Pathogenic Role of SEF14, SEF17, and SEF21 Fimbriae in Salmonella enterica Serovar Enteritidis Infection of Chickens

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Very little is known about the contribution of surface appendages of Salmonella enterica serovar Enteritidis to pathogenesis in chickens. This study was designed to clarify the role of SEF14, SEF17, and SEF21 fimbriae in serovar Enteritidis pathogenesis. Stable, single, defined sefA (SEF14), agfA (SEF17), and fimA (SEF21) insertionally inactivated fimbrial gene mutants of serovar Enteritidis were constructed. All mutant strains invaded Caco-2 and HT-29 enterocytes at levels similar to that of the wild type. Both mutant and wild-type strains were ingested equally well by chicken macrophage cell lines HD11 and MQ-NCSU. There were no significant differences in the abilities of these strains to colonize chicken ceca. The SEF14 strain was isolated in lower numbers from the livers of infected chickens and was cleared from the spleens faster than other strains. No significant differences in fecal shedding of these strains were observed.

Salmonellosis is the third most commonly reported infectious disease in the United States. Since the middle 1980s, most human cases reported to the National Salmonella Surveillance System administered by the Centers for Disease Control and Prevention have been attributed to Salmonella enterica serovar Enteritidis. More than 80% of food-borne serovar Enteritidis outbreaks reported since 1985 are associated with the consumption of raw or undercooked eggs (16).

Salmonella species enter the host by invading the gastrointestinal mucosa, a step essential for pathogenesis (13). Fimbriae have been shown to be involved in colonization and in adherence to specific host target tissues in the early stages of infection (5, 14, 15). Studies involving fimbriae have drawn considerable interest because fimbriae can serve as potential immunogens against many pathogenic bacteria that colonize epithelial surfaces (3–5, 20). Currently, we are exploring the use of fimbrial antigens as vaccine candidates. However, the role of fimbriae in pathogenesis of serovar Enteritidis is poorly understood. More knowledge is necessary to clarify the role of serovar Enteritidis fimbriae in vaccines designed to reduce the colonization and prevalence of this bacterium in poultry.

Serovar Enteritidis produces at least five distinct fimbriae, namely SEF14 and SEF17 (type 1 fimbriae) and SEF21, LPF, and PEF (28). Of all serovar Enteritidis fimbriae, SEF14 has been studied the most. SEF14 has been shown to contribute to serovar Enteritidis adherence to mouse epithelial cells, and passive administration of SEF14 antibodies is protective in mice (26). However, in vitro and in vivo studies using isogenic mutants of serovar Enteritidis have demonstrated no role for SEF14 in pathogenesis (25, 28). Information regarding the role of SEF17 and SEF21 fimbriae in serovar Enteritidis pathogenesis is lacking. Because the factors which influence fimbrial phase variation in vivo and which influence variability in the fimbriae of cells carrying an intact fimbrial operon are not known, it is difficult to evaluate the role of fimbriae in pathogenesis. Therefore, a genetic approach was used in this study to investigate the role of the three known serovar Enteritidis fimbrial operons, sefA (7), agfA (10), and fimA (22), which encode SEF14, SEF17, and SEF21 fimbriae, respectively. The ability of these sefA, agfA, and fimA mutant strains to mediate colonization and invasion was assessed in vitro and in a chicken model.

Bacterial strains, plasmids, and growth conditions. Serovar Enteritidis phage type 4 strain 4 was originally isolated from the liver of a laying chicken (18). A spontaneous nalidixic-acid-resistant (NalR) mutant was obtained by plating on a nalidixic-acid-containing Luria-Bertani (LB) agar plate and was used as the recipient strain for conjagation. Escherichia coli S17-1/pir was used as the donor strain. Inactivation of fimbrial genes was carried out by using suicide plasmid vector PKNOCK-Km (2).

Preparation of gene knockout constructs. Internal fragments (lacking sequences on 5′ and 3′ ends) of the sefA, agfA, and fimA genes were amplified from the serovar Enteritidis genomic DNA by PCR by using the following primers: for 5′ end of each primer to confer a recognition sequence.
for XhoI (underlined above). The amplification conditions were 94°C for 1.5 min, 56°C for 1 min, and 72°C for 2 min, for 30 cycles. PCR products were purified from 1% agarose gel and cloned into pGEM-T vector. Sequences of the inserts were confirmed by automated DNA sequencing. Finally, internal sequences of sefA, agfA, and fimA were subcloned into XhoI-digested pKNOCK-Km suicide vector, thus creating pKNOCK-Km/ΔsefA, pKNOCK-Km/ΔagfA, and pKNOCK-Km/ΔfimA, respectively. Resulting plasmids were electroporated into E. coli S17-1/pir donor cells. Electroporation was performed in a Gene Pulser apparatus according to the manufacturer’s instructions (Bio-Rad Laboratories, Richmond, Calif.). Recombinant clones were selected based on restriction enzyme analysis of the plasmid DNA extracted from kanamycin-resistant (Km') colonies.

**Construction of fimbrial gene knockouts.** SEF14”, SEF17”, and SEF21” strains of serovar Enteritidis were constructed by inactivation of the respective genes through homologous exchange of DNA material between the wild-type fimbrial genes and 5”, 3’-truncated fimbrial genes on pKNOCK. Suicide vector constructs pKNOCK-Km/ΔsefA, pKNOCK-Km/ΔagfA, and pKNOCK-Km/ΔfimA were mobilized from donor E. coli S17-1/pir into spontaneous Nalr serovar Enteritidis by conjugation as described (1). The Km” and Nal” bacterial colonies were selected and analyzed by PCR, nucleotide sequence analysis, and Western blotting. For each conjugation experiment, at least 20 exconjugants were tested for the presence of autonomously replicating pKNOCK plasmid. All exconjugants tested had integrated the plasmid DNA into their genome (results not shown). For PCR, regions of chromosomal DNA flanking the insertion site were amplified by using two pairs of primers for each exconjugant tested. One primer in each pair was specific to the chromosomal DNA flanking the insertion site (5’ or 3’ sequences of the fimbrial gene absent in the pKNOCK construct) and another was specific to the Km” gene (forward, 5’TGGGTTGGAGGTATTTCC and reverse, 5’CACCATGATATTCCGCAAGC). The chromosomal-DNA-specific primer sequences were as follows: for sefA, forward, 5’ATGCGTAAATACGATCTGCA and reverse, 5’TTAGTTTTTGATACTCGTGAACGTAAG; for agfA, forward, 5’ATGAAACTCTTAAAGTGCCACG and reverse, 5’AGCGCGAGCGCTAAAATTACTGCT; and for fimA, forward, 5’TGATATTCGGCAAGC). The chromosomal-DNA-specific primer sequences were as follows: for sefA, forward, 5’ATGCGTAAATACGATCTGCA and reverse, 5’TTAGTTTTTGATACTCGTGAACGTAAG; for agfA, forward, 5’ATGAAACTCTTAAAGTGCCACG and reverse, 5’AGCGCGAGCGCTAAAATTACTGCT; and for fimA, forward, 5’TGATATTCGGCAAGC). The chromosome-DNA-specific primer sequences were as follows: for sefA, forward, 5’ATGCGTAAATACGATCTGCA and reverse, 5’TTAGTTTTTGATACTCGTGAACGTAAG; for agfA, forward, 5’ATGAAACTCTTAAAGTGCCACG and reverse, 5’AGCGCGAGCGCTAAAATTACTGCT; and for fimA, forward, 5’TGATATTCGGCAAGC). The amplification conditions were 94°C for 1.5 min, 56°C for 1 min, and 72°C for 2 min, for 30 cycles. PCR products were purified from 1% agarose gel with identity confirmed by nucleotide sequence analysis. More than 90% of the clones tested from each conjugation reaction were confirmed as wild-type counterparts. No evidence for the presence of an altered sefA, agfA, or fimA allele in SEF14”, SEF17”, and SEF21” strains was observed, and PCR with wild-type-gene-specific primers failed to amplify specific DNA fragments from these strains.

For Western blot analyses, whole-cell lysates from serovar Enteritidis exconjugants were prepared as previously described (8, 9). The samples were separated on 12% polyacrylamide gels, were transferred onto a nitrocellulose membrane, and were stained with monospecific polyclonal antibodies to SEF14, SEF17, and SEF21 proteins. However, three antigens were detected in extracts of the wild-type serovar Enteritidis (Fig. 1).

The abilities of wild-type serovar Enteritidis and fimbrial mutants to invade HT-29 and Caco-2 enterocytes. The abilities of wild-type serovar Enteritidis and fimbrial mutants to invade HT-29 and Caco-2 enterocytes were compared. Individual bacterial strains were grown in static CFA or T medium for 48 to 72 h (9, 22) at 37°C, were washed, and were diluted to the appropriate concentration in tissue culture medium. Bacterial concentrations were determined by densitometry and were confirmed by serial dilution followed by viable plate counts on MacConkey agar. One milliliter of bacterial suspension con-
containing either $10^6$ or $10^7$ viable bacteria was added to each tissue culture well (15-mm-diameter, 24-well microtiter plates; Nunc) containing approximately $10^9$ enterocytes, and invasion assays were performed as previously described (31). Each bacterial strain was tested in at least three separate assays, each assay representing the average of triplicate tissue culture wells. The lower limit of assay detection was 50 bacteria; for statistical analysis, values below this limit were assigned a value equal to the lower limit of assay detection. Figure 2 shows the number of viable *Salmonella* cells internalized by HT-29 and Caco-2 enterocytes. In general, serovar Enteritidis strains were more invasive in Caco-2 cells than in HT-29 cells. The mutant strains were internalized by Caco-2 and HT-29 enterocytes as efficiently as the wild-type serovar Enteritidis. Quantitative recovery of viable intracellular bacteria was reproducible, as indicated by small standard error bars (Fig. 2). Previous studies involving SEF14 and epithelial cell monolayers have produced variable results. SEF14 fimbriae have been shown to contribute to serovar Enteritidis adherence to mouse epithelial cells (25, 26) but not to adherence and invasion of human HEp-2, INT-407, Caco-2, and HeLa cells (12, 25, 28), although a SEF14 mutant derived from avirulent serovar Enteritidis invaded HeLa cells more poorly than the wild type (25). Because HeLa cells are of cervical epithelial origin, they may have unique receptors for SEF14 compared to receptors on enterocytes. It has been suggested that SEF17 and SEF21 fimbriae of serovar Enteritidis bind basement membrane proteins (9, 21) and may thus provide a mechanism for intestinal colonization. Recent studies have shown that serovar Enteritidis cells lacking either SEF17 or SEF21 are less invasive in INT-407 (12). However, data from our study suggest that neither SEF14, SEF17, nor SEF21 augmented serovar Enteritidis internalization by enterocytes. It is possible that cell types used in our study may not express the appropriate receptor for fimbrial attachment or that bacteria might utilize multiple adhesions to attach. It has recently been shown that multiple fimbrial adhesions are required for full virulence of *Salmonella enterica* serovar Typhimurium in mice (29).

**Ingestion by chicken macrophage cell lines.** Macrophage cell lines HD-11 and MQ-NCSU (6, 27) were cultivated at 41°C in 5% CO$_2$ in either RPMI medium supplemented with 5% fetal bovine serum (HD-11) or Liebovitz-McCoy medium supplemented with 10% fetal bovine serum (MQ-NCSU). Macrophage cell suspension ($10^7$ cells/ml) was prepared in a mixture of 1% gelatin in Hank’s balanced salt solution, and viability was determined by trypan blue exclusion. Wild-type serovar Enteritidis and mutant strains were cultivated in CFA broth for 24 h at 37°C with gentle shaking, were washed, and were suspended in phosphate-buffered saline to a concentration of $10^9$ CFU/ml. Bacterial opsonization was carried out by mixing equal volumes of bacteria with 20% (vol/vol) normal chicken serum for 15 min at 37°C with shaking. Bacteria were then centrifuged at 6,000 × g for 15 min and were resuspended to the original concentration in 1% gelatin in Hank’s balanced salt solution. Ingestion of the preopsonized bacteria was measured as described (30) with modifications. Five hundred microliters of bacteria was added to 500 μl of the macrophage cell suspension, and the mixture was incubated with shaking for 45 min to compare the efficacy of bacterial ingestion of each strain. The number of bacteria in the supernatant after ingestion was verified to be at least $1 \log_{10}$ fewer than the number of internalized bacteria. Each bacterial strain was tested in at least three separate assays, each assay representing the average of triplicate samples. No significant differences in the numbers of internalized bacteria were noted with both macrophage cell lines (values ranging from 6.3 to 6.8 $\log_{10}$ CFU/5 × 10$^6$ NCSU cells or 6.0 to 6.4 $\log_{10}$ CFU/5 × 10$^6$ HD-11 cells for mutant strains and 6.3 $\log_{10}$ CFU/5 × 10$^6$ NCSU cells or 6.0 $\log_{10}$ CFU/5 × 10$^6$ HD-11 cells for the wild type). Total bacterial numbers before and after phagocytosis were similar, indicating that the bacteria did not multiply during phagocytosis. Type 1 fimbriae of serovar Typhimurium have been shown to mediate attachment, internalization, and intracellular survival in murine and porcine phagocytes (17, 23). However, in the present study, wild-type serovar Enteritidis and serovar Enteritidis lacking SEF21, SEF14, or SEF17 were internalized equally well by chicken macrophages. Thorns et al. (28) have also reported that serovar Enteritidis lacking SEF14 is internalized and persists in murine peritoneal macrophages similar to the wild-type strain.

**Invasion, persistence, and excretion of serovar Enteritidis fimbrial mutants in SPF chickens.** Groups of 25 5-day-old, specific-pathogen-free chickens (SPAFAS Inc., Roanoke, Ill.) were orally inoculated with a pure culture of approximately $10^7$ wild-type serovar Enteritidis cells or fimbrial mutant suspended in 1 ml of phosphate-buffered saline. At 1, 3, 7, 14, and 21 days postinoculation (p.i.), five chickens from each group were killed, and the liver, spleen, and cecum were aseptically excised. The number of viable bacteria per gram of tissue was determined as previously described (11). The lower limit of

**FIG. 2.** Internalization of HT-29 (A) and Caco-2 (B) enterocytes by wild-type and mutant serovar Enteritidis strains. Each bacterial strain was tested in at least three separate assays. Horizontal line indicates lower limit of assay detection. Asterisks indicate values significantly different from that of the wild type at the corresponding concentration ($P < 0.05$).
assay detection was 12 bacteria or 1.09 log_{10} cells per g of tissue. For statistical analysis, values below this limit were assigned a value equal to the lower limit of assay detection. Fecal excretion of Salmonella cells was monitored by culturing cloacal swabs. Stability of the mutants following reisolation from chickens was determined by plating onto LB agar with and without antibiotics and by PCR on genomic DNA from five individual colonies from each plate.

In general, no differences were observed among the serovar Enteritidis strains in their ability to colonize chicken ceca, to invade liver and spleen (Fig. 3), and to be shed in feces (data not shown). The only significant finding was that in birds inoculated with SEF14^- serovar Enteritidis, fewer Salmonella cells were isolated from the liver at all time points compared to the other treatment groups, and this number was significantly reduced at day 7 p.i. (Fig. 3A). Birds inoculated with SEF17^- serovar Enteritidis also had a low number of Salmonella cells in the liver at day 7 p.i. compared to birds inoculated with SEF21^- bacteria or the wild type. In addition, in birds inoculated with SEF14^- serovar Enteritidis, no Salmonella cells were recovered from the spleen at day 21 p.i. Although there was no difference in colonization of chicken ceca by the SEF14^- strain compared to other strains, the lower numbers of SEF14^- bacteria recovered from livers and spleens of infected chickens suggested that SEF14 might contribute to the persistence of serovar Enteritidis in extraintestinal tissues. Although it was also possible that these differences were due to an indirect effect of sefA gene disruption, clarification of this mechanism was beyond the scope of this study. The SEF14^-, SEF17^-, and SEF21^- mutants were stable even after reisolation from chickens.

Studies involving chickens vaccinated parenterally with recombinant SEF14 as well as with recombinant SEF17 or SEF21 and subsequently challenged with serovar Enteritidis have shown no major differences in cecal colonization or persistence of serovar Enteritidis (G. Rajashekara, S. Munir, D. A. Halvorson, and K. V. Nagaraja, unpublished data). These observations support the results of our in vitro studies using enterocytes or chicken macrophage cell lines. There is evidence that SEF14 is a T-cell immunogen (24) and that oral administration of hen egg yolk antibodies specific to SEF14 provides passive protection against experimental salmonellosis (26); using a variety of antigen delivery systems, similar results have been obtained following immunization of BALB/c mice with SEF14 (25). Differences observed in the present study could be due to differences in host species and in tissue tropism. Recent evidence that serovar Typhimurium fimbral types contribute to tissue tropism for murine small intestinal villi (3) and murine Peyer’s patches (5), as well as attachment to and invasion of epithelial cell lines (4), supports this view. Interestingly, from day 14 p.i. onwards, all three serovar Enteritidis mutant strains were recovered from chicken ceca in higher numbers than the wild type (Fig. 3C). This suggested that in the absence of these fimbral antigens, the host might be unable to efficiently clear the organisms from the cecum. Perhaps fimbral antigens might act in concert to establish infection; thus, the host immune response to multiple fimbral antigens might be necessary for clearance from the cecum. In conclusion, our data suggest no major role for SEF14, SEF17, or SEF21 fimbriae under the conditions tested. Because distinct fimbrial types contribute to tissue tropism in serovar Typhimurium (3–5) and multiple fimbral adhesins are required for full virulence of serovar Typhimurium (29), investigations using serovar Enteritidis strains lacking multiple fimbriae may provide additional information regarding the role of fimbriae in serovar Enteritidis pathogenesis.

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