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Cloning of *Salmonella enterica* Serovar Enteritidis Fimbrial Protein SefA as a Surface Protein in *Escherichia coli* Confers the Ability To Attach to Eukaryotic Cell Lines[▽]

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The gene for the *Salmonella enterica* serovar Enteritidis fimbrial protein SefA was cloned into an *Escherichia coli* surface expression vector and confirmed by Western blot assay. *E. coli* clones expressing SefA attached to avian ovary granulosa cells and HEp-2 cells, providing evidence for the involvement of SefA in the ability of *Salmonella* to attach to eukaryotic cells.

During the 1980s to 1990s, the worldwide increase in human *Salmonella enterica* serovar Enteritidis infections was associated with the consumption of contaminated eggs and egg products (13, 26, 28). In the United States, grade A shell eggs were identified as a major source contributing to *Salmonella* infections (19, 26, 29), and the percentage of *S.* Enteritidis among all *Salmonella* isolated from outbreaks increased from 5% to 26% from 1976 to 1996 (4). Although outbreak-associated cases due to *S.* Enteritidis decreased from 974 during 1998 to 2000 to 692 cases in 2004 to 2006, the 28 outbreaks in 2006 still remained above the Healthy People 2010 target of 22 (6). Despite efforts directed at reducing egg-related outbreaks, *S.* Enteritidis infections are still among those with the highest incidence of the seven most-reported serotypes of *Salmonella* (5).

The large proportion of *S.* Enteritidis serotypes involved in food-borne outbreaks is partly attributed to the adherence elicited by surface fimbriae. Fimbriae are nonflagellar filamentous surface appendages which consist of helically arranged repeating subunit proteins called fimbrins (24). Four serologically distinct fimbriae of *S.* Enteritidis have been characterized according to their size (kDa) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels: SEF21, SEF18, SEF17, and SEF14 (9–11, 21). Fimbriae can mediate the aggregation of bacteria and their attachment to inert surfaces (2) and to the surfaces of eukaryotic cells, especially to carbohydrate receptors (8, 11, 36).

SEF14 fimbriae are detected in all *S.* Enteritidis strains and are not widely distributed among the *Enterobacteriaceae* (10). These fimbriae consist of a repeating major subunit protein of 14.3 kDa (SefA) encoded by the gene *sefA* (9, 33). The results of studies by Peralta et al. (25) and Thiagarajan et al. (31) indicate that SEF14 fimbriae may have a role in pathogenesis by mediating attachment to eukaryotic cells. We focused on SEF14 fimbriae because of their limited distribution and their role as a main immunological target in the serological response to infection by *S.* Enteritidis in chickens (12). The objectives of this study were to clone and investigate the functional properties of the SEF14 fimbrin, SefA, as part of a fusion protein in *Escherichia coli* and to determine whether it could mediate adherence to tissue culture cells in vitro.

Bacterial strains, tissue culture cells, and plasmids used in this study are described in Table 1. Bacterial cultures were grown in LB medium (Fisher Scientific, Raleigh, NC) supplemented with 0.2% glucose and shaken at 220 revolutions min^{-1} or in colonization factor broth (30) at 37°C. Chloramphenicol (Cm; 10 μg ml^{-1}) and ampicillin (Amp; 50 μg ml^{-1}) were added as needed (Sigma-Aldrich, St. Louis, MO). Avian ovary granulosa cells were grown in 5% CO_2 atmosphere at 37°C in M199 medium supplemented with 26 mM $NaHCO_3$, 0.1% bovine serum albumin, 100 U ml^{-1} penicillin G, and 100 μg ml^{-1} streptomycin sulfate (Gibco-BRL, Gaithersburg, MD). HEp-2 cells were obtained from ATCC (Rockville, MD) and grown in 5% CO_2 atmosphere at 37°C in minimum essential medium (Sigma-Aldrich), pH 7.2, supplemented with 26 mM $NaHCO_3$, 7% fetal bovine serum, 100 U ml^{-1} penicillin G, and 100 μg ml^{-1} streptomycin sulfate (Gibco-BRL and Sigma-Aldrich).

Primers were designed to amplify the known *sefA* sequence (35) corresponding to the mature protein and included restriction sites to facilitate directional cloning of the amplified target to replace the β-lactamase gene in pTX101 (27). *E.* coli JM109 host cells were then transformed by electroporation (Gene Pulser, Bio-Rad Laboratories, Hercules, CA). In prior studies, derivatives of pTX101 have been used successfully to express several proteins on the outer surface of *E. coli* cells (16, 17). The Lpp portion of pTX101 serves to localize the fusion protein to the outer membrane, while the OmpA portion traverses the outer membrane and directs the product of the cloned gene (i.e., β-lactamase or SefA) to the surface.

Membrane fractions were isolated as previously described (7) with the following modifications. Cell suspensions were sonicated with an XL series sonicator (Heat Systems, Farmingdale, NY) at 100 W and then added to ice-cold Tris-Cl, and

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TABLE 1. Bacterial strains, tissue culture cells, and plasmids

Bacterial strain, tissue culture cell line, or plasmid	Relevant characteristics	Source or reference
Strains		
S. Enteritidis CDC9	Phage type 8, SEF14$^+$ *sefA*$^+$	A. M. Saeed
E. coli JM109	*endA1 recA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *mcrB*$^+$ *relA1 supE44* λ^- Δ(*lac-proAB*) *supE44* [F′ *traD36 proAB lacI*^{q}ZΔM15]	B. Olwin
Tissue culture cell lines		
HEp-2 cells	Epidermoid carcinoma isolated from human larynx	ATCC CCL23
Granulosa cells	Isolated from chicken ovaries	A. M. Saeed
Plasmids		
pTX101	12.5 kb, Amp^r Cm^r *lacI*$^{q+}$ *lpp-ompA-bla* fusion expressed through P_{lac} or P_{lpp}	G. Georgiou (15)
pDUG3A	11.5 kb, Cm^r *lacI*$^{q+}$ *lpp-ompA-sefA* fusion expressed through P_{lac} or P_{lpp}	This study

the remaining bacteria were removed by centrifugation. The total membrane was sedimented by centrifugation (45,000 $\times$ *g* at 4°C) for 1 h and then suspended in Tris-Cl, and the outer membrane was isolated by adding 0.5% (wt/vol) *N*-lauryl sarcosine (Sigma) and shaking at 200 rpm for 30 min at 22°C to dissolve the inner membrane.

Crude SefA was obtained from *S.* Enteritidis CDC9 as described previously by Feutrier et al. (14). Purified SefA was obtained by the method of Chart (7) with the following modifications. Briefly, crude SefA was run on an SDS-PAGE gel and a portion corresponding to SefA was extracted from gel slices with Z-spin microcentrifuge columns (Pall/Gelman Sciences, Ann Arbor, MI). The purified SefA was vacuum dried and suspended in phosphate-buffered saline buffer.

SDS-PAGE was performed according to the method of Laemmli (18), and proteins were visualized with Coomassie blue. An identical gel was prepared for Western blots, and proteins were transferred onto BioTrace polyvinylidene difluoride (Pall/Gelman Sciences). Western blotting was done according to the membrane manufacturer's instructions (1). SefA monoclonal mouse antibodies 69/25 supplied by C. J. Thorns (Central Veterinary Laboratories, Weybridge, Surrey; 32) were used as the primary antibody; ImmunoPure goat anti-mouse immunoglobulin G(H+L) biotin-conjugated antibodies (Pierce, Rockford, IL) were used as the secondary antibody; and AVIDX-AP, assay buffer (0.1 M diethanolamine, 1.0 mM $MgCl_2$, pH 10.0), I-Block, and CSPD (Applied Biosystems, Bedford, MA) were used in the chemiluminescent detection of the secondary antibodies using X-ray film. SDS-PAGE of total membrane fractions of *E. coli* JM109(pDUG3A) demonstrated a protein of the expected size for the Lpp-OmpA-SefA fusion protein (~31 kDa); however, similarly sized proteins were also observed in the control strains [JM109 and JM109(pTX101)] (Fig. 1A). Western blot analysis confirmed the presence of SefA in the 31-kDa band in the recombinant strain [JM109(pDUG3A)] but not in membrane protein fractions from either the host strain alone (JM109) or the host strain harboring the pTX101 vector (Fig. 1B). When outer membrane fractions were selectively isolated from the inner membrane, the presence of the 31-kDa SefA fusion protein was more readily detected over the background of proteins of similar size (Fig. 1C). The data provide further evidence for the localization of the SefA fusion protein in the outer membrane of *E. coli* JM109(pDUG3A).

Attachment assays on avian ovary granulosa and HEp-2 cells were done according to the method of Thiagarajan et al. (30), with some modifications. Briefly, granulosa cells were seeded and grown as a monolayer on sterile coverslips and then washed three times with M199 medium (Gibco), and 1×10^7 CFU of bacterial cells (grown with and without 1% D-mannose) was added to the coverslips. The coverslips were then incubated in a 5% CO_2 atmosphere at 37°C for 3 h, after which the cells were washed five times with M199 medium, fixed with methanol, stained with 10% Giemsa stain (Sigma), and examined by light microscopy. Attachment assays with HEp-2 cells were done as described above except that minimum essential medium (Sigma) was used as the wash solution.

Peralta et al. (25) showed that in vitro attachment of *S.*

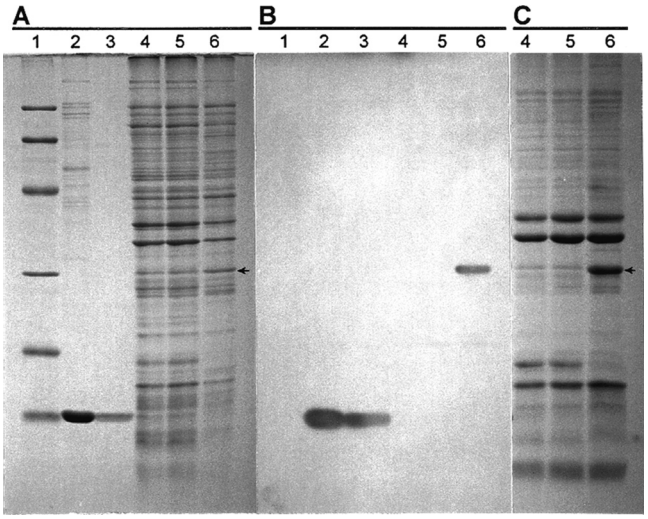

FIG. 1. SDS-PAGE and Western blot of SefA samples using SefA monoclonal antibodies. (A) SDS-PAGE of total membrane fractions collected from cells grown at 37°C. (B) Western blot of SDS-PAGE gel shown in panel A using monoclonal mouse antibodies 69/25 as an immunoprobe for SefA. (C) SDS-PAGE of outer membrane fractions collected from cells grown at 25°C. Lanes: 1, molecular mass standards (99.4, 66.2, 45, 31, 21.5, and 14.4 kDa); 2, crude SefA; 3, purified SefA; 4, *E. coli* JM109; 5, *E. coli* JM109(pTX101); 6, *E. coli* JM109(pDUG3A). Arrows indicate Lpp-OmpA-SefA fusion protein at ~31 kDa.

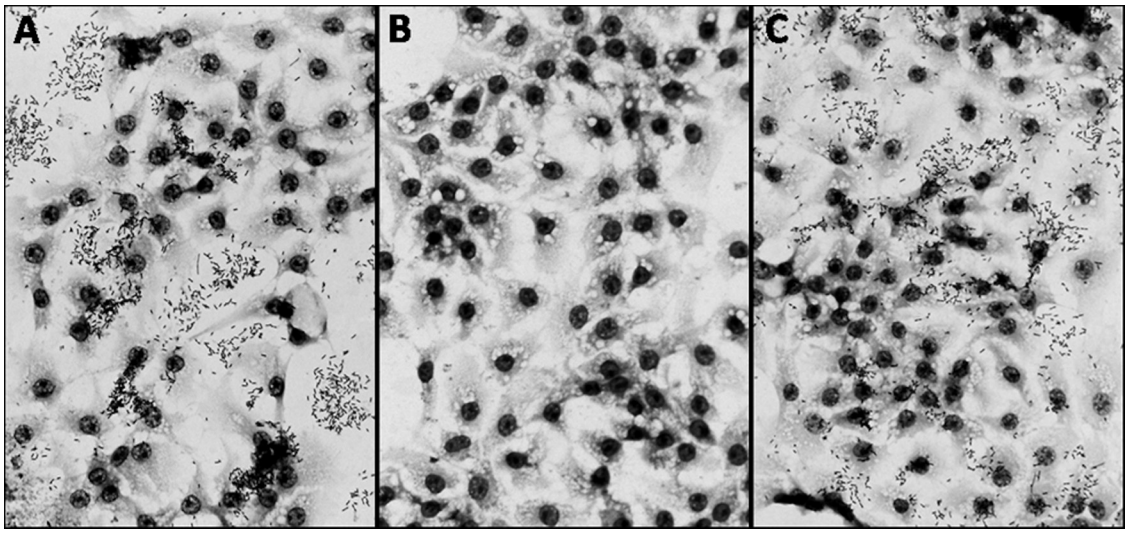

FIG. 2. Attachment of indicated bacterial cells to avian ovary granulosa cells: *S.* Enteritidis CDC9 (A), *E. coli* JM109(pTX101) (B), and *E. coli* JM109(pDUG3A) (C). Cells were stained with 10% Giemsa stain and photographed with a light microscope at ×400 magnification.

Enteritidis to murine intestinal epithelial cells was reduced by SefA antibodies, suggesting a role for SefA in attachment. Thiagarajan et al. (30) demonstrated attachment of *S.* Enteritidis to avian ovary granulosa cells that form one of the layers surrounding the yolk in a preovulatory follicle. They suggest that this attachment may be a mechanism precipitating *S.* Enteritidis infection of hens' ovaries that may subsequently lead to transovarian transmission to shell eggs. In our study, we compared the attachment of *S.* Enteritidis CDC9, *E. coli* JM109(pDUG3A) (*sefA* clone), and JM109(pTX101) (vector) to both avian granulosa (Fig. 2) and HEp-2 cells (Fig. 3). *S.* Enteritidis CDC9 demonstrated a mannose-resistant pattern of attachment to both granulosa and HEp-2 cells (Fig. 2A and 3A) while JM109(pTX101) did not attach to these cell lines (Fig. 2B and 3B). However, JM109(pDUG3A), expressing the SefA fusion protein, demonstrated mannose-sensitive attachment to both granulosa and HEp-2 cells, indicating attachment to eukaryotic cell lines attributed to the presence of SefA (Fig. 2C and 3C).

Thorns (34) reported that both a wild-type *S.* Enteritidis strain and a *sefA* mutant were able to attach to HEp-2 cells, indicating that multiple determinants may mediate attachment. This is also indicated by differences in mannose sensitivities of various *S.* Enteritidis strains (30). The results of the Western blot and cell culture attachment assays suggest that the mannose-sensitive binding of JM109(pDUG3A) is due to the presence of SefA in the outer membrane, since JM109 (pTX101) did not possess SefA or show attachment. The data also indicate that the presentation of SefA in a fimbrial structure may not be necessary for binding since nonfimbrial adhesions have been reported to mediate attachment to eukaryotic cells (22). Unfolded SefA protein in the Lpp-OmpA-SefA fusion pro-

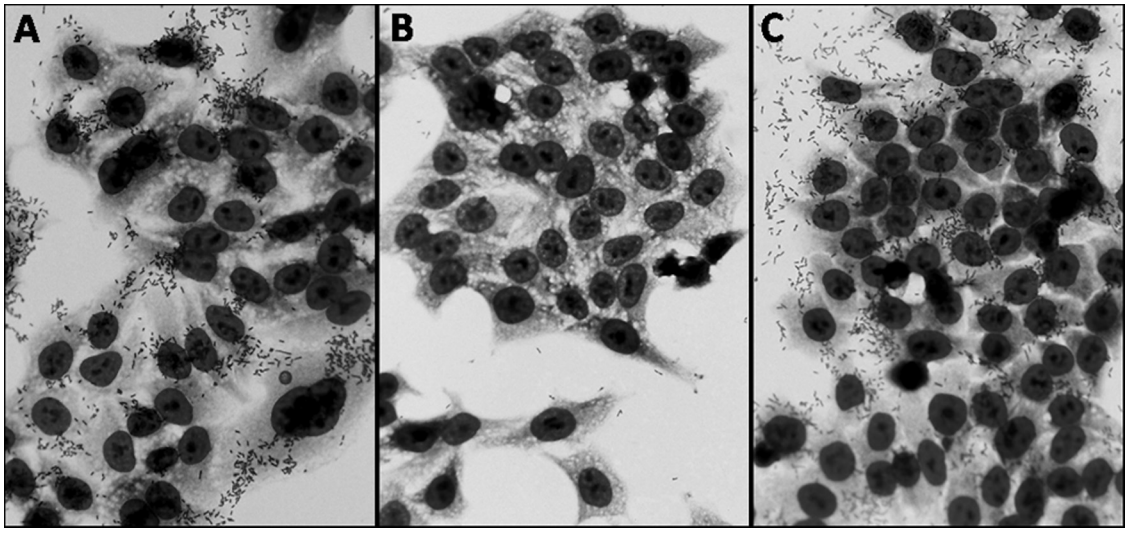

FIG. 3. Attachment of indicated bacterial cells to HEp-2 cells: *S.* Enteritidis CDC9 (A), *E. coli* JM109(pTX101) (B), and *E. coli* JM109(pDUG3A) (C). Cells were stained with 10% Giemsa stain and photographed with a light microscope at ×400 magnification.

tein may possibly allow hydrophilic or hydrophobic interactions with granulosa or HEp-2 cells, providing for mannose-sensitive adherence.

The data presented herein demonstrate that surface-expressed SefA protein may provide to nonadherent bacteria the property of adherence to eukaryotic cells and constitute further evidence for the involvement of SEF14 fimbriae in binding by *S.* Enteritidis. Heterologously expressed recombinant fimbrial antigens have been used successfully to induce immune responses in mice and pigs (3, 20). The results of previous studies have indicated that purified SefA protein is highly immunogenic and that SefA antibodies increase the survival rate of mice after challenge with *S.* Enteritidis from 32% in control mice to 78% in vaccinated mice (23, 25). The data warrant further studies to demonstrate whether the attachment observed in vitro could be demonstrated in vivo for possible applications in the competitive exclusion or induction of an immune response using this vector.

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