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Association of *Salmonella typhimurium* with, and Its Invasion of, the Ileal Mucosa in Mice

GERALD W. TANNOCK,[1] RUTH V. H. BLUMERSHINE, AND DWAYNE C. SAVAGE*
Department of Microbiology and School of Basic Medical Sciences, University of Illinois, Urbana, Illinois 61801

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A wild-type strain of *Salmonella typhimurium* and three mutant rough colonial variants of the wild type were compared for their ability to become associated with and invade the ileal mucosa of germfree and specific-pathogen-free mice. The rough-mutant strains differed from the wild type in having incomplete lipopolysaccharides lacking one or more sugars in the polysaccharide moiety. The wild-type and mutant strains also differed one from the other in the types of appendages (flagella, pili) on their surfaces. Depending upon the dosage of bacteria given, all mutant strains as well as the wild type could associate with and invade the intestinal mucosa of infected gnotobiotic mice. If the infecting dosage was high enough, at least two of the mutant strains and the wild type invade the intestinal mucosa of the specific-pathogen-free animals. O antigen, flagella, or pili do not appear to be essential for the association of *S. typhimurium* with the mucosal surface of the mouse ileum. O antigen on the bacterial cell surface may be important, but not essential, for invasion of the ileal mucosa.

Members of the genus *Salmonella* characteristically invade the ileal epithelium of a susceptible host, multiply in the mucosa, and subsequently spread throughout the host (19). Little is known about the structural or biochemical mechanisms by which pathogenic organisms invade susceptible mucosae (16). Using electron microscopy, Takeuchi (19) has observed the early stages of intestinal infection by *Salmonella typhimurium* in guinea pigs preconditioned by starvation and opium injection. These studies at the ultrastructural level show that the microvilli on epithelial cells in the ileum degenerate when a *Salmonella* cell comes within a critical distance of them (350 nm). This process is followed by degeneration of the apical cytoplasm of the epithelial cell, after which the bacterial cell is ingested. Hence, the portal of entry for the *Salmonella* bacteria is through the brush border, although entry via intercellular junctional complexes also occurs. The bacteria may elaborate substances that induce epithelial cells to ingest them. Such substances could be on the bacterial cell surface or excreted into the surrounding environment (13).

A pathogen that invades (i.e., penetrates the epithelium and reaches the lamina propria and lymphatic system) must approach the epithelium to within a critical distance so that its invasive potential can come into play. Some indigenous microorganisms are known to associate intimately with mucosal epithelia in the gastrointestinal tract (13). In some animals, certain indigenous microorganisms have special surface properties by which they attach to the intestinal epithelium (C. P. Davis, S. L. Erlandsen, and D. C. Savage, Abstr. Annu. Meet. Amer. Soc. Microbiol. G 189 p. 57, 1973; A. Takeuchi, Int. Micro-ecology Symp., Columbia, Mo., 1974). Similarly, in piglets, enteropathogenic *Escherichia coli* attach to the intestinal epithelium while inducing enteritis (7). The attachment to the epithelium of these bacteria is mediated by a surface layer of fine filaments called K88 antigen (7). We believe that *S. typhimurium* may associate with the intestinal epithelium before penetrating it, and that this association may be mediated by special surface properties of the bacteria.

In this report, we document the association of *S. typhimurium* with intestinal epithelium in the mouse. In addition we report on some factors involved in that association and on those involved in the invasion by the microbe of the intestinal mucosa. To accomplish these experiments, we used a smooth wild-type strain of *S. typhimurium* and three rough-mutant strains blocked in synthesis of specific nucleotide sugars. Lacking specific glycosyl transferase enzymes, these mutants produce incomplete lipopolysaccharide (LPS) lacking in one or more sugars in the core of the polysaccharide moiety. In addition, the mutants lack O antigen (12). During synthesis of the polysaccharide of the

[1] Present address: Department of Microbiology, University of Otago, Dunedin, New Zealand.

LPS, sugars making up the core are added sequentially by the glycosyl transferases. Thus, depending upon the transferase missing, the LPS of one mutant may lack more polysaccharide than does that of another.

The three mutant strains used were all blocked in LPS synthesis at a different point such that three levels of incomplete LPS were represented. Using these strains, then, we were able to examine how O antigen and the amount of polysaccharide in the LPS influence the association of *S. typhimurium* with, and its invasion of, the intestinal mucosa.

MATERIALS AND METHODS

Salmonella strains. The wild-type strain (LT2) and three LPS mutant strains (TV119, G30, and SL1032) of *S. typhimurium* were obtained from L. Rothfield, University of Connecticut Health Center (12). Strain TV119, the so-called "mature" rough variant, is blocked in synthesis of O antigen. Strain G30 is blocked in synthesis of uridine diphosphogalactose, whereas strain SL1032 is blocked in synthesis of uridine diphosphoglucose (12). Thus, strain SL1032 lacks the most polysaccharide from its LPS, and G30 has more polysaccharide than strain SL1032. Strain TV119 lacks only its O antigen. All strains were maintained as lyophilized cultures and grown on a minimal medium (17) containing 1% glucose. Twenty-four-hour agar cultures growing at 37 C were suspended and diluted in sterile charcoal water (15) for infection of mice.

Each strain was examined for the following characteristics: (i) absorption by cell suspensions of anti-O antibodies from serum (3); (ii) agglutination of guinea pig erythrocytes (2%) by bacterial suspensions serially subcultured in brain heart infusion broth (Difco) at 30 C (2); (iii) motility of the bacterial cultures in semisolid medium (2) at 37 C (3); (iv) colonial morphology on brain heart infusion agar incubated at 37 C for 24 h; and (v) flagella and pili (fimbriae) (see procedures for electron microscopy).

Mice. Germfree, CD-1 (Charles River, Wilmington, Mass.), male mice approximately 5 weeks old were housed in flexible plastic isolators and given sterile food (Charles River, Wilmington, Mass.) and tap water ad libitum. Fecal pellets from at least two mice in each cage were collected before experimentation and cultured in nutrient (Difco) and brain heart infusion (Difco) broths incubated at 37 C either aerobically or anaerobically in GasPak jars (BBL, Cockeysville, Md.). Sabouraud dextrose broth (Difco) cultures were incubated aerobically. The broths were subcultured to nutrient, brain heart infusion, or Sabouraud dextrose agar plates after 2 and 5 days of incubation. When cultures of fecal pellets from the animals failed to yield bacterial or fungal growth, they were considered to be germfree.

Specific-pathogen-free (SPF), CD-1 (Charles River, Wilmington, Mass.), male mice approximately 5 weeks old were housed in plastic cages fitted with Isocaps (Isocage, Carworth, New York) and given food (Lab-Blox, Allied Mills, Chicago, Ill.) and acidified water (14) ad libitum.

Inoculation of mice. The mice were inoculated intragastrically by using polyethylene tubing with either 5×10^7 or 5×10^8 bacteria of the appropriate *Salmonella* strain. Eight hours after inoculation, the mice were killed with chloroform and a portion of ileum was removed from each mouse for culture and histological examination. The mesenteric lymph nodes and spleens also were collected from each animal. The organs from mice from any one group given the same inoculum dosage were pooled. The nodes were pooled separately from the spleens.

Other groups of SPF mice were inoculated intravenously with 2×10^7 bacteria of the appropriate strain. Deaths were observed over a 2-week period. Survivors were killed at the end of this period and their spleens were cultured.

Histological examination. A portion of the ileum from each gnotobiotic mouse was frozen in 2% methylcellulose in saline at −18 C and sectioned at 4 μm by using a microtome-cryostat (14). Sections were stained by a tissue Gram stain or indirect fluorescent antibody technique (5). Antisera used in the latter method were obtained from albino rabbits injected with a series of acetone-washed bacterial preparations (3). An anti-rabbit globulin fluorescein conjugate (Difco) prepared in goats was used.

Culturing procedures. One-half gram of ileum, pooled weighed spleens, or pooled weighed mesenteric lymph nodes was collected from the gnotobiotic mice. Each specimen was homogenized with a Teflon grinder in 4.5 ml of charcoal water. A calibrated loop technique (15) was used to dilute and culture each homogenate on brain heart infusion agar. The remainder of the homogenate was cultured in 10 ml of tetrathionate broth (BBL). All cultures were incubated at 37 C for 24 h. Procedures for SPF mice were similar to those given above, except that cultures were made on brilliant green agar (BBL) and MacConkey agar (BBL).

Electron microscopy. Bacteria fixed with an equal amount of 3% glutaraldehyde in Millonigs (10) buffer (pH 7.3) at 4 C and negatively stained with an equal amount of 2% phosphotungstic acid mixed with 0.5% bovine serum albumin (5:1) were examined for flagella and pili. The cultures were from serially subcultured broths incubated at 30 C and from pellicles formed in cultures incubated at 37 C and then held at room temperature for 7 days. The preparations were examined on carbon-coated grids by transmission electron microscopy (JOEL model 6A) at 50 and 80 kV.

RESULTS

Characterization of Salmonella strains. The results from characterization of the wild type (LT2) and the three LPS mutant strains are given in Table 1. Two strains (LT2 and G30) had flagella (Fig. 1a) and two strains (LT2 and SL1032) had pili (Fig. 1b). The morphology of isolated colonies on brain heart infusion agar after 24 h of incubation at 37 C was as follows: LT2—circular entire edge, 2-mm diameter, smooth surface; TV119—circular, irregular edge, 2- to 3-mm diameter, rough surface; G30—circular, entire edge, 2-mm diameter,

TABLE 1. *Characteristics of Salmonella typhimurium strains*

Test	Strain			
	LT2	TV119	G30	SL1032
Absorption of anti-O antibodies[a]	+	−	−	−
Hemagglutination[b]	+	+	+	+
Motility	+	−	+	−
Flagella	+	−	+	−
Pili (fimbriae)[c]	+	−	−	+
Gas from glucose	+	−	+	−

[a] See ref. 3.
[b] Of guinea pig erythrocytes (2).
[c] As determined by electron microscopy.

slightly rough surface; and SL1032—circular, entire edge, 2-mm diameter, rough surface.

Intragastric inoculation of germfree mice. *S. typhimurium* strains LT2 (wild type), TV119, and G30 were present in the ilea, mesenteric lymph nodes, and spleens of infected gnotobiotic mice 8 h after intragastric inoculation with 5×10^7 bacteria (Table 2). With this inoculum, strain SL1032 could be isolated regularly only from the ileum. With an inoculum of 5×10^8 bacteria, however, this strain also could be found in the ileum, mesenteric lymph node, and spleen pools.

Intragastric inoculation of SPF mice. The LPS mutant strains of *Salmonella* were not recovered from the ilea, mesenteric lymph nodes, or spleens of the majority of SPF mice 8 h after intragastric inoculation with 5×10^7 bacteria (Table 2). The wild-type strain (LT2) was present in the ileum, mesenteric lymph nodes, and spleen, but at much lower population levels compared with the gnotobiotic mice. A 10-fold larger inoculum resulted in the isolation of two of the LPS mutants (TV119 and G30) from all organs cultured from SPF mice. SL1032 was never recovered from SPF mice.

Intravenous inoculation of SPF mice. SPF mice inoculated with 2×10^7 wild-type (LT2) bacteria by the intravenous route died within 2 weeks of inoculation (Table 3). At that dosage level, the LPS mutants were not lethal for mice observed over a 2-week period. Strains TV119 and G30 could be recovered from the spleens of survivors 2 weeks after the intravenous inoculation.

Histological examination. Light and fluorescence microscopy of histological sections prepared from gnotobiotic ilea containing *Salmonella* allowed us to determine that the wild type and the LPS mutant strains were all capable of associating with the mucosal surface of this organ (Fig. 1c–e). Bacterial cells were observed in close association with the mucosal surface, varying in position from perpendicular to parallel with respect to the epithelial surface. Bacteria were also present in the lumen. These observations were made in infected gnotobiotes because only in those animals did the populations of the *Salmonella* strains reach high enough levels for the bacteria to be seen by the techniques used (Table 2). *Salmonella* could be found microscopically only in animals in which the *Salmonella* population levels were at or exceeded 10^7 bacteria per g of intestine.

DISCUSSION

As observed in histological sections, all the *Salmonella* strains, both wild type and LPS mutant, are able to associate with the ileal mucosa. Thus, O antigen is not essential for *S. typhimurium* to come into close proximity to the mucosal surface. Moreover, since not all the strains have both flagella and pili, those structures also are not essential for such association.

Current evidence suggests that pili are important in the attachment to host surfaces of *Neisseria gonorrheae* (18) and *Moraxella bovis* (11). Duguid et al. (2) report that hemagglutination of guinea pig erythrocytes by *Salmonella* is an indication that the bacterial cells have pili on their surfaces. The *Salmonella* strains we tested all agglutinated guinea pig erythrocytes after the bacteria were serially subcultured in broth at 30 C. Nevertheless, we were only able to demonstrate pili on two of our strains (LT2 and SL1032). Cultures of *S. sendai*, although nonpiliated, can agglutinate erythrocytes, suggesting that other mechanisms of hemagglutination occur. In fact, a considerable proportion of *Salmonella* strains are nonpiliated, which suggests that pili are not essential to *Salmonella* pathogenesis (2). Our observations support this conclusion.

At the lowest inoculum level used, two of the LPS mutants as well as the wild-type strain of *S. typhimurium* could be recovered from mesenteric lymph nodes from infected gnotobiotic mice. At a higher dosage level, all three mutant strains could be found in mesenteric nodes in infected gnotobiotes. By contrast, at the lowest dosage level, bacteria of only the wild-type strain are found in the nodes of SPF mice. Moreover, population levels of *Salmonella* in the ileum of infected SPF animals were found to be much reduced compared with those in germfree mice.

Increasing the dosage of *Salmonella* overcame these inhibitory effects to some extent in SPF mice, allowing recovery of mutant strains TV119 and G30 from mesenteric lymph nodes

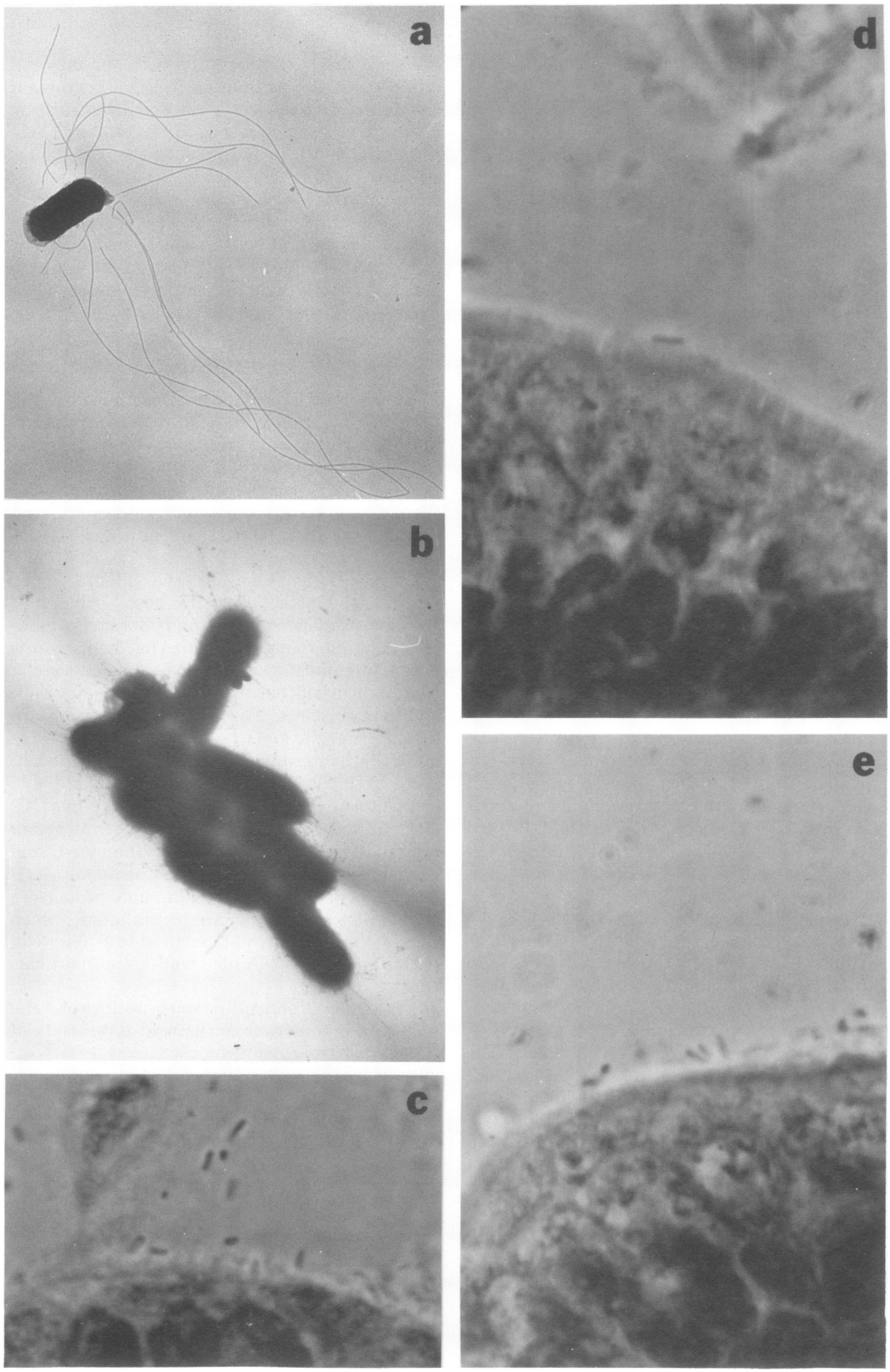
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TABLE 2. *Population levels of Salmonella typhimurium strains in SPF mice and monoinfected gnotobiotic mice 8 hours after intragastric inoculation*

Salmonella strain	Size of inoculum	SPF mice			Gnotobiotic mice		
		Ileum	Lymph node pool[a]	Spleen pool	Ileum	Lymph node pool[a]	Spleen pool
LT2	5×10^7	E (NS-3)[b]	E	E	6 (3-8)	4	E
	5×10^8	E (E-5)	E	E			
TV119	5×10^7	NS (NS-E)	NS	NS	8 (4-9)	3	4
	5×10^8	3 (E-4)	E	E			
G30	5×10^7	NS (NS-E)	NS	NS	9 (5-9)	E	E
	5×10^8	3 (E-5)	E	NS			
SL1032	5×10^7	NS	NS	NS	E (E-5)	NS	NS
	5×10^8	NS	NS	NS	5 (3-9)	4	E
Control		NS	NS	NS	NS	NS	NS

[a] Mesenteric nodes.

[b] Median and range of the $\log_{10}$ *Salmonella*/g of intestinal material; NS, no *Salmonella* detected; E, *Salmonella* detected in enrichment culture only; five mice per group.

TABLE 3. *Survival of SPF mice after intravenous inoculation with 2 × 10⁷ Salmonella typhimurium*

Strain	No. surviving[a]/ no. inoculated	$\log_{10}$ *Salmonella*/g of spleen from survivors
LT2	0/5	
TV119	5/5	E (E-4)[b]
G30	5/5	E
SL1032	5/5	NS
Control	5/5	NS

[a] At 2 weeks after inoculation.

[b] Expressed as in Table 2.

and spleen. Unlike the infected gnotobiotes, however, irrespective of the intragastric dose, SL1032 was never recovered from SPF mice. These observations may reflect interference effects of the indigenous microbiota of the gastrointestinal tract. Indigenous microbes may interfere with pathogens either directly or indirectly (13). Direct interference may be mediated by end products of the metabolism of the indigenous microbes. For example, volatile fatty acids produced by indigenous microbes are toxic to salmonella in a reducing environment such as is found in the bowel (13). Indirect interference may result from the effect on the pathogen of some property of the host that has been stimulated by the indigenous microbiota. For example, peristaltic bowel motility is slower in germfree than in conventional mice (1). Thus, pathogens such as salmonellae may multiply better in the bowels of the germfree animals than in those of conventional ones because they are not propelled so quickly through the lumen (1). In the conventional animal, the peristaltic motility is stimulated by the indigenous microbiota. Thus, the influence of the motility on the pathogen can be viewed as indirect interference by indigenous microbes (13).

An alternative explanation can be offered, however, for the LPS mutant's failure to pass to mesenteric lymph nodes as effectively in SPF mice as they did in the infected gnotobiotes. The mutant bacteria may enter the intestinal mucosa in the SPF animals but then be killed more rapidly than the wild-type strain. The gastrointestinal lamina propria is supplied more richly with cells known to be involved in immunological mechanisms in conventional than in germfree animals (6). SPF mice with their indigenous microbiotas can be viewed as conventional in this respect. Possibly, therefore, the SPF animals can respond immunologically more effectively than do the infected gnotobiotes at the immediate site of invasion of the *Salmonella* and thus restrict the bacteria from passing to the lymph nodes better than do the gnotobiotes. If this local immunological response restricts the LPS mutants to the site better than it does the wild-type bacteria, then O antigen plays an important role in the invasion of the bacteria.

After intravenous inoculation of SPF mice,

FIG. 1. *(a) Salmonella typhimurium strain LT2 cell with peritrichous flagella. Negatively stained with phosphotungstic acid-bovine serum albumin. ×14,000. (b) S. typhimurium strain SL1032 cells with pili. Negatively stained with phosphotungstic acid-bovine serum albumin. ×14,250. (c) S. typhimurium strain TV119 cells associated with ileal mucosa of ex-germfree mouse. Gram-stained section examined with phase contrast microscopy. ×4,500. (d and e) S. typhimurium strain LT2 cells associated with ileal mucosa of ex-germfree mouse. Gram-stained sections examined with phase contrast microscopy. ×4,500.*

only the wild-type strain of *S. typhimurium* killed the animals. This result also may be due to O antigen on the surface of wild-type bacteria but not on the mutant cells. Such an inference should be drawn with caution, however; the LPS mutant strains may have mutations at loci other than in genes concerned with LPS synthesis. Nevertheless, O antigen is undoubtedly an important factor in *Salmonella* virulence. Strains lacking O antigen are avirulent, strains with reduced polymeric side chains have reduced virulence, and small qualitative differences in the structure of O antigen can reduce virulence of the bacteria for mice (4, 8, 9).

We believe that the observations reported here involving mutant bacteria are not the result of the selection of revertants to the wild type in the mouse intestine. The colonial morphology of the cultures recovered from mice were typical of the mutant strain inoculated. The time between inoculation and examination of the mice was kept short (8 h) to reduce the likelihood of a wild-type population emerging should reversion have occurred. In addition, in the intravenously inoculated mice, reversion of mutants to the wild type may have led to death of the animals; at a dosage level of 2×10^7 microbes, the wild type killed all of the animals into which it had been injected intravenously.

Different properties of *Salmonella* cells may be important to the activities of the bacteria in different environments. Hence, some properties will be important in the lumen of the gastrointestinal tract, others at the mucosal surface, and yet others once the organisms have invaded the host's tissues. With respect to the ability of *S. typhimurium* to associate with and invade the ileal mucosa, a search for substances on the bacterial surface other than LPS, and excreted cell products capable of inducing ingestion of bacteria by epithelial cells, may prove worthwhile.

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