The lpf fimbrial operon mediates adhesion of *Salmonella typhimurium* to murine Peyer’s patches

(M cells/fimbriae/adhesin)

ANDREAS J. BÄumlEr*, RENÉE M. TSOLIS, AND FRED HEFFRON

Department of Molecular Microbiology and Immunology, Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Road, L220, Portland, OR 97201-3098

Communicated by Harley W. Moon, U.S. Department of Agriculture, Greenport, NY, September 14, 1995 (received for review May 8, 1995)

ABSTRACT We investigated the role of the *Salmonella typhimurium* fimbrial operon formed by the genes *lpfABCDE* in infection of mice. A mutant in *lpfC*, the gene encoding the fimbrial outer membrane usher, had an ~5-fold increased 50% lethal dose when administered orally to mice. When mice were infected with a mixture of the *lpfC* mutant and isogenic wild-type *S. typhimurium*, the *lpfC* mutant was recovered in lower numbers from Peyer’s patches, mesenteric lymph nodes, liver, and spleen. In an organ culture model using murine intestinal loops, *lpfC* mutants were shown to be associated in lower numbers than wild-type bacteria with Peyer’s patches but not with villous intestine. The defect of the *lpfC* mutant in adhesion to Peyer’s patches could be complemented by introducing *lpfABCDE* on a cosmid. Similarly, heterologous expression of the *Salmonella lpf* operon in *Escherichia coli* resulted in an increased adhesion to histological thin sections of Peyer’s patch lymph follicles. Electron microscopic analysis of histological sections taken from Peyer’s patches after intragastric infection of mice showed that, in contrast to the *S. typhimurium* wild type, the isogenic *lpfC* mutant did not destroy M cells of the follicle-associated epithelium. These data show that the *Salmonella lpf* operon is involved in adhesion to murine Peyer’s patches.

*Salmonella typhimurium* causes a systemic disease in mice known as murine typhoid fever. Before *Salmonella* can disseminate within its murine host to reach systemic sites of infection, it must invade and penetrate the epithelial lining of the alimentary tract. Carter and Collins (1) identified the terminal ileum as the main port of entry for *Salmonella* during infection of mice. In the terminal ileum, the bacteria preferentially enter areas of the intestinal wall which contain accumulations of lymph follicles, the Peyer’s patches (1, 2). Takeuchi, in 1967 (3), described the induction of membrane ruffling during invasion of intestinal epithelial cells by *S. typhimurium*. *Salmonella* induces membrane ruffling in two distinct cell types of the intestinal epithelium, absorptive epithelial cells and M cells (3–5). Bacterial invasion, however, has different consequences for these two cell types. Absorptive epithelial cells are able to recover from the gross apical membrane rearrangements induced during entry of *S. typhimurium* and seem to survive bacterial transcytosis (6). In contrast, M cells have been shown to be destroyed as early as 30 min after contact with *Salmonella* (4, 7).

While absorptive epithelial cells form the predominant cell type throughout the ileum, the presence of M cells is restricted to the follicle-associated epithelium (FAE). Adhesion to M cells would therefore result in increased attachment to Peyer’s patches. Indeed, a variety of pathogens which exhibit a tropism for Peyer’s patches seem to adhere selectively to M cells during infection of the alimentary tract (8). Following adhesion, enteroinvasive *Yersinia*, *Shigella*, and *Salmonella* species enter Peyer’s patches initially via M cells and are able to multiply within the Peyer’s patch tissue (1, 2, 9).

The only virulence genes shown to be important during this early phase of a *Salmonella* infection encode factors mediating host cell invasion (10). However, adhesion of *Salmonella* to M cells has been postulated to precede invasion (5, 7). The genes encoding this (these) adhesin(s) have so far not been identified. In this study we provide evidence that *lpfABCDE* (11), a *S. typhimurium* fimbrial operon, is involved in colonization of murine Peyer’s patches.

MATERIALS AND METHODS

Strains and Recombinant DNA Techniques. AJB23 is a spontaneous nalidixic acid-resistant derivative of χ3296 (12). Analytical grade chemicals and enzymes were purchased from SIGMA or Boehringer Mannheim, respectively. Standard methods were used for recombinant DNA techniques and Southern hybridization (13).

Virulence Studies. Six to 8-week-old female BALB/c mice were used throughout this study. The 50% lethal dose (LD₅₀) of groups of four mice infected with serial 10-fold dilutions of overnight cultures was calculated 4 weeks postinfection by the method of Reed and Muench (14). The 50% implantation dose (ID₅₀) was determined as described (15). For the course of infection studies, mice were injected intragastrically with 1.5 × 10⁷ bacteria. At appropriate times groups of four mice were sacrificed, and bacteria in internal organs were quantitated. Significance of differences observed was determined with the Wilcoxon signed rank test for a paired experiment.

Intestinal Organ Culture (IOC) Model. Bacteria were grown as standing overnight cultures in 1 ml of LB at 37°C in 5% CO₂, harvested, and resuspended in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO). The small intestine from mice starved for 24 h was collected and placed into a Petri dish containing DMEM. The intestine was ligated at the distal end, filled with 1 ml of a bacterial suspension containing 6 × 10⁷ colony-forming units (cfu), then ligated at the proximal end, and incubated for 30 min at 37°C in 5% CO₂. Nonadherent bacteria were removed by five washes in phosphate-buffered saline (PBS) and adherent bacteria were quantitated. Experiments were repeated with organs from six different animals and significance of differences measured was determined using a paired-difference test.

In Vitro Adhesion Assay to Tissue Sections. Histological sections of murine Peyer’s patches were deparaffinized and rehydrated, and nonspecific binding to sections was blocked by a 30-min incubation in 0.05% Tween 20/0.2% bovine serum albumin in PBS at 37°C. Bacteria were labeled with fluorescein isothiocyanate (FITC, Sigma) as described (16). A few drops of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: FAE, follicle-associated epithelium; ID₅₀, 50% implantation dose; IOC, intestinal organ culture.

*To whom reprint requests should be addressed.
FITC-labeled bacteria were placed directly over 8–10 tissue sections present on two microscope slides and incubated at 37°C in a moist chamber. After 30 min, nonadherent bacteria were removed by six 5-min changes in PBS and the sections were fixed for 10 min on ice in 3% paraformaldehyde. Each experiment was repeated three times using tissue from different animals.

Electron Microscopy. Groups of four mice starved for 24 h were infected intragastrically with $10^7$ and $10^8$ bacteria. The distal Peyer’s patch of each mouse was collected 90 min postchallenge, fixed in 1.5% paraformaldehyde/1.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4), and embedded in Epon 812. Ultrathin sections (110 nm) were examined on a Philips EMU 301 transmission electron microscope.

RESULTS

Construction of a S. typhimurium lpfC Mutant. The lpf operon is located at 78 minutes on the S. typhimurium chromosome and no homologue is present in the corresponding region of the Escherichia coli chromosome (Fig. 1) (11). The apparent specificity of these genes to Salmonella prompted us to study their role in murine typhoid fever.

In other pilus operons, mutations in genes encoding assembly proteins resulted in absence of fimbriae from the bacterial surface (17). Two lpf gene products, LpfB and LpfC, have homology to fimbrial periplasmic chaperones and outer membrane ushers, respectively (11). In order to inactivate lpfC, a kanamycin-resistance cassette (KIXX, Pharmacia) was introduced into the EcoRI restriction site of plasmid pMS1054 (Fig. 1) (11). The resulting plasmid, pMS1072, was digested with XbaI–EcoRIV and the insert was cloned into the suicide vector pEP185.2 (18). This construct (pMS1077) was conjugated from E. coli S17 lip (19) into S. typhimurium IR715 (20). One exconjugant sensitive to chloramphenicol (vector) and resistant to kanamycin was designated AJB1 (Fig. 1).

Virulence of the S. typhimurium lpfC Mutant in Mice. The lpfC mutation had no effect when bacteria were administered by the intraperitoneal route of infection. However, when mice were injected intragastrically, AJB1 showed a 5-fold increase

![Fig. 1.](image)

**Fig. 1.** (A) Restriction map of the lpf operon (Upper) and inserts of plasmids (Lower). Arrows and hairpins indicate genes and transcriptional terminators, respectively. Regions of homology between E. coli and S. typhimurium are indicated by black bars. H, HindIII; E, EcoRI; P, Pst I. (B) Southern blot of chromosomal DNA of AJB1 (lane 1), AJB2 (lane 2), and IR715 (lane 3) with a lpfC probe (pMS1039) (11).

in LD$_{50}$ ($2.8 \times 10^9$ as compared to $6 \times 10^8$ for IR715) and in ID$_{50}$ ($3.5 \times 10^8$ as compared to $6 \times 10^8$ for IR715). In addition, mice infected intragastrically with an LD$_{50}$ dose of AJB1 died with a delay of about 3 days as compared to mice infected with a LD$_{50}$ dose of IR715.

To further analyze the attenuation of AJB1, course of infection experiments with mice infected intragastrically with a 1:1 mixture of wild-type S. typhimurium (IR715; Nal$^+$) and the lpfC mutant (AJB1; Nal$, K$m$^r$) were performed. Bacillary numbers recovered from internal organs were recorded over time (Fig. 2). In all organs, AJB1 was consistently found in lower numbers than IR715 ($P < 0.05$), indicating that a mutation in the lpf operon causes a defect early during infection.

Adhesion to Murine Small Intestine in Vitro. The IOC allowed us to restrict bacterial contact to the luminal surface of the intestine and to distinguish between adhesion to murine Peyer’s patches and villous intestine. When a 1:1 mixture of S.

![Fig. 2.](image)

**Fig. 2.** Bacterial numbers recovered over time from Peyer’s patches (A), mesenteric lymph nodes (B), liver (C), and spleen (D) of mice after mixed oral infection with IR715 (open symbols) and AJB1 (closed symbols). Standard errors of time points are indicated as bars.

![Fig. 3.](image)

**Fig. 3.** Bacterial association with the intestinal wall after mixed infection with IR715 and AJB1 (A), AJB23 and AJB2 (B), IR715 and AJB1(pMS1000) (C), or ORN172 and ORN172(pMS1000) (D) in the IOC. Values are given as averages ± standard error.
typhimurium wild-type (IR715) and lpfC mutant (AJB1) was used as the inoculum, IR715 was recovered in larger numbers from Peyer's patches \((P < 0.05)\), while no difference in bacterial numbers associated with villous intestine could be observed \((P > 0.1)\) (Fig. 3A).

Differences in bacterial numbers recovered from the IOC could be due to adhesion, invasion, or both. To distinguish between these possibilities, we introduced the lpfC mutation into the noninvasive S. typhimurium strain AJB23. The resulting S. typhimurium inv lpfC double mutant was designated AJB2. The insertion in lpfC was confirmed by Southern hybridization (Fig. 1). Lower numbers of AJB2 than its isogenic parent AJB23 were found to be associated with Peyer's patches \((P < 0.05)\) (Fig. 3B). This difference in bacterial numbers is therefore due to a defect in adhesion caused by the lpfC mutation. However, as compared to invasive S. typhi-
murium strains, noninvasive bacteria were found to be associated in approximately 7- to 8-fold lower numbers with the intestinal wall. Thus, invasion seems to contribute to the number of bacteria recovered from the IOC.

A cosmid (pMS1000) carrying lpfABCDE was conjugated into AJB1. Strain AJB1(pMS1000) was found to be associated in larger numbers with Peyer’s patches than IR715 ($P < 0.025$). Thus, the lpfC mutation could be complemented by pMS1000 (Fig. 3C). Similarly, expression of the lpf operon in the nonpiliated E. coli strain ORN172 (21) resulted in recovery of increased bacterial numbers from Peyer’s patches ($P < 0.05$) (Fig. 3D).

Bacterial binding to thin sections from murine Peyer’s patches in vitro was analyzed by confocal laser scanning microscopy. The nonpiliated E. coli strain ORN172 bound only poorly to thin sections; however, strain ORN172(pMS1000) adhered in about 5-fold increased numbers to the surface area of lymph nodules (Fig. 4).

**DISCUSSION**

Here we provide evidence that lpfABCDE, a fimbrial operon of *S. typhimurium*, is involved in adhesion of this pathogen to thin sections of Peyer’s patches from murine ligated ileal loops (4, 7). In contrast, in sections of Peyer’s patches from AJB1 infected mice the FAE was found to be fully intact (Fig. 5B). Only in one case was a bacterium found to be associated with an M cell, which also displayed normal morphology (Fig. 5C).
murine small intestine. Moreover, lpf-mediated adhesion seems to result in binding only to certain areas of the alimentary tract, thus targeting the pathogen to its preferred port of entry, the Peyer’s patches of the ileum. We found that a mutation in lpfC, the gene encoding the fimbrial outer membrane usher, results in a 5-fold increased LD₅₀ of S. typhimurium for mice infected intragastrically. This observation was further supported by analysis of mixed oral infections of mice, showing that lpfC mutants are recovered in lower numbers from systemic sites. During the oral course of infection, the step following Salmonella adhesion is cell invasion. S. typhimurium mutants in invA, a gene required for cell invasion, have been reported to have a 50-fold increased LD₅₀ in mice (12). In comparing the LD₅₀ values of lpfC and invA mutants one has to keep in mind that Salmonella invades not only the FAE of Peyer’s patches but also villus tips in the ileum and the epithelium of cecum and rectum (1, 6). Invasion of enterocytes is thought to be required not only for penetration of the FAE of Peyer’s patches but also at these alternative sites of infection. Noninvasive mutants are therefore expected to be more attenuated than mutants which only have a decreased ability to colonize Peyer’s patches. The observed 5- and 50-fold increase in LD₅₀ of lpfC and invA mutants, respectively, is consistent with this idea.

Several enteropathogens use the Peyer’s patches of the small intestine as a port of entry into their host (2, 4, 5, 7, 22, 23). Although this trend seems to indicate similarities in their virulence strategies, the mechanisms employed during the interaction with FAE differ between species. Unlike other pathogens, at the onset of Peyer’s patch colonization Salmonella causes complete destruction of M cells (4, 7), which appears to occur in three phases. (i) Salmonella initially adheres to M cells as suggested by studies using scanning electron microscopy (5, 7). Since adhesion of S. typhimurium to M cells has been observed to precede invasion (5), it may be possible to inhibit subsequent steps by preventing M cell adhesion. Thus, the observation that S. typhimurium lpfC mutants do not destroy M cells is intriguing, since it supports the hypothesis that the lpf operon may mediate adhesion to M cells. (ii) Following adhesion, the bacteria actively invade M cells, thereby causing gross membrane rearrangements (4, 5). These disturbances in the apical membrane are reminiscent of those first described in 1967 by Takeuchi (3) during studies on Salmonella invasion. In fact, mutants in invA, a Salmonella invasion gene, were recently found not to cause apical membrane ruffling or destruction of M cells (4). (iii) Subsequent to invasion, M cells do not regain their original apical membrane morphology and are destroyed by a unknown mechanism (4, 7). During studies in ligated ileal loops bacteria were frequently observed amid cellular debris from M cells (4, 7). However, we were unable to observe bacteria at these locations. A likely explanation for this difference is that an artificially high number of bacteria is maintained in the intestinal lumen of the ligated ileal loop as compared to oral infection.

The model described above has similarities to the interaction of enteropathogenic E. coli (EPEC) with absorptive epithelial cells in that a fimbrial adhesin operates in concert with a complex bacterial machinery which induces signaling pathways in the host cell (24). In fact, genes involved in signal transduction of both S. typhimurium and EPEC were found to share homologies with type III export systems (25, 26). Interestingly, the type III export system of Yersinia species has also been found to depend on an adhesin for translocation of proteins into target cells (27). Thus, coordinated action of adhesins and type III exporters appears to be a common enterobacterial virulence strategy designed to manipulate host cells. Mutations in the lpf operon seem to prevent only destruction of M cells and, therefore, Salmonella invasion of other cell types may depend on different adhesins.

We thank R. Curtiss III for providing bacterial strains, C. J. Lipp for technical assistance, and R. Jones for performing the electron microscopy.