

A role for *Salmonella* fimbriae in intraperitoneal infections

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Enteric bacteria possess multiple fimbriae, many of which play critical roles in attachment to epithelial cell surfaces. SEF14 fimbriae are only found in *Salmonella enterica* serovar Enteritidis (*S. enteritidis*) and closely related serovars, suggesting that SEF14 fimbriae may affect serovar-specific virulence traits. Despite evidence that SEF14 fimbriae are expressed by *S. enteritidis* *in vivo*, previous studies showed that SEF14 fimbriae do not mediate adhesion to the intestinal epithelium. Therefore, we tested whether SEF14 fimbriae are required for virulence at a stage in infection after the bacteria have passed the intestinal barrier. Polar mutations that disrupt the entire *sef* operon decreased virulence in mice more than 1,000-fold. Nonpolar mutations that disrupted *sefA* (encoding the major structural subunit) did not affect virulence, but mutations that disrupted *sefD* (encoding the putative adhesion subunit) resulted in a severe virulence defect. The results indicate that the putative SEF14 adhesion subunit is specifically required for a stage of the infection subsequent to transit across the intestinal barrier. Therefore, we tested whether SefD is required for uptake or survival in macrophages. The majority of wild-type bacteria were detected inside macrophages soon after *i.p.* infection, but the *sefD* mutants were not readily internalized by peritoneal macrophages. These results indicate that the potential SEF14 adhesion subunit is essential for efficient uptake or survival of *S. enteritidis* in macrophages. This report describes a role of fimbriae in intracellular infection, and indicates that fimbriae may be required for systemic infections at stages beyond the initial colonization of host epithelial surfaces.

Fimbriae play a critical role in virulence by allowing bacteria to interact with host cells and other solid substrates (1, 2). The distribution of fimbrial operons among enteric bacteria suggests a role for fimbriae in pathogenesis; broadly distributed fimbrial operons may provide general adhesive functions, but fimbriae whose distribution is limited may provide specific functions required in virulence. For example, the common type I fimbriae found in many Gram-negative bacteria mediates adherence to the pharynx, intestinal epithelium, and bladder, whereas plasmid encoded fimbriae found only in *Salmonella* bind specifically to M cells in the intestine (1, 3).

Most fimbriae (with the notable exception of type IV pili) have a conserved mechanism of translocation to the bacterial surface. Fimbrial proteins are secreted into the periplasm by means of the general secretory system. Two accessory proteins assist in construction of the fimbrial shaft. First, the fimbrial subunits are bound by a chaperone in the periplasm to prevent premature aggregation and then the subunits are translocated across the outer membrane by an usher protein. Recently, the crystal structures of FimC–FimH and PapD–PapK complexes have been solved, demonstrating an elegantly simple mechanism for the interactions between the subunit and chaperone and between pairs of subunits (4, 5). In all fimbrial systems that have been studied, the genes encoding the chaperone and usher are located in the same operon as the major subunit. cursory examination of the *Escherichia coli*, *Yersinia pestis*, *Salmonella enterica* serovar Typhimurium (*S. typhimurium*), and *Salmonella* Typhi (*S. typhi*) genomes identified about one dozen chaperone–usher-

dependent fimbrial operons per genome, although only a handful of these have currently been characterized (R.A.E., B. M. Matlock, and S.R.M., unpublished observations). It is expected that *Salmonella enteritidis* contains a similar number of fimbrial operons to these other enteric bacteria. The *S. enteritidis* fimbriae (SEF14) is restricted to *S. enteritidis* and other closely related group D *Salmonella* (7). Therefore, an analysis of SEF14 fimbrial function may provide insight into the unique aspects of virulence that distinguish this group of *Salmonella*.

The *sef* operon is located on a small pathogenicity island. The operon contains four structural genes (*sefABCD*) required for the translocation and biogenesis of SEF14 fimbriae: *sefA* encodes the major subunit, *sefB* and *sefC* encode the chaperone and usher, respectively, and *sefD* encodes the putative adhesin. Adjacent to *sefD*, there is an AraC-like regulatory protein (encoded by *sefR*) that activates transcription of the *sef* genes (6) (Fig. 1). Some evidence suggests that SEF14 fimbriae may play a role in pathogenesis. For example, immunization of mice with purified SEF14 subunits induces a strong T lymphocyte response, and the mice present a delayed-type hypersensitive response to whole *S. enteritidis*, demonstrating that these fimbriae are expressed *in vivo* and stimulate cell-mediated immunity (8). Furthermore, pretreatment of mice with anti-SEF14 antibodies protects mice from *S. enteritidis* infection (9). However, other studies on the effect of *sef* mutants on virulence showed conflicting results (10, 11).

Because discrepancies in earlier studies may be the result of genetic differences in the mutants used, we constructed several defined *sef* mutants (R.A.E., B. M. Matlock, and S.R.M., unpublished observations; L. H. Keller, R. C. Boston, and D.M.S., unpublished observations; ref. 13). Like many other fimbrial operons, the *sefABCD* genes are cotranscribed so that insertion of a kanamycin cassette into *sefA* eliminates transcription of *sefD*, and translocation of SefD across the outer membrane is a prerequisite for translocation of SefA (6). Based on these studies, we predict that the SEF14 fimbrial proteins displayed on the surface of the *sef* mutants is as shown in Fig. 2. In this work, we assay the role of the various *sef* mutants in virulence and show that SEF14 fimbriae mediate interactions with phagocytes in the peritoneal cavity, which allows *S. enteritidis* to survive the macrophage assault. This role for fimbriae *in vivo* is in contrast to the routine *modus operandi* of previously characterized fimbriae involved in binding to host epithelial surfaces.

Materials and Methods

Bacterial Strains and Growth Conditions. All strains used in this study were derivatives of *S. enteritidis* LK5 (14) (Table 1). This prototrophic, phage type 8, *S. enteritidis* strain was isolated as a

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Abbreviations: SEF, *Salmonella enteritidis* fimbriae; Kan, kanamycin sulfate; CFA, colonization antigen factor; CFU, colony-forming unit.

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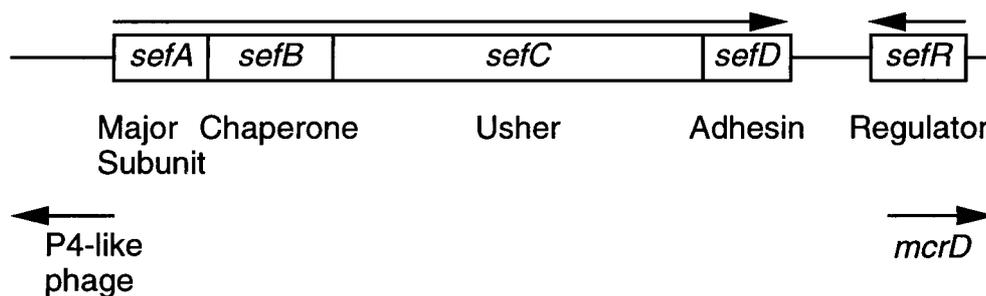


Fig. 1. The *sef* pathogenicity island. Organization of the *sef* genes, direction of transcription (arrows), and predicted function of the ORFs.

chicken pathogen. For routine growth, all strains were grown overnight at 37°C with vigorous aeration in LB broth supplemented with kanamycin sulfate (Kan) at a final concentration of 50 $\mu\text{g ml}^{-1}$ when required (15). Prior to infection of animals, bacteria were grown in conditions that optimize SEF14 fimbrial expression, colonization factor antigen (CFA) media buffered with 0.1 M Mes to pH 6.0 (16). The $\Delta sefA$ in-frame deletion removes the central 37 amino acids of the 176-aa polypeptide. The $\Delta sefD$ in-frame deletion removes the central 78 amino acids of the 149-aa polypeptide. Construction of the *sef* mutants and analysis of the exported proteins are described elsewhere (R.A.E., B. M. Matlock, and S.R.M., unpublished observations; L. H. Keller, R. C. Boston, and D.M.S., unpublished observations, ref. 13). $\Delta sefA$ and $\Delta sefD$ strains were marked with *zjg::MudJ* transduced from JS107 (17). This kanamycin-resistant insertion has been extensively tested and does not affect virulence of *Salmonella* in mice.

Mouse Studies. Female BALB/c mice between 6 and 8 wk old were purchased from Harlan Breeders (Indianapolis) and housed at the University of Illinois according to local Laboratory

Animal Care Advisory Committee guidelines. Overnight bacterial cultures grown in CFA media (pH 6.0) were diluted 5×10^5 in sterile 0.85% NaCl to yield approximately 10^4 colony-forming units (CFU) per ml, and 0.1 ml of the diluted culture was injected into the peritoneal cavity (i.p.). For competition assays, strains were grown separately overnight, diluted by 5×10^5 , and then mixed in a 50:50 ratio. To estimate the i.p. LD₅₀, 10-fold serial dilutions in 0.85% NaCl were prepared to result in a range from 10^7 to 100 bacteria per ml, and 0.1 ml of these samples were injected into the peritoneal cavity. To estimate the oral LD₅₀, a similar range of dilutions was prepared in PBS. The mice were anaesthetized by brief exposure to CO₂, and oral inoculations were performed with a feeding needle. All inocula were quantitated by plating serial 10-fold dilutions of the cultures onto LB plates and counting viable colonies. For mixed infections, the samples were replica plated onto LB and LB-Kan to quantitate total and mutant CFU. LD₅₀ values were estimated by using the method of Reed and Muench (18).

At appropriate times following injection, mice were sacrificed by CO₂ asphyxiation, and organs were aseptically removed and placed in 2 ml ice-cold, sterile, 0.85% NaCl. Tissues were

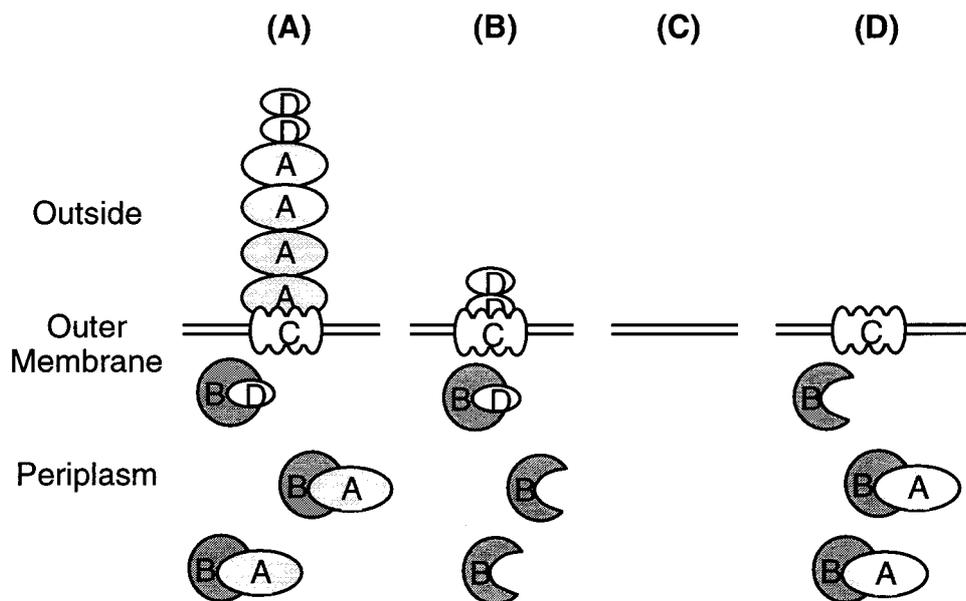


Fig. 2. Predicted export of SEF14 fimbrial subunits from the periplasm in the mutants used in this study. Note that all subunits contain signal sequences, suggesting that they are exported across the cytoplasmic membrane in a secretory-dependent manner. Wild-type SEF14 fimbriae (A) consist of the major subunit, SefA. Based on sequence homology, SefB is the chaperone that binds subunits in the periplasm and prevents premature aggregation. SefC is the membrane-located usher through which the subunits are translocated. SefD is probably a tip-located adhesin. Export of SefD occurs in the absence of SefA ($\Delta sefA$; B) and may form a fibrillar structure on the cell surface. The polar *sefA::Kan* strain (C) does not produce any subunits. Export of SefA does not occur in the absence of SefD ($\Delta sefD$; D).

Table 1. LD₅₀ values of wild-type and *sef* mutants

Strain	<i>sef</i> genotype*	IP LD ₅₀ [†]	Oral LD ₅₀ [†]
LK5	<i>sef</i> ⁺	<10	>10 ⁴
TYT3230	$\Delta sefA$	<10	>10 ⁴
LK19	<i>sefA::Kan</i>	>10 ⁴	>10 ⁷
TYT3150	$\Delta sefD$	>10 ⁴	>10 ⁷

*Only the *sef* genotype is shown.

[†]To reduce the number of mice required, we only tested two mice for each dilution of *S. enteritidis*; therefore, all LD₅₀ values are approximate.

homogenized, and then aliquots were plated onto LB plates. For mixed infections, the samples were replica plated onto LB and LB-Kan after overnight growth to allow quantitation of wild-type and mutant bacteria. The liver and spleen were routinely assayed for bacterial load; however, to study tissue tropism, other tissues were also examined (including the lungs, heart, pancreas, intestine, and kidneys).

Internalization Assays. To quantitate the internalization of *S. enteritidis* *in vivo*, overnight cultures were diluted in 0.85% NaCl, and then approximately 2,000 bacteria were injected into the peritoneal cavity of live mice. At the indicated times after infection, mice were sacrificed by CO₂ asphyxiation, surface sterilized in 70% ethanol, and the skin over the abdomen was removed leaving the peritoneum intact. An amount of 5 ml ice-cold RPMI 1640 tissue culture media (BioWhittaker) was injected into the peritoneal cavity. After 1 min, the tissue culture media was aspirated and immediately placed on ice. Two aliquots of this peritoneal lavage were compared. Gentamicin was added to a final concentration of 10 μ g/ml to one aliquot, and after a 30-min incubation on ice both samples were washed by centrifugation and resuspended in sterile 0.85% NaCl and then plated on LB agar. Viable counts from the sample that was not treated with gentamicin gave the total number of bacteria, and viable counts from the gentamicin-treated sample gave the number of internalized bacteria. There was no difference in sensitivity between any of the strains used in this study to gentamicin *in vitro* (data not shown).

Results

Virulence of a *sefA::kan* Mutant Is Attenuated *in Vivo*. To decipher the role of SEF14 fimbriae in virulence, we examined the growth of wild-type *S. enteritidis* and defined *sef* mutants *in vivo*. The i.p.

dose of wild-type *S. enteritidis* required to cause a lethal infection in 50% (LD₅₀) of BALB/c mice was <10 bacteria (Table 1). *S. enteritidis* with a polar mutation in *sefA* (*sefA::Kan*) was severely attenuated *in vivo*, resulting in an approximate 1,000-fold increase in LD₅₀. In contrast, a strain with a nonpolar, in-frame deletion in *sefA* ($\Delta sefA$) had a similar LD₅₀ to wild-type bacteria. To investigate the cause of this difference in LD₅₀, we followed the kinetics of *S. enteritidis* infections *in vivo*. Following i.p. injection, *S. enteritidis* is rapidly taken to the target organs (liver and spleen) where the bacteria multiply such that at 5 days postinoculation these organs contain between 10⁷ and 10⁸ bacteria. At this time, the mice show symptoms of advanced salmonellosis, and ultimately the mice succumb to infection approximately 6 days postinoculation. Competition assays between wild-type and mutant bacteria provide a sensitive method for analyzing the growth of *Salmonella* strains *in vivo* without requiring large numbers of animals. Equal numbers of wild-type and *sef* mutants were injected i.p. The liver and spleen were rapidly colonized; however, *sefA::Kan* mutants were unable to persist as efficiently as wild-type bacteria (Fig. 3A). In contrast, $\Delta sefA$ mutants colonized the liver and spleen as efficiently as the wild-type bacteria, indicating that the *sefA* gene product is not required for virulence (Fig. 3B). In contrast, competition assays between wild-type bacteria and *S. enteritidis* with mutations in other fimbrial systems (i.e., PEF, LPF, FIM, AGF) showed no effect on virulence when bacteria were administered by means of the i.p. route, and there was no difference during *in vitro* growth between any of the strains tested (data not shown).

The SefD Subunit Is Required for Virulence. Because the nonpolar $\Delta sefA$ mutation did not affect virulence but the polar *sefA::Kan* reduced virulence, we examined the effects of nonpolar mutations in other *sef* genes on virulence. The time course of infection of the liver and spleen was determined for each strain (Fig. 4). The results confirm that the polar *sefA::Kan* and nonpolar $\Delta sefD$ mutations attenuate growth *in vivo* but the nonpolar $\Delta sefA$ mutation has no effect on virulence. To confirm these results, approximate oral and i.p. LD₅₀ values were measured. The LD₅₀ values for the wild-type and $\Delta sefA$ strains are comparable, but both the oral and i.p. virulence of mutants lacking SefD (*sefA::Kan* and $\Delta sefD$) are greatly reduced (Table 1). Transposon mutations in *sefB* and *sefC* also showed reduced virulence as expected because these mutations are also polar on expression of *sefD* (data not shown). Thus, although SefA and SefD are

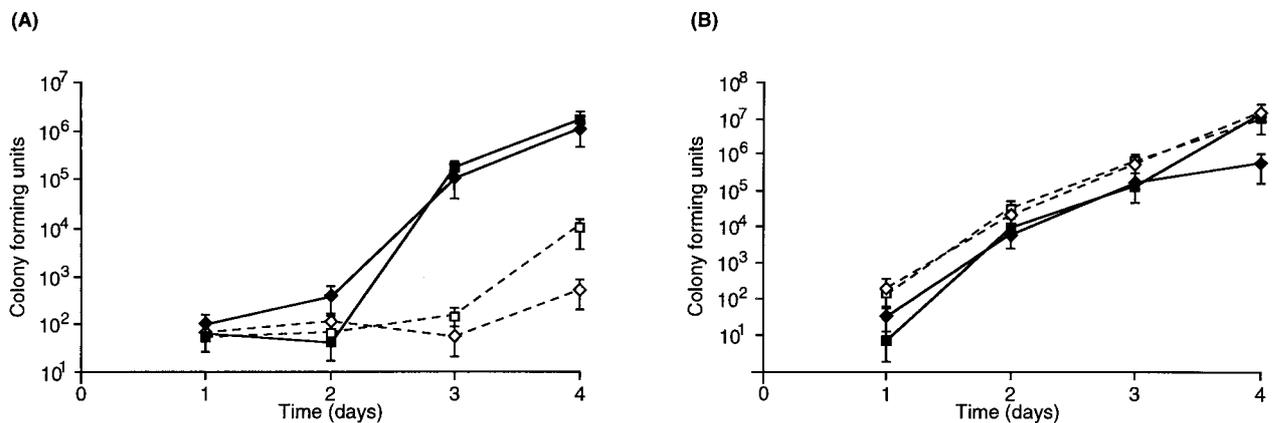


Fig. 3. Competition between wild-type and *sefA* mutants of *S. enteritidis*. Square symbols represent data from the spleens, and diamonds represent data from the livers. (A) Wild-type (closed symbols and solid lines) vs. *sefA::Kan* (open symbols and dashed lines). (B) Wild-type (closed symbols and solid lines) vs. $\Delta sefA$ (open symbols and dashed lines). All data are the average of at least three independent samples, and error bars represent the SEM.

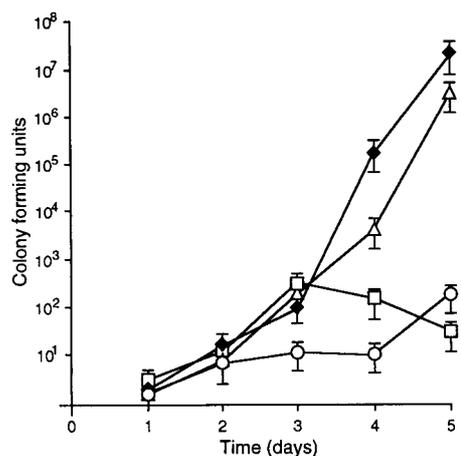


Fig. 4. Comparison of virulence of individual *S. enteritidis* strains in the liver. ◆, Wild-type *S. enteritidis*. △, $\Delta sefA$ mutants. ○, $sefA::Kan$ mutants. □, $\Delta sefD$ mutants. Similar data were obtained from the spleen but are not shown. All data are the average of at least three independent samples, and error bars represent the SEM.

components of the same fimbriae (6), these results show that only the SefD subunit is required for virulence.

Tissue Tropism Is Not Affected by *sef* Mutations. Previous studies on the role of fimbriae in virulence have shown that different fimbriae bind to specific types of host cells (1, 2, 19, 20). Thus, it seemed possible that the SEF14 fimbriae were required for binding to different tissues during the course of infection. Examination of the tissue distribution of mutant and wild-type bacteria indicated that there was no difference in the ability of wild-type and $\Delta sefA$ strains to colonize any tissues. Although much lower numbers of $sefA::Kan$ and $\Delta sefD$ mutants were recovered than the wild-type bacteria, equally low numbers were recovered from all tissues and there did not appear to be any difference in the colonization of any tissue examined (data not shown). Hence, SEF14 fimbriae are probably not responsible for tissue tropism.

SefD Is Required for Efficient Internalization into Macrophages. Soon after injection of bacteria into the peritoneal cavity, they are engulfed by phagocytic cells as part of the host immune response. Phagocytes kill most bacteria, however *Salmonella* have evolved elaborate mechanisms to evade cytotoxicity and can persist inside the phagocyte. To investigate the uptake of *S. enteritidis* by macrophages *in vivo*, we compared the localization of wild-type bacteria and $\Delta sefD$ mutants in the peritoneal cavity at different times following injection (Fig. 5). The majority of wild-type bacteria are internalized rapidly after injection (within 10 min; Fig. 5), and subsequently the total number of bacteria in the peritoneum declines as bacteria are taken from the peritoneal cavity to the liver and spleen. In contrast, the $\Delta sefD$ mutants are not internalized as efficiently and the majority of viable mutant bacteria remain extracellular. The $\Delta sefA$ mutant behaved like wild-type, remaining intracellular. In contrast, the $sefA::Kan$ mutant behaved like the $\Delta sefD$ mutant, remaining extracellular (data not shown).

Discussion

These results indicate that the SEF14 fimbriae are essential for full virulence of *S. enteritidis in vivo*, and that the fimbriae mediate interactions between the bacteria and host phagocytes. Using a genetic approach with well defined, isogenic mutants, we have shown that the minor subunit, SefD, is essential for SEF14

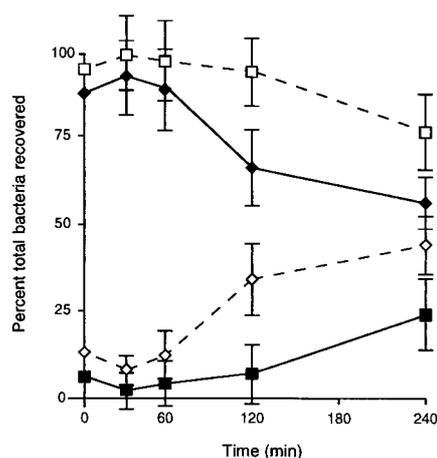


Fig. 5. Location of *S. enteritidis* in the peritoneal cavity. Solid lines and solid symbols represent intracellular bacteria, and dashed lines and open symbols represent extracellular bacteria. Diamonds represent wild-type bacteria, and squares represent $\Delta sefD$ mutants. Each time point is an average from at least three independent samples, and error bars represent the SEM.

fimbrial function *in vivo*. Based on these results and sequence homology between SefD and other fimbrial systems, we propose that SefD encodes the adhesin subunit of SEF14 fimbriae. The translocation of SefD is a prerequisite for the export of SefA across the outer membrane (R.A.E., B. M. Matlock, and S.R.M., unpublished observations); thus, SefD is probably located at the tip of the fimbrial shaft. The major SEF14 subunit (SefA) was not required for the virulence of *S. enteritidis*, indicating that the tip of the fimbrial structure composed of SefD subunits is probably sufficient for successful interactions with phagocytes. It is possible that either the major subunit is redundant and SefD is presented on another fimbrial shaft, or that SefD tip structures can successfully fulfill all the functions required of the SEF14 fimbriae in the absence of SefA. When there is insufficient SefA, SefD may form a fibrillar structure on the bacterial cell surface (Fig. 2B) similar to that described for other fimbriae (21).

The role of SEF14 fimbriae in virulence has been controversial. For example, it has been shown that SEF14 fimbriae are not involved in primary attachment to intestinal epithelia (22), but SEF14 fimbriae can illicit a strong, protective, immune response (8, 11). The results presented here explain this paradox: SEF14 fimbriae are essential for proper interactions with macrophages subsequent to the colonization and penetration of the intestinal barrier.

In addition to demonstrating a role for SEF14 fimbriae in virulence, the results provide insight into the growth of *Salmonella in vivo*. It has previously been shown that rapidly after infection *S. enteritidis* transit from the peritoneum to the liver and spleen (23, 24), where they infect Kupffer cells, macrophages associated with liver sinusoids (25). For approximately 2–3 days, the number of bacteria increase slowly. During this time, *Salmonella* multiply inside the Kupffer cells, and any free bacteria released into the sinusoids by macrophage lysis are killed by neutrophils (25). The number of bacteria seen over the first 3 days presumably represents the equilibrium established between growth inside Kupffer cells and bactericidal activity of neutrophils (26). This cycle of infection of macrophages, release into the environment, and uptake into either neutrophils (lethal) or macrophages (safe havens) is thought to allow *Salmonella* to persist for up to 48 hr at the site of infection (27, 28). During this time, the infective foci include a large number of neutrophils and a small number of macrophages. After 48–72 hr, the neutrophils in the infective foci are replaced with activated macrophages,

which in a normal host would be able to ultimately clear the infection (29, 30). However, BALB/c mice contain a mutation in NRAMP-1 (natural resistance associated macrophage protein) that makes them susceptible to *Salmonella* infections by reducing the efficacy of the activated macrophage onslaught (31). The data shown in Figs. 3 and 4 demonstrate that *S. enteritidis* persists in the liver and spleen for the first 3 days but is not able to grow rapidly. However, as resident macrophages and neutrophils are replaced by activated macrophages, it is clear that *S. enteritidis* begins growing at an almost exponential rate until the mice succumb. In contrast, *S. enteritidis* that contain either the *sefA::Kan* or the Δ *sefD* mutations are no longer able to compete once the neutrophils are replaced by activated macrophages. Unless the immune system is overwhelmed with very large numbers of bacteria, mutant bacteria are eventually cleared and the mice survive the infections. The difference between wild-type and mutant bacteria becomes accentuated starting 3 days postinfection (Fig. 4), indicating that SefD is required for *S. enteritidis* to survive the assault by activated macrophages.

An initial step in virulence of *Salmonella* in mice involves attachment to the phagocyte surface before the bacteria are ingested and transported by the lymph system to the liver and spleen (23). SEF14 fimbriae could be involved in either attachment to the macrophage surface or survival after uptake. Attachment mediated between adhesins on the bacterial surface and receptors on the phagocyte surface has been characterized

in many bacteria (32–34). SefD may bind to a receptor on the macrophage surface and alter the uptake of *S. enteritidis* into the phagocyte so that *S. enteritidis* can survive in the intracellular environment. In contrast, mutants lacking *sefD* may be taken up by pathways that result in a hostile response from the phagocyte and lead to bacterial cell death. Alternatively, SefD may be required subsequent to uptake into the phagosome in a similar manner to that which has been proposed for the *Y. pestis* (pH 6) antigen (6, 12).

This is the first *Salmonella* fimbriae that has been shown to play an essential role in interactions with macrophages. All other *Salmonella* fimbriae that have been shown to be required for virulence are involved in binding to epithelial surfaces. Since *S. typhimurium* does not contain the *sef* operon, it seems likely that one or more of the currently uncharacterized fimbrial operons, or another adhesive structure (such as an afimbrial adhesin) may perform the functions in *S. typhimurium* that SEF14 fimbriae perform in *S. enteritidis*. The specific interactions between fimbrial adhesins and macrophages may be important determinants in the different etiology and epidemiology of the variety of *S. enterica* serovars.

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