 2×10^3 to 3×10^5 CFU/mg were recovered from the feces over the first 21 days postinoculation (Fig. 3A). The phase ON/OFF ratio in the inoculum was approximately 3:1, and a similar ratio was recovered at day 1 postinoculation. However, on subsequent days the proportion of phase ON cells decreased steadily. At day 21 postinoculation the phase ON/OFF ratio was close to 1:100 (Fig. 3B). No *lpf* phase ON colonies were detectable at later time points, and the experiment was therefore discontinued.

In addition to colonization of the intestine and subsequent shedding in fecal pellets, serotype Typhimurium also invades deeper tissues of the murine host. In our experience, CFU are commonly recoverable from Peyer's patches, mesenteric lymph nodes, and livers of CBA/J mice between days 4 and 9 postinoculation. To investigate the *lpf* phase ON/OFF ratio in these tissues, five mice were inoculated with an *lpf* phase ON culture of AJB33 (*lpfABCDE::lacZYA*), and the phase ON and phase OFF CFU were enumerated on day 9 postinoculation (Fig. 4A and B). The bacterial numbers recovered from feces were similar to those recovered in the previous experiment. The *lpf* phase ON/OFF ratio was determined daily in fecal pellets until day 9, when the CFU of AJB33 were enumerated in Peyer's patches of the terminal ileum, mesenteric lymph nodes, and livers. Again a decrease in the proportion of *lpf* phase ON cells in fecal pellets was observed over time (Fig. 4B). On day 9 postinoculation, the phase ON/OFF ratio in the Peyer's patches, mesenteric lymph nodes, and livers was significantly greater than that in fecal pellets ($P < 0.05$) (Fig. 4). The experiment was repeated by inoculating six mice with an *lpf* phase OFF culture of AJB33 (the phase ON/OFF ratio was approximately 1:1,000). No *lpf* phase ON cells were recovered

FIG. 3. Mean recovery and *lpf* phase ON/OFF ratio from the feces of mice inoculated with *S. enterica* serotype Typhimurium strain AJB33 (*lpfABCDE::lacZYA*). (A) Number of bacteria (CFU) recovered from feces over time. Data are means from five mice $\pm$ standard errors. (B) Ratio of AJB33 *lpf* phase ON to *lpf* phase OFF variants recovered from the feces during the first 21 days after oral inoculation. The first data point (0) represents the *lpf* phase ON/OFF ratio in the inoculum. The means from five mice ($\pm$ standard deviation) are plotted.

from feces or organs of mice, suggesting that the *lpf* phase ON/OFF ratio stayed below 1:100, the approximate limit of detection, throughout the 9-day experiment (data not shown).

We considered three alternative hypotheses which could explain the progression to predominantly *lpf* phase OFF variants in the feces of mice inoculated with a predominantly phase ON inoculum (Fig. 3 and 4). First, an adaptive immune response against LP fimbrial proteins may generate a selection against *lpf* phase ON cells over time, thereby resulting in the presence of predominantly *lpf* phase OFF variants in the feces. Second, preferential adhesion of *lpf* phase ON cells to host tissue may result in the presence of predominantly *lpf* phase OFF variants in the feces. Third, similar to growth on LB agar plates in vitro, f_{OFF} may be greater than f_{ON} in vivo, resulting in an *lpf* phase equilibrium culture containing a large proportion of *lpf* phase OFF cells.

To distinguish between these possibilities, five mice were infected with a phase ON culture of serotype Typhimurium strain RAK49 (Δ *lpfABCDE* *P*_{*lpf*}::*lacZYA*). We reasoned that the deletion of *lpf* fimbrial biosynthesis genes in RAK49 would eliminate any selection for (adhesion) or against (immune response) *lpf* phase ON cells. The phase ON/OFF ratio recovered from mice infected with RAK49 was thus expected to be only a function of the *lpf* phase transition frequencies in vivo. On day 1 postinoculation, mice inoculated with 10^9 CFU of RAK49 shed numbers of bacteria with their feces similar to those of mice inoculated with a similar dose of AJB33 (Fig. 4A). However, by 2 days postinoculation the mean number of CFU per milligram of feces was approximately 100-fold higher in mice inoculated with AJB33 (*lpfABCDE::lacZYA*) than in mice inoculated with RAK49, and this difference remained

significant ($P < 0.05$) for the remainder of the experiment. The phase ON/OFF ratio of RAK49 recovered from feces was also on average lower than that of AJB33 on days 2 through 9 postinoculation (Fig. 4B). Similarly, the RAK49 *lpf* phase ON/OFF ratio in the internal organs at day 9 postinoculation was on average lower than the ratio determined for AJB33, although this difference was not significant (Fig. 4B). These data suggested that expression of LP fimbriae may provide a selective advantage for serotype Typhimurium during colonization of mice, which is consistent with previous reports (4, 5, 17). However, the phase ON/OFF ratio recovered from feces of mice infected with a phase ON culture of strain RAK49 decreased during the 9 days postinoculation with a slope similar to that for mice inoculated with a phase ON culture of strain AJB33 (Fig. 4B). These data suggested that the decrease in the *lpf* phase ON/OFF ratio in the feces of mice infected with an *lpf* phase ON culture is likely the result of f_{OFF} being greater than f_{ON} in vivo. The RAK49 phase ON/OFF ratios in the Peyer's patches, mesenteric lymph nodes, and livers on day 9 postinoculation were greater than the ratio in the feces on the same day (Fig. 4). These data were therefore similar to those obtained with strain AJB33, in which *lpf* phase ON/OFF ratio in tissue was greater than that in feces (Fig. 4B).

DISCUSSION

Phase variation was first described in 1922 by Andrews as the oscillation between H1 and H2 expression states of flagellar antigens in *S. enterica* serotype Typhimurium (1). Stocker calculated the flagellar phase transition frequencies of serotype Typhimurium to be 8.6×10^{-4} per generation for transition from phase H2 to phase H1 and 4.7×10^{-3} per generation for transition from phase H1 to phase H2 (22). The proportion of phase H1 cells present in broth culture after 600 generations is approximately 14%, regardless of whether the culture is initially inoculated with a phase H1 or phase H2 colony (22). This work demonstrates that after a serotype Typhimurium culture went through a sufficient number of generations to attain equilibrium, flagellar phase variation gave rise to a characteristic phase H1/H2 ratio. Similarly, we found that after passage of serotype Typhimurium in LB broth for approximately 120 generations, the culture is composed of approximately 96% *lpf* phase ON cells, regardless of whether it was initially inoculated with an *lpf* phase ON or a phase OFF colony (Fig. 2A). However, a different *lpf* phase equilibrium was attained after passage of serotype Typhimurium in LB agar plates. That is, a serotype Typhimurium culture grown on LB agar plates for approximately 500 generations contained approximately 2% *lpf* phase ON cells at equilibrium, regardless of whether it was initially inoculated with an *lpf* phase ON or a phase OFF colony (Fig. 2B). These data showed that the proportion of phase variants present in a serotype Typhimurium population at phase equilibrium is strongly influenced by culture conditions.

Growth conditions are known to alter the proportion of type 1 fimbriate serotype Typhimurium and *E. coli* cells in vitro (7, 13, 19, 20). Growth in static broth culture increases the proportion of type 1 fimbriate cells, whereas growth in roller culture or on agar plates decreases it. There is good evidence that changes in the proportion of type 1 fimbriate cells induced

FIG. 4. Mean recovery and *lpf* phase ON/OFF ratio recovered from the feces, Peyer's patches, mesenteric lymph nodes, and livers of mice inoculated with *S. enterica* serotype Typhimurium strain AJB33 (*lpfABCDE::lacZYA*) or RAK49 (Δ *lpfABCDE::lacZYA*). (A) (Left) Total CFU of strain AJB33 (closed squares) and RAK49 (open circles) recovered from fecal pellets during the first 9 days after oral inoculation. (Right) Total CFU of strains AJB33 (closed bars) and RAK49 (open bars) recovered from Peyer's patches, mesenteric lymph nodes, and livers at day 9 after oral inoculation. Data are means from five mice $\pm$ standard errors. (B) (Left) Phase ON/OFF ratios of strain AJB33 (closed squares) and RAK49 (open circles) recovered from feces of mice over time. (Right) Phase ON/OFF ratio of strain AJB33 (closed bars) and RAK49 (open bars) recovered from the Peyer's patches, mesenteric lymph nodes, and livers at day 9 after oral inoculation. Data are means from five mice $\pm$ standard errors.

by growth under different culture conditions are at least in part due to selective outgrowth of one phase (20). However, in *E. coli*, the proportion of type 1 fimbriated cells is influenced by culture conditions, including temperature and medium, and this phenomenon can be observed even in the absence of intact fimbrial biosynthesis genes (i.e., a strain carrying a *fimA::lacZYA* transcriptional fusion) (9). Similarly, we found that differences in the phase equilibrium attained as a result of serotype Typhimurium passage in broth or on plates were not caused by an outgrowth of *lpf* phase ON or *lpf* phase OFF cells, since deletion of *lpf* biosynthesis genes did not alter phase equilibrium points (Fig. 2). Growth of *E. coli* *fimA::lacZYA* at 28°C in rich medium results in a 125-fold increase in the *fim* phase ON-to-OFF transition frequency (f_{OFF}) compared to that determined after growth at 42°C in minimal medium (9). However, no obvious differences in *fim* switching frequencies are observed during growth of *E. coli* *fimA::lacZYA* on agar or in liquid medium (9). In contrast, the f_{OFF} of *lpf* decreased by

a factor of 150 during growth of serotype Typhimurium in LB broth, and f_{ON} increased 8-fold compared to that determined for growth on LB agar plates (Table 1). These changes in transition frequency were not due to alterations in temperature, since agar and liquid medium were both incubated at 37°C. Furthermore, f_{OFF} was higher than f_{ON} during growth of serotype Typhimurium on plates, whereas f_{ON} was higher than f_{OFF} during growth in broth (Table 1), leading to phase equilibrium cultures that were predominantly phase OFF or phase ON (Fig. 2).

Studies on phase variation in vivo show that it is difficult to predict whether the proportion of phase ON variants is likely to decrease or increase during an infection. For example, the proportion of MR/P-fimbriated cells increases during transurethral infection of mice with *Proteus mirabilis* (25), while the proportion of type 1-fimbriated cells of *E. coli* decreases (12), despite the fact that both adhesins are virulence factors in this cystitis model. The proportion of *E. coli* cells expressing an-

other adhesin, S fimbriae, increases from 3 to 85% during intraperitoneal infection of mice (18). The different *lpf* phase equilibrium points attained under different in vitro growth conditions (Fig. 2) raised the question of whether in vivo passage through the mouse intestine would result in a serotype Typhimurium culture which is predominantly composed of *lpf* phase ON or phase OFF cells. We found that the *lpf* phase ON/OFF ratio in the murine intestine gradually decreased over time (Fig. 3) even in the absence of intact fimbrial biosynthesis genes (Fig. 4), suggesting that f_{OFF} was higher than f_{ON} during growth in vivo. We were unable to determine the phase equilibrium point attained during in vivo passage, since it was above 99% *lpf* phase OFF, the approximate limit of detection, after 21 days postinoculation. Similarly, more than 98% of cells recovered from the urine of women with cystitis carry the type 1 fimbrial operon of *E. coli* in the phase OFF expression state (12).

Two days after transurethral infection of mice with *E. coli*, the majority of cells recovered from the urine carry the type 1 fimbrial operon in the phase OFF expression state, whereas 33% of *fim* phase ON cells are recovered from murine bladder tissue (12). It is currently not known whether or not the higher proportion of *fim* phase ON cells in tissue compared to urine is the result of selection for type 1 fimbriated *E. coli* cells in the bladder tissue. Similarly, we found that at 9 days after inoculation of mice with a predominantly *lpf* phase ON inoculum, approximately 27% of serotype Typhimurium cells recovered from feces were phase ON, whereas more than 82% of bacteria recovered from liver tissue carried the *lpf* operon in the phase ON expression state (Fig. 4). Even in the absence of *lpf* fimbrial biosynthesis genes, lower proportions of *lpf* phase ON cells were recovered from feces than from organs (Fig. 4). Two possibilities could account for these findings. First, different *lpf* phase transition frequencies may be encountered during growth in feces compared with murine tissues. Second, a different proportion of *lpf* phase ON cells in feces and murine tissues may arise because bacteria grow with different generation times at these sites. Different generation times for growth in feces compared with murine tissues may result in the recovery of different proportion of *lpf* phase ON cells from these sites, since a decrease in the *lpf* phase ON/OFF ratio is initially proportional to the number of generations a culture went through (Fig. 2).

Our data indicate that the major factor determining the proportion of *lpf* phase ON cells attained after passage of serotype Typhimurium through naïve mice were the *lpf* phase transition frequencies and not selection. However, the phase transition frequencies are not the only factor that can alter the proportion of *lpf* phase ON cells during in vivo passage. That is, previous exposure of mice to LP fimbrial proteins markedly reduces the proportion of *lpf* phase ON cells recovered from animals during a subsequent challenge (15–17). Thus, an adaptive immune response generated by previous exposure results in a strong selection against LP fimbriated cells, thereby reducing the proportion of *lpf* phase ON cells during a subsequent challenge with serotype Typhimurium. Collectively, these data suggest that the adaptive immune response does not result in marked selection against LP fimbriated bacteria during a primary infection, although it does so during a secondary exposure.

ACKNOWLEDGMENTS

We thank Renée M. Tsolis for critical comments on the manuscript. Work in the laboratory of Andreas Bäumler is supported by the Texas Advanced Research (Technology) Program under grant 000089-0051-1999 and Public Health Service grants AI40124 and AI44170.

REFERENCES

- Andrews, F. W. 1922. Studies in group-agglutination. I. The *Salmonella* group and its antigenic structure. *J. Pathol. Bacteriol.* **25**:515–521.
- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1994. Current protocols in molecular biology. J. Wiley & Sons, New York, N.Y.
- Bäumler, A. J., and F. Heffron. 1995. Identification and sequence analysis of *lpfABCDE*, a putative fimbrial operon of *Salmonella typhimurium*. *J. Bacteriol.* **177**:2087–2097.
- Bäumler, A. J., R. M. Tsolis, and F. Heffron. 1996. The *lpf* fimbrial operon mediates adhesion of *Salmonella typhimurium* to murine Peyer's patches. *Proc. Natl. Acad. Sci. USA* **93**:279–283.
- Bäumler, A. J., R. M. Tsolis, P. J. Valentine, T. A. Ficht, and F. Heffron. 1997. Synergistic effect of mutations in *invA* and *lpfC* on the ability of *Salmonella typhimurium* to cause murine typhoid. *Infect. Immun.* **65**:2254–2259.
- Bäumler, A. J., R. M. Tsolis, A. W. M. van der Velden, I. Stojiljkovic, S. Anic, and F. Heffron. 1996. Identification of a new iron regulated locus of *Salmonella typhi*. *Gene* **193**:207–213.
- Brinton, C. C. 1959. Non-flagellar appendages of bacteria. *Nature* **183**:782–786.
- Eisenstein, B. I. 1981. Phase variation of type 1 fimbriae in *Escherichia coli* is under transcriptional control. *Science* **214**:337–339.
- Gally, D. L., J. A. Bogan, B. I. Eisenstein, and I. C. Blomfield. 1993. Environmental regulation of the *fim* switch controlling type 1 fimbrial phase variation in *Escherichia coli* K-12: effects of temperature and media. *J. Bacteriol.* **175**:6186–6193.
- Grant, S. G. N., J. Jessee, F. R. Bloom, and D. Hanahan. 1990. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. *Proc. Natl. Acad. Sci. USA* **87**:4645–4649.
- Kinder, S. A., J. L. Badger, G. O. Bryant, J. C. Pepe, and V. L. Miller. 1993. Cloning of the *YenI* restriction endonuclease and methyltransferase from *Yersinia enterocolitica* serotype O:8 and construction of a transformable R-M+ mutant. *Gene* **136**:271–275.
- Lim, J. K., N. W. Gunther, I. V., H. Zhao, D. E. Johnson, S. K. Keay, and H. L. T. Mobley. 1998. In vivo phase variation of *Escherichia coli* type 1 fimbrial genes in women with urinary tract infection. *Infect. Immun.* **66**:3303–3310.
- Maccacaro, G. A., and W. Hayes. 1961. The genetics of fimbriation in *Escherichia coli*. *Genet. Res.* **2**:394–405.
- Nicholson, B., and D. Low. 2000. DNA methylation-dependent regulation of *pef* expression in *Salmonella typhimurium*. *Mol. Microbiol.* **35**:728–742.
- Nicholson, T. L., and A. J. Bäumler. 2001. *Salmonella enterica* serotype Typhimurium elicits cross-immunity against a *Salmonella enterica* serotype Enteritidis strain expressing LP fimbriae from the *lac* promoter. *Infect. Immun.* **69**:204–212.
- Norris, T. L., and A. J. Bäumler. 1999. Phase variation of the *lpf* fimbrial operon is a mechanism to evade cross immunity between *Salmonella* serotypes. *Proc. Natl. Acad. Sci. USA* **96**:13393–13398.
- Norris, T. L., R. A. Kingsley, and A. J. Bäumler. 1998. Expression and transcriptional control of the *Salmonella typhimurium lpf* fimbrial operon by phase variation. *Mol. Microbiol.* **29**:311–320.
- Nowicki, B., J. Vuopio-Varkila, P. Viljanen, T. K. Korhonen, and P. H. Mäkelä. 1986. Fimbrial phase variation and systemic *E. coli* infection studies in the mouse peritonitis model. *Microb. Pathog.* **1**:335–347.
- Old, D. C., I. Corneil, L. F. Gibson, A. D. Thomson, and J. P. Duguid. 1968. Fimbriation, pellicle formation and the amount of growth of salmonellas in broth. *J. Gen. Microbiol.* **51**:1–16.
- Old, D. C., and J. P. Duguid. 1970. Selective outgrowth of fimbriate bacteria in static liquid medium. *J. Bacteriol.* **103**:447–456.
- Simon, R., U. Priefer, and A. Puhler. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Bio/Technology* **1**:784–791.
- Stocker, B. A. D. 1949. Measurement of the rate of mutation of flagellar antigenic phase in *Salmonella typhimurium*. *J. Hyg.* **47**:398–413.
- Stojiljkovic, I., and K. Hantke. 1995. Functional domains of the *Escherichia coli* ferric uptake regulator (Fur). *Mol. Gen. Genet.* **247**:199–205.
- Tsolis, R. M., A. J. Bäumler, I. Stojiljkovic, and F. Heffron. 1995. Fur regulon of *Salmonella typhimurium*: identification of new iron-regulated genes. *J. Bacteriol.* **177**:4628–4637.
- Zhao, H., D. E. Johnson, I. Blomfield, and H. L. T. Mobley. 1997. In vivo phase variation of MR/P fimbrial gene expression in *Proteus mirabilis* infecting the urinary tract. *Mol. Microbiol.* **23**:1009–1019.