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## In Vivo Colonization of the Mouse Large Intestine and In Vitro Penetration of Intestinal Mucus by an Avirulent Smooth Strain of *Salmonella typhimurium* and Its Lipopolysaccharide-Deficient Mutant

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The relative abilities of an avirulent *Salmonella typhimurium* strain with wild-type lipopolysaccharide (LPS) character, SL5319, and a nearly isogenic LPS-deficient mutant, SL5325, to colonize the large intestines of streptomycin-treated CD-1 mice in vivo and to penetrate colonic mucus in vitro were studied. Previously it had been shown that, when fed simultaneously to streptomycin-treated mice (approximately  $10^{10}$  CFU each), the *S. typhimurium* strain with wild-type LPS colonized at  $10^8$  CFU/g of feces indefinitely, whereas the LPS-deficient mutant dropped within 3 days to a level of only  $10^4$  CFU/g of feces. In the present investigation, when SL5325 was allowed to colonize for 8 days before feeding mice SL5319 or when it was fed to mice simultaneously with an *Escherichia coli* strain of human fecal origin ( $10^{10}$  CFU each), both strains colonized indefinitely at  $10^7$  CFU/g of feces. Moreover, when the wild-type and LPS-deficient mutant strains were fed to mice simultaneously in low numbers (approximately  $10^5$  CFU each) the strains survived equally well in the large intestines for 8 days, after which the LPS-deficient mutant was eliminated ( $<10^2$  CFU/g of feces), whereas the wild-type colonized at a level of  $10^7$  CFU/g of feces. In addition although both strains were able to adhere to mucus and epithelial cell preparations in vitro, the wild-type strain was shown to have greater motility and chemotactic activity on CD-1 mouse colonic mucus in vitro and to more rapidly penetrate and form a stable association with immobilized colonic mucosal components in vitro. Based on these data, we suggest that the ability of an *S. typhimurium* strain to colonize the streptomycin-treated mouse large intestine may, in part, depend on its ability to penetrate deeply into the mucus layer on the intestinal wall and subsequently, through growth, colonize the mucosa.

A wide variety of bacteria colonize the large intestines of humans and animals. The bacterial populations involved consist of hundreds of species which for any given mammalian host interact in such a way as to achieve a relatively stable numerical balance (8, 17). Freter and co-workers have performed elegant experiments both in vivo and in continuous-flow cultures in vitro which suggest that the ability of a bacterium to persist in the large intestine under limiting nutrient conditions in part depends on its ability to colonize the intestinal wall (14, 15, 17). According to the model, if two nearly isogenic strains are able to grow equally well in the lumen of the intestine but only one is able to colonize the intestinal wall, that one would be the better large intestine colonizer since under limiting nutrient conditions it would be protected against washout in feces.

In a few instances intestinal wall mucus has been implicated in either the normal bacterial colonization process or in disease states caused by gram-negative intestinal pathogens. For example, the ability of *Vibrio cholerae* to colonize mouse and rabbit small intestine mucosa has been positively correlated with its ability to associate with and grow in the mucus layer (13, 16). Furthermore, the ability of a normal human fecal *Escherichia coli* strain to colonize the mouse large intestine has been positively correlated with its ability to bind to mucus glycoprotein (4), and enterotoxigenic *E. coli* strains have been shown to bind avidly to intestinal mucus (24, 25). In addition, the ability of *Shigella flexneri* to invade HeLa cells in vitro has recently been shown to be

inhibited by guinea pig but not monkey colonic mucus (6), and some virulent *Campylobacter* strains have been shown to bind avidly to both rabbit small and large intestine mucus (26).

Antibiotic treatment of animals is known to increase their susceptibility to colonization by exogenous antibiotic-resistant, enteric gram-negative bacteria in general (10, 11); in the case of *S. typhimurium* it has been shown that streptomycin treatment increases its ability to colonize the mouse large intestine about 100,000-fold (3, 33). Taking advantage of this fact, we were recently able to show that when an avirulent *S. typhimurium* strain and lipopolysaccharide (LPS)-deficient mutants derived from it were placed in direct competition with one another in streptomycin-treated mice, their relative intestinal colonizing abilities were found to decrease as their LPS structure became more defective (30). In the present investigation, we show that the defect in the ability of an LPS-deficient mutant to colonize is in initiating and not in maintaining that state. Furthermore we show that both the wild-type *S. typhimurium* strain and its LPS-deficient mutant grow equally well together in the streptomycin-treated mouse large intestine, but that differences in their relative abilities to initiate colonization may be related to differences in their relative abilities to form a stable association with the mucosal surface.

### MATERIALS AND METHODS

**Bacteria.** *S. typhimurium* SL5316, which contains wild-type LPS, is streptomycin resistant (Str<sup>r</sup>), requires histidine

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and leucine for growth, and is avirulent because of an *aroA* defect (30). Strain SL5319 is a tetracycline-resistant (Tet<sup>r</sup>) derivative of SL5316 caused by insertion of Tn10 into its chromosome and also has the wild-type LPS character (30). SL5316 and SL5319 have identical growth requirements and colonize streptomycin-treated mice equally well (30), and both will hereafter be referred to as wild type. Strain SL5325 is an *rfaJ* derivative of SL5316 (30). In the experiments reported here, a spontaneous nalidixic acid-resistant (Nal<sup>r</sup>) mutant of SL5325 was used (30). This strain, SL5325 (Str<sup>r</sup> Nal<sup>r</sup>), and its parent, SL5325 (Str<sup>r</sup>), have identical colonizing abilities in streptomycin-treated mice (30). The *Salmonella* strains used here are of the FIRN biotype, which does not have type 1 pili (7). *E. coli* F-18 (Str<sup>r</sup> Rif<sup>r</sup>) is a mutant of a normal fecal strain isolated from a healthy University of Rhode Island student in 1977.

**Mouse colonization experiments.** We routinely used male CD-1 mice (5 to 8 weeks old), which were given drinking water containing streptomycin sulfate (5 g/liter). After 1 day of streptomycin treatment, the count of facultative bacteria dropped from about 10<sup>8</sup> CFU/g of feces to less than 10<sup>2</sup> CFU/g of feces, whereas the count of anaerobic bacteria remained constant at about 10<sup>9</sup> CFU/g of feces (30). Mice were then held overnight without food or water. Depending on the experiment, one of the following three protocols was followed thereafter.

(i) **Precolonization with *S. typhimurium* SL5325 followed by challenge with *S. typhimurium* SL5319.** The morning after starvation, three individually housed mice were fed approximately 10<sup>10</sup> CFU of the LPS-deficient mutant strain SL5325 in 1 ml of sterile 20% (wt/vol) sucrose. The mice drank the bacterial suspension almost immediately and were then returned to and maintained on their normal diet (Charles River Valley Rat, Mouse, Hamster Formula), including sterile drinking water containing streptomycin (5 g/liter). The next day and at 48-hour intervals thereafter, 1 g of feces, no older than 24 h, was collected from each mouse, homogenized in 10 ml of L broth, diluted, and plated on selective agar media as described previously (30). On day 7, the mice were again held overnight without food and water, and the next morning they were fed about 10<sup>10</sup> CFU of the wild-type strain, SL5319, as described above. The mice were then returned to their normal diet, including water containing streptomycin, and the next day and at 48-h intervals thereafter fecal samples were diluted and plated as described above.

(ii) **Simultaneous feeding of *S. typhimurium* SL5325 and *E. coli* F-18.** The morning after food and water were withheld, three individually housed mice were fed about 10<sup>10</sup> CFU each of the LPS-deficient mutant *S. typhimurium* SL5325 and *E. coli* F-18. The mice were then returned to their normal diet, and the next morning and at 48-h intervals fresh fecal samples were homogenized, diluted, and plated as described above.

(iii) **Wild-type *S. typhimurium* and LPS mutant strain SL5325 competition in mice fed 10<sup>5</sup> CFU of each strain.** The morning after food and water were withheld, three individually housed mice were fed about 10<sup>5</sup> CFU each of the wild-type strain SL5319 and the LPS-deficient mutant SL5325. The mice were then returned to their normal diet as described above, and the next morning and at 24-h or 48-h intervals thereafter fecal samples were collected, homogenized, diluted, and plated as described above. Plates in all colonization experiments were incubated at 37°C from 18 to 24 h before counting. Colonizing ability was assessed by the level at which a strain persisted in feces. Each colonization

experiment was performed at least twice with essentially identical results.

**Media and antibiotics.** L broth was made as described by Revel (34). L agar is L broth containing 12 g of Bacto-Agar (Difco Laboratories, Detroit, Mich.) per liter. Streptomycin sulfate, nalidixic acid, tetracycline, and rifampin were purchased from Sigma Chemical Co., St. Louis, Mo. Modified Davis minimal medium is the sulfur-free medium described previously (4), but in addition it contained 50 µg each of L-histidine, L-leucine, L-phenylalanine, L-tryptophan, and L-tyrosine per ml and 1 µg each of *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and 2,3-dihydroxybenzoic acid per ml all necessary for growth of the *S. typhimurium* strains (30). L motility agar is L broth containing 3.5 mg of Bacto-Agar per ml of water. Mucus motility agar contains 3 mg of colonic mucus, 1 µg each of *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and 2,3-dihydroxybenzoic acid, and 3.5 mg of Difco agar purified per ml of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) plus Hanks balanced salt solution (HEPES-Hanks buffer), pH 7.4.

**Crude mucus isolation.** Crude mucus was isolated from the colons of 5- to 8-week-old male CD-1 mice by scraping the walls of excised colon into HEPES-Hanks buffer (pH 7.4) as described previously (4) and centrifuging away (4°C) the epithelial cells and membranes twice at 12,000 × *g* for 10 min and once at 27,000 × *g* for 15 min. Crude mucus preparations were microscopically (970×) free of visible epithelial cells.

**Colonic epithelial cell preparation.** Intestinal cells from the mouse colon were prepared by the method of Weiser (37). Twenty-four hours before use the mice were deprived of food and given sterile water containing streptomycin sulfate (5 g/liter). Routinely 10 to 15 mice were employed. The final suspension was 4.0 × 10<sup>6</sup> epithelial cells per ml in 24.5 mM HEPES buffer (pH 7.5) containing 2.5 mM monosodium phosphate and 98 mM sodium chloride.

Although the method of Weiser was originally developed for isolation of epithelial cells from the rat small intestine (37), the method works well for isolation of epithelial cells from both the CD-1 mouse small intestine (25) and the colon. Typically, an entire colon yields about 5 × 10<sup>6</sup> cells. The majority (at least 75 to 80%) appear to be columnar epithelial cells. As pointed out previously (12), it is difficult to ensure that the epithelial cells are free of mucus; indeed, microscopically the cells appear to have a small amount of mucus associated with them.

**Mucus and epithelial cell immobilization.** Mucus was immobilized as described previously (4). Briefly, 0.25-ml samples of mucus (0.5 mg of protein per ml) were added to multiwell polystyrene tissue culture plates (Linbro 24-well flat-bottom tissue culture plates with a well diameter of 1.6 cm; Flow Laboratories, McLean, Va.), and the plates were incubated overnight at 4°C. After incubation for 1 h at 37°C, the wells were washed twice with 0.5 ml of HEPES-Hanks buffer (pH 7.4) to remove unbound protein. Plates containing bovine serum albumin (BSA) were prepared identically, except that 0.25 ml of BSA (0.5 mg/ml) was added to the wells. Colonic epithelial cells (4.0 × 10<sup>6</sup> cells per ml, 0.25 ml per well) were immobilized as described above for mucus. The epithelial cells, although not viable as determined by trypan blue uptake, remained intact upon immobilization and formed a homogeneous layer of cells, each surrounded by mucus, on the bottom of each well. Wells containing either immobilized colonic epithelial cells or colonic mucus, when treated with Coomassie brilliant blue to detect immobilized proteins, stained uniformly blue, whereas untreated wells remained colorless after staining.

**Radioactive labeling of *S. typhimurium*.** Cultures of the *S. typhimurium* strains were labeled in modified Davis minimal medium as described previously (4).  $^{35}\text{SO}_4$ -labeled cultures were washed twice with HEPES-Hanks buffer (pH 7.4) at 4°C and suspended in the same buffer at 37°C at about  $5 \times 10^8$  cells per ml. The specific activity of the cells was routinely between  $2 \times 10^{-3}$  and  $1 \times 10^{-2}$  cpm/CFU, depending on the age of the  $^{35}\text{SO}_4$ .

**Adhesion assay.** The adhesion assay has been described previously (4, 24, 25). Prewarmed (37°C) radioactive *S. typhimurium* cells (0.2 ml,  $5.0 \times 10^8$  cells per ml) were added to each well, and the plates were incubated for 1 h at 37°C. The wells were then washed twice with 0.5-ml samples of HEPES-Hanks buffer (pH 7.4) to remove nonadherent bacteria. Adherent bacteria were recovered by adding 0.5 ml of 5% sodium dodecyl sulfate to each well and reincubating the plates at 37°C for 3 h. The sodium dodecyl sulfate was removed from each well, and the level of radioactivity was determined by scintillation counting.

Radioactive bacteria released less than 0.5% of the total incorporated label during the 1-h incubation period. Furthermore, subsequent incubation of supernatants failed to produce significant levels of radioactivity adhering to mucus (<250 cpm).

**In vitro penetration of *S. typhimurium* through mucus.** Polystyrene wells containing immobilized colonic epithelial cells were prepared as described above. Colonic mucus (0.5 ml, 4.1 mg of protein per ml) was then added to each well above the epithelial cells, forming a distinct layer. Samples of an *S. typhimurium* strain (0.2 ml,  $5 \times 10^8$  CFU/ml) labeled with  $^{35}\text{SO}_4$  were then carefully layered atop the mucus in the wells. The samples were then incubated at 37°C for 1.5, 3.0, 5.0, 7.0, and 18.0 h. At each time, a set of two wells was aspirated once to remove bacteria still in the mucus layer but leave any bacteria which had penetrated through and reached the mucus-epithelial cell interface, and a second set of two wells was aspirated as above and washed twice with 1 ml of HEPES-Hanks buffer (pH 7.4) to remove all bacteria which had penetrated mucus but were not firmly bound to the epithelial cell layer. The adherent bacteria remaining in each set of wells were then collected and counted as described above.

As a control, polystyrene wells containing immobilized colonic epithelial cells were exposed to the same  $^{35}\text{SO}_4$ -labeled *S. typhimurium* strain (0.2 ml per well); at 1.5 and 18 h, the bacteria at the HEPES-Hanks buffer (pH 7.4)-epithelial cell interface and the bacteria firmly bound to the epithelial cell layer were collected and counted as described above.

**Motility.** Strains to be tested for motility were streaked on L agar and incubated for 18 h at 37°C. At that time, samples of each strain were transferred with a sterile toothpick from L agar to L motility agar and mucus motility agar. Plates were incubated at 37°C, and diameters of visible spread were measured (30).

**Statistics.** Where indicated below, groups of duplicate or triplicate values were compared by Student's *t* test (*P* values), and the arithmetic means of specific time points were compared by linear regression analysis (correlation coefficients). Bars in the graphs represent standard errors of the means.

## RESULTS

**In vivo colonization.** Recently, we reported that when  $10^{10}$  CFU each of an avirulent *S. typhimurium* strain with wild-

type LPS character and an LPS-deficient mutant derived from it, SL5325, were fed simultaneously to streptomycin-treated mice, the wild-type strain persisted in feces indefinitely at  $10^8$  CFU/g of feces, whereas the LPS-deficient mutant dropped from  $10^8$  to  $10^4$  CFU/g of feces within 3 days and colonized indefinitely thereafter at a level of  $10^3$  CFU/g of feces (30). When fed alone to mice the LPS-deficient mutant colonized at a level of about  $10^7$  CFU/g of feces (30). To learn more about the relative colonizing abilities of the wild-type *S. typhimurium* and its LPS-deficient mutant three additional colonization experiments were performed.

(i) **Precolonization of streptomycin-treated mice with an LPS-deficient mutant before challenge with the wild-type strain.** Streptomycin-treated mice were fed  $10^{10}$  CFU of the LPS-deficient mutant *S. typhimurium* SL5325 and 8 days later were fed  $10^{10}$  CFU of the wild-type strain. Under these conditions, over the next 13 days the LPS mutant remained at about  $10^7$  CFU/g of feces, and the wild-type strain colonized at about the same level (Fig. 1). Thus, when the LPS-deficient mutant was given time to successfully colonize, it had no trouble maintaining that state in the face of competition from its wild-type parent.

(ii) ***S. typhimurium* SL5325 competition with *E. coli* F-18 in streptomycin-treated mice.** Streptomycin-treated mice were simultaneously fed  $10^{10}$  CFU each of the LPS-deficient mutant strain SL5325 and *E. coli* F-18, a normal human fecal *E. coli* strain (4, 5). Each strain colonized at a level of about  $10^7$  CFU/g of feces indefinitely (Fig. 2). The same result was obtained when SL5325 competed against a different human fecal *E. coli* strain, *E. coli* F-17 (28); i.e., both colonized at  $10^7$  CFU/g of feces (data not shown). Therefore, the LPS-deficient mutant is a defective colonizer only when competing against a related *Salmonella* strain, not when competing against unrelated *E. coli* strains.

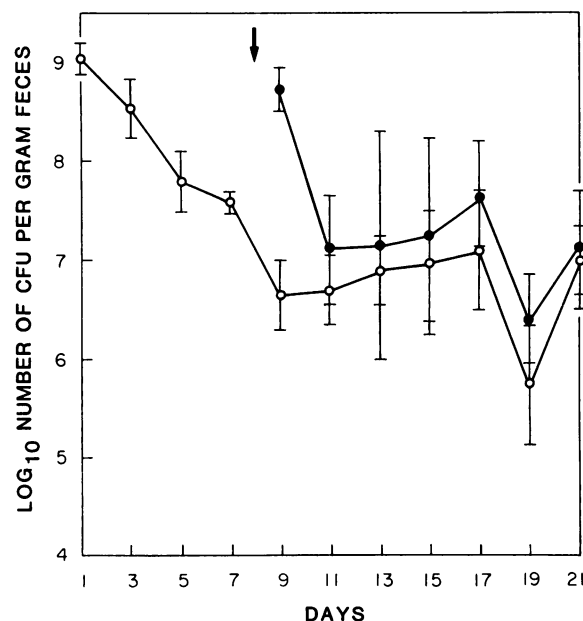


FIG. 1. Stability of SL5325 in vivo when fed to mice before SL5319. Three streptomycin-treated mice were fed  $10^{10}$  CFU of the LPS-deficient mutant (○) SL5325 (Str<sup>r</sup> Nal<sup>r</sup>). At the indicated time (arrow), the same mice were fed  $10^{10}$  CFU of the wild-type strain (●) SL5319 (Str<sup>r</sup> Tet<sup>r</sup>). Fecal samples were plated as described in Materials and Methods. Counts (mean values  $\pm$  standard errors) are expressed as log<sub>10</sub> numbers of CFU per gram of feces.

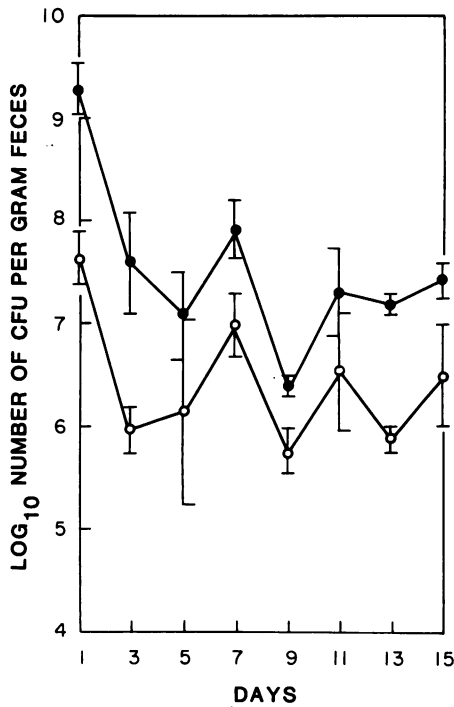


FIG. 2. Colonization of streptomycin-treated mice with *E. coli* F-18 and *S. typhimurium* SL5325. *E. coli* F-18 (Str<sup>r</sup> Rif<sup>r</sup>) (●) and *S. typhimurium* SL5325 (Str<sup>r</sup> Nal<sup>r</sup>) (○) were fed simultaneously to each of three streptomycin-treated mice (10<sup>10</sup> CFU each). At the indicated times, fecal samples were plated as described in Materials and Methods.

(iii) **Wild-type *S. typhimurium* strain SL5319 and LPS-deficient mutant strain SL5325 competition in mice fed 10<sup>5</sup> CFU of each strain.** When streptomycin-treated mice were simultaneously fed 10<sup>5</sup> CFU each of the wild type and the LPS-deficient mutant strain, i.e., 100,000-fold fewer than in previous experiments, both strains persisted in about equal numbers in feces for about 8 days (Fig. 3), i.e., about three times as long as when mice were fed 10<sup>10</sup> CFU of each strain (30). After 8 days, however, the wild-type strain increased 100-fold in numbers, whereas the LPS mutant was rapidly eliminated, i.e., to <10<sup>2</sup> CFU/g of feces (Fig. 3). These data suggested that some factor other than the relative abilities of the two strains to withstand the environment in the mouse large intestine was responsible for their disparate colonizing abilities.

**Adhesion and the ability of the *S. typhimurium* strains to form a close association with the mucosal surface in vitro.** It is becoming increasingly clear that association with the intestinal mucosal surface may be an important factor in colonization (14, 15, 17). Although undoubtedly a complex process, the establishment of such an association presumably involves penetration to the deeper portions of the mucus layer and perhaps adhesion to the underlying epithelial cells. With this in mind, we attempted, first, to assess the abilities of the strains to adhere to mucosal components immobilized on polystyrene and, second, to assess in vitro the ability of each strain to adhere to and form a stable association with immobilized components after passing through a layer of colonic mucus. Each experiment in this section was performed at least three times. Typical results are shown in Table 1 and Fig. 4 and 5.

Both strains were capable of binding to mucus and epithe-

TABLE 1. Adhesion of the *S. typhimurium* wild-type and LPS-deficient mutant strains to immobilized colonic mucus, epithelial cell preparation, and BSA

Prepn	Adhesion (mean cpm ± SE) <sup>a</sup>		Adhesion ratio <sup>b</sup>
	Wild type	LPS-deficient mutant	
Colonic mucus	3,311 ± 489	17,016 ± 1,880	5.14
Epithelial cells	2,445 ± 222	12,258 ± 689	5.01
BSA	1,222 ± 144	4,444 ± 460	3.64

<sup>a</sup> Data are from triplicate samples. The specific activities of the wild-type strain and the LPS-deficient mutant were essentially identical (i.e., 1.1 × 10<sup>-2</sup> cpm/CFU).

<sup>b</sup> Ratio of adhesion of the LPS mutant to that of the wild type.

lial cell preparations immobilized on polystyrene (Table 1). In the case of the wild-type strain, binding to the mucus or epithelial cell preparations was greater than twice (2.7- and 2.0-fold, respectively) that detected on BSA-treated control wells (*P* < 0.05), whereas binding of the LPS-deficient mutant was approximately three (3.8- and 2.7-fold, respectively) times greater than that of the BSA-treated control wells (*P* < 0.05). When adhesion of the two strains was compared directly, it appeared that the LPS-deficient mutant bound to all of the immobilized preparations, including the BSA, at levels three to five times higher than the wild-type strain (*P* < 0.05). The apparently greater binding ability of the LPS-deficient mutant may be due to the fact that the LPS-deficient mutants are more hydrophobic than the wild-type strain (19). Evidence for such hydrophobicity in the present case was seen microscopically in the tendency of the LPS-deficient mutant to form aggregates of 5 to 10 cells when in suspension, and it may be that the overall higher levels of adhesion are due, at least in part, to the presence of bound aggregates.

In the second portion of the in vitro studies, a related but more complex assay was employed. In this case, the system employed was designed to measure the relative abilities of the two strains to travel through a layer of colonic mucus and subsequently adhere to and establish a stable association

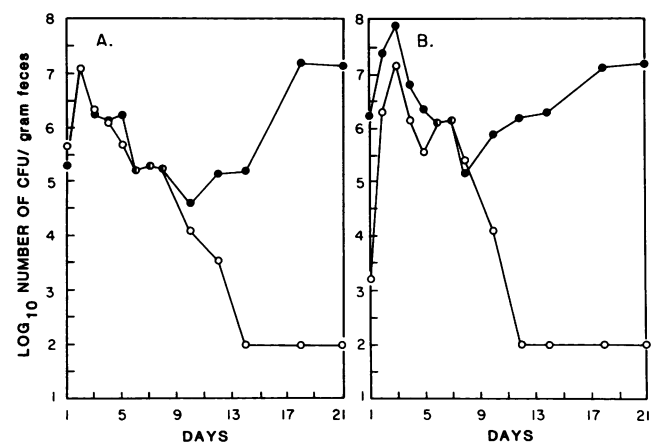


FIG. 3. Colonization of SL5319 and SL5325 in mice fed 10<sup>5</sup> CFU of each strain. Streptomycin-treated mice were simultaneously fed 10<sup>5</sup> CFU each of the wild-type strain (●) SL5319 (Str<sup>r</sup> Tet<sup>r</sup>) and the LPS mutant (○) SL5325 (Str<sup>r</sup> Nal<sup>r</sup>). At the indicated times, fecal samples were plated as described in Materials and Methods. For clarity, the data from two animals (A and B) are shown individually. Counts lower than 10<sup>2</sup> CFU/g of feces are presented as 10<sup>2</sup> CFU/g of feces, the lower limit of the assay.

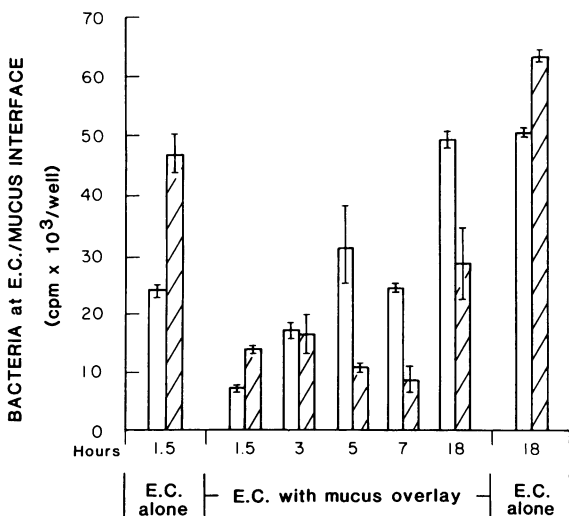


FIG. 4. Penetration of *S. typhimurium* strains through mucus to the mucus-colonic epithelial cell (E.C.) interface in vitro. Data are presented as the means and standard errors of duplicate samples. In this experiment the specific activity of the two strains were essentially identical (i.e.,  $1.1 \times 10^{-2}$  cpm/CFU). Symbols: □, wild type; ▨, LPS mutant.

with an underlying immobilized epithelial cell preparation. Penetration was assessed by first layering the wild-type strain or LPS-deficient mutant atop a layer of mucus. At the times indicated the mucus was aspirated, and the total number of bacteria at the mucus-epithelial cell interface, i.e., adherent and nonadherent, was determined (Fig. 4). To assess the level of bacteria closely associated with (adhering to) the epithelial preparation, the mucus was overlaid and aspirated as above, and the number of bacteria adhering to the epithelial cell preparation was determined after two additional washes (Fig. 5).

During the first 3 h the overall number of wild-type and LPS-deficient mutant cells continuously increased at the mucus-epithelial cell interface; however between 1.5 and 7 h

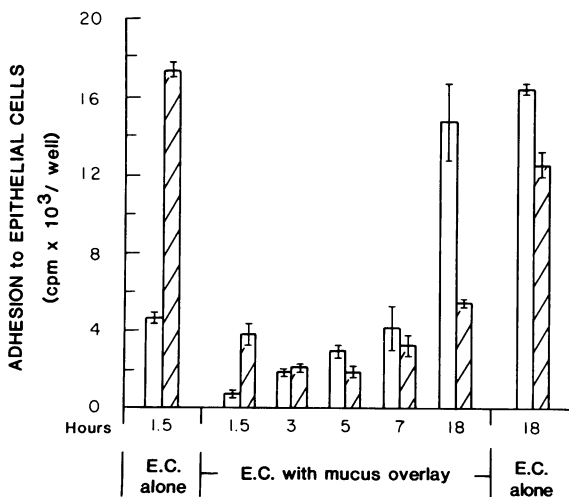


FIG. 5. Adhesion of *S. typhimurium* strains to the colonic epithelial cell (E.C.) layer after penetration through mucus in vitro. Data are presented as the means and standard errors of duplicate samples. The bacterial cultures used are those in Fig. 4. Symbols: □, wild type; ▨, LPS mutant.

about 50% of the LPS-deficient mutants returned to the mucus layer (correlation coefficient, 0.83), whereas the number of wild-type cells generally increased (correlation coefficient, 0.81), so that by 7 h there were almost three times as many wild-type cells as LPS-deficient mutant cells at the mucus-epithelial cell interface ( $P < 0.05$ ) (Fig. 4). At 18 h the numbers of wild-type cells at the mucus-epithelial cell interface were still greater than the number of LPS-deficient mutants ( $P < 0.05$ ), although the ratio had dropped from about 3 to 1 to about 1.8 to 1 (Fig. 4). A similar pattern was observed in wells which were washed twice to remove unbound bacteria (Fig. 5). Initially (1.5 h) there appeared to be more LPS-deficient mutants bound ( $P < 0.05$ ). However, once again about 50% of the LPS-deficient mutant bound appeared to return to the mucus layer during the next 3.5 h, whereas the number of wild-type cells bound to the epithelial cell preparation increased continuously throughout the experiment (correlation coefficient, 1.0). By 18 h nearly three times as many wild-type cells as mutant cells were bound to the epithelial cell preparation ( $P < 0.05$ ).

**Motility.** SL5325, the LPS-deficient mutant, has been reported to be far less motile on L motility agar than its smooth parent, SL5316, presumably due to a defect in either flagellar or chemotactic activity (30). In the present study, to determine whether this defect was also apparent on colonic mucus, we measured the rate of spreading the two wild-type strains SL5316 and SL5319 and the LPS-deficient mutant SL5325 on both L motility agar and mucus motility agar (see Materials and Methods). In both instances at either time SL5316 and SL5319 spread far more rapidly ( $P < 0.01$ ) than SL5325 (Table 2).

## DISCUSSION

In a previous report (30) a series of LPS-deficient mutants derived from *S. typhimurium* were shown to be poor colonizers of the streptomycin-treated mouse large intestine when placed in direct competition with the parent strain, which possessed wild-type LPS. In the present investigation, in vivo studies designed to further characterize the colonizing properties of the wild-type strain and an LPS-deficient mutant were conducted. Evidence is presented which illustrates several important points regarding the relative colonizing abilities of these strains.

First, when mice are simultaneously fed low numbers of each strain, the LPS-deficient mutant can grow in the streptomycin-treated mouse large intestine as well as the wild-type parent (Fig. 3). Only after 8 days does the wild-type parent become the dominant organism. This effectively rules out the possibility that the wild-type strain is a better colonizer of the streptomycin-treated mouse large intestine

TABLE 2. *S. typhimurium* spreading on motility agar<sup>a</sup>

Agar	Time (h) of spreading	Spread (mean mm $\pm$ SE) <sup>b</sup>		
		SL5316 <sup>c</sup>	SL5319 <sup>c</sup>	SL5325 <sup>d</sup>
L motility	8	24.3 $\pm$ 3.1	21.0 $\pm$ 1.7	4.3 $\pm$ 0.6
	12	44.7 $\pm$ 3.2	41.7 $\pm$ 2.1	12.3 $\pm$ 0.6
Mucus motility	10	7.0 $\pm$ 1.0	6.7 $\pm$ 0.6	2.3 $\pm$ 0.6
	23	14.7 $\pm$ 1.2	12.7 $\pm$ 1.2	3.7 $\pm$ 0.6

<sup>a</sup> Motility assays were performed in triplicate (see Materials and Methods).

<sup>b</sup> Measurements are diameters of spread after incubation for the indicated times at 37°C.

<sup>c</sup> Wild type LPS.

<sup>d</sup> *rfaJ* mutant.

because it possesses an intrinsic advantage relating to the environmental conditions of the large intestine, e.g., resistance to hydrogen sulfide (14), bacteriocins (22), short-chain fatty acids (18, 33), or low pH and  $E_h$  (3, 27) or an advantage in competing for limiting nutrients (14, 15), factors suggested to be operative in the untreated mouse large intestine. Second, the data indicate (Fig. 2) that there is a degree of specificity to the competitive aspects of the colonization process. Although the LPS-deficient mutant is a defective colonizer relative to the wild-type parent strain, when competing against an unrelated *E. coli* strain, the LPS-deficient mutant is an effective colonizer (Fig. 2). Third, it appears that the defect of the LPS-deficient mutant is related to its relative ability to initiate colonization in the face of competition from large numbers of the parent strain, rather than in its ability to maintain colonization. That is, if given an opportunity to become well established (i.e., 8 days), the LPS-deficient mutant appears to be able to maintain itself in relatively high numbers over a significant period of time in the face of subsequent competition from the wild-type strain (Fig. 1).

The wild-type strain, in addition to being a better colonizer, appears to have an advantage over the LPS-deficient mutant in penetrating mucus *in vitro*, although this process and subsequent adhesion to the underlying epithelial cell layer *in vitro* appear to be complex processes (Fig. 4 and 5). That is, at first (1.5 to 3 h) a significant number of LPS-deficient mutants penetrated the mucus layer rapidly (Fig. 4) and bound to the underlying epithelial cell layer (Fig. 5), but both penetration and adhesion of these cells seemed to be reversible in that a large proportion returned to the mucus layer during the next few hours (Fig. 4 and 5). It is possible that the initial rapid penetration of the LPS-deficient mutants was due to aggregates sinking through the mucus, binding, and subsequently breaking up and returning to the mucus layer. During and after this time penetration of remaining LPS-deficient mutants through the mucus to the mucus-epithelial cell interface was extremely slow (Fig. 4). In contrast, the wild-type strain, consisting primarily of single cells, penetrated the mucus layer and bound to the epithelial cell layer continuously such that by 18 h nearly three times as many wild-type cells as LPS-deficient mutant cells were found bound to the underlying epithelial cell layer (Fig. 4 and 5). In any case, the data suggest a difference in both the rate at which the two strains can penetrate the mucus layer and, perhaps more importantly, the rate at which the two strains form a stable association with the underlying mucosal components.

It should be emphasized that we do not mean to imply that either the wild-type or LPS-deficient mutant strain necessarily binds specifically to the epithelial cells after penetrating the mucus layer *in vitro*. We simply do not know whether the *S. typhimurium* strains bind to the epithelial cells, mucus associated with the epithelial cells, or both. Experiments designed to resolve this issue are in progress.

How might the relative abilities of the wild-type and LPS-deficient strains to penetrate mucus and form a stable association with underlying mucosal components help explain the *in vivo* colonization data? It is known that both the large and small intestine walls consist of an epithelium containing brush border epithelial cells and secretory goblet cells which synthesize and secrete a relatively thick (up to 400  $\mu$ m), viscous, mucus covering (2, 29). The mucus layer contains mucin, a 2-megadalton, gel-forming glycoprotein and a large number of smaller glycoproteins, proteins, glycolipids, and lipids (1, 23, 35). Presumably, shed epithe-

lial cells are the source of many of the smaller components of mucus (9, 31, 32, 38). The intestinal mucus layer itself is in a dynamic state, continuously being synthesized and then degraded to a large extent by indigenous intestinal microflora (20, 29). Degraded mucus components are shed into the lumen of the intestine and eventually find their way into feces (20, 21, 36, 39). We suggest that the LPS-deficient mutant penetrates relatively slowly through mucus toward the underlying epithelial cell layer *in vivo*, perhaps due to relatively poor flagellar chemotactic activity (Table 2). Additionally, it is possible that some of the LPS-deficient mutants which do penetrate mucus *in vivo* return to the mucus layer as they do *in vitro* (Fig. 4 and 5) and are subsequently eliminated as older mucus is sloughed into the lumen of the intestine. In contrast, the wild-type strain, perhaps because it has greater flagellar chemotactic activity (Table 2), moves more quickly through the mucus layer to the epithelial cell layer (Fig. 4 and 5), where it binds to specific *Salmonella* receptors and grows by using the mucus layer as a source of carbon and nitrogen. If so, when the wild-type strain and the LPS-deficient mutant are fed to mice simultaneously, the wild-type strain would eventually occupy the majority of the available salmonella-specific sites at the mucus epithelial cell interface and would therefore be the better colonizer since, as suggested by Freter et al. (14, 15, 17), it would be protected against washout in feces.

We further suggest that when the LPS-deficient mutant is allowed to colonize for 8 days before mice are fed the wild-type strain, it has enough time in the absence of competitor to slowly penetrate the mucus layer, bind at the mucus-epithelial cell interface, grow, occupy a significant percentage of *Salmonella* mucosal receptor sites, and thereby colonize the intestinal wall. Thus, as observed here (Fig. 1), when the wild-type strain is subsequently fed to the mice at day 8 it does not cause elimination of the LPS mutant.

At the present time we do not understand why feeding mice  $10^{10}$  CFU each of the wild-type strain and the LPS-deficient mutant results in an almost immediate elimination of the LPS-deficient mutant (30) whereas feeding only  $10^5$  CFU of each strain results in their existing in equal numbers for 8 days before the LPS-deficient mutant is eliminated (Fig. 3). However, it is possible that when mice are fed  $10^5$  CFU of each strain we observe the results of growth in the intestinal lumen for the first 8 days and only thereafter the effects of disparate colonizing abilities of the intestinal mucosa. In contrast, when mice are fed  $10^{10}$  CFU of each strain, limiting nutrients in the lumen might prevent further luminal growth, and we therefore observe an almost immediate effect of disparate colonization abilities of the intestinal mucosa.

Finally, we suggest that *E. coli* strains do not cause elimination of the *Salmonella* LPS-deficient mutant (Fig. 2), because they bind to different receptors within the intestinal mucosa and therefore do not prevent one another from colonizing the intestinal wall. Experiments designed to test these hypotheses are currently in progress.

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