The Streptomycin-Treated Mouse Intestine Selects Escherichia coli envZ Missense Mutants That Interact with Dense and Diverse Intestinal Microbiota

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The Streptomycin-Treated Mouse Intestine Selects Escherichia coli envZ Missense Mutants That Interact with Dense and Diverse Intestinal Microbiota

Mary P. Leatham-Jensen, Jakob Frimodt-Møller, Jimmy Adedin, Matthew E. Mokszytcki, Megan E. Banner, Joyce E. Caughron, Karen A. Krosgfalt, Tyrrell Conway, and Paul S. Cohen

Department of Cell and Molecular Biology, University of Rhode Island, Kingston, Rhode Island, USA; Department of Microbiological Surveillance and Research, Statens Serum Institut, Copenhagen S, Denmark; and Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma, USA

Previously, we reported that the streptomycin-treated mouse intestine selected nonmotile Escherichia coli MG1655 flhDC deletion mutants of E. coli MG1655 with improved colonizing ability that grow 15% faster in vitro in mouse cecal mucus and 15 to 30% faster on sugars present in mucus (M. P. Leatham et al., Infect. Immun. 73:8039–8049, 2005). Here, we report that the 10 to 20% remaining motile E. coli MG1655 are envZ missense mutants that are also better colonizers of the mouse intestine than E. coli MG1655. One of the flhDC mutants, E. coli MG1655 ΔflhD, and one of the envZ missense mutants, E. coli MG1655 mot-1, were studied further. E. coli MG1655 mot-1 is more resistant to bile salts and colicin V than E. coli MG1655 ΔflhD and grows ca. 15% slower in vitro in mouse cecal mucus and on several sugars present in mucus compared to E. coli MG1655 ΔflhD but grows 30% faster on galactose. Moreover, E. coli MG1655 mot-1 and E. coli MG1655 ΔflhD appear to colonize equally well in one intestinal niche, but E. coli MG1655 mot-1 appears to use galactose to colonize a second, smaller intestinal niche either not colonized or colonized poorly by E. coli MG1655 ΔflhD. Evidence is also presented that E. coli MG1655 is a minority member of mixed bacterial biofilms in the mucus layer of the streptomycin-treated mouse intestine. We offer a hypothesis, which we call the “Restaurant” hypothesis, that explains how nutrient acquisition in different biofilms comprised of different anaerobes can account for our results.

When a bacterial species indefinitely persists in stable numbers in the intestine of an animal, without repeated introduction, the animal is, by definition, colonized by the bacterium. Commensal Escherichia coli strains colonize the human intestine in the presence of a dense and diverse intestinal microbiota comprised of at least 500 cultivable species and 10^{13} to 10^{14} total bacteria (15). Unfortunately, E. coli colonization cannot be studied experimentally in conventional animals due to colonization resistance, which results when all niches are filled by the microbiota (10). Such experiments require an animal model with open niches for E. coli to colonize in relatively high numbers but must also have a dense and diverse anaerobic community that matches as closely as possible the native microbiota of the conventional animal. The streptomycin-treated mouse model is used routinely for this purpose (11, 34).

In mammals, a large fraction of the intestinal microbiota belongs to either of two phyla, the Firmicutes and the Bacteroidetes (16, 37, 58). Extensive microbiological characterization of the microbial community in the streptomycin-treated mouse intestine, published in the classic paper by Hentges et al. (28), demonstrated that streptomycin treatment (5 mg/ml in drinking water) selectively removes the facultative anaerobes, with much less impact on the anaerobes. Streptomycin treatment does not alter the total numbers of Bacteroides (Bacteroidetes), Eubacterium (Firmicutes), Bifidobacterium (Actinobacteria), and Clostridium (Firmicutes), although there is a large decrease in Lactobacillus (Firmicutes) and Actinomyces (Actinobacteria) with a corresponding increase in Fusobacterium (Fusobacteria) and an apparent 50% decline in anaerobic species, as indicated by anaerobic colony morphologies (28). In general, more recent studies using 16S rRNA gene sequencing confirmed these results (23, 58, 68). It is now clear that any strategy to overcome colonization resistance will impact community structure (12, 57). While the streptomycin-treated mouse model is not perfect, it allows competition of a variety of E. coli strains against a dense and diverse anaerobe population over extended time periods.

Previously, we found that when E. coli MG1655 was fed to streptomycin-treated mice, 80 to 90% of the population lacked flagella and became nonmotile within 7 to 9 days postfeeding (24, 36). We showed that these nonmotile better colonizing mutants have deletions of various sizes beginning downstream of an IS1 element in the flhDC regulatory region and extending into or beyond the flhDC structural genes (24, 36). FlhD and FlhC form the FlhD\_C\_complex (5, 40, 66), which activates transcription of class II flagellar genes that encode components of the flagellar basal body and export machinery (51). The IS1 element upstream of the E. coli MG1655 flhDC promoter increases expression of the flhDC operon and makes E. coli MG1655 hypermotile (4, 24).
MG1655-IS strain used in the present investigation is isogenic to the Strr Nalr Spontaneous nalidixic acid-resistant mutant of MG1655 -IS

sdhCDAB (succinate dehydrogenase),

BW37751(pKD267)::Knr The plasmid in this strain contains

MG1655 Strr Spontaneous streptomycin resistant mutant of MG1655 MG1655 45

MG1655 Strr Nalr mot-1 Spontaneous nalidixic acid-resistant mutant of MG1655 Strr

MG1655 Strr mot-2 P148S F-18 Strr Rifr Spontaneous streptomycin- and nalidixic acid-resistant

MG1655 Strr Nalr mot-1

TABLE 1

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Genotype and/or phenotype*</th>
<th>Abbreviated form</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1655 Strr</td>
<td>Spontaneous streptomycin resistant mutant of MG1655</td>
<td>MG1655</td>
<td>45</td>
</tr>
<tr>
<td>MG1655 Strr ΔflhD::cat</td>
<td>546-bp deletion beginning immediately downstream of IS1 in the regulatory region of flhD and ending in flhD, streptomycin and chloramphenicol resistant</td>
<td>MG1655 ΔflhD</td>
<td>36</td>
</tr>
<tr>
<td>MG1655-IS/I Strr NaI</td>
<td>Spontaneous nalidixic acid-resistant mutant of MG1655 -IS/I Strr, no IS1 element in the flhDC promoter</td>
<td>MG1655-IS/I</td>
<td>24</td>
</tr>
<tr>
<td>MG1655 Strr mot-1</td>
<td>P41L envZ missense mutant of MG1655 Strr</td>
<td>MG1655 mot-1</td>
<td>This study</td>
</tr>
<tr>
<td>MG1655 Strr mot-2</td>
<td>P148S envZ missense mutant of MG1655 Strr</td>
<td>MG1655 mot-2</td>
<td>This study</td>
</tr>
<tr>
<td>MG1655 Strr mot-3</td>
<td>V33E envZ missense mutant of MG1655 Strr</td>
<td>MG1655 mot-3</td>
<td>This study</td>
</tr>
<tr>
<td>MG1655 Strr NaI mot-1</td>
<td>Spontaneous nalidixic acid-resistant mutant of MG1655 Strr mot-1</td>
<td>MG1655 mot-1</td>
<td>This study</td>
</tr>
<tr>
<td>MG1655 Strr NaI mot-1 restored</td>
<td>MG1655 Strr mot-1 restored to wild type with respect to EnvZ</td>
<td>MG1655 mot-1 restored</td>
<td>This study</td>
</tr>
<tr>
<td>MG1655 Strr NaI mot-1 ΔgalK::cat</td>
<td>912-bp deletion in galactokinase gene replaced by a chloramphenicol resistance cassette</td>
<td>MG1655 mot-1 ΔgalK</td>
<td>This study</td>
</tr>
<tr>
<td>MG1655 Strr NaI mot-1 ΔgalK::cat restored</td>
<td>MG1655 Strr NaI mot-1 ΔgalK::cat restored to wild type with respect to galK</td>
<td>MG1655 mot-1 ΔgalK restored to wild type with respect to galK</td>
<td>This study</td>
</tr>
<tr>
<td>F-18 Strr Rif</td>
<td>Spontaneous streptomycin- and nalidixic acid-resistant mutant of F-18</td>
<td>F-18</td>
<td>47</td>
</tr>
<tr>
<td>BW37751(pKD267)::Knr</td>
<td>The plasmid in this strain contains parE under the control of the rhamnose promoter and the kanamycin resistance gene</td>
<td>pKD267</td>
<td>Barry Wanner</td>
</tr>
</tbody>
</table>

* Strr, streptomycin resistance; Knr, kanamycin resistance; NaI, nalidixic acid resistance; Rif, rifampin resistance.

explains why they were selected. Additional studies suggested the E. coli MG1655 flhDC operon deletion mutants utilize sugars better than their parent because a number of metabolic genes are repressed by the FlhDC_Cr regulatory complex, including gltA (citrate synthase), sdhCDAB (succinate dehydrogenase), mdh (malate dehydrogenase), mglBAC (galactose transport), and a large number of sugar catabolism operons (20, 52, 53). Also, increased energy is available for other cellular processes in the absence of hyper-flagellum synthesis and rotation (24).

Despite the strong selection for nonmotile E. coli MG1655 flhDC deletion mutants by the streptomycin-treated mouse intestine, 10 to 20% of the E. coli MG1655 remained motile over a 15 day period (24), suggesting either that they too were better colonizing mutants or that an as-yet-undefined intestinal niche exists in which motility is an advantage. Here, we report that the 10 to 20% of E. coli MG1655 that remain motile in the streptomycin-treated mouse intestine are envZ missense mutants that display increased colonizing ability.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in the present study are listed in Table 1. The original E. coli strain K-12 was obtained from a stool sample from a convalescing diphtheria patient in Palo Alto, CA, in 1922 (2). The sequenced E. coli MG1655 strain (CGSC 7740) was derived from the original K-12 strain, having only been cured of the temperate bacteriophage lambda and the F plasmid by means of UV light and acidine orange treatment (2). It has an IS1 element in the flhDC promoter (6). The E. coli MG1655-IS1 strain used in the present investigation is isogenic to the MG1655 strain but lacks the IS1 element in the flhDC promoter (24). The allelic-exchange method described by Datsenko and Warner (13) was used to construct E. coli MG1655 ΔgalK, which contains a 912-bp deletion, replaced by a chloramphenicol cassette beginning 133 bp downstream of the ATG start codon and ending 78 bp upstream of the TGA stop codon. As expected, the E. coli MG1655 mot-1 ΔgalK strain failed to grow in M9 minimal medium containing 0.4% (wt/vol) galactose as the sole carbon and energy source. E. coli MG1655 mot-1 ΔgalK was restored to Gal+ by allelic replacement (13), selecting for restored growth on M9 minimal galactose agar plates. The restored Gal+ E. coli MG1655 mot-1 grew at the same rate as E. coli MG1655 mot-1 in M9 galactose minimal medium. The mutated envZ gene in E. coli MG1655 mot-1 was replaced with the wild-type envZ gene using an unpublished allelic replacement strategy developed by Barry Wanner and Kiryl Datsenko at Purdue University (unpublished data). The first step replaced the E. coli MG1655 mot-1 envZ::ompR locus with a cassette encoding kanamycin resistance (kan) and parE under the control of the rhamnose promoter (kan-rha-::parE). The parE gene encodes a DNA gyrase inhibitor, i.e., the toxin of a toxin anti-toxin pair that kills the cell when produced in the absence of its cognate antitoxin (31). The second step replaced the kan-rha-::parE cassette with the wild-type envZ allele by selecting for growth on M9 minimal agar plates containing 1.0% (wt/vol) rhamnose (which would otherwise kill when rhamnose induces ParE). The double recombinant event leaves no scars on the genome. All constructions were confirmed by sequencing.

Media and growth conditions. LB broth Lennox (Difco Laboratories, Detroit, MI), LB agar Lennox (Difco), and MacConkey agar (Difco) were used for routine cultivation. SOC medium was prepared as described by Datsenko and Wanner (13). For testing carbon and energy source utilization, M9 minimal medium (44) was modified by addition of 120 mM NaCl to more closely approximate the sodium chloride concentration in the intestine (3). Growth also was tested in cecal mucus (2.5 mg of protein/ml) in HEPES-Hanks buffer (pH 7.0), which contains 137 mM NaCl, the sodium chloride concentration in jejunal and ileal intestinal fluid (3). Cultures were prepared, and growth was monitored as described previously (36).

Isolation of cecal mucus. Mouse cecal mucus was isolated as previously described (9). Briefly, mice (5 to 8 weeks old) were fed Teklad mouse and rat diet (Harlan Laboratories, Madison, WI) for 5 days after being sacrificed. The drinking water was then replaced with sterile distilled water containing streptomycin sulfate (5 gl/iter). After 24 h, the mice were sacrificed by CO2 asphyxiation, and their ceca were removed. The cecal contents were washed out with sterile distilled water, and the cecal mucus was scraped into HEPES-Hanks buffer (pH 7.0), centrifuged, and sterilized by UV irradiation as described previously (9). All animal protocols were

Downloaded from http://iai.asm.org on November 26, 2018
approved by the University Committee on Use and Care of Animals at the University of Rhode Island.

**Motility.** The motility of *E. coli* MG1655 strains was assayed by toothpick-colonies from LB agar Lennox onto Luria motility agar (3.5 g/liter) containing 200 mM NaCl (3). The plates were incubated for 6 h at 37°C, after which spreading was measured from the edge of each colony. For testing the motility of fecal isolates, 50 colonies from fecal platings of each mouse were toothpicked to motility agar made from LB broth Lennox containing 85.5 mM NaCl. The plates were incubated overnight at room temperature. For testing motility of bacteria from intestinal segments, the ileum, cecum, and colon were removed from each mouse and washed extensively with HEPES-Hanks buffer (pH 7.0), and the mucus was scraped into 5 ml of HEPES-Hanks buffer (pH 7.0), as described previously (9). Samples were homogenized by vortexing, plated on MacConkey agar with appropriate antibiotics, and toothpicked onto motility agar, as described above for fecal isolates.

**Growth in the presence of 5% bile salts.** Strains to be tested for bile sensitivity were grown overnight in LB broth Lennox, diluted to an A600=0.1 to 0.1 into fresh LB broth Lennox containing 5% (wt/vol) Bacto-Bile Salts No. 3 (Difco Laboratories), and incubated at 37°C with shaking in 125-ml tissue culture bottles.

**Mouse colonization experiments.** The specifics of the streptomycin-treated mouse model used to compare the large intestine colonizing abilities of *E. coli* strains in mice have been described previously (42, 47, 59, 60, 65). Briefly, sets of three male CD-1 mice (5 to 8 weeks old) were given drinking water containing streptomycin sulfate (5 g/liter) for 24 h to eliminate resident facultative bacteria (43). After 18 h of starvation for food and water, the mice were fed 1 ml of 20% (wt/vol) sucrose containing 10^5 CFU or 10^10 CFU of LB broth Lennox-grown *E. coli* strains, as described in Results. After ingesting the bacterial suspension, the food (Teklad mouse and rat diet; Harlan Laboratories) and streptomycin-water were returned to the mice, and 1 g of feces was collected after 5 h, 24 h, and on odd-numbered days thereafter at the indicated times. Mice were housed individually in cages without bedding and were placed in clean cages at 24-h intervals. Individual fecal pellets were therefore no older than 24 h. Each fecal sample (1 g) was homogenized in 10 ml of 1% Bacto tryptone (Difco), diluted in the same medium, and plated on MacConkey agar plates with appropriate antibiotics. When appropriate, 1 ml of a fecal homogenate (sampled after the feces had settled) was centrifuged at 12,000 x g, resuspended in 100 ml of 1% Bacto tryptone, and plated on a MacConkey agar plate with the appropriate antibiotics. This procedure increases the sensitivity of the assay from 10^5 CFU/g of feces to 10^6 CFU/g of feces. To distinguish the various *E. coli* strains in feces, dilutions were plated on lactose MacConkey agar containing either streptomycin sulfate (100 μg/ml), streptomycin sulfate (100 μg/ml) and nalidixic acid (50 μg/ml), or streptomycin sulfate (100 μg/ml) and chloramphenicol (30 μg/ml). Streptomycin sulfate, chloramphenicol, and nalidixic acid were purchased from Sigma-Aldrich (St. Louis, MO). All plates were incubated for 18 to 24 h at 37°C prior to counting. When necessary to distinguish strains, 100 colonies from plates containing streptomycin were toothpicked onto MacConkey agar plates containing streptomycin and nalidixic acid or onto MacConkey agar plates containing streptomycin and chloramphenicol. Each colonization experiment was performed at least twice, with essentially identical results. Pooled data from at least two independent experiments (a total of six mice) are presented in the figures.

**Sequencing.** DNA sequencing was done at the URI Genomics and Sequencing Center, University of Rhode Island, Kingston, using an Applied Biosystems 3130xl genetic analyzer (Applied Biosystems, Foster City CA). A BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) was used in the sequencing reactions. The primers used to amplify the PCR product (2,241 bp) for sequencing the ompB locus included the forward primer (upstream of ompB) 5'-TAGCTGGTGAGCGAAGGTGA G-3' and the reverse primer (downstream of envZ) 3'-TGATCCGCGTGG TCCGAA-3'. The same primers, as well as 5'-ATCTCTGCTGCTATCA

**RESULTS**

**Isolation of *E. coli* MG1655 mot-1, mot-2, and mot-3 from the mouse intestine.** Previously, we showed that despite the strong selection for nonmotile *E. coli* MG1655 flhDC deletion mutants by the streptomycin-treated mouse intestine, 10 to 20% of the *E. coli* MG1655 cells remained motile over a 15-day period (36). To determine the nature of the population that remained motile, three mice were fed 10^10 CFU of *E. coli* MG1655 and 10^10 CFU of *E. coli* MG1655 ΔflhD. At 5 h and day 1 postfeeding, the level of *E. coli* MG1655 ΔflhD in feces was ~5 orders of magnitude lower than *E. coli* MG1655, but by day 7 *E. coli* MG1655 ΔflhD had grown to a population of only 1.5 orders of magnitude lower than *E. coli* MG1655 and remained at that level thereafter for the 15 days of the experiment (Fig. 1A). As a control, mice were fed 10^10 CFU of *E. coli* MG1655 ΔflhD and 10^5 CFU of *E. coli* MG1655. As expected, the better colonizing *E. coli* MG1655 ΔflhD completely eliminated *E. coli* MG1655 within 3 days postfeeding (Fig. 1B). At day 15, the mice fed high numbers of *E. coli* MG1655 and low numbers of *E. coli* MG1655 ΔflhD (Fig. 1A) were sacrificed, and the numbers of *E. coli* MG1655 and *E. coli* MG1655 ΔflhD in ileal mucus, cecal mucus, and colonic mucus were determined. In each mucus preparation, the numbers of *E. coli* MG1655 ΔflhD were 1.5 to 2 orders of magnitude lower than *E. coli* MG1655, reflecting the results observed in feces (Table 2). Although less than 1 in 6,300 CFU of the *E. coli* MG1655 fed to the mice were nonmotile (24) and less than 1 in 100 CFU in feces were nonmotile at both 5 h and 1 day postfeeding, by day 3 postfeeding 17% ± 7.9% (mean ± the SEM) in feces were nonmotile, by day 7 postfeeding 65% ± 15% in feces were nonmotile, by day 13 postfeeding 75% ± 18% in feces were nonmotile, and on day 15 postfeeding 76% ± 13% in feces were nonmotile. Therefore, despite competition from the better-colonizing *E. coli* MG1655 ΔflhD and the...
selection by the intestine of nonmotile \textit{E. coli} MG1655, ca. 25% of the \textit{E. coli} MG1655 remained motile within each section of the intestine. On day 13 postfeeding, 1 motile \textit{E. coli} MG1655 colony was selected from the feces of each mouse for further study. These colonies were designated \textit{E. coli} MG1655 mot-1, mot-2, and mot-3, respectively.

\textit{E. coli} MG1655 mot-1, mot-2, and mot-3 are \textit{envZ} missense mutants. \textit{E. coli} MG1655 mot-1, mot-2, and mot-3 were less motile than the hypermotile \textit{E. coli} MG1655 (Table 3). Previously, we reported that when the IS element was removed from the \textit{flhDC} regulatory region of \textit{E. coli} MG1655, the resulting strain, \textit{E. coli} MG1655-\textit{IS}, was a better colonizer and remained motile, although far less so than its parent (24). However, \textit{E. coli} MG1655 mot-1, mot-2, and mot-3 retained the \textit{IS} element, as determined by PCR (data not shown).

Giraud et al. reported that when germfree mice were fed \textit{E. coli} MG1655, the mouse intestine selected better-colonizing, less-motile \textit{E. coli} MG1655 mutants that contained point mutations in the \textit{envZ-ompR} locus (25). \textit{EnvZ}, a histidine kinase, and OmpR, its cognate response regulator, comprise a two-component signal transduction system that modulates gene expression in response to osmolality (17). When we sequenced the \textit{envZ} and \textit{ompR} genes of the three mot mutants—\textit{E. coli} MG1655 \textit{ΔflhD}, \textit{E. coli} MG1655-\textit{IS}, and \textit{E. coli} MG1655—we found that the \textit{ompR} sequences of the six strains were identical, as were the \textit{envZ} sequences of \textit{E. coli} MG1655 \textit{ΔflhD}, \textit{E. coli} MG1655-\textit{IS}, and the wild-type \textit{E. coli} MG1655. Importantly, \textit{E. coli} MG1655 mot-1, mot-2, and mot-3 were found to be \textit{envZ} missense mutants: mot-1 a P41L mutant (CCG to CTG) in the N-terminal transmembrane domain, mot-2 a P148S mutant (CCG to TCG) in the periplasmic osmolarity sensing domain, and mot-3 a V33E mutant (GTG to GAG) in the N-terminal transmembrane domain (21, 69). That the \textit{envZ} missense mutation in mot-1 was responsible for reduced motility was shown by the fact that when \textit{E. coli} MG1655 mot-1 was restored to the wild type with respect to \textit{envZ}, it regained hypermotility (Table 3).

The \textit{E. coli} mot mutants are resistant to bile salts and colicin V. Among the many activities controlled by the two component EnvZ/OmpR signal transduction system are transcription of \textit{ompF} and \textit{ompC}, which encode \textit{E. coli} outer membrane porins that allow passage of many small molecules (≤600 Da) from the environment into the periplasm (1). Bile salts, which are present in the human small intestine at a concentration of ca. 0.3% (64) and can inhibit \textit{E. coli} growth at 5% concentration \textit{in vitro} (25), pass readily through the OmpF porin but much less so through the OmpC porin (62). High levels of OmpR–P downregulate tran-

### TABLE 2

<table>
<thead>
<tr>
<th>Location</th>
<th>Mean (\log_{10}) CFU ± SEM(^a)</th>
<th>% Nonmotile \textit{E. coli} MG1655(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ileal mucus</td>
<td>4.81 ± 0.57</td>
<td>75 ± 14</td>
</tr>
<tr>
<td>Cecal mucus</td>
<td>7.15 ± 0.27</td>
<td>83 ± 7.9</td>
</tr>
<tr>
<td>Colonic mucus</td>
<td>6.73 ± 0.27</td>
<td>72 ± 11</td>
</tr>
<tr>
<td>Feces</td>
<td>8.39 ± 0.07</td>
<td>76 ± 13</td>
</tr>
</tbody>
</table>

\(^a\) The values are means for three mice. Mucus preparations were isolated on day 15 postfeeding. The CFU values for the mucus preparations are corrected for the entire volume of each mucus preparation. The fecal values are the CFU/g of feces at 15 days after feeding.

\(^b\) A total of 50 \textit{E. coli} MG1655 colonies isolated from the 15-day mucus preparations and feces of each of the three mice were tested for motility as described in Materials and Methods. The values are means for three mice.

### TABLE 3 Motility of \textit{E. coli} MG1655 strains

<table>
<thead>
<tr>
<th>\textit{E. coli} strain</th>
<th>Expt 1 Mean spread (mm) ± SEM (^b)</th>
<th>Expt 2 Mean spread (mm) ± SEM (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1655</td>
<td>8.3 ± 0.4</td>
<td>9.6 ± 0.2</td>
</tr>
<tr>
<td>MG1655 (\Delta flhD)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MG1655-\textit{IS}1</td>
<td>1.8 ± 0.4 ((&lt;0.005))</td>
<td>1.6 ± 0.3 ((&lt;0.001))</td>
</tr>
<tr>
<td>MG1655 mot-1</td>
<td>3.7 ± 0.4 ((&lt;0.005))</td>
<td>3.1 ± 0.4 ((&lt;0.002))</td>
</tr>
<tr>
<td>MG1655 mot-1 restored</td>
<td>ND</td>
<td>8.8 ± 0.3 ((&gt;0.05))</td>
</tr>
<tr>
<td>to wild type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1655 mot-2</td>
<td>3.7 ± 0.7 ((0.005))</td>
<td>ND</td>
</tr>
<tr>
<td>MG1655 mot-3</td>
<td>4.4 ± 0.8 ((&lt;0.01))</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^b\) That is, the spread of \textit{E. coli} MG1655 strains on Luria motility agar containing 200 mM NaCl after 6 h at 37°C as described in Materials and Methods. Values represent the mean of three samples. \(P\) values, determined using the Student t test and indicated in parentheses, were calculated for each strain relative to \textit{E. coli} MG1655 in the same experiment. ND, not done.
and an 

scription of ompF and upregulate transcription of ompC, resulting in decreased OmpF and increased OmpC in the outer membrane (55). The E. coli MG1655 envZ missense mutants described by Girard et al. as being selected in germfree mice monoassociated with E. coli MG1655 were reported to have decreased OmpF and increased OmpC and were found to be more resistant to 5% bile salts than their parent (25), suggesting that the envZ mutants had higher levels of OmpR–P than E. coli MG1655. Here, we report that E. coli MG1655 mot-1, mot-2, and mot-3 are far more resistant to 5% bile salts than E. coli MG1655, E. coli MG1655 ΔflhD, and an E. coli MG1655 mot-1 strain restored to wild type (Fig. 2A and 2B). It should also be noted that the E. coli MG1655 and E. coli MG1655 ΔflhD strains are still relatively resistant to bile salts, i.e., their growth is unaffected by 0.3% bile salts (data not shown), the concentration reported to be present in the human small intestine (64).

In addition to regulating ompF and ompC, the EnvZ/OmpR signal transduction system also positively regulates the transcription of omrA and omrB, which encode two small RNAs that negatively regulate the expression of a number of outer membrane proteins, including CirA, the receptor for colicin V (7). Since it appeared likely that the E. coli MG1655 mot mutants have higher than normal levels of OmpR–P, it seemed possible that they would have lower levels of CirA in the outer membrane and therefore be more resistant to the action of colicin V. Indeed, E. coli F-18, which produces colicin V (44), inhibited the growth of E. coli MG1655 (zone of inhibition, 2.01 ± 0.11 mm [n = 8]), E. coli MG1655 ΔflhD (zone of inhibition, 2.03 ± 0.11 mm [n = 8]), and E. coli MG1655 mot-1 restored to wild type (zone of inhibition, 1.98 ± 0.21 mm [n = 8]) to a greater extent than it inhibited the growth of E. coli MG1655 mot-1 (zone of inhibition, 0.40 ± 0.07 mm [n = 8]), E. coli MG1655 mot-2 (zone of inhibition, 0.53 ± 0.13 mm [n = 8]), and E. coli MG1655 mot-3 (zone of inhibition, 0.31 ± 0.08 mm [n = 8]) (P < 0.0005 in each case), suggesting that the mot mutants have less-than-normal levels of CirA in their outer membranes. Furthermore, since omrA and omrB also negatively regulate expression of a number of other outer membrane proteins (26), such as CsgD (regulator of Curli biosynthesis), it is likely that the outer membranes of the E. coli MG1655 mot mutants differ markedly from those of E. coli MG1655 and E. coli MG1655 ΔflhD.

E. coli MG1655 mot-1 is a better mouse intestinal colonizer than E. coli MG1655 and equal to E. coli MG1655 ΔflhD. The P41L replacement in EnvZ previously was shown to result in higher than normal levels of OmpR–P in E. coli AT142 (63). We therefore chose to study E. coli MG1655 mot-1 in more detail since it has the same envZ missense mutation. We found that when 105 CFU each of E. coli MG1655 mot-1 and E. coli MG1655 were fed to mice simultaneously, E. coli MG1655 mot-1 colonized at a level of ~108 CFU per g of feces, whereas E. coli MG1655 colonized at a level of between 105 and 106 CFU per g of feces (Fig. 3A). The enhanced colonization of E. coli MG1655 mot-1 could be specifically attributed to the P41L replacement in EnvZ, since E. coli MG1655 mot-1 restored to wild type and E. coli MG1655 cocolonized at equal population sizes (data not shown). Moreover, E. coli MG1655 mot-1 was as good a colonizer as E. coli MG1655 ΔflhD (Fig. 3B). Therefore, E. coli MG1655 mot-1 is a far better colonizer than E. coli MG1655 wild type and as competitive as E. coli MG1655 ΔflhD, which explains why the mot-1 strain can remain in the intestine at high levels despite conversion of the majority of the wild-type E. coli MG1655 to the ΔflhD genotype with its superior colonization phenotype.

Growth of E. coli MG1655 mot-1 on sugars in vitro. To address the question of whether E. coli MG1655 mot-1 and E. coli MG1655 ΔflhD are equally good colonizers because they grow equally well on sugars present in the mouse intestine, experiments were performed to compare the growth rates of E. coli MG1655 mot-1, E. coli MG1655 ΔflhD, and E. coli MG1655 on a variety of sugars as the sole source of carbon and energy. E. coli MG1655 mot-1 and E. coli MG1655 ΔflhD did not grow equally well on all of the sugars tested. In fact, E. coli MG1655 mot-1 grew 30% faster than E. coli MG1655 ΔflhD on galactose (P < 0.0005) and 5% faster on N-acetylglucosamine (P < 0.01) but grew slower than E. coli MG1655 ΔflhD on arabinose (15%, P < 0.005), fucose (14%, P < 0.01), glucose (10%, P < 0.01), maltose (27%, P < 0.001), and mannose (5%, P < 0.025) and at the same rate as E. coli MG1655 ΔflhD on fructose (P = 0.15), glucosamine (P > 0.2), and ribose (P > 0.05) (Table 4). Also, E. coli MG1655 mot-1 grew faster than E. coli MG1655 on fructose (16%, P < 0.01), galactose (34%, P < 0.0005), glucosamine (11%, P < 0.005), mannose (9%, P < 0.05), and N-acetylglucosamine (21%, P < 0.001), slower than E. coli
Three sets of three mice were fed 10^5 CFU of MG1655 mot-1 Strr (NAG, GlcNac) (Fig. 3). At the indicated times, fecal samples were homogenized, diluted, and plated as described in Materials and Methods. When necessary, i.e., to distinguish strains, 100 colonies from plates containing streptomycin were toothpicked onto MacConkey agar plates containing streptomycin and nalidixic acid or onto MacConkey plates containing streptomycin and chloramphenicol. Bars represent standard errors of the log_{10} means of CFU per g of feces.

**FIG 3** Colonization of the mouse intestine by *E. coli* MG1655, *E. coli* MG1655 mot-1, *E. coli* MG1655 mot-1 restored to wild type, and *E. coli* MG1655 ΔflhD. Three sets of three mice were fed 10^7 CFU of MG1655 mot-1 Strr (■) and 10^5 CFU of *E. coli* MG1655 Strr Na^+ (▲) (A), and three sets of three mice were fed 10^3 CFU of *E. coli* MG1655 mot-1 Strr (▲) and 10^5 CFU of *E. coli* MG1655 Strr ΔflhD::cat (■) (B). At the indicated times, fecal samples were homogenized, diluted, and plated as described in Materials and Methods. When necessary, i.e., to distinguish strains, 100 colonies from plates containing streptomycin were toothpicked onto MacConkey agar plates containing streptomycin and nalidixic acid or onto MacConkey agar plates containing streptomycin and chloramphenicol. Bars represent standard errors of the log_{10} means of CFU per g of feces.

**TABLE 4** Growth of MG1655 strains on various carbon sources

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>MG1655</th>
<th>MG1655 mot-1</th>
<th>MG1655 ΔflhD</th>
<th>MG1655 mot-1 restored to wild type</th>
<th>MG1655 mot-1 ΔgalK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>98 ± 1.0</td>
<td>97 ± 0.3</td>
<td>83 ± 1.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Fucose</td>
<td>115 ± 3.1</td>
<td>118 ± 1.2</td>
<td>102 ± 2.9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Fructose</td>
<td>139 ± 1.8</td>
<td>117 ± 3.5</td>
<td>114 ± 1.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Galactose</td>
<td>204 ± 3.5</td>
<td>135 ± 1.2</td>
<td>201 ± 1.2</td>
<td>207 ± 1.1</td>
<td>ND</td>
</tr>
<tr>
<td>Gluconate</td>
<td>87 ± 0.7</td>
<td>77 ± 1.3</td>
<td>76 ± 0.3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Glucose</td>
<td>83 ± 1.2</td>
<td>86 ± 1.8</td>
<td>77 ± 0.9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Maltose</td>
<td>112 ± 2.0</td>
<td>127 ± 1.6</td>
<td>101 ± 0.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mannose</td>
<td>192 ± 4.2</td>
<td>175 ± 2.9</td>
<td>166 ± 0.3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NAG*</td>
<td>107 ± 1.5</td>
<td>85 ± 0.4</td>
<td>89 ± 0.8</td>
<td>105 ± 1.3</td>
<td>ND</td>
</tr>
<tr>
<td>Ribose</td>
<td>181 ± 1.2</td>
<td>179 ± 0.3</td>
<td>174 ± 1.3</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Carbon source utilization was determined in M9 minimal medium (0.4% [wt/vol]) in 125-ml tissue culture flasks with shaking. Inocula were grown on M9 glycerol (0.4% [vol/vol]) and then overnight on the specified carbon source.

*Generation times were calculated from semilogarithmic plots. ND, not done; NG, no growth.

MG1655 on maltose (12%, *P* < 0.005), and at the same rate as *E. coli* MG1655 on arabinose (*P* > 0.10), fucose (*P* > 0.10), glucose (*P* > 0.05), and ribose (*P* > 0.05) (Table 4). In addition, *E. coli* MG1655 ΔflhD grew 4 to 18% faster than *E. coli* MG1655 on all of the sugars tested as the sole carbon and energy sources (*P* < 0.0005 depending on the sugar) except for galactose, on which the two strains grew equally well (*P* > 0.15) (Table 4). That the 34% faster growth rate of *E. coli* MG1655 mot-1 on galactose relative to wild-type *E. coli* MG1655 was due to the P41L mutation in EnvZ was shown by the fact that the growth rates of *E. coli* MG1655 mot-1 restored to wild type and *E. coli* MG1655 on galactose were identical (*P* > 0.15) (Table 4).

**Growth of *E. coli* MG1655 mot-1 in cecal mucus in vitro.** *E. coli* MG1655 simultaneously metabolizes a number of sugars when it colonizes the streptomycin-treated mouse intestine (8, 19). That *E. coli* MG1655 mot-1 grew slower than *E. coli* MG1655 ΔflhD on arabinose, fucose, and maltose, three sugars used by *E. coli* MG1655 for growth in the mouse intestine (8, 19, 33), but faster on galactose and N-acetylglucosamine could explain why *E. coli* MG1655 mot-1 colonizes mice as well as *E. coli* MG1655 ΔflhD. Since *E. coli* colonizes the intestine by growing in intestinal mucus (39, 45, 46, 48, 59, 60, 65), which contains at least 13 different monosaccharides that can be used by *E. coli* MG1655, and *E. coli* MG1655 ΔflhD in mouse cecal mucus standing cultures (2.5 mg/ml with respect to protein). Under these conditions, *E. coli* MG1655 mot-1 grew ca. 15% slower than *E. coli* MG1655 ΔflhD (*P* < 0.025) but at about the same rate as *E. coli* MG1655 (P > 0.15) and *E. coli* MG1655 mot-1 restored to wild type (*P* = 0.50) (Table 5). Therefore, the differences in growth rates of the strains in cecal mucus do not reflect their relative colonizing abilities, i.e., *E. coli* MG1655 mot-1 = *E. coli* MG1655 ΔflhD > *E. coli* MG1655 (Fig. 3).

**E. coli** MG1655 mot-1 resides in all intestinal niches occupied by *E. coli* MG1655 ΔflhD. Although low numbers of *E. coli* MG1655 ΔflhD grew to higher numbers in the presence of high numbers of *E. coli* MG1655 in the mouse intestine (Fig. 1), it did not grow up relative to *E. coli* MG1655 mot-1, i.e., the two strains, fed to mice in numbers 5 orders of magnitude apart, remained 5 to 5.
6 orders of magnitude apart throughout the 15 days of the experiment (Fig. 4A). *E. coli* MG1655 mot-1 did not prevent low numbers of *E. coli* MG1655 ΔflhD from growing to higher numbers due to *flhDC* deletion mutants arising in the *E. coli* MG1655 mot-1 population, since at 7 and 15 days postfeeding 50 *E. coli* MG1655 mot-1 colonies from each mouse were tested, and all remained motile. Nor was it due to an inhibitor made by *E. coli* MG1655, since *E. coli* MG1655 ΔflhD growth was unaffected by *E. coli* MG1655 mot-1 in colicin tests (data not shown). Clearly, the enhanced colonization of *E. coli* MG1655 mot-1 was due specifically to the P41L envZ mutation, since *E. coli* MG1655ΔflhD grew from low to high numbers in the mouse intestine in the presence of high numbers of *E. coli* MG1655 mot-1 restored to wild type (Fig. 4B). Therefore, it is likely that high numbers of *E. coli* MG1655 mot-1 prevented low numbers of *E. coli* MG1655 ΔflhD from growing to higher numbers because *E. coli* MG1655 mot-1 resides in and competes effectively in all intestinal niches occupied by *E. coli* MG1655 ΔflhD.

*E. coli* MG1655 does not use galactose to colonize the streptomycin-treated mouse intestine, i.e., an *E. coli* MG1655ΔgalK mutant that is unable to grow on galactose as a sole carbon source cocolonizes equally well with *E. coli* MG1655 wild type in the streptomycin-treated mouse intestine (19). However, it appears that the ability of *E. coli* MG1655 mot-1 to grow 30% faster on galactose than *E. coli* MG1655 ΔflhD contributes in part to it preventing low numbers of *E. coli* MG1655 ΔflhD from growing to higher numbers. When *E. coli* MG1655 ΔflhD was fed to mice at 5 orders of magnitude lower than *E. coli* MG1655 mot-1 ΔgalK, it colonized at a level 4 orders of magnitude lower (Fig. 4C), rather than the 5 to 6 orders of magnitude lower in competition with *E. coli* MG1655 mot-1 (Fig. 4A) or *E. coli* MG1655 mot-1 ΔgalK restored to wild type with respect to galK (data not shown). It should be emphasized, however, that *E. coli* MG1655 mot-1 ΔgalK still prevented *E. coli* MG1655 ΔflhD from growing to a level any higher than 4 orders of magnitude below it, despite growing 25% slower than *E. coli* MG1655 mot-1 in cecal mucus in vitro (P < 0.01) (Table 5).

*E. coli* MG1655 mot-1 may colonize an intestinal niche that *E. coli* MG1655 ΔflhD colonizes poorly. That *E. coli* MG1655 mot-1 resides in all intestinal niches occupied by *E. coli* MG1655 ΔflhD does not mean *E. coli* MG1655 ΔflhD resides in all intestinal niches occupied by *E. coli* MG1655 mot-1. Since *E. coli* MG1655 mot-1 and *E. coli* MG1655 ΔflhD are equally good colonizers when 10⁶ CFU of each is fed to mice (Fig. 3C) and since high numbers of *E. coli* MG1655 mot-1 prevent low numbers of *E. coli* MG1655 ΔflhD from growing to high numbers in the intestinal niches they occupy together (Fig. 4A), it would be expected that if *E. coli* MG1655 ΔflhD resides in all intestinal niches occupied by *E. coli* MG1655 mot-1 that high numbers of *E. coli* MG1655 ΔflhD would prevent low numbers of *E. coli* MG1655 mot-1 from growing to higher numbers in the streptomycin-treated mouse intestine. To test that possibility, mice were fed 10¹⁰ CFU of *E. coli* MG1655 ΔflhD and 10⁵ CFU of *E. coli* MG1655 mot-1. Surprisingly, *E. coli* MG1655 mot-1 grew from about 5 orders of magnitude lower than *E. coli* MG1655 ΔflhD to within 2 orders of magnitude of *E. coli* MG1655 ΔflhD by 9 days postfeeding (Fig. 5A). Therefore, it appears that *E. coli* MG1655 mot-1 not only resides in all the intestinal niches occupied by *E. coli* MG1655 ΔflhD but may also colonize a smaller niche that *E. coli* MG1655 ΔflhD is unable to colonize or colonizes poorly. Moreover, it appears that *E. coli* MG1655 mot-1 uses its superior ability to metabolize galactose to colonize that niche, since low numbers of *E. coli* MG1655 mot-1 ΔgalK failed to grow to higher numbers in the presence of high numbers of *E. coli* MG1655 ΔflhD (Fig. 5B), whereas low numbers of *E. coli* MG1655 mot-1 ΔgalK restored to wild type with respect to galactose utilization grew to higher numbers (data not shown). To summarize, *E. coli* MG1655 mot-1 and *E. coli* MG1655 ΔflhD appear to colonize one intestinal niche equally well and *E. coli* MG1655 mot-1 appears to need galactose for maximum growth in that niche. In addition, it appears that *E. coli* MG1655 mot-1 also uses galactose to colonize a second smaller intestinal niche that *E. coli* MG1655 ΔflhD either fails to colonize or colonizes poorly.

### TABLE 5 Growth of MG1655 strains in cecal mucus in vitro

<table>
<thead>
<tr>
<th>Carbon source (cecal mucus sample)</th>
<th>Mean rate of growth (ΔA_{600/n}) ± SD</th>
<th>MG1655</th>
<th>MG1655 mot-1 ΔflhD</th>
<th>MG1655 restored to wild type</th>
<th>MG1655 mot-1 ΔgalK</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.037 ± 0.002</td>
<td>0.038 ± 0.0006</td>
<td>0.045 ± 0.0006</td>
<td>0.037 ± 0.003</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>ND</td>
<td>0.032 ± 0.002</td>
<td>0.037 ± 0.002</td>
<td>0.028 ± 0.001</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a* Cultures were grown overnight in LB broth Lennox, washed in HEPES-Hanks buffer (pH 7.0), to inoculate 1 ml of cecal mucus (2.5 mg/ml) in HEPES-Hanks buffer, and then incubated at 37°C in polystyrene cuvettes. The ΔA_{600/n} of each culture was determined at 30-min intervals. The results of independent experiments (n = 3) are shown.

*b* Cecal mucus cultures grew linearly. The slope of the line was used to measure the growth rate.
minority members of dense mixed biofilms (Fig. 6B). At neither 24 h nor 48 h postfeeding was *E. coli* MG1655 found to be associated with the epithelium (Fig. 6).

**DISCUSSION**

Previously, we reported that the streptomycin-treated mouse intestine selects nonmotile *E. coli* MG1655 *flhDC* deletion mutants that have improved colonizing ability (24, 36). These mutants grew 15% faster than their parent in mouse cecal mucus *in vitro* and 15 to 30% faster on several sugars found in the mouse intestine, suggesting that their better colonizing ability was due to their more efficient utilization of sugars for growth (36). In our previous studies, despite the strong selection for nonmotile *flhDC* deletion mutants, 10 to 20% of the *E. coli* MG1655 cells remained motile (24). In the present study, we demonstrate that these motile *E. coli* MG1655 contain missense mutations in *envZ* that make them less motile and better colonizers than wild-type *E. coli* MG1655 and more resistant to bile salts and colicin V than *E. coli* MG1655 and *E. coli* MG1655 *flhD*, suggesting that they have higher levels of OmpR.

Among the many activities controlled by EnvZ/OmpR is tran-
scription of the flhDC operon, i.e., OmpR−P is a negative regulator of the operon in E. coli strains that lack the IS1 element in the flhDC promoter (56). Such strains are not hypermotile and become even less motile at higher medium osmolarity because the level of OmpR−P under these conditions is sufficient to further repress the flhDC operon (4, 24). In contrast, E. coli MG1655, which contains the IS1 element in the flhDC promoter, remains hypermotile under high-osmolarity conditions (4, 24), suggesting the possibility that the IS1 element reduces the affinity of OmpR−P for the OmpR binding sites in the flhDC promoter. The fact that the E. coli MG1655 envZ missense mutants are far less motile than E. coli MG1655 (Table 3) suggests that the even higher level of OmpR−P in these mutants allows some level of binding to the OmpR binding sites in the flhDC regulatory region, resulting in less transcription of the operon and consequently less FlhD4C2, despite the presence of the IS1 element. Also, since FlhD4C2 represses a number of sugar catabolism operons (20), less FlhD4C2 in E. coli MG1655 mot-1 than in E. coli MG1655 but more than in E. coli MG1655 ΔflhD would be consistent with it growing faster than E. coli MG1655 on a number of sugars and slower than E. coli MG1655 ΔflhD on a number of sugars, as we observed (Table 4).

Although E. coli MG1655 mot-1 grew slower than E. coli MG1655 ΔflhD on several sugars, it did grow ca. 30% faster than E. coli MG1655 ΔflhD and E. coli MG1655 on galactose in vitro (Table 4). In this regard, it is interesting that there is a putative OmpR binding site upstream of the galR promoter between galR and omrB (26). GalR is the major repressor of both galactose transport and catabolism (67). Although it is known that OmpR−P positively regulates transcription of omrB (26), it is not known whether it also negatively regulates galR transcription. Negative regulation of galR transcription by increased levels of OmpR−P in E. coli MG1655 mot-1 could explain why it grows faster than E. coli MG1655 and E. coli MG1655 ΔflhD on galactose as the sole source of carbon and energy.

Giraud et al. (25) demonstrated that when germfree mice were fed the same wild-type E. coli MG1655 strain as used here, the mouse intestine selected better-colonizing, less-motile E. coli MG1655 envZ missense mutants. More recently, De Paepe et al. (14) demonstrated that several days after the envZ missense mutants reached maximum numbers in germfree mice monoassociated with E. coli MG1655, mutants with flhDC deletions appeared and increased in numbers thereafter, showing that the monoaossoicated mouse intestine can select E. coli MG1655 flhDC deletion mutants in the presence of high numbers of E. coli MG1655 envZ missense mutants. Interestingly, our data show that the monoaossoicated ex-germfree mouse intestine and the streptomycin-treated mouse intestine are different in this regard; flhDC mutants would not be selected after envZ missense mutants reach maximum numbers in the streptomycin-treated mouse intestine since in this model high numbers of an E. coli MG1655 envZ missense mutant prevented growth of an E. coli MG1655 flhDC deletion mutant from lower to higher numbers (Fig. 4A).

It was also demonstrated by De Paepe et al. (14) that their in vitro results could be reproduced in chemostats containing 0.8% bile salts, a concentration reasonable for the intestine of a germfree mouse (18), but ~2.7-fold higher than the 0.3% concentration of bile salts reported to be in the human small intestine (64). In fact, the presence of a conventional microbiota decreases the concentration of bile salts in the mouse intestine, i.e., in the conventional mouse cecum and colon the concentration of bile salts is known to be ~2.5-fold lower than in the germfree mouse cecum and colon (18). It is therefore unlikely that bile salts play as much of a role in E. coli MG1655 colonization of the streptomycin-treated mouse intestine, which contains a large and diverse microbiota, as they do in its colonization of the monoaossoicated mouse intestine. In support of this view, despite E. coli MG1655 ΔflhD being more sensitive to bile salts than E. coli MG1655 mot-1, the two strains grew equally well from low to high numbers in the streptomycin treated mouse intestine when 10^5 CFU each of the two strains were fed to mice simultaneously (Fig. 3B). It therefore appears likely that the differences between our results and those of De Paepe et al. (14) reflect physical and nutritional interaction of the E. coli MG1655 strains with other members of the complex microbial community present in the streptomycin-treated mouse intestine but absent in the monoaossoicated, formerly germfree mouse intestine.

The concept that nutrient availability dictates the community structure of the intestine was originally presented in Freter’s nutrient-niche hypothesis, which states that species coexist in the intestine because each is able to grow faster than all others on one
or a few limiting nutrients and that the rate of growth of each species during the colonization process is at least equal to its washout rate from the intestine (22). The hypothesis assumes that all nutrients are perfectly mixed and that they are equally available to all species present in the intestine. According to the hypothesis, two strains cannot coexist in the intestine when one competes less well than the other for the same nutrient(s) unless the metabolically less efficient one adheres to the intestinal wall (22). Adhesion of a bacterium to the intestinal wall is not restricted to the epithelium, it could, for example, also be achieved by adhesion of that bacterium to mucin strands in the outer loose mucus layer where commensal strands reside (32, 50) that might be anchored in the relatively bacteria-free inner dense mucus layer or, alternatively by attachment to other members of the microbiota that adhere to mucin strands in the outer loose mucus layer. In either case, such attachment could delay the metabolically less efficient strain from being washed out as the loose mucus layer at the luminal surface is sloughed into luminal contents and eliminated in feces.

We found no evidence that E. coli MG1655 adheres to the intestinal epithelium but did find that it binds to other members of the microbiota as a minority member of mixed biofilms in cecal mucus (Fig. 6B). We also found that E. coli MG1655 mot-1 and E. coli MG1655 flhD each use some sugars for growth better than the other in vitro (Table 4) and that they co-colonize the mouse intestine equally well when equal numbers of each strain are fed to mice (Fig. 3B), indicating that sufficient levels of the sugars that each strain uses better are available to each strain when they co-colonize. As such, it would be expected according to the nutrient-niche hypothesis that low numbers of each strain would be able to grow to higher numbers in the intestine in the presence of high numbers of the other strain. Indeed, by using its superior ability to grow on galactose, low numbers of E. coli MG1655 mot-1 grew to higher numbers in the presence of high numbers of E. coli MG1655 flhD (Fig. 5A). However, contrary to expectations, high numbers of E. coli MG1655 mot-1 prevented low numbers of E. coli MG1655 flhD from growing to higher numbers (Fig. 4A). Below we outline a modified version of the Freter nutrient-niche hypothesis to explain our results based on the finding that E. coli MG1655 appears to reside in mixed biofilms in the streptomycin-treated mouse intestine.

Mono- and disaccharides and maltodextrins are absorbed in the small intestine, whereas dietary fiber reaches the large intestine later in the intestinal tract. In contrast to the anaerobes (29), E. coli does not secrete extracellular polysaccharide hydrolases (27, 30) and therefore cannot degrade dietary fiber-derived and mucin-derived oligo- and polysaccharides. Commensal E. coli strains colonize the mouse large intestine by growing in intestinal mucus (39, 45, 46, 48, 59, 60, 65). It therefore appears likely that E. coli depends on the anaerobes present in mucus that can degrade oligo- and polysaccharides to provide them with the mono- and disaccharides they need for growth. We hypothesize that the anaerobes in the mixed biofilms inhabited by E. coli provide them with mono- and disaccharides locally, rather than from a perfectly mixed pool available to all species, which is an assumption of the nutrient-niche hypothesis. We further hypothesize that one strain of E. coli can have a higher affinity for binding sites on mixed biofilms than a second strain due to differences between the outer surfaces of the two strains. In addition, we hypothesize that E. coli cells that detach from a mixed biofilm either spontaneously or through replication and hence free in mucus are not exposed to monosaccharides to provide them with the mono- and disaccharides because those sugars are produced and used locally within the mixed biofilms and are therefore not available to detached E. coli cells. Consequently, E. coli cells not attached to a mixed biofilm will not grow or will grow slower than the washout rate and will be eliminated in feces unless they bind to and become part of other mixed biofilms that are constantly forming in the mucus layer.

How does the hypothesis account for our results? Our results suggest that the outer membranes of E. coli MG1655 mot-1 and E. coli MG1655 flhD differ not only with respect to the presence or absence of flagella and amounts of OmpF and OmpC but also with respect to the amounts of a number of proteins present in their outer membranes, including CirA and CsgD (see Results and reference 26), which we hypothesize results in E. coli MG1655 mot-1 having a higher affinity than E. coli MG1655 flhD for the E. coli binding sites on the mixed biofilms they inhabit. In addition, E. coli MG1655 mot-1 grows slower than E. coli MG1655 flhD on a number of sugars present in mucus and in mouse cecal mucus in vitro (Table 5). Accordingly, we suggest that the lower growth rate of E. coli MG1655 mot-1 within a biofilm is compensated for by its higher affinity for the biofilm. If so, it would then be expected that if E. coli MG1655 mot-1 and E. coli MG1655 flhD were fed to mice in equal numbers they would co-colonize in about equal numbers, as observed (Fig. 3B), and if mice were fed E. coli MG1655 mot-1 and E. coli MG1655 flhD at a ratio of 10^5:1, that ratio would also be maintained throughout the duration of the experiment, as observed (Fig. 4A). However, it would also be expected that if mice were fed E. coli MG1655 mot-1 and E. coli MG1655 flhD at a ratio of 10^5:1, that ratio would be maintained throughout the duration of the experiment, when in fact, under these conditions, low numbers of E. coli MG1655 mot-1 grow to higher numbers (Fig. 5A). This can be explained if E. coli MG1655 mot-1 also grows in a second niche that E. coli MG1655 flhD cannot colonize or colonizes poorly. That second niche could be another mixed biofilm consisting of a different group of anaerobes in which E. coli MG1655 mot-1 takes advantage not only of its higher affinity for binding sites but also of its superior growth rate on galactose and thereby prevents E. coli MG1655 flhD from occupying that niche. Alternatively, that second niche could be another mixed biofilm that contains an anaerobe that makes a bacteriocin that inhibits E. coli MG1655 flhD growth far more than it inhibits E. coli MG1655 mot-1 growth, much in the same way that E. coli MG1655 flhD is more sensitive to colicin V than E. coli MG1655 mot-1. Most bacterial species, including several that reside in the mammalian intestine, are known to make bacteriocins, many of which kill across species (54). Finally, our hypothesis raises the possibility that E. coli strains with vastly different surfaces (e.g., fimbriae, O, K, and H antigens etc) reside in biofilms made up of different anaerobes that supply different sugars to those strains, which could explain why different E. coli strains display different nutritional programs in the intestine (19, 35). Whether our hypothesis, which we call the “Restaurant” hypothesis, has merit will be the focus of future research.

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