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Roles of Motility, Chemotaxis, and Penetration through and Growth in Intestinal Mucus in the Ability of an Avirulent Strain of *Salmonella typhimurium* To Colonize the Large Intestine of Streptomycin-Treated Mice

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Previously, it had been shown that an avirulent strain of *Salmonella typhimurium*, SL5316, with wild-type lipopolysaccharide (LPS) was a far better colonizer of the streptomycin-treated CD-1 mouse large intestine, was far more motile, did not bind to mouse intestinal mucus nearly as well as, but penetrated through a layer of intestinal mucus in vitro far better than an almost isogenic LPS-deficient transductant, SL5325. In the present investigation, a nonflagellated transductant, SL5681, and a nonchemotactic transductant, SL5784, were isolated from SL5316 and tested for relative colonizing ability versus SL5316 (smooth) and SL5325 (rough) in streptomycin-treated mice. In addition, the *Salmonella* strains were tested for their ability to grow together in cecal intestinal mucus and in cecal luminal contents, for their tumbling and swimming activities after growth in cecal mucus, and for their ability to adhere to and travel through cecal mucus in vitro. The data show that the nonflagellated and nonchemotactic derivatives colonized large intestine nearly as well as their parent and were far better colonizers than the LPS-deficient mutant, that all the strains grew equally well in cecal mucus but did not grow in cecal luminal contents, and that cecal mucus-grown strains lost tumbling and swimming activities. Furthermore, the LPS-deficient strain adhered to cecal mucus far better but penetrated mucus far worse than did the nonflagellated transductant, the nonchemotactic transductant, and the parent. Thus, motility and chemotaxis do not appear to play a major role in the ability of the avirulent *S. typhimurium* strains to colonize the mouse large intestine, colonization may require growth in cecal mucus but does not depend on growth in cecal luminal contents, growth in cecal mucus inhibits *S. typhimurium* motility, and increased adhesion of the LPS-deficient mutant to cecal mucus and its poor ability to penetrate cecal mucus may play a role in its poor intestine-colonizing ability.

Recently, we reported that the ability of a *Salmonella typhimurium* strain to colonize the large intestine of a streptomycin-treated mouse decreased as its lipopolysaccharide (LPS) structure became more defective (22). That is, when an avirulent smooth (wild-type LPS) *S. typhimurium* strain, SL5316, and its nearly isogenic derivatives SL5318 (*rfb* deletion, Ra LPS), SL5325 (*rfaJ* mutant, Rb2 LPS), and SL5326 (*rfa-990* mutant, with LPS less complete than Rb2 chemotype) were fed individually to streptomycin-treated mice, each strain colonized at between 10^7 and 10^8 CFU/g of feces; however, when fed to mice in pairs, their relative colonizing abilities were observed to be wild-type > *rfb* >> *rfaJ* > *rfa-990* (22).

LPS-defective mutants of *Escherichia coli* and *Salmonella typhimurium* are known to be less motile than their wild-type parents (9, 15), and the LPS-defective derivatives of strains SL5316, scored for motility by testing the ability to spread during growth in semisolid motility agar, conformed to this rule (22). The rate of spread through semisolid medium depends not only on the presence of functional (*mot*$^+$) flagella but also on chemotaxis (Che$^+$ character). It was thus theoretically possible that the nonspreading or slow-spreading LPS-defective derivatives of strain SL5316 colonized poorly because they had fewer (functional) flagella or less chemotactic ability than their parent strain, SL5316. In the present study, two transductional derivatives of SL5316, one lacking flagella (Fla$^-$) and the other motile but chemotaxis deficient (Che$^-$) were found to colonize the large intestine of streptomycin-treated mice nearly as well as did their parent strain, SL5316, which has wild-type motility and chemotaxis ability. Both the Fla$^-$ and the Che$^-$ transductants colonized much more efficiently than did the *rfaJ* mutant of the same parent strain. These results indicate that the defect in colonization of the LPS mutant results from causes other than defective motility or chemotaxis.

We also show that strain SL5316 and its derivatives can grow in cecal mucus but not in cecal luminal contents from streptomycin-treated mice. Furthermore, the LPS-defective *rfaJ* mutant showed much stronger adhesion to and far less ability to penetrate through cecal mucus in vitro than did the related "smooth" strains. We suggest that this strong adhering ability and weak penetrating ability may cause or contribute to its colonizing inefficiency.

MATERIALS AND METHODS

Bacteria. *S. typhimurium* SL5316, which contains wild-type LPS, is of biotype FIRN (4) and so is unable to produce type 1 pili and is streptomycin resistant, histidine and leucine dependent and, in addition, is aromatic dependent and nonvirulent because of an *aroA* mutation (12, 22). Strain SL5316 Nal^r, a spontaneous nalidixic acid-resistant mutant of SL5316, unaltered in other characters including colonizing ability, was used in place of SL5316 itself. These strains are able to colonize the streptomycin-treated CD-1 mouse large

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intestine despite the *aroA* defect (21, 22), presumably because the indigenous anaerobes which continue to colonize the intestine in the presence of streptomycin (21, 22) provide the *p*-aminobenzoate and 2,3-dihydroxybenzoate that *S. typhimurium* strains require for growth. SL5319 is strain SL5316 made *hisD8557*::Tn*10* by transduction and therefore tetracycline resistant but otherwise unaltered in phenotype and was shown previously to colonize as efficiently as its parent (22). SL5325 is a spontaneous rough mutant, *rfaJ989*, isolated from SL5316 (22). In the present work we used SL5325 Nal^r, a spontaneous nalidixic acid-resistant mutant, instead of SL5325 itself. SL5325 and SL5325 Nal^r are identical in colonizing ability in streptomycin-treated mice (22). Strain SL5681 is strain SL5316 made Δ*flaFV1704 zcd-907*::Tn*10* by transduction, as described in Results; this strain is nonflagellate and tetracycline resistant. Strain SL5784 is strain SL5316 made *che-107 zca-1*::Tn*10* by transduction. Mutation *che-107* (29) causes the chemotaxis-negative character (of the never-tumbling or counterclockwise-bias type), but is now known to affect gene *flaAII.2*.

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 facultative bacteria dropped from about 10^8 CFU/g of feces to less than 10^2 CFU/g of feces, whereas the count of anaerobic bacteria remained constant at about 10^9 CFU/g of feces (22). Mice were then held overnight without food or water.

The morning after food and water were withheld, a group of three individually housed mice were fed either 10^4 or 10^{10} CFU each of two strains to be tested in 1 ml of sterile 20% (wt/vol) sucrose. The mice drank the bacterial suspension almost immediately and were then returned to their normal diet and provided drinking water with streptomycin as before, and the next morning and at 48-h intervals, fresh fecal samples were homogenized, diluted, and plated on selective L-agar medium as described previously (22). All colonization experiments were repeated at least twice with essentially identical results.

Determination of *S. typhimurium* strains on cecal epithelial cells and in cecal luminal contents. After food and water were withheld as described above, nine individually housed mice were fed 10^{10} CFU of either SL5316 Nal^r (wild type) and SL5681 (Fla^-), SL5316 Nal^r and SL5784 (Che^-), or SL5325 (*rfaJ*) alone. The animals were then returned to their normal diet, including drinking water containing streptomycin sulfate. On day 10 postfeeding, three animals were sacrificed and their ceca were removed. Cecal luminal contents from each mouse were collected, homogenized, and plated on selective agar medium (22). Epithelial cells from the entire cecum of each mouse were prepared by the method of Weiser (30). As seen under the microscope, each epithelial cell was surrounded by mucus. Each sample was finally suspended in 2 ml of phosphate-buffered saline, pH 7.4. Typically, an entire cecum yielded about 10^7 epithelial cells. Epithelial cells were homogenized, diluted, and plated on selective agar medium (22). The plates were incubated at 37°C for 18 to 24 h.

Media and antibiotics. L-broth was made as described by Revel (24). L-agar is L-broth containing 12 g of Bacto-agar (Difco Laboratories, Detroit, Mich.) per liter. Cecal mucus agar is cecal mucus (2 mg of protein per ml in HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]-Hanks buffer [pH 7.4]) containing 12 g of purified agar (Difco) per liter. Streptomycin sulfate, nalidixic acid, and tetracycline were purchased from Sigma Chemical Co., St. Louis, Mo. Modified Davis minimal medium (MDMM) is the sulfur-free minimal medium described previously (3) but in addition contains 50 μg/ml each of L-histidine, L-leucine, L-phenylalanine, L-tryptophan, L-tyrosine, and 1 μg/ml each of *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and 2,3-dihydroxybenzoic acid, all necessary for growth of the *S. typhimurium* strains (22). L-motility agar is L-broth containing 3.5 mg of Bacto-agar per ml of water. Cecal mucus motility agar is the same as cecal mucus agar but contains only 3.5 mg of purified agar.

Crude mucus isolation for adhesion assays. Crude mucus was isolated from the ceca of 5- to 8-week-old male CD-1 mice as described previously (3) by scraping the walls of excised cecum into HEPES plus Hanks balanced salt solution (HEPES-Hanks buffer, pH 7.4) 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.

Cecal epithelial cell preparation. Intestinal cells from the mouse cecum were prepared as described above. 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 contained 4.0×10^6 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 used to isolate epithelial cells was originally developed for isolation from the rat small intestine (30), the method works well for isolation of epithelial cells from the CD-1 mouse small intestine (17), colon (21), and cecum. Typically, an entire cecum yields about 10^7 cells. The majority (at least 75 to 80%) appear to be columnar epithelial cells. It is difficult to ensure that the epithelial cells are free of mucus; indeed, as seen microscopically, the cells appear to have a small amount of mucus associated with them.

Mucus and epithelial cell immobilization. Cecal mucus and epithelial cell preparations were immobilized as described previously (3, 16, 17, 21). Briefly, 0.20-ml samples of cecal mucus (0.5 mg of protein per ml) or epithelial cells (4×10^6 cells per ml) were added to multiwell polystyrene tissue culture plates (Linbro 24-well flat-bottomed 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 mucosal components. Wells containing immobilized cecal mucus or epithelial cells, when treated with Coomassie brillant blue to detect immobilized material, stained uniformly blue, whereas untreated wells remained colorless after staining.

Radioactive labeling of *S. typhimurium*. Cultures of the *S. typhimurium* strains were labeled in MDMM as described previously (21). $^{35}SO_4$-labeled cultures were washed twice with HEPES-Hanks buffer (pH 7.4) at 4°C and resuspended in the same buffer at 37°C at about 5×10^8 cells per ml. The specific activity of the cells was routinely between 2×10^{-3} and 1×10^{-2} cpm/CFU, depending on the age of the $^{35}SO_4$.

Adhesion assay. The assay for bacterial adhesion to mucus or epithelial cell preparations has been described previously (3, 16, 17, 21). Warm (37°C), live, radioactive *S. typhimurium* cells (0.2 ml, 5.0×10^8 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 (SDS) to each well and reincubating the plates at 37°C for 3 h. The SDS, containing mucosal components and bacteria, 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 bacteria-free supernatants failed to produce significant levels of radioactivity adhering to mucus or epithelial cell preparations (<250 cpm).

In vitro penetration of the *S. typhimurium* strains through mucus. The in vitro penetration assay has been described previously (21). Briefly, polystyrene wells containing immobilized cecal epithelial cells were prepared as described above. Cecal mucus (0.5 ml, 4.0 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, 5×10^8 CFU/ml) labeled with $^{35}SO_4$ were then carefully layered atop the mucus in the wells. The samples were then incubated at 37°C for 1.0, 3.0, 5.0, and 7.0 h. At each time, a set of two wells were aspirated once to remove bacteria still in the mucus layer but leave any bacteria which had penetrated and reached the mucus-epithelial cell interface, and a second set of two wells were aspirated as above and washed twice with 1 ml of HEPES-Hanks buffer (pH 7.4) to remove all bacteria which had penetrated the mucus but were not firmly bound to the epithelial cell layer. The bacteria remaining in each set of wells were then collected and counted as described above.

***S. typhimurium* growth in cecal luminal contents and cecal mucus in vitro.** The ceca from 16 streptomycin-treated CD-1 male mice (5 to 8 weeks old) were removed and their contents were collected. Routinely, the yield of cecal contents was about 10 ml and the pH was 6.4. To obtain mucus, the ceca were then rinsed briefly in sterile distilled water to remove remaining fecal matter and debris and drained, and the walls of the tissue were gently scraped. Typically, 2 ml of crude, extremely viscous mucus, pH 7.1, was obtained. These preparations were each inoculated with about 10^4 CFU of each of two strains to be tested and incubated at 37°C, and at 0, 6, and 18 h a portion was removed, homogenized, diluted, and plated on selective agar medium. Plates were incubated at 37°C for 18 to 24 h prior to counting.

Motility after growth in L-broth and cecal mucus. The avirulent *S. typhimurium* strains were inoculated at about 10^4 CFU/ml into either L-broth or cecal mucus diluted to 2 mg of protein per ml in HEPES-Hanks buffer (pH 7.4). Tumbling and swimming activities were observed in wet mounts (1,000×) under an Olympus BH-2 phase-contrast microscope. The strains were then streaked from L-broth onto L-agar containing 100 μg of streptomycin sulfate per ml or from cecal mucus to cecal mucus agar containing 100 μg of streptomycin sulfate per ml, and the plates were incubated for 18 h at 37°C. At that time, samples of each strain on L-agar were moved with a toothpick to L-motility agar and cecal mucus motility agar, and samples of each strain on cecal mucus agar were moved with a toothpick to L-motility agar and cecal mucus motility agar. Plates were incubated at 37°C, and at hourly intervals, the diameters of visible spread were measured.

Statistics. Where indicated in the text, means derived from triplicate samples were compared by Student's *t* test (*P* values).

RESULTS

Construction of nearly isogenic nonflagellate and nonchemotactic derivatives of a streptomycin-resistant, smooth, nonvirulent strain. Phage P22 HT105/1 *int* (27) grown on a Δ*flaFV1704 zcd-907*::Tn*10* strain (received from M. Carsiotis, Department of Microbiology, College of Medicine, University of Cincinnati) was used to evoke tetracycline-resistant transductants from strain SL5316. Of 11 tetracycline-resistant clones picked to semisolid medium with tetracycline (25 μg/ml), 2 gave nonspreading growth. One such clone, reisolated from a single colony and confirmed to be unaltered in nutritional character and phage sensitivity pattern and inferred to have incorporated both its mutations from the donor, was labeled SL5681. Gene *flaFV* specifies the constituent protein of the proximal (hook) part of the flagellum, and strain SL5681 had the phenotype expected of a nonflagellate strain, i.e., no spread in semisolid medium and no more than Brownian movement of bacteria seen by low-power dark-field microscopy of a broth culture.

To construct a chemotaxis-deficient derivative, we used a stock of the same phage grown on strain SL5288, which is the *che-107* chemotaxis-deficient mutant of Tsui-Collins and Stocker (28) made *zea-1*::Tn*10* by transduction. This lysate was applied to strain SL5316 to evoke tetracyline-resistant clones. Of 15 tested in semisolid medium with tetracycline, 3 gave only slow-spreading growth, presumably by incorporation of *che-107* as well as *zea-1*::Tn*10*. One such transductant, confirmed to be unaltered in nutritional character and phage sensitivity pattern, was labeled SL5784. Its motility phenotype was that expected of a *che-107* strain: very slow spreading in semisolid medium and many bacteria in translational motility not showing tumbles at detectable frequency. Many such motile cells were followed for 30 s or longer without any sudden change in direction of travel being observed.

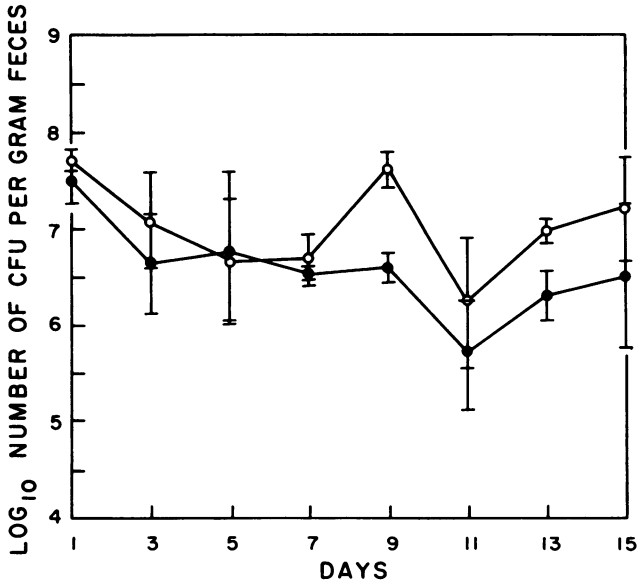

FIG. 1. Colonization of SL5316 Nal^r and SL5784 (Che$^-$) in mice fed 10^{10} CFU of each strain. *S. typhimurium* SL5316 Nal^r (○) and *S. typhimurium* SL5784 (Tet^r) (●) were fed simultaneously to each of three streptomycin-treated mice (10^{10} CFU each). At the indicated times, fecal samples were plated as described in Materials and Methods. Bars representing standard error of the $\log_{10}$ mean are presented for each point.

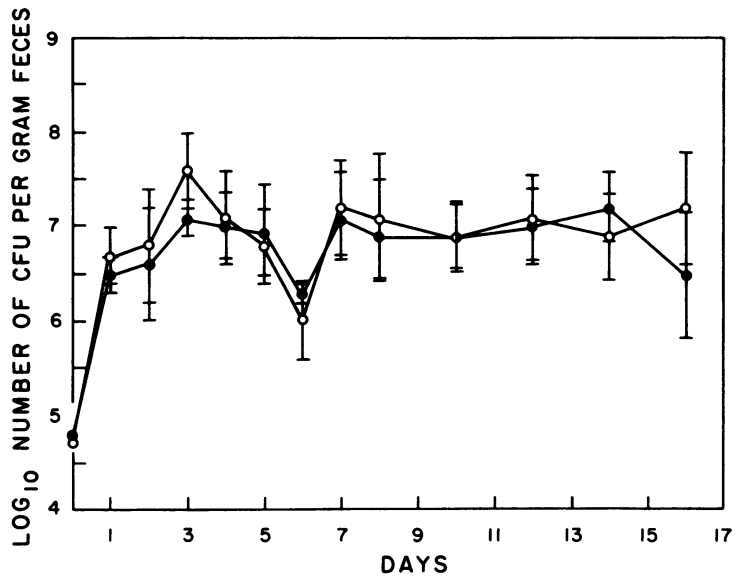

FIG. 2. Colonization of SL5316 Nal^r and SL5784 (Che$^-$) in mice fed 10^4 CFU of each strain. Three streptomycin-treated mice were simultaneously fed 10^4 CFU each of SL5316 Nal^r (○) and SL5784 (Tet^r) (●). At the indicated times, fecal samples were plated as described in Materials and Methods. Bars are as described in the legend to Fig. 1. Viable counts on day 0 represent the number of CFU of each strain fed to each mouse.

Relative colonizing abilities of SL5316 Nal^r and SL5784 (Che$^-$) and of SL5316 Nal^r and SL5681 (Fla$^-$). When mice were fed 10^{10} CFU each of SL5316 Nal^4, wild type for motility and chemotaxis, and SL5784, the nonchemotactic (Che$^-$) derivative, both strains colonized at about 10^7 CFU/g of feces (Fig. 1). Similarly, when mice were fed 10^{10} CFU each of SL5316 Nal^r and SL5681, the nonflagellated (Fla$^-$) transductant, the wild-type strain colonized at between 10^7 and 10^8 CFU/g of feces, whereas the nonflagellated derivative colonized at between 10^6 and 10^7 CFU/g (data not shown).

When mice were fed 10^4 CFU (instead of 10^{10} CFU) each of SL5316 Nal^r and SL5784 (Che$^-$), both strains grew equally well in vivo and both colonized at levels between 10^7 and 10^8 CFU/g of feces (Fig. 2). Similarly, when mice were fed 10^4 CFU each of SL5316 Nal^r and SL5681 (Fla$^-$), both strains grew equally well in vivo, and again SL5316 Nal^r colonized at a level of between 10^7 and 10^8 CFU/g of feces, whereas SL5681 colonized at a level of between 10^6 and 10^7 CFU/g of feces (data not shown).

Several colonies from each mouse were tested for motility on semisolid L-motility agar on the last day of each colonization experiment. The spread of each colony was that expected, i.e., no spread of SL5681 (Fla$^-$), very slow spread of SL5784 (Che$^-$), and rapid spread of the fully motile and normally chemotactic parent, SL5316 Nal^r (data not shown). Therefore, these experiments suggest that neither motility nor chemotaxis plays a major role in the ability of *S. typhimurium* to grow in and colonize the streptomycin-treated mouse large intestine.

Relative colonizing abilities of SL5784 (Che$^-$) and SL5325 Nal^r and of SL5681 (Fla$^-$) and SL5325 Nal^r. When mice were fed 10^{10} CFU each of SL5784 (Che$^-$) and SL5325 Nal^r, the LPS-deficient mutant (*rfaJ*), SL5784 colonized and persisted at a level between 10^7 and 10^8 CFU/g of feces, whereas SL5325 Nal^r declined to a level between 10^2 and 10^3 CFU/g of feces by day 7 (Fig. 3). Similarly, when mice were fed 10^{10} CFU each of SL5681 (Fla$^-$) and SL5325 Nal^r, the LPS-deficient mutant (*rfaJ*), SL5681 colonized at a level between

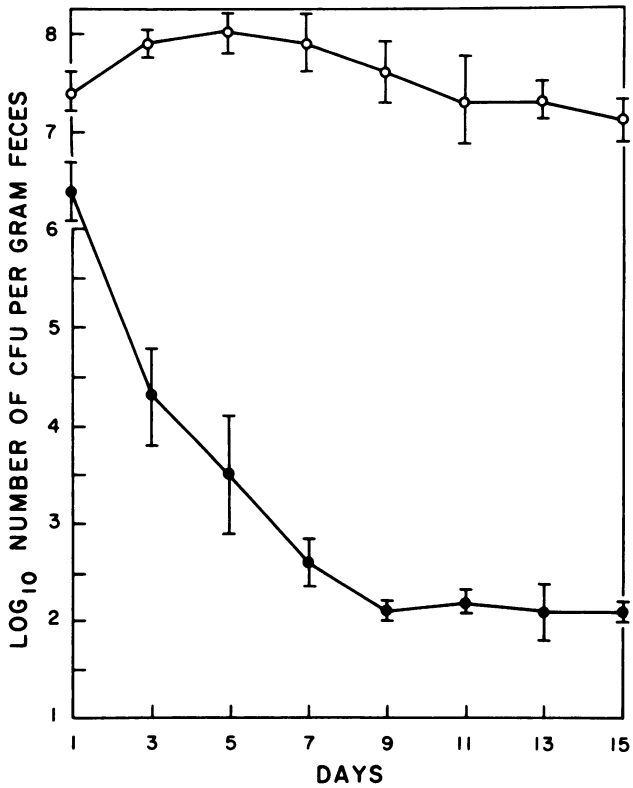

FIG. 3. Colonization of streptomycin-treated mice with *S. typhimurium* SL5784 (Tet^r Che$^-$) (○) and *S. typhimurium* SL5325 Nal^r (*rfaJ*) (●). The strains were fed simultaneously to each of three streptomycin-treated mice (10^{10} CFU of each). At the indicated times, fecal samples were plated as described in Materials and Methods. Bars are as described in the legend to Fig. 1.

TABLE 1. Distribution of *S. typhimurium* strains between cecal epithelial cells and cecal luminal contents 10 days postfeeding[a]

Sample tested	Mean $\log_{10}$ CFU ± SE		
	SL5316 Nal^r and SL5681 (Fla$^-$)	SL5316 Nal^r and SL5784 (Che$^-$)	SL5325 Nal^r
Cecal epithelial cells	5.32 ± 0.24 and 4.29 ± 1.44	4.71 ± 0.33 and 5.23 ± 0.13	5.27 ± 0.46
Cecal luminal contents	6.78 ± 0.44 and 5.81 ± 1.20	6.58 ± 0.62 and 6.94 ± 0.40	7.05 ± 0.72
Feces (per g)	6.94 ± 0.15 and 6.81 ± 0.45	6.41 ± 0.62 and 6.93 ± 0.47	7.13 ± 0.68

[a] Three mice in each group were fed 10^{10} CFU each of the indicated strains and sacrificed 10 days postfeeding, and cecal epithelial cells, cecal luminal contents, and feces were plated as described in Materials and Methods.

10^7 and 10^8 CFU/g of feces, whereas SL5325 Nal^r colonized at a level of only 10^2 to 10^3 CFU/g of feces (data not shown). Clearly, the nonflagellated and nonchemotactic derivatives were far better colonizers than the LPS-deficient mutant, suggesting that the LPS-deficient strain is not a poor colonizer due to impaired motility or chemotaxis ability.

Location of the *S. typhimurium* strains in the mouse cecum in vivo. Streptomycin-treated mice were fed 10^{10} CFU each of either SL5316 Nal^r and SL5681 (Fla$^-$), SL5316 Nal^r and SL5784 (Che$^-$), or SL5325 Nal^r (*rfaJ*) alone. At 10 days postfeeding, the mice were sacrificed and cecal epithelial cell preparations and cecal luminal contents were assayed for viable counts (see Materials and Methods). In each case, between 1 and 3% of the total cecal CFU were found firmly associated with the epithelial cell preparations (Table 1).

Growth in cecal mucus and cecal contents in vitro. The smooth avirulent strain SL5316 Nal^r was inoculated into cecal mucus and cecal luminal contents (see Materials and Methods) along with either SL5681 (Fla$^-$) or SL5784 (Che$^-$), and SL5319, also smooth and avirulent, was inoculated along with SL5325 Nal^r, the LPS-deficient mutant, at about 10^4 CFU of each strain per ml. In all cases, both strains grew equally well and rapidly in cecal mucus to about 10^9 CFU/ml, but did not grow in cecal luminal contents (Fig. 4).

Motility and spread on motility agar after growth in cecal mucus. Strains SL5316 Nal^r (wild type), SL5319 (wild type)

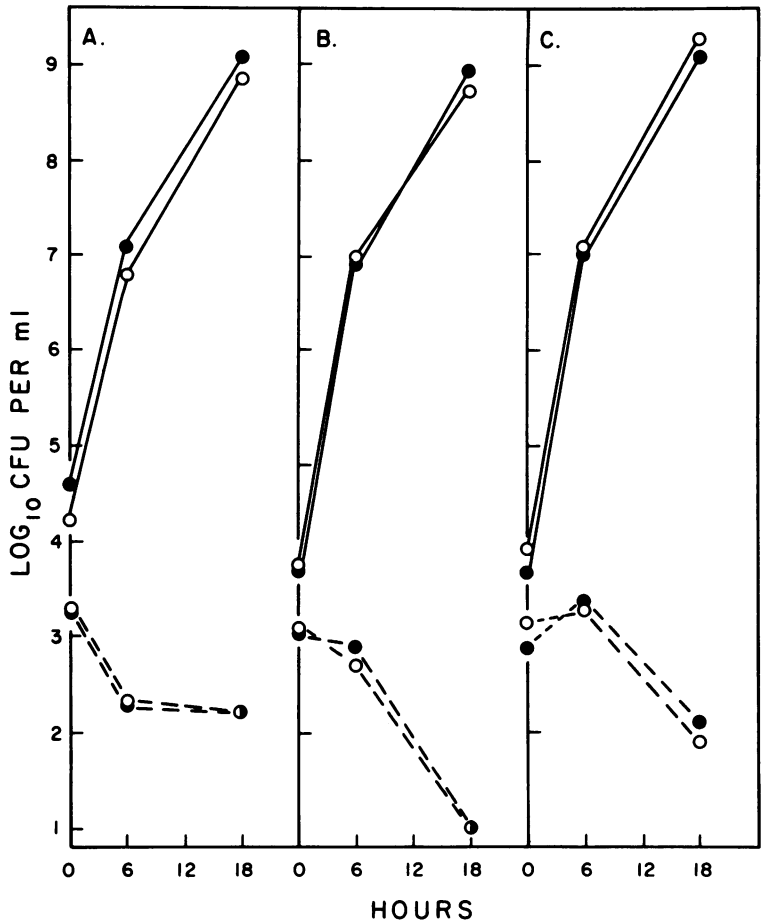

FIG. 4. Growth of the *S. typhimurium* strains in cecal mucus and cecal luminal contents. The *S. typhimurium* strains were inoculated together into cecal mucus (——) and cecal luminal contents (– – –) and incubated and plated as described in Materials and Methods. (A) SL5316 Nal^r (●) and SL5681 (Fla$^-$ Tet^r) (○); (B) SL5316 Nal^r (●) and SL5784 (Che$^-$ Tet^r) (○); (C) SL5319 (Tet^r) (●) and SL5325 Nal^r (○).

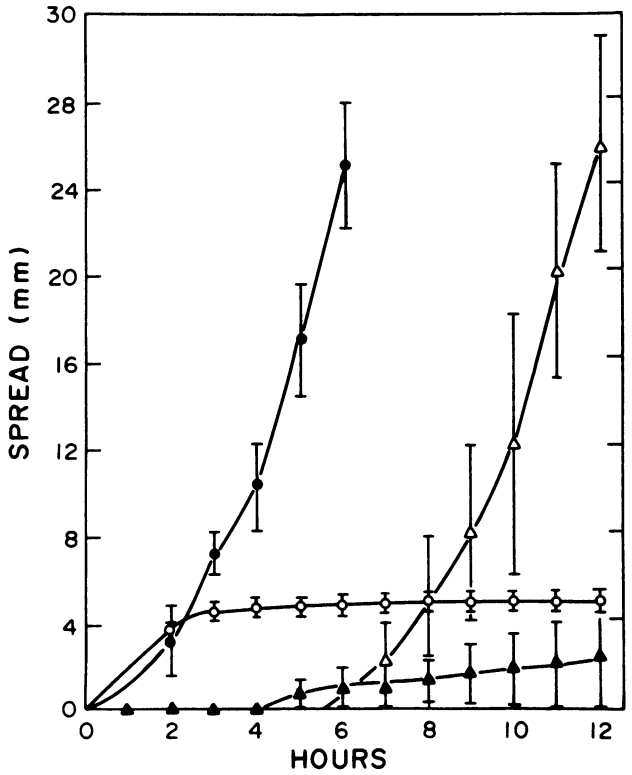

FIG. 5. Spread of *S. typhimurium* SL5316 Nal^r on L-motility agar and cecal mucus motility agar. Individual SL5316 Nal^r colonies grown on L-agar from L-broth were moved with a toothpick onto L-motility agar and cecal mucus motility agar as described in Materials and Methods. Similarly, SL5316 Nal^r colonies grown on cecal mucus agar from liquid cecal mucus were moved with a toothpick onto L-motility agar and cecal mucus motility agar. At the indicated times, diameters of spread were measured. Symbols: ●, from L-agar to L-motility agar; ○, from L-agar to cecal mucus motility agar; △, from cecal mucus agar to L-motility agar; ▲, from cecal mucus agar to cecal mucus motility agar. Bars represent the standard error of the mean for triplicate samples.

and SL5325 Nal^r (LPS deficient) were inoculated into either L-broth or cecal mucus diluted in HEPES-Hanks buffer (pH 7.4) (2 mg of protein per ml) at about 10^4 CFU/ml (see Materials and Methods). Under these conditions, each strain grew rapidly and equally well to a final yield of about 2.0×10^9 CFU/ml (data not shown). Microscopic examination revealed that L-broth-grown strains were fully motile, whereas their cecal mucus-grown counterparts showed both greatly reduced tumbling and swimming activities. Furthermore, strains from L-broth spread rapidly on L-motility agar but slowly on cecal mucus motility agar (Fig. 5). Moreover, when grown in cecal mucus, the strains grew well but spread poorly on cecal mucus motility agar and grew well on L-motility agar but spread only after a 5-h lag period (Fig. 5). Only the data obtained for SL5316 Nal^r, the avirulent smooth strain, are presented in Fig. 5; however, strains SL5319 and SL5325 Nal^r behaved in a similar manner. These data suggest that growth in cecal mucus severely limits *S. typhimurium* motility and chemotaxis.

Adhesion to immobilized cecal mucus and cecal epithelial cells in vitro. Recently, we showed that the LPS-deficient mutant, SL5325 Nal^r, bound far better to both immobilized mouse colonic mucus and immobilized colonic epithelial cell preparations than did the smooth avirulent strain (21). Here we have extended those studies to the cecum, using not only the wild type and LPS-deficient mutant, but the Che$^-$ and Fla$^-$ derivatives as well. The LPS-deficient mutant was found to bind three to four times better to immobilized mouse cecal mucus and immobilized cecal epithelial cell preparations than either the avirulent smooth parent ($P < 0.001$), the nonflagellated derivative ($P < 0.001$), or the nonchemotactic derivative ($P < 0.001$) (Table 2). That is, adhesion of all three better-colonizing strains to both cecal mucus and epithelial cell preparations was far less than that of the poorly colonizing, LPS-deficient strain.

Ability of the strains to travel through cecal mucus in vitro. Recently, we developed an in vitro assay designed to measure the relative abilities of the *S. typhimurium* wild-type smooth and LPS-deficient mutants to travel through colonic mucus and subsequently adhere to an underlying colonic epithelial cell preparation (21). Penetration was assessed by first layering the wild-type smooth strain or LPS-deficient mutant atop a layer of colonic mucus. At various times the mucus was aspirated, and the total number of bacteria at the mucus-epithelial cell interface, adherent and nonadherent, was determined. To assess the level of bacteria closely associated with (adhering to) the colonic epithelial cell preparation, colonic mucus was overlaid and aspirated as above, and the number of bacteria adhering to the epithelial cell preparation was determined after two additional washes. At that time, we reported that the wild-type strain penetrated the colonic mucus layer more rapidly than the LPS-deficient mutant (21).

Here we have extended the in vitro colonic mucus penetration studies to the cecum, using not only the wild type and LPS deficient mutant but the Che$^-$ and Fla$^-$ derivatives as well. The LPS-deficient mutant did not penetrate the cecal mucus layer nearly as well as the wild-type strain, as measured by either the number of bacterial cells at the mucus-epithelial cell interface (Fig. 6A) or the number firmly bound to the epithelial cell layer (Fig. 6B). In contrast, the nonchemotactic derivative was at least as good as the wild-type strain in penetrating the cecal mucus layer (Fig. 7), as was the nonflagellated derivative (data not shown). Therefore, each of the three better-colonizing strains traveled through a layer of cecal mucus in vitro far more rapidly than the poorly colonizing, LPS-deficient mutant.

DISCUSSION

The present study was undertaken to determine whether avirulent *S. typhimurium* LPS-deficient mutants are poor colonizers of the mouse streptomycin-treated large intestine because they are less motile and chemotactic, as judged by

TABLE 2. Adhesion of avirulent *S. typhimurium* strains to immobilized cecal mucus and cecal epithelial cells[a]

Strain	Relevant phenotype	Mean adhesion (cpm) ± SE to:	
		Cecal mucus	Cecal epithelial cells
SL5316 Nal^r	Wild type	1,909 ± 54	1,991 ± 10
SL5681	Nonflagellated	1,500 ± 76	1,832 ± 190
SL5784	Nonchemotactic	1,620 ± 84	2,223 ± 138
SL5325	LPS deficient	9,451 ± 313	7,484 ± 89

[a] Data are presented as the mean and standard error of triplicate samples. The specific activities of the four strains were essentially identical within each experiment and were 2.9×10^{-3} cpm/CFU and 3.0 ± 10^{-3} cpm/CFU for the cecal mucus and cecal epithelial cell experiments, respectively.

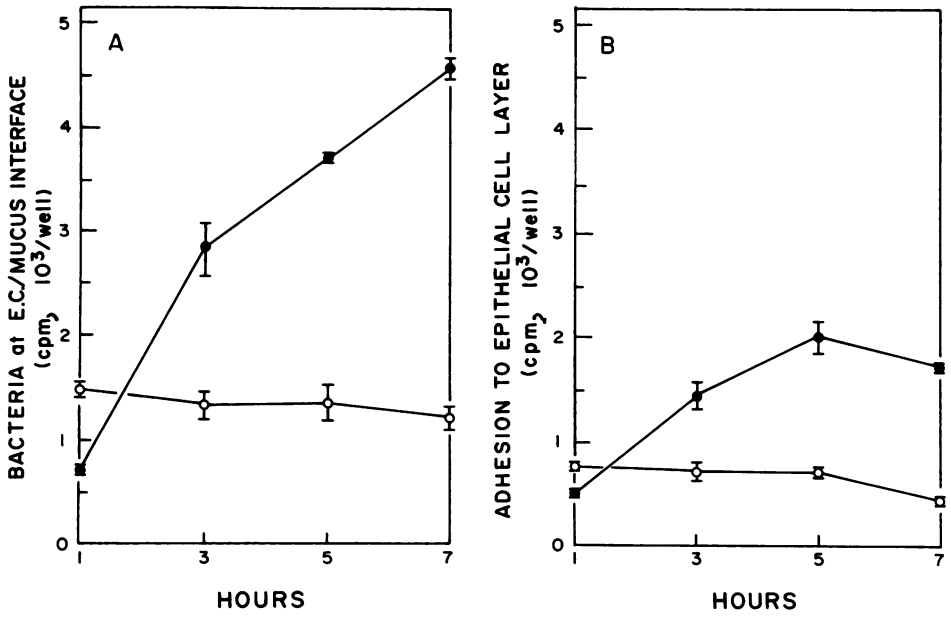

FIG. 6. Penetration of *S. typhimurium* SL5319 (wild type) and SL5325 Nal^r (LPS-deficient mutant) through cecal mucus in vitro. (A) Penetration to the cecal mucus-cecal epithelial cell interface. The mucus layer was aspirated, and the *S. typhimurium* cells at the interface were taken into 5% SDS and counted (see Materials and Methods). Data are presented as the mean and standard errors of duplicate samples. In this experiment the specific activities of the two strains were essentially identical (2.7×10^{-3} cpm/CFU. Symbols: ●, SL5319; ○, SL5325 Nal^r. (B) Adhesion to the cecal-epithelial cell layer. As in panel A, except that wells were aspirated and washed twice prior to counting (see Materials and Methods).

ability to spread in a semisolid medium, than their smooth parent (22). The data presented here show that motility and chemotaxis, as we know them in vitro, play no major role in colonization of the streptomycin-treated mouse large intestine in vivo by avirulent *S. typhimurium*. That is, both the nonflagellated (Fla$^-$) derivative, SL5681, and the nonchemotactic (Che$^-$) derivative, SL5784, colonized the CD-1 mouse large intestine in large numbers in the presence of the avirulent smooth parent, strain SL5316 Nal^r (Fig. 1 and 2); however, both strains were shown to be far better colonizers than SL5325 Nal^r, the LPS-deficient (*rfaJ*) mutant (Fig. 3). These data are consistent with those of Weinstein et al. (29), who could find no statistically significant difference between the ability of virulent flagellated and nonflagellated (Δ*flaF25*)

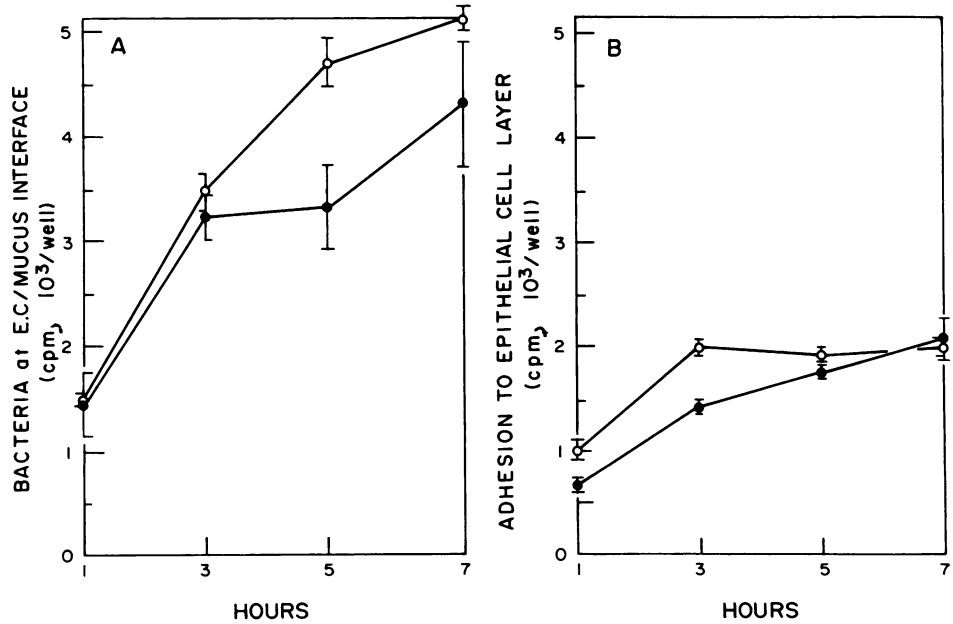

FIG. 7. Penetration of *S. typhimurium* SL5316 Nal^r (wild type) and SL5784 (Che$^-$) through cecal mucus in vitro. Panels are as in Fig. 6. In this experiment the specific activities of the two strains were essentially identical (3.0×10^{-3} cpm/CFU). Symbols: ●, SL5316 Nal^r; ○, SL5784.

S. typhimurium strains to colonize the entire bowel of conventional C57BL/6J mice.

It is likely that motility and chemotaxis play no major role in avirulent *S. typhimurium* large intestinal colonization because growth in cecal mucus severely depresses avirulent *S. typhimurium* motility and chemotaxis activities (Fig. 5) and that growth in mucus appears to be required for colonization (i.e., the strains grew rapidly in cecal mucus but poorly in cecal luminal contents in vitro [Fig. 4]). Presently, we do not know why growth in mucus causes avirulent *S. typhimurium* to be less motile than L-broth-grown cells; however it appears that the mechanism is not due to catabolite repression of flagella synthesis (18). That is, even though mucus-grown cells grew immediately on L-motility agar, they did not begin to spread for at least 5 h (Fig. 5), i.e., they presumably divided many more times than would be required to reverse the effects of catabolite repression.

At present, we also do not know why the *S. typhimurium* strains do not grow in cecal luminal contents. However, several conditions in cecal luminal contents have been reported to inhibit bacterial growth, including the presence of hydrogen sulfide (6), bacteriocins (7), short-chain fatty acids (10, 23), and low Eh and pH (2, 19). Whether any of these conditions is indeed responsible for the observed poor growth of the strains in cecal luminal contents is presently under investigation.

Intestinal mucus is in a dynamic state, constantly being synthesized by specialized mucosal goblet cells, degraded by indigenous microorganisms, and sloughed into the intestinal lumen (13, 14, 20). In order for a bacterium to colonize mucus, it seems reasonable that it must grow in it faster than it is sloughed into the lumen (25, 26). Furthermore, since the intestinal mucus layer in vivo can be as much as 400 μm in width (1, 20) and because it is constantly being synthesized and sloughed, of two microorganisms which can metabolize mucus equally well, the one that can more easily penetrate and colonize deep within the mucus layer should have an advantage in colonizing the intestine.

In a recent report we showed that SL5325 Nal^r, the LPS-deficient (*rfaJ*) mutant, did not travel through a layer of colonic mucus in vitro nearly as rapidly as its wild-type parent (21) and suggested that the ability of *S. typhimurium* to colonize the streptomycin-treated mouse large intestine may depend on its ability to penetrate deeply into the mucus layer on the intestinal wall and subsequently, through growth, colonize the mucosa (21). In support of this view, when streptomycin-treated mice were fed SL5325 Nal^r alone, to give it time to penetrate the mucus layer, it was not eliminated when challenged 8 days later with its wild-type parent (21). The data presented here are consistent with this hypothesis. That is, upon colonization, the *S. typhimurium* strains were found on the cecal wall in vivo (Table 1) and were shown to grow equally well in cecal mucus in vitro (Fig. 4). However, the three better colonizers, i.e. the wild-type, the nonflagellated, and the nonchemotactic strains, all penetrated cecal mucus in vitro far better (Fig. 7) than the poorly colonizing LPS-deficient (*rfaJ*) mutant (Fig. 6).

Presently, we do not know how the *S. typhimurium* strains would travel through mucus in vivo in a nonmotile state. However, it is possible that *S. typhimurium* cells are passively taken into the mucus layer as water is resorbed to form the fecal pellets found in the proximal colon. If so, it is possible that the LPS-deficient (*rfaJ*) mutant, due to its greater hydrophobicity (11), is retarded in this process by binding either specifically or nonspecifically to the "receptors" that we have observed to be present in cecal mucus (Table 2).

In summary, contrary to what has been observed during *Vibrio cholerae* infection of the mouse and rabbit small intestine (5, 8), colonization of the streptomycin-treated mouse large intestine by *S. typhimurium* does not require that the bacterium be either flagellated or chemotactic. Furthermore, it appears highly likely that avirulent *S. typhimurium* colonization of the streptomycin-treated mouse large intestine requires growth in intestinal mucus. Presumably, the bacterium then continuously enters cecal luminal contents in sloughed mucus but does not grow, and eventually finds its way into the feces.

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