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Salmonella enterica Serovars Gallinarum and Pullorum Expressing *Salmonella enterica* Serovar Typhimurium Type 1 Fimbriae Exhibit Increased Invasiveness for Mammalian Cells

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***Salmonella enterica* serovars Gallinarum and Pullorum are *S. enterica* biotypes that exhibit host specificity for poultry and aquatic birds and are not normally capable of causing disease in mammalian hosts. During their evolution toward host restriction serovars Gallinarum and Pullorum lost their ability to mediate mannose-sensitive hemagglutination (MSHA), a phenotype correlated with adherence to certain cell types. Because adherence is an essential requirement for invasion of cells by bacterial pathogens, we examined whether MHSA type 1 fimbriae would increase the ability of serovars Pullorum and Gallinarum to invade normally restrictive cells. Serovars Gallinarum and Pullorum expressing *S. enterica* serovar Typhimurium strain LT2 type 1 fimbriae exhibited a 10- to 20-fold increased ability to adhere to and a 20- to 60-fold increased invasion efficiency of the human epithelial HEp-2 cell line. Invasion was accompanied by extensive ruffling of the membranes of the HEp-2 cells. In a murine ligated ileal loop model, a 32% increase in the number of M-cell ruffles was seen when serovar Gallinarum expressed serovar Typhimurium type 1 fimbriae.**

The gram-negative bacteria of the genus *Salmonella* are the causative agents of numerous diseases, such as bacteremia, enteric fever, and enterocolitis in a broad range of organisms. Although some serovars of *S. enterica*, such as serovars Typhimurium and Enteritidis, can cause disease in a wide range of hosts, other strains have a greatly reduced range of host specificity. Two such serotypes are Gallinarum and Pullorum, which show specificity for poultry and aquatic birds (3). Several factors are most likely involved in determining the host specificity of a certain *Salmonella* biotype. One set of factors, for example, are the *spv* (for *Salmonella* plasmid virulence) genes which are required for the systemic phase of disease in numerous hosts (reviewed in reference 14). In addition to the virulence plasmid, it is presumed that the acquisition of several chromosomal genes has expanded the host repertoire of certain biotypes (4). Another property likely to be involved in host adaptation is the ability of the bacteria to adhere to mucosal surfaces. Most members of the *Salmonella* species display numerous appendages, termed fimbriae or pili, upon their cell surface that are thought to enhance the ability of the bacteria to attach to various substrates.

Type 1 fimbriae are expressed by most *Salmonella* serovars and are characterized by their ability to bind to mannose derivatives found on eukaryotic cells. Such binding can be inhibited by the addition of exogenous α-D-mannose, a phenotype termed mannose-sensitive hemagglutination (MSHA). It has been shown by immunoelectron microscopy and DNA hybridization techniques that serovars Pullorum and Gallinarum encode fimbriae that are closely related to the type 1 fimbriae of serovar Typhimurium (9, 22). Although closely related, the fimbriae of serovars Gallinarum and Pullorum do not exhibit MSHA (12). However, the introduction of the serovar Typhimurium LT2 fimbrial adhesin into serovar Pullorum enables this strain to exhibit MSHA and adherence to HEp-2 cells (15). To examine the relationship between MSHA, adherence, and invasion, serovar Pullorum 297 (obtained from James Duguid) and serovar Gallinarum 2933 (obtained from Bruce Stocker) were transformed with either plasmid pISF156, which confers expression of the serovar Typhimurium LT2 type 1 fimbriae (22), or, as a control, plasmid pACYC184. The ability of these transformants to agglutinate guinea pig erythrocytes and to adhere to HEp-2 cells was tested as described previously (16, 22). As shown in Table 1, the introduction of plasmid pISF156 into either serovar Gallinarum or Pullorum allowed the transformed strains to agglutinate guinea pig erythrocytes. In addition, adherence to HEp-2 cells was increased at least 10- to 20-fold when serovar Gallinarum or Pullorum contained the plasmid that confers expression of the LT2 type 1 fimbriae. The presence of 0.3% mannose abolished the abilities of these strains to hemagglutinate and to adhere to HEp-2 cells (data not shown).

Although serovar Typhimurium SL1344 and LT2 can both mediate MSHA, the LT2 strain adheres at much higher levels (15-fold more) to HEp-2 cells than does SL1344 (not shown). Similarly, Baumler et al. observed differences in *fim*-mediated adherence to HeLa cells by the serovar Typhimurium isolates SR-11 and ATCC 14028 (5). Interestingly, the deduced amino acid sequence of the serovar Typhimurium LT2 fimbrial adhesin protein, FimH, (15) is slightly different from the deduced serovar Typhimurium SL1344 FimH amino acid sequence (J.

TABLE 1. The presence of plasmid pISF156 in serovars Gallinarum and Pullorum increases hemagglutination (HA) and adherence to HEp-2 cells

Bacterium	HA	No. of adherent bacteria/HEp-2 cell
Serovar Typhimurium LT2	+	16
Serovar Pullorum(pACYC184)	−	<1
Serovar Pullorum(pISF156)	+	19
Serovar Gallinarum(pACYC184)	−	<1
Serovar Gallinarum(pISF156)	+	10

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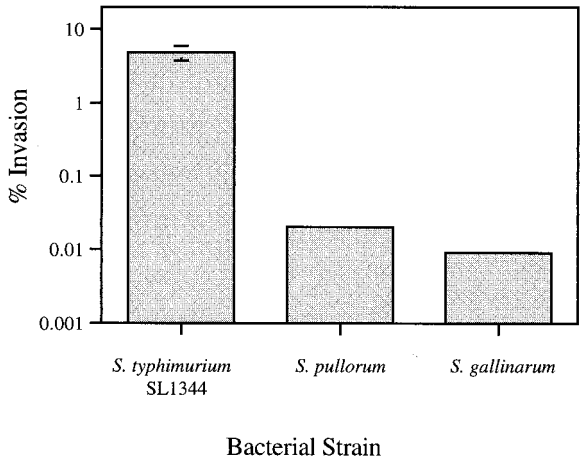

FIG. 1. Invasion of HEp-2 cells by serovar Typhimurium SL1344, serovar Pullorum, and serovar Gallinarum. Bacteria were grown statically at 37°C in Luria broth for 48 h before infecting 5×10^4 HEp-2 cells at a multiplicity of infection of 400 in a 24-well culture dish. After the addition of bacteria, the cell culture plates were centrifuged at $750 \times g$ for 15 min at 25°C. Data are CFU presented as the mean ± standard deviation (error bar) of triplicate wells and are presented as the percentage of the inoculum surviving gentamicin treatment. Data are representative of at least three independent experiments.

TABLE 2. Plasmid pISF156 increases invasion of HEp-2 cells by serovars Gallinarum and Pullorum

Bacterial strain	% Invasion[a] with:	
	No mannose	Mannose
Serovar Pullorum pACYC184	0.011 ± 0.008	0.006 ± 0.001
Serovar Pullorum pISF156	0.651 ± 0.202	0.003 ± 0.000
Serovar Gallinarum pACYC184	0.013 ± 0.005	0.009 ± 0.001
Serovar Gallinarum pISF156	0.306 ± 0.059	0.009 ± 0.005
Serovar Typhimurium *hilA*(pACYC184)	0.038 ± 0.002	0.038 ± 0.012
Serovar Typhimurium *hilA*(pISF156)	0.057 ± 0.007	0.015 ± 0.005

[a] Bacteria were grown and invasion assays were performed as described in the legend to Fig. 1. Data are CFU presented as the means ± standard deviations of triplicate wells and are presented as the percentage of the inoculum surviving gentamicin treatment.

Boddicker, unpublished data). It is possible that small differences in the adhesin molecules among the serovar Typhimurium strains may lead to differences in the ability of these strains to attach to the mannosyl derivatives of a certain cell type. Alternatively, as was shown by Thankavel et al. (23), the composition of the fimbrial shaft of the type 1 fimbriae can influence the adhesion of serovar Typhimurium to different cell types, perhaps by constraints imposed by the fimbrial shaft on the adhesin. In these studies we have not determined whether the serovar Typhimurium LT2 fimbriae conferred by pISF156 are solely responsible for the MSHA phenotype or whether hybrid fimbriae are produced by expression of the serovar Gallinarum and Pullorum chromosomally encoded type 1 fimbriae. Further studies are necessary to elucidate the molecular mechanisms by which the type 1 fimbriae of different serovar Typhimurium strains mediate attachment to different cell types.

Compared to the virulent serovar Typhimurium SL1344 strain, serovar Pullorum is approximately 80-fold less efficient, and serovar Gallinarum is about 200-fold less efficient at invading HEp-2 cells in an in vitro invasion assay (Fig. 1). To determine whether the increased adherence of the pISF156-transformed serovar Gallinarum and Pullorum strains also led to an increased ability of these strains to invade HEp-2 cells, invasion assays were performed as described previously (19). Serovar Gallinarum and Pullorum strains transformed with

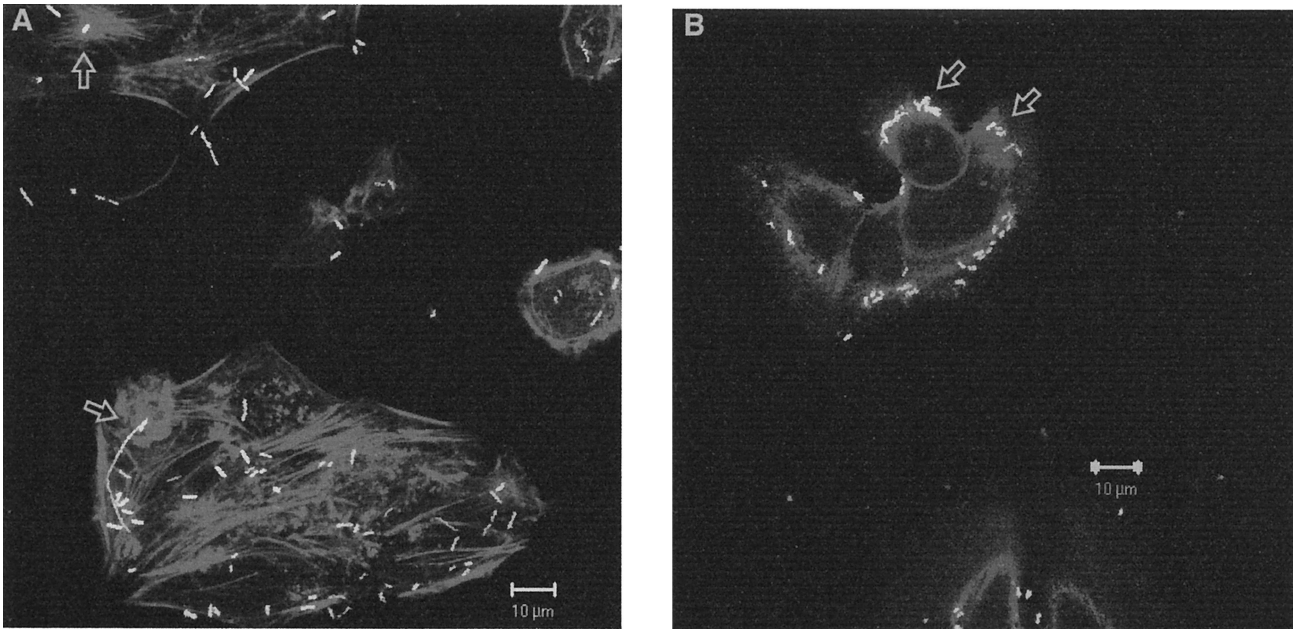

FIG. 2. Serovars Gallinarum and Pullorum can induce actin rearrangement upon invasion of HEp-2 cells. HEp-2 cells (5×10^4) were infected at a multiplicity of infection of 400 with either serovar Gallinarum (A) or serovar Pullorum (B) containing a green fluorescent protein-expressing plasmid and pISF156. The plates were centrifuged at 1,400 rpm for 15 min. After 1 h of incubation actin was visualized using rhodamine phalloidin (Molecular Probes, Eugene, Oreg.) and coverslips viewed with a Zeiss LSM 510 confocal microscope. The arrows denote the actin rearrangement of HEp-2 cells and associated bacteria.

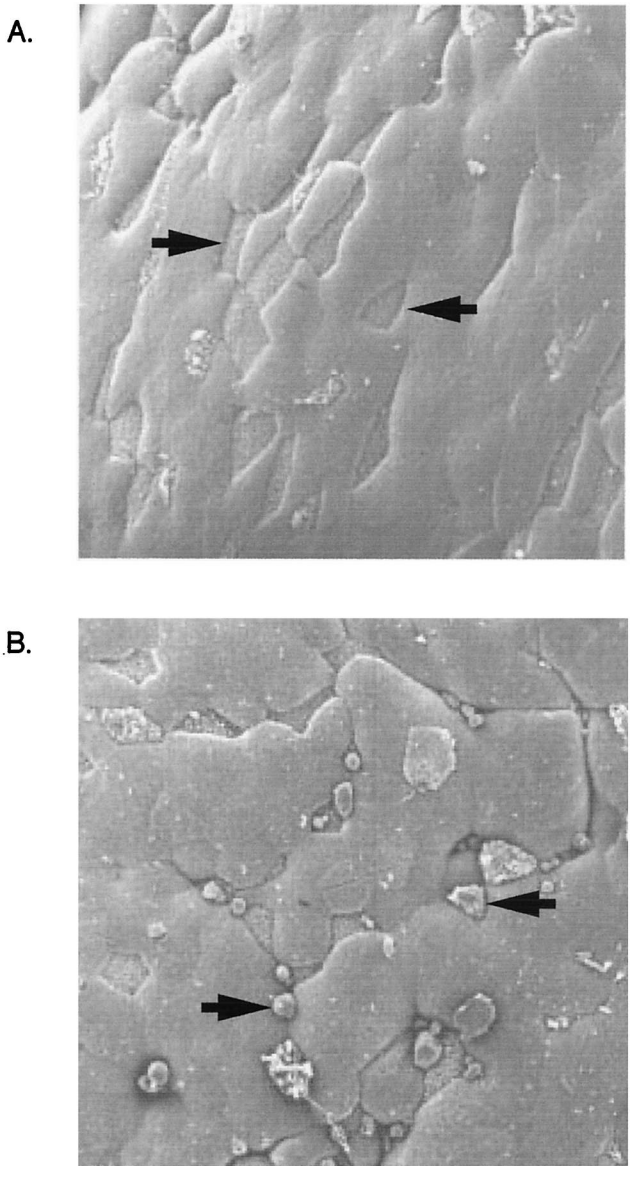

FIG. 3. Serovar Typhimurium type 1 fimbriae increase M-cell disruption by serovar Gallinarum. Approximately 4×10^9 serovar Gallinarum bacteria transformed with either vector alone (A) or pISF156 (B) were grown under fimbria-inducing conditions and were used to inoculate BALB/C ligated ileal loops. Scanning electron microscopy was used to examine M cells (denoted by arrows) in the Peyer's patch tissue. Magnification is 1,000-fold.

pACYC184 showed very low levels of invasion, while the pISF156 transformants exhibited a 20- to 60-fold increase in invasion (Table 2). The addition of 0.3% mannose reduced invasion levels of the pISF156 transformants to levels similar to those of the strains transformed with the vector alone. The presence of 0.3% glucose had no effect on invasion levels (not shown), indicating that the presence of sugars other than mannose did not inhibit invasion. These results underscore the importance of cellular adherence in the infective process and suggest a role for fimbriae in the expansion or restriction of host specificity of a *Salmonella* biotype.

To examine whether this enhanced invasion of HEp-2 cells could be due to a result of host cell responses triggered by increased adherence of the bacteria, the serovar Typhimurium SL1344 *hilA* mutant BJ70 was transformed with plasmid pISF156. HilA is a positive regulator of *Salmonella* pathogenicity island 1 (SPI-1) virulence gene expression, and serovar Typhimurium strains carrying mutations in *hilA* are 100-fold less invasive for HEp-2 cells than wild-type serovar Typhimurium (2). As is the case with SL1344, BJ70 is not very adherent to HEp-2 cells (fewer than two adherent bacteria per cell) (not shown). If BJ70(pISF156) were to exhibit increased invasion of HEp-2 cells, it would suggest that the invasion exhibited by serovar Gallinarum(pISF156) and serovar Pullorum(pISF156) is a result of nonspecific responses, such as pinocytosis, by the host cell. Invasion assays were performed using wild-type BJ70 transformed with either plasmid pISF156 or pACYC184. BJ70(pISF156) exhibited extremely high levels of MSHA and adherence to HEp-2 cells (>50 adherent bacteria per cell) (not shown). However, BJ70 expressing the serovar Typhimurium LT2 type 1 fimbriae exhibits less than a twofold increase in invasion of HEp-2 cells (Table 2). This slight increase in invasion is significantly less than the 20- to 60-fold increase shown by the pISF156-transformed serovars Pullorum and Gallinarum. This suggests that the presence of LT2 type 1 fimbriae alone is insufficient to increase invasion levels; rather, an active, bacterium-dependent uptake mechanism is also required. The efficiency of such a mechanism may be increased by fimbria-mediated adherence that facilitates a close proximity between the bacterium and the host cell.

Several of the genes on SPI-1 (i.e., *prgH*, *prgK*, *orgA*, and *invH*) encode components of a type III secretion system that directs the export of bacterial proteins across the inner and outer membranes and are involved in delivering bacterial effector proteins (SipA, SipC, SptP, and AvrA) into eukaryotic cells. Delivery of these proteins leads to the induction of cytoskeletal rearrangements in the host cell, ruffling of the cell membrane, and macropinocytosis of the invading bacterium (reviewed in reference 10). DNA hybridization studies using probes specific for *invH* (1) and PCR analysis using *invA*-specific primers (20) have indicated that serovars Gallinarum and Pullorum possess at least some SPI-1 genes. To determine whether additional genes spanning the length of the island are present in these strains, PCR was performed on DNA isolated from serovars Pullorum and Gallinarum using primers specific for the serovar Typhimurium *hilA*, *invH*, *orgA*, and *sipC* genes. The presence of the *hilA*, *invH*, *orgA*, and *sipC* SPI-1 genes in serovars Pullorum and Gallinarum was detected by the generation of DNA products the same size as those from invasive serovar Typhimurium (data not shown). In contrast, PCR performed on DNA isolated from serovar Litchfield (obtained from Kris Rahn), which does not possess the SPI-1 genes (13), did not generate any DNA products.

Because serovars Gallinarum and Pullorum appear to possess several of the SPI-1 genes, we wanted to determine whether the invasion of HEp-2 cells by the pISF156-transformed serovar Gallinarum and Pullorum strains might occur in an actin-dependent manner similar to that of serovar Typhimurium. In order to visualize the bacteria using fluorescent microscopy, these strains were also transformed with a plasmid carrying the gene for green fluorescent protein (8) in vector pKSV7 (21). After allowing the doubly transformed strains to invade HEp-2 tissue culture cells for 1 h, actin was visualized using rhodamine phalloidin. Ruffling events were seen in both the serovar Pullorum- and serovar Gallinarum-invaded HEp-2 cells, and green fluorescent bacteria were visualized near the ruffling (Fig. 2). Because actin rearrangement could occasionally be visualized after invasion of HEp-2 cells with the pACYC184-transformed parent strains but at a much lower frequency (not shown), we interpret this result to mean that all

of the components necessary for the serovar Gallinarum- or serovar Pullorum-induced ruffling are intact in the wild-type strains and that plasmid pISF156 simply promotes the contact of the bacteria with the HEp-2 cells to allow the exchange of invasion signals to occur at a higher frequency. Studies using SPI-1 mutants of serovars Gallinarum and Pullorum are needed to confirm this interpretation.

A previous study examining the ability of serovar Gallinarum to invade murine M cells showed no evidence of entry into or destruction of M cells (18). However, the results of our in vitro invasion assay led us to examine whether the presence of the serovar Typhimurium LT2 type 1 fimbriae might also lead to an ability of serovar Gallinarum to invade murine M cells in a ligated ileal loop model. Approximately 4×10^9 serovar Gallinarum bacteria transformed with either pISF156 or vector alone were grown under fimbria-inducing conditions and were used to inoculate ligated ileal loops according to a previously published protocol (17) using 8- to 12-week-old BALB/c mice. The number of M-cell ruffles seen by electron microscopy increased from 0% (0 of 50) to 32% (16 of 50) when serovar Gallinarum expressed the serovar Typhimurium type 1 fimbrial genes as compared to serovar Gallinarum transformed with vector alone (Fig. 3). Wild-type serovar Typhimurium SL1344 induces ruffles in about 50 to 60% of the M cells in ligated ileal loops (unpublished data). These results could explain why Pascopella et al. did not see entry of serovar Gallinarum into murine M cells (18). It is possible that expression of serovar Typhimurium LT2 type 1 fimbriae by serovar Gallinarum facilitated contact of the bacteria with the M cells in order for the invasion process to occur. This would mean that, in addition to *lpf* (6), under certain conditions the serovar Typhimurium type 1 fimbriae may be important for the attachment of *Salmonella* to the small intestine.

Because it appeared that the pISF156-transformed serovar Gallinarum was able to invade murine M cells, we were interested in determining whether the transformants would also be lethal in mice. BALB/c mice were orally inoculated with 2×10^9 pACY184- or pISF156-transformed serovar Gallinarum organisms. There were no obvious signs of disease, nor did any deaths occur, in either group of mice (not shown). Some isolates of serovar Gallinarum 2933 lack the *spv* virulence plasmid (4), and most have been found to be nonflagellate (7, 11). Therefore, decreased virulence in the mouse model of typhoidal infection by avian-adapted serovars is undoubtedly due to the lack of numerous factors. Additional studies are needed to define the components required for the expansion or restriction of the host repertoire by a specific *Salmonella* biotype.

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