

## Multiple Fimbrial Adhesins Are Required for Full Virulence of *Salmonella typhimurium* in Mice

ADRIANUS W. M. VAN DER VELDEN,<sup>1</sup> ANDREAS J. BÄUMLER,<sup>1,2</sup> RENÉE M. TSOLIS,<sup>1,3</sup> AND FRED HEFFRON<sup>1\*</sup>

Department of Molecular Microbiology and Immunology, Oregon Health Sciences University, Portland, Oregon 97201,<sup>1</sup>  
Department of Medical Microbiology and Immunology, Texas A&M University Health Science Center,  
College Station, Texas 77843-1114,<sup>2</sup> and Department of Veterinary Pathobiology,  
Texas A&M University, College Station, Texas 77843-4467<sup>3</sup>

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**Adhesion is an important initial step during bacterial colonization of the intestinal mucosa. However, mutations in the *Salmonella typhimurium* fimbrial operons *lpf*, *pef*, or *fim* only moderately alter mouse virulence. The respective adhesins may thus play only a minor role during infection or *S. typhimurium* may encode alternative virulence factors that can functionally compensate for their loss. To address this question, we constructed mutations in all four known fimbrial operons of *S. typhimurium*: *fim*, *lpf*, *pef*, and *agf*. A mutation in the *agfB* gene resulted in a threefold increase in the oral 50% lethal dose (LD<sub>50</sub>) of *S. typhimurium* for mice. In contrast, an *S. typhimurium* strain carrying mutations in all four fimbrial operons (quadruple mutant) had a 26-fold increased oral LD<sub>50</sub>. The quadruple mutant, but not the *agfB* mutant, was recovered in reduced numbers from murine fecal pellets, suggesting that a reduced ability to colonize the intestinal lumen contributed to its attenuation. These data are evidence for a synergistic action of fimbrial operons during colonization of the mouse intestine and the development of murine typhoid fever.**

*Salmonella enterica* serotype Typhimurium (*S. typhimurium*) causes murine typhoid fever. This systemic infection is initiated by colonization and penetration of the intestinal mucosa, which is commonly accepted as a necessary first step in the establishment of infection. Indeed, recent evidence suggests that fimbrial adhesins of *S. typhimurium* play a role during bacterial attachment to and invasion of the intestinal mucosa in vitro and in vivo (3, 5, 6, 16). For instance, attachment mediated by fimbrial adhesins appears to be important for invasion of cultured epithelial cell lines in vitro (4, 10, 11). In addition, a mutation in *pefC*, encoding the putative outer membrane usher of plasmid-encoded (PE) fimbriae, reduces the ability of *S. typhimurium* to attach to the murine villous small intestine (3). Furthermore, insertional inactivation of *lpfC*, encoding the putative outer membrane usher of long polar (LP) fimbriae, impairs colonization of murine Peyer's patches by *S. typhimurium* (5, 6). However, since mutations in fimbrial biosynthesis genes cause only a subtle decrease (3, 5) or even a slight increase (16) in mouse virulence, it is not evident from these data that adhesion mediated by fimbriae is essential during the development of murine typhoid.

Since blockage of individual adhesins does not strongly reduce mouse virulence of *S. typhimurium*, it has been speculated that attachment is not essential during murine typhoid (14). However, more recent evidence suggests an alternative interpretation of these data, namely that *S. typhimurium* encodes alternate pathways for intestinal penetration (6, 16). The presence of additional entry mechanisms may mask the effect of mutations in individual virulence genes of a single pathway. For example, a synergy of virulence factors involved in penetrating the intestinal mucosa is suggested by the fact that an *S. typhimurium lpfC invA* double mutant has a 150-fold increased oral 50% lethal dose (LD<sub>50</sub>). In contrast, isogenic

strains carrying a single insertion in either *lpfC* or *invA* are only 5- or 15-fold attenuated in mouse virulence, respectively (6). In addition, a similar synergistic effect has been observed for motility and type I fimbriation. Loss of motility has no effect on mouse virulence, and deletion of the *fim* operon, encoding type I fimbriae, results in a modest decrease in LD<sub>50</sub>. However, an *S. typhimurium* mutant that is both nonmotile and lacks type I fimbriae is 150-fold attenuated (16).

The presence of at least four distinct fimbrial operons in *S. typhimurium*, *fim* (8), *lpf* (2), *pef* (12), and *agf* (9), raises the possibility that *S. typhimurium* compensates for a functional defect of any individual fimbrial adhesin by producing alternate attachment elements. Redundancy in virulence determinants involved in intestinal colonization may explain why mutations that affect the expression of only one fimbrial structure have little to no effect on the ability of *S. typhimurium* to cause a lethal systemic infection in mice. Thus, a simultaneous loss of several fimbrial adhesins would be expected to reduce *S. typhimurium* virulence to a greater degree than mutations in individual fimbrial operons. To investigate whether inactivation of the genes essential to assembling distinct fimbrial adhesins has a synergistic effect on the ability of *S. typhimurium* to cause murine typhoid, we determined the virulence properties of strains carrying mutations in one or more fimbrial operons.

### MATERIALS AND METHODS

**Bacterial strains, bacteriophages, and recombinant DNA techniques.** Bacteria were grown overnight in Luria-Bertani broth at 37°C. Antibiotics, when required, were incorporated into the medium at the following concentrations: naladixic acid, 50 mg/liter; kanamycin, 60 mg/liter; chloramphenicol, 30 mg/liter; and carbenicillin, 100 mg/liter. Analytical-grade chemicals were purchased from Sigma (St. Louis, Mo.) or Boehringer Mannheim (Indianapolis, Ind.). AJB3 is a fully mouse virulent naladixic acid-resistant derivative of *S. typhimurium* SR-11 (3). SR-11 derivatives carrying a *pefC*::Tet<sup>r</sup> allele (AJB9) or a deletion of the *fim* operon (AJB4) have been described previously (3, 4). Bacteriophage KB1int or P22HTint was used to transduce a *pefC*::Tet<sup>r</sup> or *lpfC*::Kan<sup>r</sup> mutation from *S. typhimurium* AJB7 (3) or AJB1 (5), respectively, into the desired SR-11 background. Recombinant DNA techniques and Southern hybridizations were performed by using standard protocols (1).

A 927-bp fragment internal to *agfB* was amplified from  $\chi$ 252 (wild-type SR-11 [16]) with primers 5'-CTGACAGATGTTGCACTGCTGTG-3' and 5'-TTCGC

\* Corresponding author. Mailing address: Department of Molecular Microbiology and Immunology, Oregon Health Sciences University, 3181 SW Sam Jackson Park Rd., L220, Portland, OR 97201. Phone: (503) 494-6841. Fax: (503) 494-6862. E-mail: heffronf@ohsu.edu.

TABLE 1. Bacterial strains used in this study

Strain	Genotype	Source or reference
<i>E. coli</i>		
DH5 $\alpha$	<i>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 <math>\Delta</math>(lacZYA-argF)U169 deoR</i> [ $\phi$ 80 <i>dlac <math>\Delta</math>(lacZ)M15</i> ]	Laboratory collection
S17 $\lambda$ pir	<i>pro thi recA hsdR</i> ; chromosomal RP4-2 ( <i>TnI::ISR1 tet::Mu Km::Tn7</i> ); $\lambda$ pir	15
<i>S. typhimurium</i>		
AJB3	Wild-type (SR11 $\chi$ 4252 Nal <sup>r</sup> )	3
AJB4	$\Delta$ [ <i>fim-aph-11::Tn10</i> ]-391 Nal <sup>r</sup>	3
AJB5	$\Delta$ [ <i>aph-11::Tn10</i> ]-251 Nal <sup>r</sup> <i>lpfC::Kan<sup>r</sup></i>	This study
AJB6	$\Delta$ [ <i>fim-aph-11::Tn10</i> ]-391 Nal <sup>r</sup> <i>lpfC::Kan<sup>r</sup></i>	This study
AJB9	$\Delta$ [ <i>aph-11::Tn10</i> ]-251 Nal <sup>r</sup> <i>pefC::Tet<sup>r</sup></i>	3
AJB11	$\Delta$ [ <i>aph-11::Tn10</i> ]-251 Nal <sup>r</sup> <i>lpfC::Kan<sup>r</sup></i> <i>pefC::Tet<sup>r</sup></i>	This study
AJB12	$\Delta$ [ <i>fim-aph-11::Tn10</i> ]-391 Nal <sup>r</sup> <i>lpfC::Kan<sup>r</sup></i> <i>pefC::Tet<sup>r</sup></i>	This study
AWM394	$\Delta$ [ <i>aph-11::Tn10</i> ]-251 Nal <sup>r</sup> <i>agfB::Cam<sup>r</sup></i>	This study
AWM400	$\Delta$ [ <i>aph-11::Tn10</i> ]-251 Nal <sup>r</sup> <i>lpfC::Kan<sup>r</sup></i> <i>pefC::Tet<sup>r</sup></i> <i>agfB::Cam<sup>r</sup></i>	This study
AWM401	$\Delta$ [ <i>fim-aph-11::Tn10</i> ]-391 Nal <sup>r</sup> <i>lpfC::Kan<sup>r</sup></i> <i>pefC::Tet<sup>r</sup></i> <i>agfB::Cam<sup>r</sup></i>	This study
IR715	ATCC 14028 Nal <sup>r</sup>	22
AJB1	IR715 <i>lpfC::Kan<sup>r</sup></i>	5
AJB7	IR715 <i>pefC::Tet<sup>r</sup></i>	3
SR-11 $\chi$ 4252	Wild-type $\Delta$ [ <i>aph-11::Tn10</i> ]-251	16

CCGATTATTTCTCC-3'. This PCR product was cloned into the *EcoRV* site of pBluescript SK to yield plasmid pAV326. The *agfB* allele was inactivated upon insertion of a chloramphenicol resistance gene (a 1.2-kb *SmaI* fragment from pCMXX [6]) into a unique *NruI* site (nucleotide 466). This plasmid was digested with *SacI* and *KpnI*, and a 2.2-kb fragment was cloned into suicide vector pGP704 (19). The resulting plasmid (pAV328) was transformed into *Escherichia coli* S17 $\lambda$ pir (15) and conjugated into *S. typhimurium* AJB3 (wild type) and AJB12 ( $\Delta$ *fim lpfC pefC*). A double cross-over was obtained by homologous recombination. Chloramphenicol-resistant, carbenicillin-sensitive (loss of vector pGP704) exconjugants were screened for and named AWM394 (*agfB*) and AWM401 ( $\Delta$ *fim lpfC pefC agfB*).

DNA probes specific for *fim*, *lpf*, *pef*, and *agf* were used as probes for Southern hybridization. In brief, a *SphI* fragment of pISF101 (7) and a *SacI-KpnI* fragment of pMS1054 (2) served as probes to detect *fim*- and *lpf*-specific loci, respectively. A 520-bp fragment internal to *pefA* was amplified by PCR with primers 5'-GG GAATTCCTTGCTTCCATTATTGCACTGGG-3' and 5'-TCTGTCCGACGGG GATTATTTGTAAGCCACT-3' and cloned into the *EcoRV* site of pBluescript (21) to give rise to plasmid pAV323. The *EcoRI*- and *ClaI*-restricted insert of pAV323 was labeled and used as a *pef*-specific probe. A *SacI-KpnI* fragment of pAV326 was used to generate an *agf*-specific probe. Restriction enzyme-digested chromosomal DNA was separated on an agarose gel and transferred onto a positively charged membrane (Boehringer Mannheim). The predicted sizes of hybridizing fragments were as follows. A *fim*-specific probe detected a 13.7-kb fragment in *SphI*-restricted chromosomal DNA of *fim*<sup>+</sup> strains (AJB3, AJB5, AJB9, AJB11, AWM394, and AWM400) and 10.5- and 3.1-kb fragments in *SphI*-restricted chromosomal DNA of *fim* mutants (AJB4, AJB6, AJB12, and AWM401). An *lpf*-specific probe detected a 3.7-kb fragment in *PstI*-restricted chromosomal DNA of *lpfC*<sup>+</sup> strains (AJB3, AJB4, AJB9, and AWM394) and 2.8- and 1.7-kb fragments in *PstI*-restricted chromosomal DNA of *lpfC* mutants (AJB5, AJB6, AJB11, AJB12, AWM400, and AWM401). A *pef*-specific probe detected a 3.6-kb fragment in *EcoRI*- and *HindIII*-restricted chromosomal DNA of *pefC*<sup>+</sup> strains (AJB3, AJB4, AJB5, AJB6, and AWM394) and a 2.8-kb fragment in *EcoRI*- and *HindIII*-restricted chromosomal DNA of *pefC* mutants (AJB9, AJB11, AJB12, AWM400, and AWM401). An *agf*-specific probe detected a 1.8-kb fragment in *EcoRI*- and *SalI*-restricted chromosomal DNA of *agfB*<sup>+</sup> strains (AJB3, AJB4, AJB5, AJB6, AJB9, AJB11, and AJB12) and a 3.0-kb fragment in *EcoRI*- and *SalI*-restricted chromosomal DNA of *agfB* mutants (AWM394, AWM400, and AWM401). Detection was performed by using the Renaissance random primer fluorescent dUTP labeling and detection system from DuPont NEN (Boston, Mass.).

**Mouse experiments.** Six- to eight-week-old female BALB/c mice (Jackson Laboratories, Bar Harbor, Maine) were used throughout this study. To determine the (two-step) LD<sub>50</sub>, a series of 10-fold dilutions of overnight cultures in a 0.2-ml volume were injected intragastrically into groups of four mice. The LD<sub>50</sub>s were calculated 28 days postinfection by the method of Reed and Muench (20). For course of infection studies, approximately 10<sup>8</sup> bacteria were administered to groups of four mice by intragastric injection. Five days postinfection, the animals

were sacrificed, after which internal organs (Peyer's patches, villous intestinal tissues, mesenteric lymph nodes, spleens, and livers) were collected and homogenized in 5 ml of phosphate-buffered saline (PBS) by using a stomacher (Tekmar, Cincinnati, Ohio). To test the ability of a particular strain to colonize the intestinal lumen, fecal pellets were collected at days 1, 3, and 5 postinfection and homogenized in 5 ml of PBS. A 10-fold dilution series was plated on Luria-Bertani agar plates containing the appropriate antibiotics to determine the number of CFU. Results are reported in CFU per organ or per gram of feces (single strain infections) or as percentages of the total number of bacteria recovered (mixed infections). A paired *t* test was used to calculate statistical differences between arithmetic means.

**Electron microscopy.** Bacterial strains were grown as 3-ml static broth cultures to promote expression of fimbrial structures. Subsequently, 15  $\mu$ l of bacterial suspension was pipetted onto a Formvar-coated grid (Ted Pella Inc., Redding, Calif.). Bacteria were allowed to adhere for 2 min and then were fixed for 1 min with 1.5% glutaraldehyde in sodium cacodylate buffer (100 mM, pH 7.4). The grids were rinsed twice with water and negatively stained with 0.75% (wt/vol) uranyl acetate (pH 6.4) for 1 min. The grids were drained and subjected to microscopic studies.

## RESULTS

**Construction of *S. typhimurium* fimbrial mutants.** Mutations in three fimbrial operons, *fim*, *lpf*, and *pef*, have been reported previously (3, 5, 16) and were used to construct a set of isogenic *S. typhimurium* mutants that carried deletions of and/or insertions in essential fimbrial biosynthesis genes (Table 1). The *lpfC::Kan<sup>r</sup>* allele of *S. typhimurium* ATCC 14028 derivative

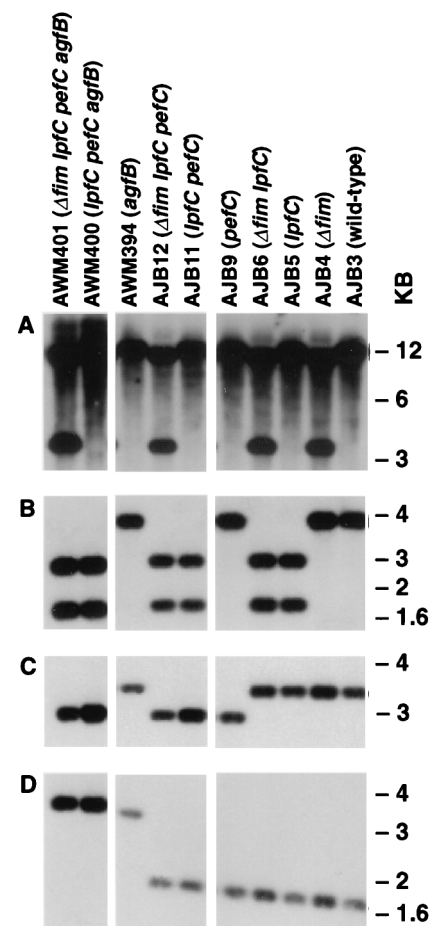


FIG. 1. Southern blot analyses of chromosomal DNA digested with *SphI* using a *fim*-specific probe (A), of chromosomal DNA digested with *PstI* using an *lpf*-specific probe (B), of chromosomal DNA digested with *EcoRI* and *HindIII* using a *pef*-specific probe (C), and of chromosomal DNA digested with *EcoRI* and *SalI* using an *agf*-specific probe (D). For further details, see Materials and Methods. Molecular sizes in kilobases (kb) are shown at right.

TABLE 2. Virulence properties of fimbrial mutants of *S. typhimurium* when administered orally to BALB/c mice

Strain	Relevant genotype	i.g. <sup>a</sup> LD <sub>50</sub>	Fold attenuation	Reference
AJB3	Wild type	$5.8 \times 10^5$	1.0	4
AJB4	$\Delta fim$	$1.5 \times 10^5$	0.3	4
AJB5	<i>lpfC</i>	$2.8 \times 10^6$	4.8	This study
AJB9	<i>pefC</i>	$1.4 \times 10^6$	2.4	3
AWM394	<i>agfB</i>	$1.9 \times 10^6$	3.3	This study
AJB12	$\Delta fim$ <i>lpfC</i> <i>pefC</i>	$1.7 \times 10^5$	0.3	This study
AWM400	<i>lpfC</i> <i>pefC</i> <i>agfB</i>	$1.7 \times 10^7$	>29	This study
AWM401	$\Delta fim$ <i>lpfC</i> <i>pefC</i> <i>agfB</i>	$1.5 \times 10^7$	26.4	This study

<sup>a</sup> i.g., intragastric.

AJB1 (5) was transduced into SR-11 derivatives AJB3 (wild type) and AJB4 ( $\Delta fim$ ) (3), yielding strains AJB5 (*lpfC*) and AJB6 ( $\Delta fim$  *lpfC*), respectively. The *pefC*::Tet<sup>r</sup> allele of strain AJB7 (3) was transduced into AJB3 and AJB6 to give rise to strains AJB9 (*pefC*) and AJB12 ( $\Delta fim$  *lpfC* *pefC*), respectively (Table 1). The *lpfC*::Kan<sup>r</sup> allele of AJB1 was then transduced into AJB9 to give rise to AJB11 (*lpfC* *pefC*) (Table 1). All mutants were confirmed by Southern blot analysis with the appropriate DNA probes (Fig. 1).

Thin aggregative fimbriae, which are encoded by the *S. typhimurium* *agf* operon, are assembled by an export machinery

that is distinct from the chaperone- and usher-dependent transport systems of type 1 fimbriae, PE fimbriae, or LP fimbriae. Curli, encoded by the *csg* operon in *E. coli*, is the prototypic member of this novel pilus assembly class. Recent evidence by Hammar et al. suggests that CsgB, a membrane-associated nucleator protein, is required for the assembly of curli fimbriae on the bacterial cell surface (13). It was therefore decided to inactivate *agfB*, the *csgB* homolog in *S. typhimurium* (9). An *agfB* allele (carried on plasmid pAV328) was inactivated by insertion of a 1.2-kb chloramphenicol resistance cassette and introduced into strains AJB3 (wild type), AJB11 (*lpfC* *pefC*), and AJB12 ( $\Delta fim$  *lpfC* *pefC*). Double cross-over events were obtained by homologous recombination, and the resulting strains were designated AWM394 (*agfB*), AWM400 (*lpfC* *pefC* *agfB*), and AWM401 ( $\Delta fim$  *lpfC* *pefC* *agfB*), respectively (Table 1). All three mutants were confirmed by Southern blot analysis with an *agfB*-specific DNA probe (Fig. 1).

**Synergistic effect of mutations in fimbrial operons on mouse virulence.** LD<sub>50</sub> studies were conducted to investigate the effect of mutations in fimbrial operons on mouse virulence (Table 2) (20). Strains carrying mutations in a single fimbrial operon were either more virulent (AJB4,  $\Delta fim$ ) or less than fivefold attenuated (AJB5, *lpfC*; AJB9, *pefC*; and AWM394, *agfB*) in comparison with the wild type (AJB3). Strain AJB12 ( $\Delta fim$  *lpfC* *pefC*) also exhibited slightly increased virulence, sug-

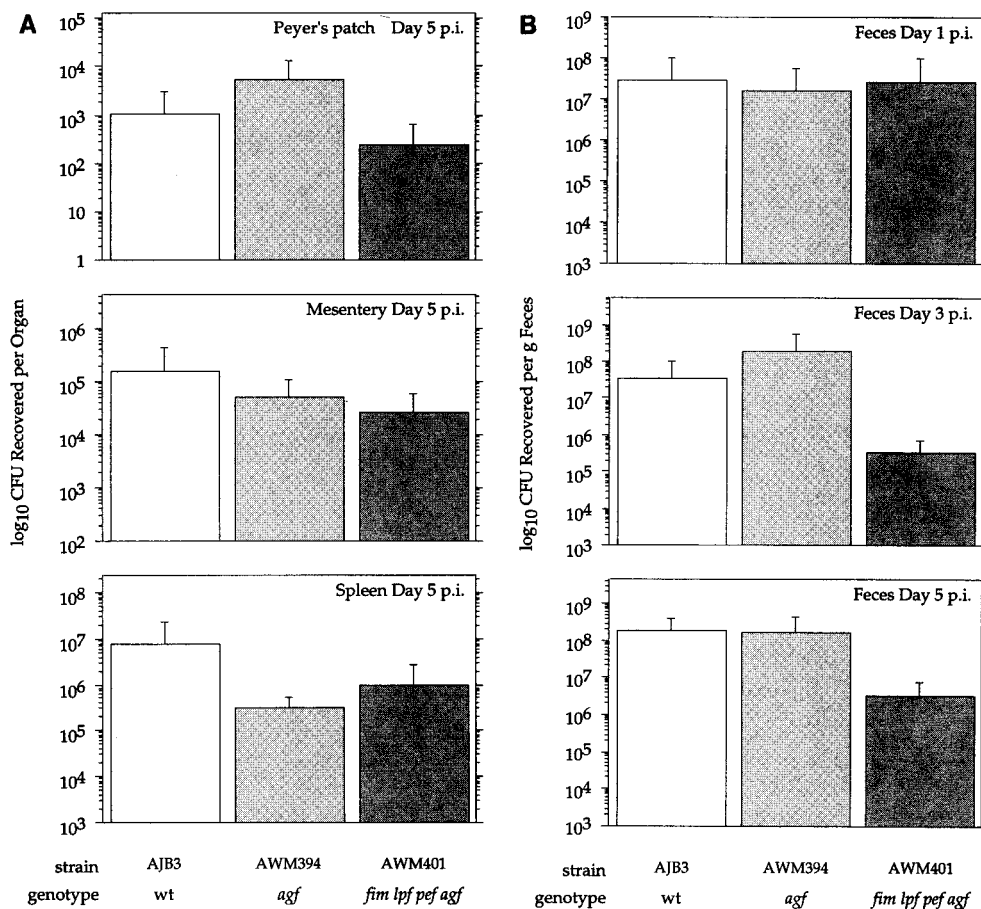


FIG. 2. Bacterial recovery from internal organs 5 days postinfection (p.i.) (A) and feces 1, 3, and 5 days postinfection (B) reported in CFU per organ (three Peyer's patches in the terminal ileum, close to the cecum, were collected and pooled for each mouse) or CFU per gram of feces. Three groups of four mice each were orally infected with  $10^8$  CFU of AJB3 (wild type [wt]), AWM394 (*agfB*), or AWM401 ( $\Delta fim$  *lpfC* *pefC* *agfB*). Data are arithmetic means. Error bars indicate standard deviations.

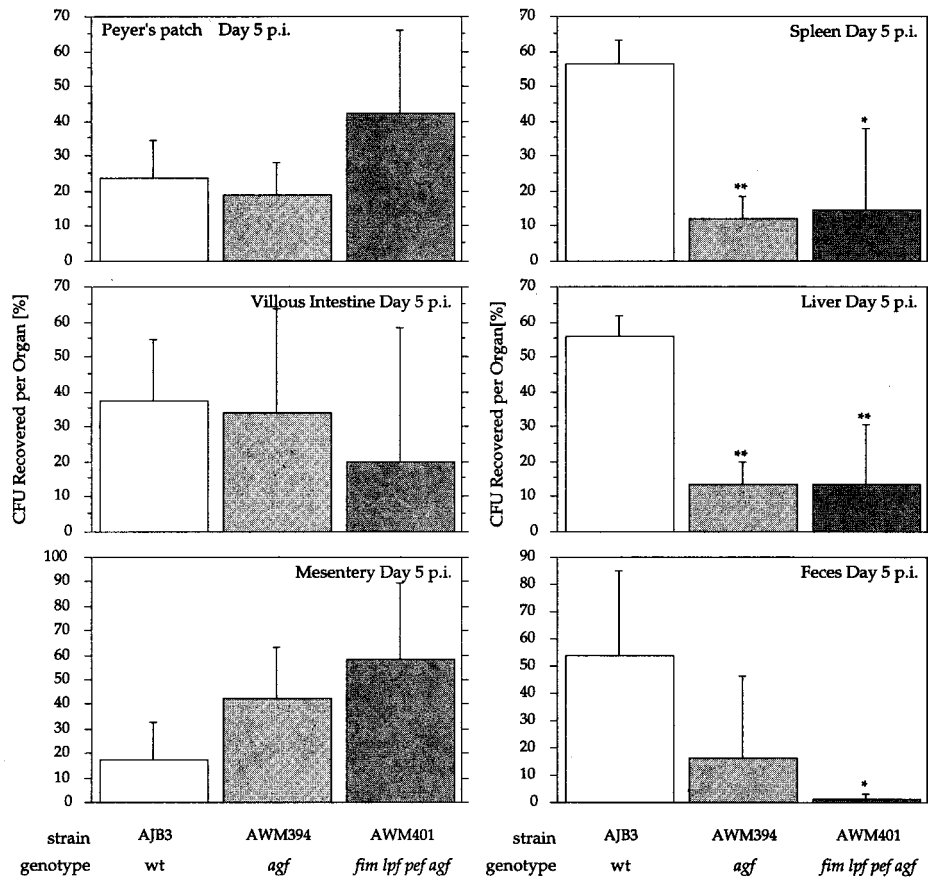


FIG. 3. Bacterial recovery from internal organs and feces 5 days postinfection (p.i.) reported as percentages of the total number of bacteria recovered. Three groups of four mice each were orally infected with a 1:1:1 mixture of three strains, AJB3 (wild type [wt]), AWM394 (*agfB*), and AWM401 ( $\Delta$ *fim lpf pefC agfB*), respectively, for a total of  $10^8$  CFU per mouse. Three Peyer's patches in the terminal ileum, close to the cecum, were collected and pooled for each mouse. Data are arithmetic means. Error bars indicate standard deviations. \*,  $P < 0.05$  (paired *t* test); \*\*,  $P < 0.01$  (paired *t* test).

gesting that the phenotype of a *fim* deletion mutant is dominant over the attenuating effect of mutations in *lpf* and *pef*. Interestingly, AWM400 (*lpfC pefC agfB*) is more strongly attenuated (>29-fold) than AWM401 ( $\Delta$ *fim lpfC pefC agfB*) (see below and Table 2). We and others have observed that all *fim* mutants tested had a slight increase in virulence compared to that of the wild type (16) (Table 2 and our unpublished results). These data suggest a dominant phenotype for mutant *fim* alleles.

AWM401 ( $\Delta$ *fim lpfC pefC agfB*), a quadruple fimbrial mutant, was more strongly attenuated (26-fold) than AJB12 ( $\Delta$ *fim lpfC pefC*) or any of the strains carrying a single fimbrial mutation. This result suggested an additive attenuating effect of these mutations on the ability of *S. typhimurium* to cause murine typhoid (Table 2). Furthermore, the increased virulence of strain AJB12 ( $\Delta$ *fim lpfC pefC*) compared to that of AWM401 ( $\Delta$ *fim lpfC pefC agfB*) supports the idea that the insertional inactivation of *agfB* is one of the mutations responsible for the strong attenuation of the quadruple mutant.

**The quadruple mutant has a reduced ability to colonize liver, spleen, and intestine.** To investigate at which step during the infection process AWM401 ( $\Delta$ *fim lpfC pefC agfB*) is impaired, course of infection studies were conducted. Since our mouse virulence data (Table 2) suggested that a mutation in *agfB* in combination with a mutation in at least one other fimbrial operon is responsible for the attenuation of AWM401 ( $\Delta$ *fim lpfC pefC agfB*), strain AWM394 (*agfB*) was included in these studies. Groups of four mice were orally infected with  $10^8$  CFU, and bacteria were recovered from Peyer's patches,

mesenteric lymph nodes, and spleens on days 3 (data not shown) and 5 postinfection (Fig. 2A). In addition, bacteria were recovered from the feces on days 1, 3, and 5 postinfection to monitor intestinal colonization (Fig. 2B). Compared to the wild type (AJB3), reduced numbers of both AWM401 ( $\Delta$ *fim lpfC pefC agfB*) and AWM394 (*agfB*) were recovered from internal organs and fecal pellets. However, these differences proved not to be statistically significant ( $P > 0.05$ ). As bacterial numbers recovered from individual animals may vary greatly during infection, small differences between wild type and mutant may go undetected. In order to control for the variability between experimental animals, mixed infections with AJB3 (wild type), AWM394 (*agfB*), and AWM401 ( $\Delta$ *fim lpfC pefC agfB*) were performed, permitting a direct comparison between wild type and mutants. A group of four mice was orally infected with  $10^8$  CFU of a mixture containing approximately equal amounts of AJB3 (wild type), AWM394 (*agfB*), and AWM401 ( $\Delta$ *fim lpfC pefC agfB*). On day 5 postinfection, CFU in internal organs (Peyer's patches, villous intestinal tissues, mesenteric lymphs, spleens, and livers) were determined. In addition, bacteria were recovered from the feces up to 5 days postinfection to monitor intestinal colonization (Fig. 3). Both AWM394 (*agfB*) and AWM401 ( $\Delta$ *fim lpfC pefC agfB*) were able to compete with the wild type (AJB3) for colonization of Peyer's patches and villous intestinal tissues in the terminal ileum. Interestingly, increased numbers of both the quadruple mutant (AWM401) and the *agfB* mutant (AWM394) were recovered from the mesenteric lymph nodes compared to that

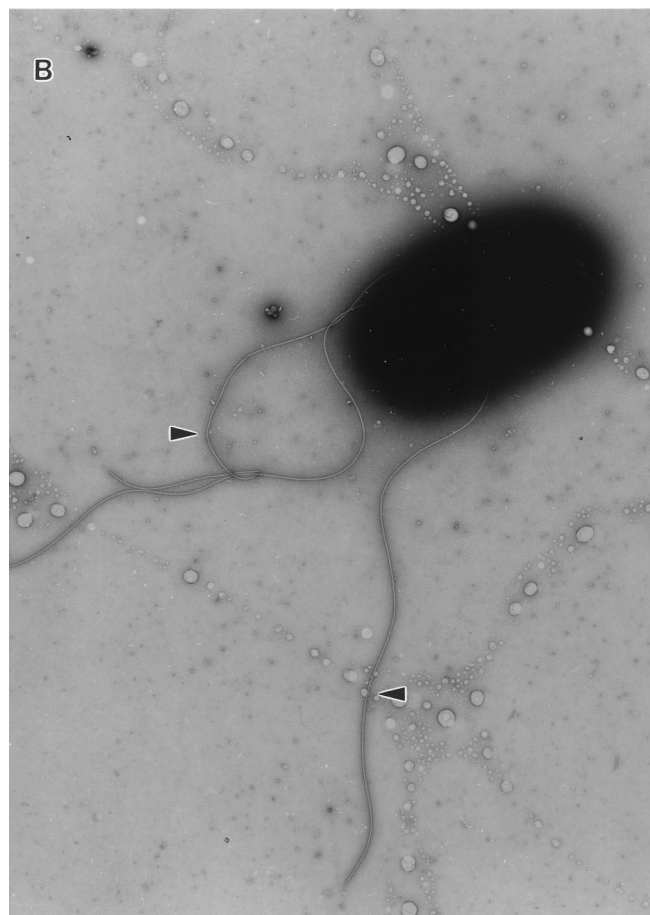
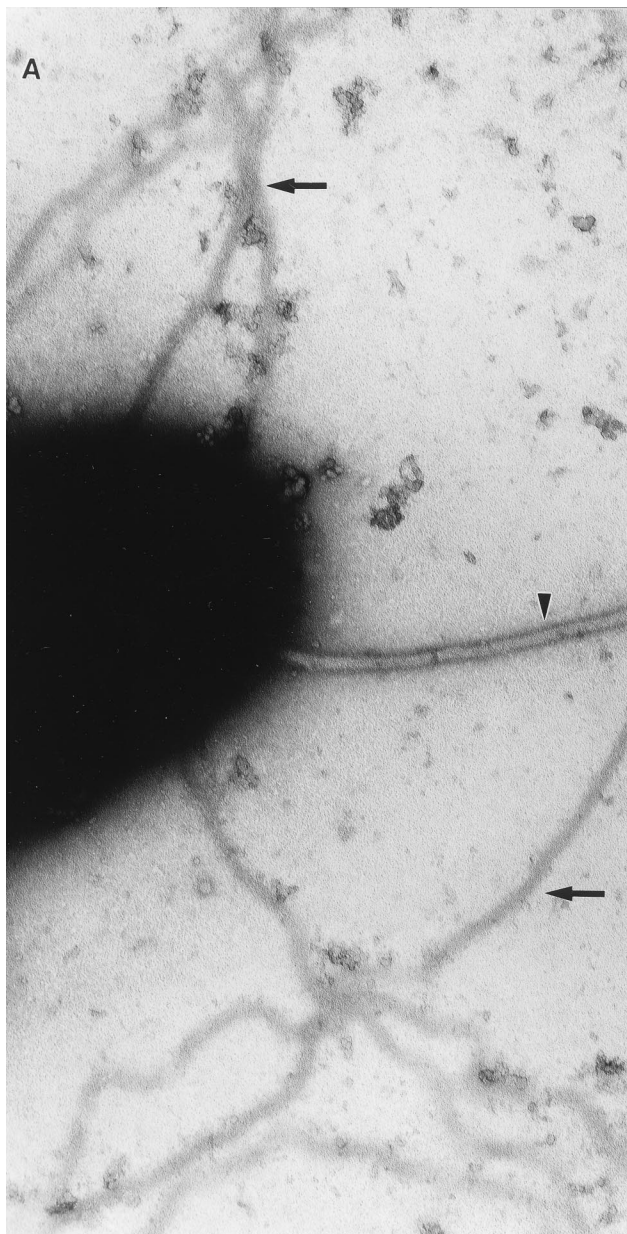


FIG. 4. Electron micrographs of AWM401, which harbors mutations in the *fim*, *lpf*, *pef*, and *agf* fimbrial operons. This quadruple mutant expresses a thus far uncharacterized fimbrial structure (A, arrows) that can be distinguished from flagellar filaments (A and B, arrowheads). Magnification,  $\times 35,000$  (A) and  $\times 8,000$  (B).

of the wild type (AJB3). These differences were not statistically significant ( $P > 0.05$ ). However, both AWM394 (*agfB*) and AWM401 ( $\Delta fim\ lpfC\ pefC\ agfB$ ) were outcompeted by the wild type (AJB3) for colonization of the liver and spleen ( $P < 0.05$  and  $P < 0.01$ , respectively). In addition, AWM401 ( $\Delta fim\ lpfC\ pefC\ agfB$ ) failed to compete with the wild type for colonization of the intestine, as suggested by recovery of significantly reduced numbers of AWM401 from fecal pellets ( $P < 0.05$ ). These results provide evidence that fimbrial adhesins act synergistically during colonization of the mouse intestinal tract.

**Identification of new fimbrial structures.** Although we have demonstrated that fimbrial adhesins of *S. typhimurium* play an important role during infection (Table 2), AWM401 ( $\Delta fim\ lpfC\ pefC\ agfB$ ) was still able to cause a lethal systemic illness in mice when administered at higher doses. These data suggest that AWM401 ( $\Delta fim\ lpfC\ pefC\ agfB$ ) may express yet other factors for intestinal attachment. To investigate this possibility,

we examined strain AWM401 ( $\Delta fim\ lpfC\ pefC\ agfB$ ) by electron microscopy for the presence of fimbriae. Interestingly, this mutant (AWM401) expressed thus far uncharacterized fimbrial structures (Fig. 4A), which could easily be distinguished from flagellar filaments required for cell motility (Fig. 4). Flagellar filaments varied in length from 5 to 10  $\mu\text{m}$ , with a diameter of approximately 20 nm (18). Fimbriae could be distinguished from flagella by means of morphology and diameter (typically between 2 and 8 nm [17]). These data provide direct evidence for the expression of at least one yet uncharacterized fimbrial structure in *S. typhimurium* which may contribute to the redundancy of virulence factors involved in colonization of the intestinal mucosa.

## DISCUSSION

Our results demonstrate that despite the moderate effect on mouse virulence of individual mutations in fimbrial operons, the simultaneous inactivation of genes involved in the biosynthesis of four distinct fimbrial adhesins markedly attenuates *S. typhimurium*. To our knowledge, this is the first study to provide direct evidence for a synergistic effect of fimbrial adhesins during infection. Previous studies have shown that inactivation of biosynthetic genes for type 1 fimbriae, LP fimbriae, or PE fimbriae attenuate *S. typhimurium* mouse virulence only fivefold or less (3, 5, 16). Here, we report that a

mutation in a fourth *S. typhimurium* fimbrial operon, *agf*, resulted in a threefold reduction in mouse virulence. A recent study indicated that thin aggregative fimbriae mediate adhesion to murine small intestinal epithelial cells (23). We have observed that strains carrying the *agfB* mutation have an altered colony morphology (data not shown). A pleiotropic effect for *agf* mutants regarding colony morphology has also been reported by others (23). However, our virulence data strongly suggests that this pleiotropic effect does not reduce the ability to cause murine typhoid (Table 2 and Fig. 2, AWM394 [*agfB*]). Furthermore, from these data it is evident that inactivation of individual adhesins does not strongly reduce the ability of *S. typhimurium* to cause a lethal systemic infection in mice. However, strain AWM401, in which all four known fimbrial operons are inactivated, was 26-fold attenuated when orally administered to mice. These results are consistent with the idea that mutations in individual *S. typhimurium* fimbrial operons have only moderate effects on mouse virulence because the lack of a single attachment factor can be compensated for by the presence of other adhesins.

Because a strain carrying mutations in *fim*, *lpf*, and *pef* (AJB12) was not attenuated, insertional inactivation of *agfB* must be partly responsible for the strong attenuation of AWM401 ( $\Delta$ *fim lpfC pefC agfB*). Neither the *agfB* mutant (AWM394) nor the quadruple mutant (AWM401) were able to compete with the wild type (AJB3) for colonization of the liver and spleen ( $P < 0.05$  and  $P < 0.01$ , respectively). However, during mixed infection experiments, only AWM401 ( $\Delta$ *fim lpfC pefC agfB*) was recovered in reduced numbers from fecal pellets ( $P < 0.05$ ), suggesting that the decreased virulence of AWM401, compared to that of AWM394 (*agfB*), is caused by a defect in intestinal colonization. From these results, we conclude that the absence of at least two fimbrial structures may significantly decrease adherence to murine intestinal tissue and further reduce mouse virulence. Additional studies are needed to identify which combination of mutations in fimbrial operons reduces virulence.

The ability of AWM401 ( $\Delta$ *fim lpfC pefC agfB*) to cause a lethal systemic infection in mice upon intragastric injection of large inocula suggested that a quadruple mutant might express additional means of adhesion and colonization. Electron microscopic studies demonstrated that, in addition to flagellar filaments, AWM401 ( $\Delta$ *fim lpfC pefC agfB*) expresses at least one additional, yet uncharacterized, fimbrial structure. Thus, this fimbrial structure, and possibly others, may be the adhesive organelle(s) that allows residual colonization of the mouse intestine by *S. typhimurium* in the absence of type 1, LP, PE, and thin aggregative fimbriae.

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