IAI Journals.ASM.org

An *Escherichia coli* Nissle 1917 Missense Mutant Colonizes the Streptomycin-Treated Mouse Intestine Better than the Wild Type but Is Not a Better Probiotic

Jimmy Adediran,[a] Mary P. Leatham-Jensen,[a] Matthew E. Mokszycki,[a] Jakob Frimodt-Møller,[b] Karen A. Krogfelt,[b] Krystyna Kazmierczak,[c,d,e*] Linda J. Kenney,[c,d,e] Tyrrell Conway,[f] Paul S. Cohen[a]

Department of Cell and Molecular Biology, University of Rhode Island, Kingston, Rhode Island, USA[a]; Department of Microbiological Surveillance and Research, Statens Serum Institut, Copenhagen, Denmark[b]; Jesse Brown Veterans Administration Medical Center, Chicago, Illinois, USA[c]; Department of Microbiology, University of Illinois—Chicago, Chicago, Illinois, USA[d]; Mechanobiology Institute, National University of Singapore, Singapore, Singapore[e]; Department of Microbiology and Plant Biology, University of Oklahoma, Norman, Oklahoma, USA[f]

Previously we reported that the streptomycin-treated mouse intestine selected for two different *Escherichia coli* MG1655 mutants with improved colonizing ability: nonmotile *E. coli* MG1655 *flhDC* deletion mutants that grew 15% faster *in vitro* in mouse cecal mucus and motile *E. coli* MG1655 *envZ* missense mutants that grew slower *in vitro* in mouse cecal mucus yet were able to cocolonize with the faster-growing *flhDC* mutants. The *E. coli* MG1655 *envZ* gene encodes a histidine kinase that is a member of the *envZ-ompR* two-component signal transduction system, which regulates outer membrane protein profiles. In the present investigation, the $envZ_{P41L}$ gene was transferred from the intestinally selected *E. coli* MG1655 mutant to *E. coli* Nissle 1917, a human probiotic strain used to treat gastrointestinal infections. Both the *E. coli* MG1655 and *E. coli* Nissle 1917 strains containing $envZ_{P41L}$ produced more phosphorylated OmpR than their parents. The *E. coli* Nissle 1917 strain containing $envZ_{P41L}$ also became more resistant to bile salts and colicin V and grew 50% slower *in vitro* in mucus and 15% to 30% slower on several sugars present in mucus, yet it was a 10-fold better colonizer than *E. coli* Nissle 1917. However, *E. coli* Nissle 1917 $envZ_{P41L}$ was not better at preventing colonization by enterohemorrhagic *E. coli* EDL933. The data can be explained according to our "restaurant" hypothesis for commensal *E. coli* strains, i.e., that they colonize the intestine as sessile members of mixed biofilms, obtaining the sugars they need for growth locally, but compete for sugars with invading *E. coli* pathogens planktonically.

Previously we reported that when streptomycin-treated mice are fed wild-type *Escherichia coli* MG1655, the intestine selects for nonmotile *flhDC* deletion mutants (1, 2) and *envZ* missense mutants (3), both of which are better colonizers than the wild type. The *flhDC* mutants have deletions of various sizes, beginning downstream of an IS*1* element in the *flhDC* regulatory region and extending into or beyond the *flhDC* structural genes (1, 2). FlhD and FlhC form the $FlhD_4C_2$ complex (4), which activates transcription of class II flagellar genes that encode components of the flagellar basal body and export machinery (5). The IS*1* element immediately upstream of the *E. coli* MG1655 *flhDC* promoter increases expression of the *flhDC* operon and makes *E. coli* MG1655 hypermotile (1, 6). One of the better-colonizing nonmotile *flhDC* deletion mutants, *E. coli* MG1655 Δ*flhD*, grew 15% faster *in vitro* in mouse cecal mucus and 15% to 30% faster on several sugars present in cecal mucus than *E. coli* MG1655 (1), presumably explaining its better colonizing ability. Additional studies suggested that the *E. coli* MG1655 *flhDC* operon deletion mutants utilize sugars better than their parent at least in part because a number of metabolic genes are repressed by the $FlhD_4C_2$ regulatory complex, including *gltA* (citrate synthase), *sdhCDAB* (succinate dehydrogenase), *mdh* (malate dehydrogenase), *mglBAC* (galactose transport), and a large number of sugar catabolism operons (7–9).

EnvZ, a histidine kinase, and OmpR, its cognate response regulator, comprise a two-component signal transduction system that modulates gene expression in response to osmolarity (10). Among the many activities controlled by EnvZ-OmpR are transcription of *ompF*, which encodes an *E. coli* outer membrane porin that allows passage of bile salts as well as other small molecules from the environment into the periplasm (11); transcription of the *flhDC* operon (12); and transcription of *omrA* and *omrB*, which encode two small RNAs that regulate the expression of a number of outer membrane proteins, including CirA, the receptor for colicin V (13). The better-colonizing *E. coli* MG1655 *envZ* missense mutants were more resistant to bile salts and colicin V than *E. coli* MG1655 and *E. coli* MG1655 Δ*flhD* and were less motile than *E. coli* MG1655, consistent with them containing a higher level of phosphorylated OmpR (OmpR~P) (3). One better-colonizing *E. coli* MG1655 *envZ* missense mutant which was studied further, i.e., *E. coli* MG1655 mot-1 (referred to here as *E. coli* MG1655 $envZ_{P41L}$), grew about 15% slower in mouse cecal mucus *in vitro* and on several sugars present in mucus than *E. coli* MG1655 Δ*flhD*, but it grew 30% faster on galactose (3). In addition, despite growing slower in mouse cecal mucus *in vitro*, *E. coli* MG1655 $envZ_{P41L}$ appeared to colonize one intestinal niche in the cecum as well as *E. coli* MG1655 Δ*flhD*, suggesting that factors other than competition for nutrients were responsible, and it ap-

Received 9 September 2013 **Returned for modification** 11 October 2013
Accepted 18 November 2013
Published ahead of print 25 November 2013
Editor: B. A. McCormick
Address correspondence to Paul S. Cohen, pco1697u@mail.uri.edu.
* Present address: Krystyna Kazmierczak, IHMA Inc., Schaumburg, Illinois, USA.

doi:10.1128/IAI.01149-13

TABLE 1 Bacterial strains used in this study[a]

E. coli strain	Description	Name used in text	Source or reference
EDL933 Str^r Rif^r	Spontaneous rifampin-resistant mutant of EDL933 Str^r	EDL933	34
MG1655 Str^r	Spontaneous streptomycin-resistant mutant of MG1655	MG1655	3
MG1655 Str^r Nal^r	Spontaneous nalidixic acid-resistant mutant of MG1655 Str^r	MG1655	3
MG1655 Str^r mot-1	P41L *envZ* missense mutant of MG1655 Str^r	MG1655 $envZ_{P41L}$	3
MG1655 Str^r mot-2	P148S *envZ* missense mutant of MG1655 Str^r	MG1655 $envZ_{P148S}$	3
MG1655 Str^r mot-3	V33E *envZ* missense mutant of MG1655 Str^r	MG1655 $envZ_{V33E}$	3
MG1655 Str^r Nal^r mot-1	Spontaneous nalidixic acid-resistant mutant of MG1655 Str^r mot-1	MG1655 $envZ_{P41L}$	3
MG1655 Str^r Nal^r mot-1 restored	MG1655 Str^r mot-1 restored to wild type with respect to EnvZ	MG1655 $envZ_{P41L}$ restored to wild type with respect to EnvZ	3
MG1655 Str^r $envZ_{Nis}$	MG1655 Str^r with the Nissle 1917 *envZ* gene	MG1655 $envZ_{Nis}$	This study
MG1655 Str^r Nal^r $envZ_{Nis}$	Spontaneous nalidixic acid-resistant mutant of MG1655 Str^r $envZ_{Nis}$	MG1655 $envZ_{Nis}$	This study
Nissle 1917 Str^r	Spontaneous streptomycin-resistant mutant of Nissle 1917	Nissle 1917	34
Nissle 1917 Str^r Nal^r	Spontaneous nalidixic acid-resistant mutant of Nissle 1917 Str^r	Nissle 1917	34
Nissle 1917 Str^r $envZ_{P41L}$	Nissle 1917 Str^r with the $envZ_{P41L}$ gene in place of its own	Nissle 1917 $envZ_{P41L}$	This study
Nissle 1917 Str^r Nal^r $envZ_{P41L}$	Spontaneous nalidixic acid-resistant mutant of Nissle 1917 Str^r $envZ_{P41L}$	Nissle 1917 $envZ_{P41L}$	This study
Nissle 1917 Str^r $envZ_{P41L}$ restored	Nissle 1917 Str^r $envZ_{P41L}$ restored to wild type with respect to EnvZ	Nissle 1917 $envZ_{P41L}$ restored to wild type with respect to EnvZ	This study
Nissle 1917 Str^r Δ*galK*::*cat*	912-bp deletion in galactokinase gene replaced by a chloramphenicol resistance cassette	Nissle 1917 Δ*galK*::*cat*	This study
Nissle 1917 Str^r $envZ_{P41L}$ Δ*galK*::*cat*	Same deletion as in Nissle 1917 Str^r Δ*galK*::*cat*	Nissle 1917 $envZ_{P41L}$ Δ*galK*::*cat*	This study
Nissle 1917 Str^r $envZ_{P41L}$ Δ*galK*::*cat* restored	Nissle 1917 Str^r $envZ_{P41L}$ Δ*galK*::*cat* restored to wild type with respect to *galK*	Nissle 1917 $envZ_{P41L}$ Δ*galK*::*cat* restored to wild type with respect to *galK*	This study
Nissle 1917 Str^r Nal^r $envZ_{P41L}$ Δ*galK*::*cat* restored	Spontaneous nalidixic acid-resistant mutant of Nissle 1917 Str^r $envZ_{P41L}$ Δ*galK*::*cat* restored	Nissle 1917 $envZ_{P41L}$ Δ*galK*::*cat* restored to wild type with respect to *galK*	This study
F-18 Str^r Rif^r	Spontaneous streptomycin- and rifampin-resistant mutant of F-18	F-18	31
BW37751(pKD267)::Kan^r	The plasmid in this strain contains *parE* under the control of the rhamnose promoter and the kanamycin resistance gene	pKD267	Barry Wanner

[a] Str^r, streptomycin resistant; Rif^r, rifampin resistant; Nal^r, nalidixic acid resistant; Kan^r, kanamycin resistant.

peared to use galactose to colonize a second intestinal niche in the cecum that was either not colonized or poorly colonized by *E. coli* MG1655 Δ*flhD* (3).

E. coli Nissle 1917 is a commensal strain that has been used as a probiotic agent to treat gastrointestinal infections in humans since the early 1920s (14). Several features of *E. coli* Nissle 1917 have been proposed to be responsible for its probiotic nature, including its ability to express two microcins (15), the absence of known protein toxins, its semirough lipopolysaccharide, and hence serum sensitivity (16, 17), and the presence of six iron uptake systems (18). To study the *envZ* derivative encoding the P41L mutation ($envZ_{P41L}$) further, we constructed an $envZ_{P41L}$ derivative of *E. coli* Nissle 1917 and in the present study characterized the strain and tested whether it has improved mouse intestinal colonizing ability and whether it is better at limiting colonization of *E. coli* EDL933, a human enterohemorrhagic strain. We found that *E. coli* Nissle 1917 $envZ_{P41L}$ is not a better probiotic, but like *E. coli* MG1655 $envZ_{P41L}$, it colonized an intestinal niche not colonized by wild-type *E. coli* Nissle 1917. We discuss the results in terms of the restaurant hypothesis.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this study are listed in Table 1. The original *E. coli* K-12 strain was obtained from a stool sample from a convalescing diphtheria patient in Palo Alto, CA, in 1922 (19). 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 acridine orange treatment (19). It has an IS*1* element in the *flhDC* promoter (20). *E. coli* Nissle 1917 was originally isolated during World War I from a soldier who escaped a severe outbreak of diarrhea (14). It has a beneficial effect on several types of intestinal disorders, is well tolerated by humans, and has been marketed as a probiotic remedy against intestinal disorders in several European countries since the 1920s (14). The allelic exchange method described by Datsenko and Wanner (21) was used to construct *E. coli* Nissle 1917 Str^r $envZ_{P41L}$ Δ*galK*::*cat* (Table 1), which contains a 912-bp deletion replaced by a chloramphenicol cassette beginning 153 bp downstream of the ATG start codon and ending 78 bp upstream of the TGA stop codon. As expected, *E. coli* Nissle 1917 Str^r $envZ_{P41L}$ Δ*galK*::*cat* failed to grow in M9 minimal medium (22) containing 0.4% (wt/vol) galactose as the sole carbon and energy source. *E. coli* Nissle 1917 Str^r $envZ_{P41L}$ Δ*galK*::*cat* was restored to the Gal$^+$ phenotype by allelic replacement (21),

with selection for restored growth on M9 minimal galactose agar plates. The restored Gal$^+$ strain grew at the same rate as *E. coli* Nissle 1917 Str^r *envZ*$_{P41L}$ in M9 galactose minimal medium. The *E. coli* MG1655 *envZ* gene was replaced with the *E. coli* Nissle 1917 wild-type *envZ* gene by an unpublished allelic replacement strategy developed by Barry Wanner and Kiryl Datsenko at Purdue University, as described previously (3). The same method was used to replace the *E. coli* Nissle 1917 *envZ* gene with the *envZ*$_{P41L}$ gene. All constructions were confirmed by sequencing.

Growth of strains and sample preparation for detection of phosphorylated OmpR. Bacteria were grown overnight in the presence of 100 μg/ml of streptomycin sulfate in M9 minimal medium (22) supplemented with 0.2% glucose or M63 medium (23) supplemented with 0.2% glucose and 0.0005% vitamin B_1. Cells were diluted 100- to 200-fold in the same medium or medium supplemented with 150 mM NaCl or 15% sucrose, respectively. Cultures (32 to 35 ml) were grown to an optical density at 600 nm (OD_{600}) of ≈0.5 to 0.7 and harvested by centrifugation at 14,500 × *g* for 10 min at 4°C. Samples were prepared for Phos-tag analysis as described by Wayne et al. (24), with the following changes. For samples grown in M63 medium, cell pellets were suspended in 1 ml of cold 20 mM Tris-HCl, pH 7.0, supplemented with 15% sucrose and EDTA-free bacterial protease inhibitor cocktail (RPI Corp., Mount Prospect, IL). Suspensions were transferred to chilled tubes of lysing matrix B (MP Biomedicals, Solon, OH), and cells were disrupted by two 40-s cycles of homogenization at a speed of 6.0 m/s in a FastPrep-24 instrument (MP Biomedicals). Samples were chilled on ice for 5 min between disruption cycles. For samples grown in M9 medium, cell pellets were suspended in 1 ml of cold BugBuster master mix (Novagen, Darmstadt, Germany) supplemented with EDTA-free bacterial protease inhibitor cocktail (RPI Corp.). Suspensions were transferred to chilled tubes of lysing matrix B (MP Biomedicals) and incubated on a platform shaker for 15 min at 4°C. Cells were disrupted by one 20-s cycle of homogenization at a speed of 4.0 m/s in a FastPrep-24 instrument (MP Biomedicals). After cell disruption, all samples were centrifuged at 10,000 rpm for 1 min at 4°C, and 75 μl of each cleared lysate was mixed with cold 4× Laemmli buffer (Bio-Rad, Hercules, CA). Samples were frozen on dry ice and stored at −80°C until analysis.

SDS-PAGE, Western blotting, and detection of OmpR and OmpR~P. SDS-PAGE and Western blotting were performed as described by Wayne et al. (24), with the following changes. Samples were thawed briefly on ice. Approximately 16 OD_{600} units of each sample was loaded into each well of a chilled 10% SDS-polyacrylamide gel containing 25 μM or 50 μM Phos-tag acrylamide (AAL-107; Wako Chemicals USA Inc., Richmond, VA) and then separated by electrophoresis at 140 V for 2.25 h at 4°C. Proteins were transferred to a 0.45-μm Immobilon-P membrane (polyvinylidene difluoride [PVDF]; EMD Millipore, Billerica, MA) at 30 V for 15 h, followed by an additional 1 h at 100 V. Membranes were blocked in 3% (wt/vol) Amersham ECL blocking agent (GE Healthcare Biosciences, Pittsburgh, PA) in 1× Tris-buffered saline (TBS), 0.5% (vol/vol) Tween 20, incubated in a 1:50 to 1:80 dilution of affinity-purified rabbit anti-OmpR antibody for 24 h at 4°C, and then incubated in a 1:100,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Pierce Antibodies, Rockford, IL) for 1 h at room temperature (RT). Signals were detected using SuperSignal West Pico chemiluminescent substrate (Pierce Antibodies) followed by exposure to Hyblot-CL film (Denville Scientific Inc., Plainfield, NJ).

Outer membrane protein analysis. Outer membrane fractions were prepared as described by Morona and Reeves (25), with the following modifications. Briefly, bacteria were grown overnight in LB broth supplemented with 100 μg/ml of streptomycin sulfate. Cells were diluted 100- to 200-fold in medium A (26) and medium A supplemented with 15% sucrose and grown to an OD_{600} of ≈0.6 to 1.0. Cultures (25 to 30 ml) were harvested by centrifugation at 3,000 × *g* for 15 min at 4°C, and cell pellets were frozen on dry ice. After storage at −20°C, pellets were thawed on ice and suspended in 0.2 ml of 20% (wt/vol) sucrose in 30 mM Tris-HCl, pH 8.8. Fifty microliters of lysozyme solution (5 mg/ml) in 5 mM Tris-HCl, pH 8.0, 0.1 M EDTA, pH 8.0, was added to each suspension. After 30 min of incubation on ice, 5 ml of cold 3 mM EDTA was added to the suspensions, which were mixed by vortexing and sonicated three times for 30 s at power setting 7, using a Fisher Scientific model 100 Sonic Dismembrator instrument. Cellular debris was removed by centrifugation at 3,000 × *g* for 15 min at 4°C. Supernatants were centrifuged at 21,000 × *g* for 1 h at 4°C, and membrane pellets were suspended in 100 μl of 4× Laemmli buffer. Samples were heated at 95°C for 10 min before electrophoresis at 125 V on 4 M urea, 10% SDS-polyacrylamide gels.

Other 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 (21). For testing of carbon and energy source utilization, M9 minimal medium (22) was modified by addition of 120 mM NaCl to more closely approximate the sodium chloride concentration in the intestine (27). Growth was also tested in cecal mucus (2.5 mg protein/ml) in HEPES-Hanks buffer (pH 7.0), which contains 137 mM NaCl, the sodium chloride concentration in jejunal and ileal intestinal fluid (27). Cultures were prepared and growth was monitored as described previously (1).

Isolation of cecal mucus. Mouse cecal mucus was isolated as previously described (28). Briefly, mice (5 to 8 weeks old) were fed Teklad mouse and rat diet (Harlan Laboratories, Madison, WI) for 5 days after being received. The drinking water was then replaced with sterile distilled water containing streptomycin sulfate (5 g/liter). Twenty-four hours later, the mice were sacrificed by CO_2 asphyxiation, and their ceca were removed. The cecal contents were washed out with sterile distilled water, and cecal mucus was scraped into HEPES-Hanks buffer (pH 7.0), centrifuged, and sterilized by UV irradiation as described previously (28). All animal protocols were approved by the University Committee on Use and Care of Animals at the University of Rhode Island.

Motility. Motility of *E. coli* Nissle 1917 strains was assayed by transferring colonies by toothpick from LB agar Lennox onto Luria motility agar as described previously (3). Plates were incubated at 37°C for 6 h, after which spreading was measured from the edge of each colony.

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 OD_{600} of about 0.1 in 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. Growth was monitored as described previously (1).

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 (3). 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 (29). Following 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 ingestion of the bacterial suspension, both the food (Teklad mouse and rat diet; Harlan Laboratories, Madison, WI) and streptomycin-water were returned to the mice, and 1 g of feces was collected after 5 h and 24 h and on odd-numbered days, 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 × *g*, resuspended in 100 μl of 1% Bacto tryptone, and plated on a MacConkey agar plate with appropriate antibiotics. This procedure increased the sensitivity of the assay from 10^2 CFU/g of feces to 10 CFU/per g of feces. To distinguish the various *E. coli* strains in feces, dilutions were plated on lactose MacConkey agar containing streptomycin sulfate (100 μg/ml), streptomycin sulfate (100 μg/ml) and nalidixic acid (50 μg/ml),

streptomycin sulfate (100 μg/ml) and chloramphenicol (30 μg/ml), or streptomycin sulfate (100 μg/ml) and rifampin (50 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 transferred by toothpick 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 (for a total of six mice) are presented in the figures.

Sequencing and primers. DNA sequencing was done at the URI Genomics and Sequencing Center, University of Rhode Island, Kingston, RI, using an Applied Biosystems 3130xl genetic analyzer (Applied Biosystems, Foster City, CA). A BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) was used for the sequencing reactions. Primers were designed using the *E. coli* K-12 genome sequence (20) or the *E. coli* CFT073 genome sequence (30), which is closely related to the *E. coli* Nissle 1917 genome sequence (18).

The *galK* deletion primers used to make *E. coli* Nissle 1917 Str^r Δ*galK*::*cat* (uppercase letters, *E. coli* Nissle 1917 DNA; lowercase letters, chloramphenicol resistance cassette DNA) were as follows: primer 1, 5′-AAACG TAAAAAGTCTCTTTAATACCTGTTTTTGCTTCATATTGTTCAGC gtgtaggctggagctgcttcg-3′; and primer 2, 5′-CGACTACAACGAC GGTTTCGTTCTGCCCTGCGCGATTGATTATCAAACCcatatgaata tcctccttagt-3′. The primers used for both amplifying the *galK*::*cat* deletion for allelic exchange into *E. coli* Nissle 1917 Str^r *envZ*$_{P41L}$ and sequencing to confirm its presence in both *E. coli* Nissle 1917 Str^r Δ*galK*::*cat* and *E. coli* Nissle 1917 Str^r *envZ*$_{P41L}$ Δ*galK*::*cat* were as follows: primer 1 (43 bp upstream of the *galK* coding sequence), 5′-AACAGGCAGCAGAGCGTT TGC-3′; and primer 2 (53 bp downstream of the *galK* coding sequence), 5′-AGTCCATCAGCGTGACTACCATC-3′. The same primers were used for replacement of the Δ*galK*::*cat* mutation in *E. coli* Nissle 1917 Str^r *envZ*$_{P41L}$ Δ*galK*::*cat* with the wild-type *galK* gene and for confirmation of the replacement by sequencing.

Replacing the *E. coli* MG1655 *envZ* gene with the *E. coli* Nissle 1917 *envZ* gene was done in two steps. First, to replace the *E. coli* MG1655 *envZ* gene with the cassette encoding kanamycin resistance (*kan*) and with *parE* under the control of the rhamnose promoter (*kan-rha$_P$-parE*) (3), the following primers were used on pKD267 DNA (uppercase letters, *E. coli* MG1655 DNA; lowercase letters, *kan-rha$_P$-parE* DNA): primer 1, 5′-GT ATCTTATAGAAAGCAAAACGGGAGGCACCTTCGCCTCCCGTTTA TTTACCCTCTTTTGTCtcccgctcagaagaactcgt-3′; and primer 2, 5′-GC TTCTCGCCACGAAGTTCATTTGCCCGTACGTTATTGCTCAT CGTCACCTTGCTGTTCGgatccaatcagcccttgag-3′. Second, to replace the *kan-rha$_P$-parE* cassette in *E. coli* MG1655 with the *E. coli* Nissle 1917 *envZ* gene, the following primers were used on *E. coli* Nissle 1917 DNA (uppercase letters, sequence common to *E. coli* MG1655 and *E. coli* Nissle 1917; lowercase letters, sequence found only in *E. coli* MG1655): primer 1, 5′-tatctatccagtatcttatagaaagcAAAACGGGAGGCACCTTCGCCT CCCGTTTATTTACCCTTCTTTTGTC-3′; and primer 2, 5′-TCTAAAG CATGAGGCGATTGCGCTTCTCGCCACGAAGT-3′.

Replacing the *E. coli* Nissle *envZ* gene with the *envZ*$_{P41L}$ gene was also done in two steps. First, to replace the *E. coli* Nissle 1917 *envZ* gene with the cassette encoding kanamycin resistance (*kan*) and *parE* under the control of the rhamnose promoter (*kan-rha$_P$-parE*), the following primers were used on pKD267 DNA (uppercase letters, *E. coli* Nissle 1917 DNA; lowercase letters, *kan-rha$_P$-parE* DNA): primer 1, 5′-TTCATTAT CAATCAATTGAAAACAATCTAAAAACGGGAGGCACCTTCACC TCCCGTTTTTTTACCCTTCTTTTGTCtcccgctcagaagaactcgtc-3′; and primer 2, 5′-GCTTCTCGCCACGAAGTTCATTTGCCCGTACGTTATT GCTCATCGTCACCTTGCTGTTCGgatccaatcagcccttgag-3′. Second, to replace the *kan-rha$_P$-parE* cassette in *E. coli* Nissle 1917 with the *envZ*$_{P41L}$ gene, the following primers were used on *E. coli* MG1655 *envZ*$_{P41L}$ DNA: primer 1 (uppercase letters, *E. coli* Nissle 1917 DNA sequence; lowercase letters, *envZ*$_{P41L}$ sequence), 5′-CTTTTGCAAGCGAATCCTT TCATTATCAATCAATTGAAAACAATCTAAAAACGGGAGGCACCT TCACCTCCCGTTTTTTtaccctcttttgtcgtgc-3′; and primer 2 (sequence common to both *E. coli* MG155 and *E. coli* Nissle 1917), 5′-TCGACGTG CAGATTTCGCGT-3′. The primers used for confirmation of the *envZ* replacements by sequencing were as follows: primer 1, 5′-GTAGCGGCT GGTCCGAA-3′; and primer 2, 5′-TAGCTGGTGACGAACGTGAG-3′.

Colicin V sensitivity. *E. coli* F-18 produces colicin V (31). *E. coli* MG1655 strains to be tested for sensitivity to colicin V were grown overnight in LB broth Lennox at 37°C with shaking in 125-ml tissue culture bottles. The next day, 10^6 CFU of each strain to be tested was added to 3 ml of Luria broth Lennox containing 7 g/liter Difco agar (soft agar), which was then poured onto a 20-ml Luria broth Lennox agar plate. After the soft agar on each plate solidified, *E. coli* F-18 colonies were transferred by toothpick to each plate and incubated for 18 h at 37°C, and zones of growth inhibition were measured.

FISH. Fluorescence *in situ* hybridization (FISH) was performed as described previously (3, 32). Briefly, the cecum of a mouse designated for FISH was cut into 1- to 2-cm pieces, suspended in Tissue-Tek OCT compound (Sakura, Torrance, CA), and snap-frozen in 2-methylbutane suspended in liquid nitrogen. Pieces were then stored at −80°C until ready for sectioning. For sectioning, an UltraPro 5000 cryostat (Vibratome, St. Louis, MO) was used to cut 10-μm sections, which were adhered to poly-L-lysine-treated slides for visualization. Slides were fixed in 4% paraformaldehyde for 1 h at room temperature, followed by a wash in phosphate-buffered saline for 10 min. Slides were allowed to air dry overnight prior to hybridization. Fluorescent probes were diluted in hybridization solution (0.1 M Tris-HCl buffer, pH 7.2, 0.9 M NaCl, and 0.1% SDS). The *E. coli* 23S rRNA-specific Cy-3 probe (5′-5Cy3-CAC CGT AGT GCC TCG TCA TCA-3′) (red) and the eubacterial 23S rRNA-specific FitC probe (5′-GCT GCC TCC CGT AGG AGT-36-FAM-3′) (green) were used at concentrations of 5 ng/μl and 25 ng/μl, respectively. Subsequently, 10 μl of diluted probes in hybridization solution was pipetted onto each slide. Each slide was then covered with a HybriSlip hybridization cover (Life Technologies, Carlsbad, CA) and allowed to incubate in the dark for 2 h at 50°C. The slides were then placed in a wash buffer (0.1 M Tris-HCl buffer, pH 7.2, and 0.9 M NaCl) and allowed to soak for 20 min at 50°C. Slides were then rinsed with distilled water and air dried overnight. Sections were viewed by confocal microscopy. Prior to viewing, sections on the poly-L-lysine-treated slides were treated with Vectashield mounting medium (Vector Laboratories, Burlingame, CA), an antibleaching agent which helps to prolong fluorescence for imaging, and covered with a coverslip.

Statistics. Means and standard deviations derived from the indicated numbers of samples in Tables 2 and 3 were compared by two-tailed Student's *t* test (*P* values). The colonization data on *E. coli* Nissle 1917 *envZ*$_{P41L}$ between days 3 and 15 in Fig. 5A were combined, as were those of *E. coli* Nissle 1917, and the means for the two strains were compared by two-tailed Student's *t* test (see Fig. 5A). The same analysis was applied to *E. coli* Nissle 1917 *envZ*$_{P41L}$ restored to the wild type with respect to *envZ*, as well as *E. coli* Nissle 1917 (see Fig. 5B). *P* values of >0.05 were interpreted as indicating no significant difference, and *P* values of <0.05 were interpreted as indicating a significant difference.

RESULTS

The *E. coli* Nissle 1917 *envZ* gene bestows no colonization advantage to *E. coli* MG1655. The *E. coli* Nissle 1917 EnvZ protein differs from that of *E. coli* MG1655 in two places: the V25A and M446T changes. Since *E. coli* Nissle 1917 is a probiotic strain considered to have enhanced colonization properties, it was possible that the *E. coli* Nissle 1917 *envZ* gene (called *envZ*$_{Nis}$ here) might confer increased colonization ability to *E. coli* MG1655. To test this possibility, the *E. coli* MG1655 *envZ* gene was replaced with *envZ*$_{Nis}$ to create *E. coli* MG1655 *envZ*$_{Nis}$, and the colonization ability of this strain was examined. *E. coli* MG1655 and *E. coli* MG1655 *envZ*$_{Nis}$ were found to be identical in colonization ability,

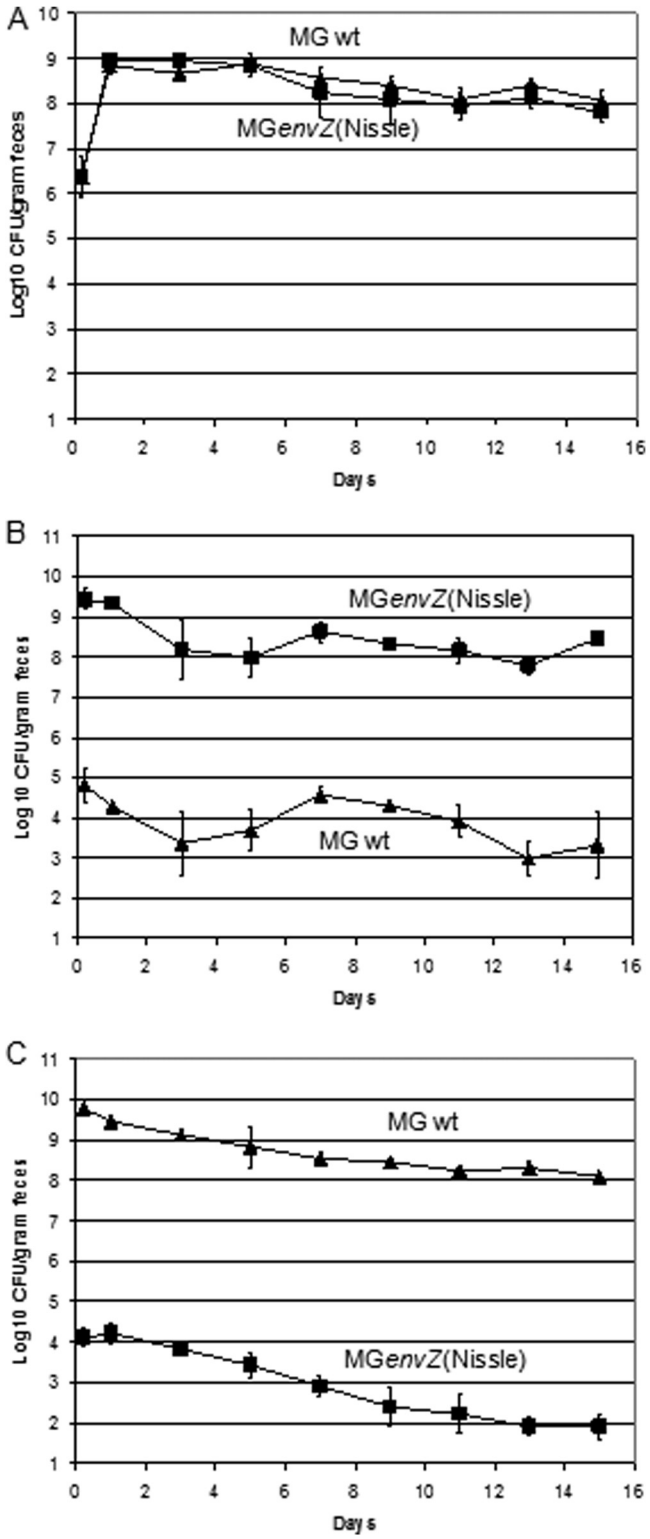

FIG 1 Colonization of the mouse intestine by *E. coli* MG1655 and *E. coli* MG1655 $envZ_{Nis}$. (A) Two sets of three mice were fed 10^5 CFU of *E. coli* MG1655 Str^r Nal^r $envZ_{Nis}$ (■) and 10^5 CFU of *E. coli* MG1655 Str^r (▲). (B) Two sets of three mice were fed 10^{10} CFU of *E. coli* MG1655 Str^r $envZ_{Nis}$ (■) and10^5 CFU of *E. coli* MG1655 Str^r Nal^r (▲). (C) Two sets of three mice were fed 10^{10} CFU of *E. coli* MG1655 Str^r (▲) and 10^5 CFU of *E. coli* MG1655 Str^r $envZ_{Nis}$ (■). 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 transferred by toothpick onto MacConkey agar plates containing streptomycin and nalidixic acid. Error bars represent standard errors of the $\log_{10}$ mean CFU per gram of feces.

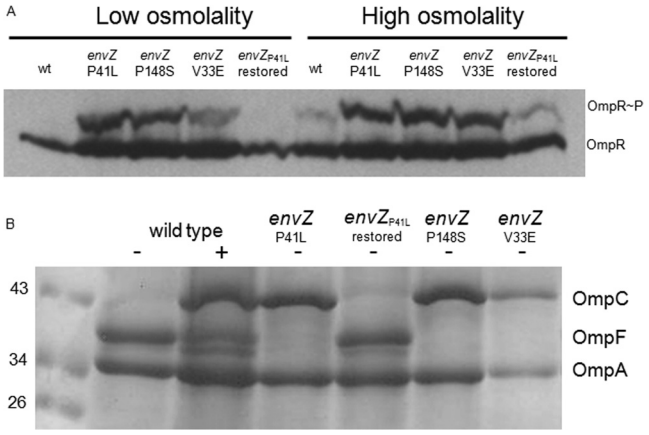

FIG 2 (A) OmpR∼P levels in *E. coli* MG1655 (wt), the MG1655 *envZ* missense mutants, and MG1655 $envZ_{P41L}$ restored to the wild type, grown in the absence (low osmolality) or presence (high osmolality) of 15% sucrose. (B) OmpC and OmpF in the outer membranes of the *E. coli* MG1655 strains grown in the absence (−) or presence (+) of 15% sucrose. Sizes of prestained molecular standards are indicated on the left.

i.e., when 10^5 CFU of each strain was fed to mice, the strains cocolonized equally well (Fig. 1A); when 10^{10} CFU of *E. coli* MG1655 $envZ_{Nis}$ and 10^5 CFU of *E. coli* MG1655 were fed to mice, the 10^5 ratio of the input CFU values of the 2 strains in feces was maintained throughout the 15-day duration of the experiment (Fig. 1B); and when 10^{10} CFU of *E. coli* MG1655 and 10^5 CFU of *E. coli* MG1655 $envZ_{Nis}$ were fed to mice, the 10^5 ratio of the input CFU values of the 2 strains was maintained in feces throughout the 15-day duration of the experiment (Fig. 1C). Therefore, despite the two amino acid differences between the wild-type *envZ* genes of *E. coli* Nissle 1917 and *E. coli* MG1655, the *E. coli* Nissle 1917 *envZ* gene conferred no colonization advantage to *E. coli* MG1655.

Effects of *envZ* missense mutations on OmpF, OmpC, and phosphorylated OmpR (OmpR∼P) in *E. coli* MG1655 and *E. coli* Nissle 1917. The phenotypic effects of the P41L missense mutation in the *E. coli* MG1655 *envZ* gene ($envZ_{P41L}$) reported previously were consistent with it containing a higher level of OmpR∼P than that in *E. coli* MG1655 (3). In order to test this possibility, we examined OmpR∼P levels in these strains. At low osmolality, the wild-type *E. coli* MG1655 strain had no detectable OmpR∼P, and at high osmolality, the OmpR∼P level in the wild-type strain was barely detectable (Fig. 2A). In contrast, *E. coli* MG1655 $envZ_{P41L}$ exhibited an extremely high level of OmpR∼P at low osmolality that did not appear to increase further at high osmolality (Fig. 2A). P148S and V33E mutants behaved similarly (Fig. 2A).

The elevated levels of OmpR∼P observed in the *E. coli* MG1655 *envZ* mutant strains even at low osmolality (Fig. 2A) suggested that OmpC levels should also be elevated (10). We prepared outer membranes and examined the porin content of cells grown at low and high osmolalities (Fig. 2B). As expected (10), in the wild-type strain, OmpF was the major porin at low osmolality

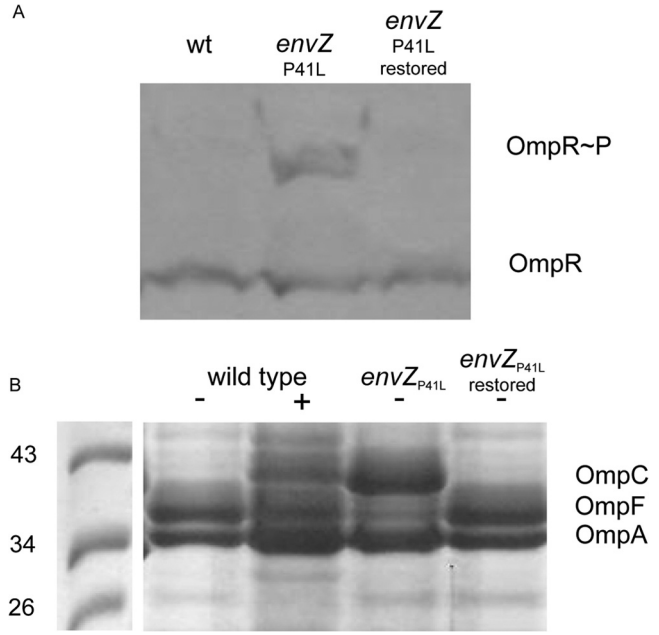

FIG 3 (A) OmpR~P levels in *E. coli* Nissle 1917 (wt), Nissle 1917 $envZ_{P41L}$, and Nissle 1917 $envZ_{P41L}$ restored to the wild type. Bacteria were grown in glucose M9 minimal medium modified by addition of 150 mM NaCl to approximate the sodium chloride concentration in intestinal fluids. (B) OmpC and OmpF in the outer membranes of the *E. coli* Nissle 1917 strains grown in the absence (−; low osmolality) or presence (+; high osmolality) of 15% sucrose. Sizes of prestained molecular standards are indicated on the left.

(Fig. 2B), and OmpC was the major porin at high osmolality (Fig. 2B). In the P41L background, OmpC was present at low osmolality, and OmpF was completely absent (Fig. 2B). OmpF expression was normal in the P41L strain restored to the wild type (Fig. 2B). The P148S and V33E mutants behaved similarly to the P41L strain (Fig. 2B). Thus, the *E. coli* MG1655 *envZ* missense mutants express OmpC at low osmolality and repress OmpF, consistent with the elevated OmpR~P levels shown in Fig. 2A (10).

We were next interested in determining whether $envZ_{P41L}$ would have the same effect on *E. coli* Nissle 1917 and therefore replaced the *E. coli* Nissle 1917 *envZ* gene with $envZ_{P41L}$ to create *E. coli* Nissle 1917 $envZ_{P41L}$. Indeed, $envZ_{P41L}$ had the same effect in *E. coli* Nissle 1917 $envZ_{P41L}$ as in *E. coli* MG1655 $envZ_{P41L}$, i.e., under conditions of low osmolality, *E. coli* Nissle 1917 $envZ_{P41L}$ contained a much higher level of OmpR~P than *E. coli* Nissle 1917 (Fig. 3A) and, as a result, a higher level of OmpC and a far lower level of OmpF in its outer membrane (Fig. 3B).

Effects of $envZ_{P41L}$ on *E. coli* Nissle 1917 motility, sensitivity to bile salts and colicin V, and growth rate on galactose. The mutant $envZ_{P41L}$ gene in *E. coli* MG1655 $envZ_{P41L}$ causes it to be less motile than *E. coli* MG1655, because the higher level of OmpR~P negatively regulates production of $FlhD_4C_2$, the master regulator of flagellum biosynthesis (12). *E. coli* MG1655 $envZ_{P41L}$ is more resistant to bile salts than *E. coli* MG1655 and *E. coli* MG1655 Δ*flhD*, because higher levels of OmpR~P negatively regulate production of OmpF, the porin that transports bile salts (33) (Fig. 2B). A higher level of OmpR~P also causes *E. coli* MG1655 $envZ_{P41L}$ to be more resistant to colicin V than *E. coli* MG1655 and *E. coli* MG1655 Δ*flhD*, because OmpR~P positively regulates the transcription of *omrA* and *omrB*, which encode two small RNAs

TABLE 2 Motility and colicin V sensitivity of *E. coli* Nissle 1917 strains

Strain	Colicin V sensitivity (mean zone of inhibition [mm] ± SD) ($n = 12$)[a]	Mean motility spread (mm) ± SD ($n = 6$)[a]
E. coli Nissle 1917	2.06 ± 0.47	1.08 ± 0.31
E. coli Nissle 1917 $envZ_{P41L}$	0.44 ± 0.37*	0.60 ± 0.20***
E. coli Nissle 1917 restored to wild type with respect to EnvZ	2.41 ± 0.24**	1.02 ± 0.15**

[a] Spread of *E. coli* Nissle 1917 strains on Luria motility agar containing 200 mM NaCl after 6 h at 37°C or zones of inhibition of growth of the strains caused by colicin V produced by *E. coli* F-18 overnight at 37°C, as described in Materials and Methods. Student's *t* test *P* values were calculated for each strain relative to *E. coli* Nissle 1917 in the same experiment. *, $P < 0.00001$; **, $P > 0.10$; ***, $P < 0.01$.

that negatively regulate the expression of a number of outer membrane proteins, including CirA, the receptor for colicin V (13). Finally, for as yet unknown reasons, the $envZ_{P41L}$ gene causes *E. coli* MG1655 $envZ_{P41L}$ to grow 30% faster on galactose as the sole carbon and energy source than *E. coli* MG1655 and *E. coli* MG1655 Δ*flhD* (3). We were interested in determining whether $envZ_{P41L}$ would have the same effect on *E. coli* Nissle 1917, and therefore *E. coli* Nissle 1917 $envZ_{P41L}$ and *E. coli* Nissle 1917 were tested for motility, resistance to bile salts, resistance to colicin V, and growth rate on galactose. Indeed, *E. coli* Nissle 1917 $envZ_{P41L}$ was less motile (Table 2), more resistant to colicin V (Table 2), and more resistant to bile salts than *E. coli* Nissle 1917 (Fig. 4). However, *E. coli* Nissle 1917 $envZ_{P41L}$ grew about 15% slower than *E. coli* Nissle 1917 on galactose (Table 3). Still, three of the four phenotypic characteristics ascribed to the $envZ_{P41L}$ gene were transferred to *E. coli* Nissle 1917. The hypothesis that the phenotypic changes in *E. coli* Nissle 1917 $envZ_{P41L}$ were due to the $envZ_{P41L}$ gene was sup-

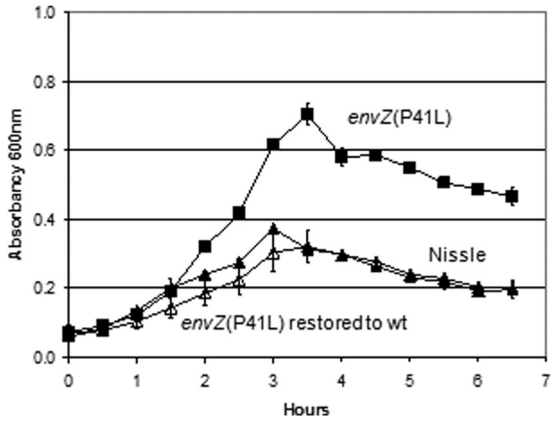

FIG 4 Growth of *E. coli* Nissle 1917 strains in the presence of 5% bile salts. *E. coli* Nissle 1917 Str^r (▲), *E. coli* Nissle 1917 Str^r $envZ_{P41L}$ (■), and *E. coli* Nissle 1917 Str^r $envZ_{P41L}$ restored to the wild type with respect to *envZ* (△) were grown in Luria broth Lennox containing 5% (wt/vol) bile salts. Incubation was done at 37°C with aeration. The means and standard deviations of the A_{600} values at the indicated times are presented for three independent cultures of each strain. In Student's *t* tests on each time point between 3.5 h and 6.5 h for *E. coli* Nissle 1917 Str^r $envZ_{P41L}$ versus *E. coli* Nissle 1917 Str^r and *E. coli* Nissle 1917 Str^r $envZ_{P41L}$ versus *E. coli* Nissle 1917 Str^r $envZ_{P41L}$ restored to the wild type with respect to *envZ*, $P < 3 \times 10^{-4}$. In Student's *t* tests on each time point between 3.5 h and 6.5 h for *E. coli* Nissle 1917 Str^r versus *E. coli* Nissle 1917 Str^r $envZ_{P41L}$ restored to the wild type with respect to *envZ*, $P > 0.80$.

TABLE 3 Generation times of *E. coli* Nissle 1917 and *E. coli* Nissle 1917 $envZ_{P41L}$ on various sugars[a]

Carbon source[b]	Mean generation time (min) ± SD ($n = 3$)	
	E. coli Nissle 1917	*E. coli* Nissle 1917 $envZ_{P41L}$[c]
Arabinose	66 ± 1	64 ± 1*
Fructose	96 ± 4	99 ± 1**
Fucose	83 ± 1	79 ± 4**
Galactose	69 ± 1	81 ± 4***
Gluconate	61 ± 2	67 ± 1***
Glucose	89 ± 2	92 ± 2**
Maltose	85 ± 3	120 ± 2****
Mannose	129 ± 7	164 ± 7†
N-Acetylglucosamine	84 ± 1	83 ± 1**
Ribose	104 ± 7	108 ± 8**
Cecal mucus	52 ± 3	100 ± 3****

[a] Generation times were calculated from semilogarithmic plots.
[b] Carbon source utilization was determined in M9 minimal medium (0.4% [wt/vol]) in 125-ml tissue culture flasks with shaking after inocula were grown on M9 glycerol (0.4% [vol/vol]) and then overnight on the specified carbon source.
[c] Each *P* value refers to a comparison between the generation times of *E. coli* Nissle 1917 and *E. coli* Nissle 1917 $envZ_{P41L}$ growing on a specific sugar: *, $P > 0.05$; **, $P > 0.10$; ***, $P < 0.05$; ****, $P < 0.001$; †, $P = 0.0036$.

ported by the fact that when the $envZ_{P41L}$ gene in *E. coli* Nissle 1917 $envZ_{P41L}$ was replaced with the wild-type *E. coli* Nissle 1917 *envZ* gene, the restored *E. coli* Nissle 1917 strain was more motile (Table 2), more sensitive to colicin V (Table 2), and more sensitive to bile salts (Fig. 4), and grew faster on galactose, than *E. coli* Nissle 1917 $envZ_{P41L}$ (not shown).

Growth of *E. coli* Nissle 1917 $envZ_{P41L}$ on a variety of sugars present in mucus *in vitro*. We previously reported that *E. coli* MG1655 $envZ_{P41L}$ grew slower than *E. coli* MG1655 Δ*flhD* on arabinose, fucose, glucose, maltose, and mannose, at the same rate as *E. coli* MG1655 Δ*flhD* on fructose, gluconate, and ribose, and faster than *E. coli* MG1655 Δ*flhD* on galactose and *N*-acetylglucosamine (3). In contrast, *E. coli* Nissle 1917 $envZ_{P41L}$ did not grow faster than *E. coli* Nissle 1917 on any of the sugars tested and grew slower than *E. coli* Nissle 1917 on a number of sugars, i.e., with doubling times about 15% greater than that of *E. coli* Nissle 1917 on galactose, 10% greater on gluconate, 40% greater on maltose, and 30% greater on mannose (Table 3). Apparently, the effect of $envZ_{P41L}$ on sugar metabolism is strain specific. Of the sugars tested, *E. coli* Nissle 1917 is known to use arabinose, fucose, galactose, gluconate, and mannose to colonize the streptomycin-treated mouse large intestine (34).

Growth of *E. coli* Nissle 1917 $envZ_{P41L}$ in cecal mucus *in vitro*. *E. coli* colonizes the intestine by growing in intestinal mucus (35, 36), which contains at least 13 different sugars that can be used by *E. coli* as sole sources of carbon. When *E. coli* MG1655 and *E. coli* Nissle 1917 colonize the streptomycin-treated mouse intestine, they simultaneously metabolize a number of these sugars (37, 38). Colonization requires bacteria to penetrate the mucus layer, compete for nutrients with the microbiota, and divide at a rate that is at least equal to the washout rate caused by sloughing of the mucus layer into feces (35, 36). We previously reported that despite being as good an intestinal colonizer, *E. coli* MG1655 $envZ_{P41L}$ grew 15% slower than *E. coli* MG1655 Δ*flhD* in mouse cecal mucus *in vitro* (3). Even more dramatically, the doubling time of *E. coli* Nissle 1917 $envZ_{P41L}$ was twice that of *E. coli* Nissle 1917 in mouse cecal mucus in standing cultures *in vitro* (Table 3), i.e., it grew far slower than *E. coli* Nissle 1917 ($P < 0.001$).

***E. coli* Nissle 1917 $envZ_{P41L}$ is a better mouse intestinal colonizer than *E. coli* Nissle 1917.** Since *E. coli* Nissle 1917 $envZ_{P41L}$ grew slower than *E. coli* Nissle on a number of sugars present in cecal mucus and in cecal mucus itself *in vitro*, it would be expected that if colonizing ability was based solely on the ability of a strain to utilize sugars present in mucus for growth, *E. coli* Nissle $envZ_{P41L}$ would be the poorer colonizer. Yet *E. coli* Nissle 1917 $envZ_{P41L}$ was about a 10-fold better colonizer than wild-type *E. coli* Nissle 1917 when 10^5 CFU of each strain was fed to mice ($P = 3.49 \times 10^{-7}$) (Fig. 5A). The hypothesis that the better colonizing ability of *E. coli* Nissle 1917 $envZ_{P41L}$ was due to the $envZ_{P41L}$ gene was supported by the fact that when the $envZ_{P41L}$ gene in *E. coli* Nissle 1917 $envZ_{P41L}$ was replaced with the wild-type *E. coli* Nissle 1917 *envZ* gene, the restored *E. coli* Nissle 1917 and wild-type *E. coli* Nissle 1917 strains had identical colonizing abilities ($P = 0.16$) (Fig. 5B).

We found previously that despite *E. coli* MG1655 $envZ_{P41L}$ growing about 15% slower than *E. coli* MG1655 Δ*flhD* in mouse cecal mucus *in vitro*, large numbers of *E. coli* MG1655 $envZ_{P41L}$ (10^{10} CFU) prevented small numbers of *E. coli* MG1655 Δ*flhD* (10^5 CFU) from growing to larger numbers in the mouse intestine, whereas small numbers of *E. coli* MG1655 $envZ_{P41L}$ were able to grow to larger numbers in the presence of large numbers of *E. coli* MG1655 Δ*flhD* (3). Similarly, despite *E. coli* Nissle 1917 $envZ_{P41L}$ growing far slower than *E. coli* Nissle 1917 in mouse cecal mucus *in vitro* and on a number of sugars present in mucus, large numbers of *E. coli* Nissle 1917 $envZ_{P41L}$ prevented small numbers of *E. coli* Nissle 1917 from growing to larger numbers in the mouse intestine (Fig. 5C), suggesting that *E. coli* Nissle 1917 $envZ_{P41L}$ resides in and competes effectively in all intestinal niches occupied by *E. coli* Nissle 1917. In contrast, despite its growing 50% slower than *E. coli* Nissle 1917 in mouse cecal mucus *in vitro*, small numbers of *E. coli* Nissle 1917 $envZ_{P41L}$ grew to larger numbers in the presence of large numbers of *E. coli* Nissle 1917 (Fig. 5D), suggesting that *E. coli* Nissle 1917 $envZ_{P41L}$ resides in at least one intestinal niche that *E. coli* Nissle 1917 fails to colonize or colonizes poorly. Moreover, the growth of *E. coli* Nissle 1917 $envZ_{P41L}$ from small to large numbers in the presence of large numbers of *E. coli* Nissle 1917 was not restricted to the mucus layer of any one section of the intestine, i.e., it did so in ileal mucus, cecal mucus, and colonic mucus *in vivo* (Table 4).

***E. coli* Nissle 1917 $envZ_{P41L}$ uses galactose to grow from small to large numbers *in vivo* in the presence of large numbers of *E. coli* Nissle 1917.** Previously we showed that *E. coli* MG1655 $envZ_{P41L}$, which grows 30% faster than *E. coli* MG1655 on galactose, uses galactose to grow from small to large numbers in the mouse intestine in the presence of large numbers of *E. coli* MG1655 Δ*flhD* (3). Here we show that *E. coli* Nissle 1917 $envZ_{P41L}$ also uses galactose to grow from small to large numbers in the mouse intestine in the presence of large numbers of *E. coli* Nissle 1917, despite growing 15% slower than *E. coli* Nissle 1917 *in vitro* on galactose (Table 3). That is, small numbers of *E. coli* Nissle 1917 $envZ_{P41L}$ Δ*galK* failed to grow to larger numbers in the presence of large numbers of *E. coli* Nissle 1917 (Fig. 6A), whereas small numbers of *E. coli* Nissle 1917 Δ*galK* restored to the wild type with respect to galactose utilization did so (Fig. 6B). These data suggest that *E. coli* Nissle 1917 $envZ_{P41L}$ uses galactose to colonize an in-

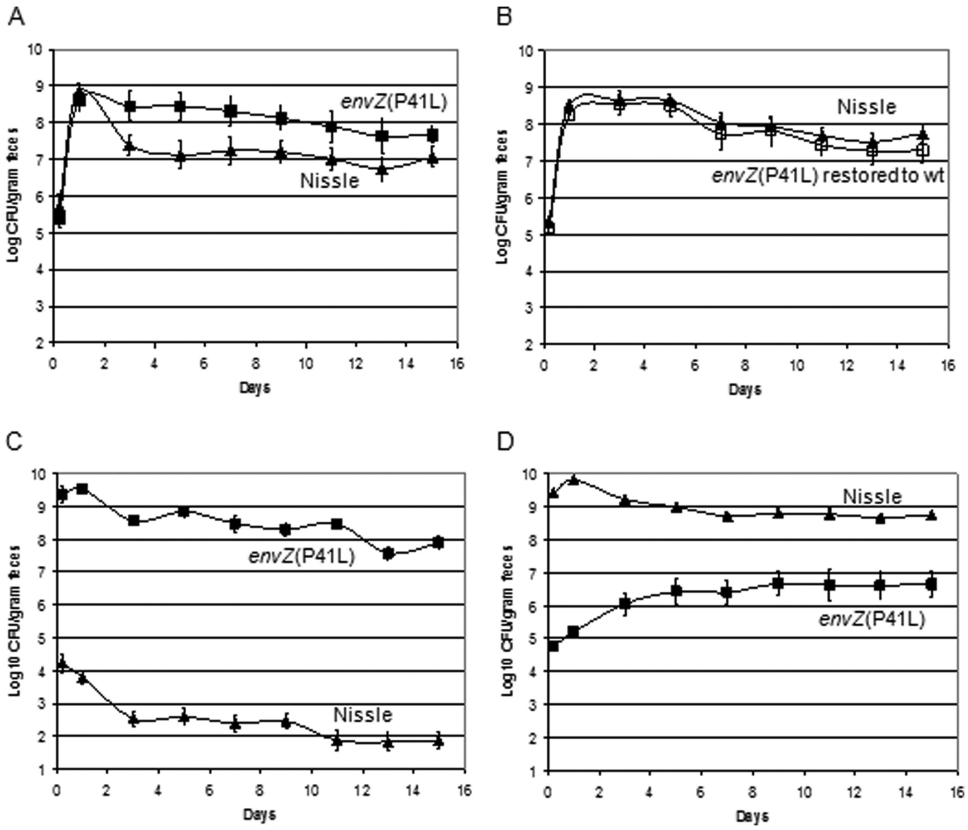

FIG 5 Colonization of the mouse intestine by *E. coli* Nissle 1917, *E. coli* Nissle 1917 $envZ_{P41L}$, and *E. coli* Nissle $envZ_{P41L}$ restored to the wild type. (A) Three sets of three mice were fed 10^5 CFU of *E. coli* Nissle 1917 Str^r Nal^r (▲) and 10^5 CFU of *E. coli* Nissle 1917 Str^r $envZ_{P41L}$ (■). (B) Two sets of three mice were fed 10^5 CFU of *E. coli* Nissle 1917 Str^r Nal^r (▲) and 10^5 CFU of *E. coli* Nissle Str^r $envZ_{P41L}$ restored to the wild type (□). (C) Two sets of three mice were fed 10^5 CFU of *E. coli* Nissle 1917 Str^r Nal^r (▲) and 10^{10} CFU of *E. coli* Nissle 1917 Str^r $envZ_{P41L}$ (■). (D) Six sets of three mice were fed 10^5 CFU of *E. coli* Nissle 1917 Str^r (▲) and 10^5 CFU of *E. coli* Nissle 1917 Str^r Nal^r $envZ_{P41L}$ (■). 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 transferred by toothpick onto MacConkey agar plates containing streptomycin and nalidixic acid. Error bars represent standard errors of the $\log_{10}$ mean CFU per gram of feces.

testinal niche that *E. coli* Nissle 1917 fails to colonize or colonizes poorly.

***E. coli* Nissle 1917 resides closely associated with other members of the microbiota in the mouse intestine.** Since commensal *E. coli* strains colonize the streptomycin-treated mouse intestine by growing in the intestinal mucus layer of the large intestine (35, 36), and since it has been shown that *E. coli* MG1655 resides in mixed biofilms in the mouse cecal mucus layer (3), we examined whether *E. coli* Nissle 1917 also resides in mixed biofilms in mucus *in vivo*. Indeed, as shown in Fig. 7A, *E. coli* Nissle 1917 appears to reside in the cecal mucus layer, closely associated with other members of the microbiota. As expected, *E. coli* was not found in the cecal mucus of mice not fed *E. coli* Nissle 1917 (Fig. 7B).

TABLE 4 *E. coli* Nissle 1917 and *E. coli* Nissle 1917 $envZ_{P41L}$ populations in mouse ileal, cecal, and colonic mucus at 15 days postfeeding

Location	Log_{10} CFU[a]		Log_{10} ratio of Nissle 1917 to Nissle 1917 $envZ_{P41L}$
	Nissle 1917	Nissle 1917 $envZ_{P41L}$	
Ileal mucus	5.01 ± 0.71	3.18 ± 0.57	1.83
Cecal mucus	7.51 ± 0.24	6.30 ± 0.15	1.21
Colonic mucus	6.96 ± 0.33	5.71 ± 0.21	1.25
Feces	8.88 ± 0.07	7.82 ± 0.38	1.06

[a] Mice were fed 10^{10} CFU of *E. coli* Nissle 1917 and 10^5 CFU of *E. coli* Nissle 1917 $envZ_{P41L}$ as described in the legend to Fig. 4D, and the animals were sacrificed on day 15. The values are means ± standard errors of the means for three mice. Mucus preparations were isolated on day 15 postfeeding. The CFU values for the mucus preparations were corrected for the entire volume of each mucus preparation. The fecal values are numbers of CFU/g of feces 15 days after feeding.

Protection against *E. coli* EDL933 colonization by *E. coli* Nissle 1917 and *E. coli* Nissle 1917 $envZ_{P41L}$. Different resident commensal *E. coli* strains vary by as much as 10,000-fold with respect to the level of colonization of the prototypic enterohemorrhagic *E. coli* strain EDL933 that they allow in the streptomycin-treated mouse intestine (38). Among several commensal strains previously tested, *E. coli* Nissle 1917 was one of the best at limiting *E. coli* EDL933 colonization, i.e., in mice precolonized with *E. coli* Nissle 1917 and then fed 10^5 CFU of *E. coli* EDL933 10 days later, *E. coli* EDL933 dropped from 10^5 CFU/g of feces and colonized at a level between 10^3 and 10^4 CFU/g of feces (39). While this level of colonization is low, as long as the relatively few *E. coli* EDL933 cells are healthy and able to persist in the intestine at that low level, they might also be able to initiate the pathogenic process in humans by damaging the mucosa, resulting in subsequent disease. It was therefore of interest to determine whether *E. coli* Nissle 1917

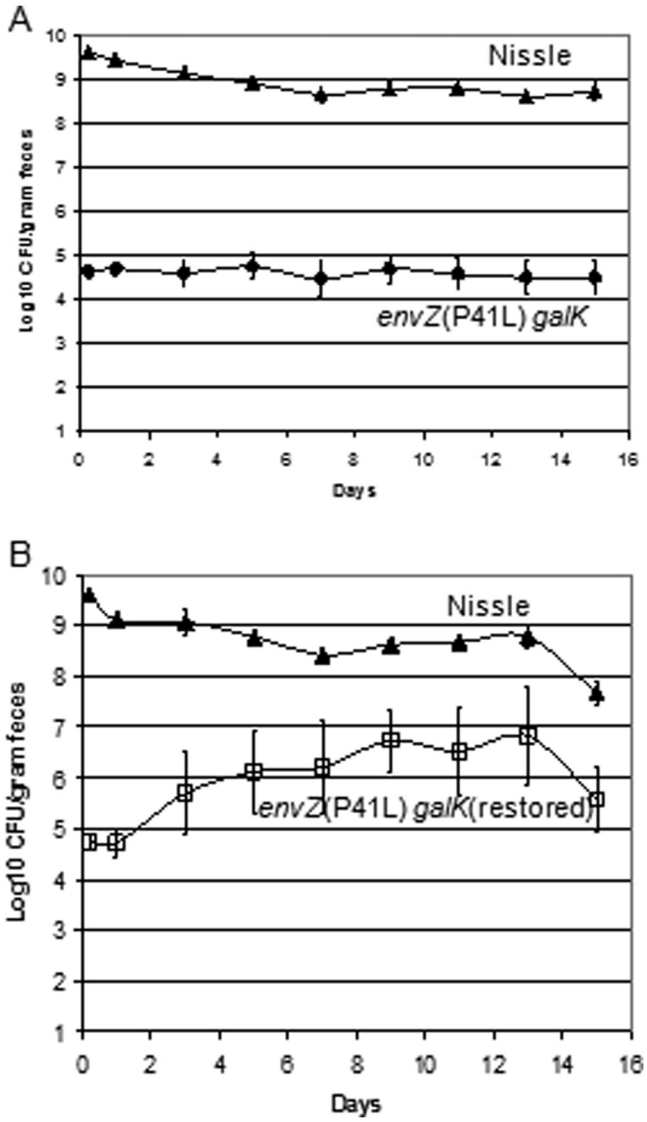

FIG 6 Colonization of the mouse intestine by *E. coli* Nissle 1917 $envZ_{P41L}$, *E. coli* Nissle 1917 $envZ_{P41L}$ Δ*galK*, and *E. coli* Nissle 1917 $envZ_{P41L}$ Δ*galK* restored to the wild type. (A) Three sets of three mice were fed 10^{10} CFU of *E. coli* Nissle 1917 Str^r (▲) and 10^5 CFU of *E. coli* Nissle 1917 Str^r Δ*galK*::*cat* (●). (B) Two sets of three mice were fed 10^{10} CFU of *E. coli* Nissle 1917 Str^r (▲) and 10^5 CFU of *E. coli* Nissle 1917 Str^r Nal^r Δ*galK*::*cat* restored to the wild type (□). At the indicated times, fecal samples were homogenized, diluted, and plated as described in Materials and Methods. Error bars represent standard errors of the $\log_{10}$ mean CFU per gram of feces.

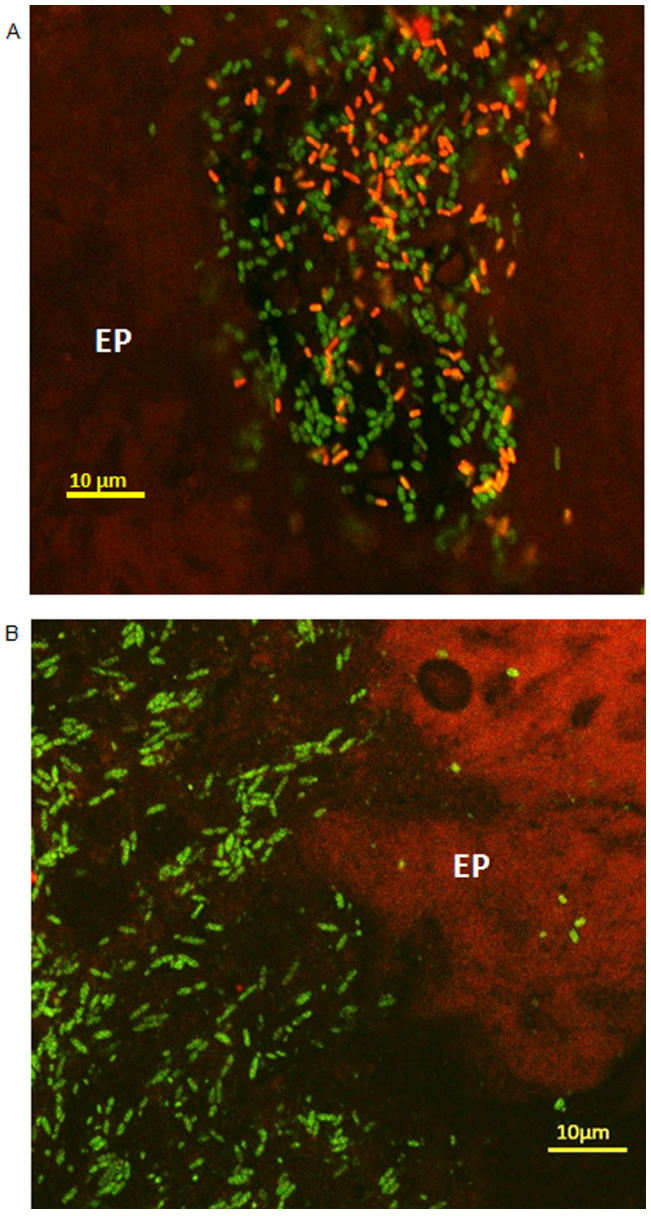

FIG 7 *In situ* hybridization with fluorescence-labeled oligonucleotide probes. Ten days after feeding mice *E. coli* Nissle 1917 Str^r (A) and 5 days after sham feeding mice without *E. coli* (B), cecal mucosal sections were hybridized with an *E. coli*-specific oligonucleotide probe (red) and a eubacterium-specific oligonucleotide probe (green). EP, epithelium. *E. coli* cells appear red, while all other bacteria appear green.

$envZ_{P41L}$, the better colonizer, was better than *E. coli* Nissle 1917 at limiting *E. coli* EDL933 colonization. It was not. In fact, although not statistically significant, it appeared that *E. coli* Nissle 1917 $envZ_{P41L}$ was not quite as effective as *E. coli* Nissle 1917 in limiting *E. coli* EDL933 growth in the intestine (compare Fig. 8A and B).

DISCUSSION

When a bacterial species persists indefinitely in stable numbers in the intestine of an animal, without repeated introduction, the animal is, by definition, colonized by the bacterium. Commensal *E. 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 (40). 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 (41). Such experiments require an animal model with open niches for *E. coli* to colonize in relatively large numbers, but the animal model intestine should have a dense and diverse anaerobic community that matches as closely as possible the native microbiota of the conventional animal in order to examine interactions among members of the microbiota. The streptomycin-treated mouse model is used routinely for this purpose because it fulfills these criteria (35, 36).

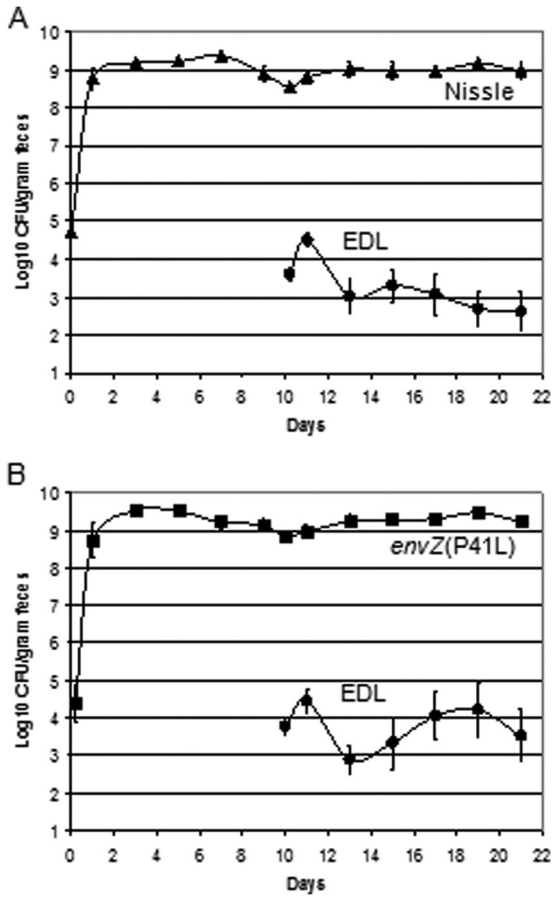

FIG 8 *E. coli* EDL933 colonization of the mouse intestine precolonized with either *E. coli* Nissle 1917 or *E. coli* Nissle 1917 $envZ_{P41L}$. (A) Three sets of three mice were fed 10^5 CFU of *E. coli* Nissle 1917 Str^r Nal^r (▲) and, 10 days later, were fed 10^5 CFU of *E. coli* EDL933 Str^r Rif^r (●). (B) Three sets of three mice were fed 10^5 CFU of *E. coli* Nissle 1917 Str^r Nal^r $envZ_{P41L}$ (■) and, 10 days later, were fed 10^5 CFU of *E. coli* EDL933 Str^r Rif^r (●). Data were collected and plotted as described in the legend to Fig. 4.

This study was undertaken to determine whether the $envZ_{P41L}$ gene, selected in *E. coli* MG1655 by the streptomycin-treated mouse intestine (3), would make the probiotic strain *E. coli* Nissle 1917 a better mouse intestine colonizer and better able to resist colonization by *E. coli* EDL933, an enterohemorrhagic strain. The data presented here clearly show that despite being a 10-fold better colonizer than *E. coli* Nissle 1917 and despite appearing to colonize an intestinal niche not colonized by *E. coli* Nissle 1917, *E. coli* Nissle 1917 $envZ_{P41L}$ is not better than *E. coli* Nissle 1917 at limiting *E. coli* EDL933 growth in the intestine (Fig. 8).

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 (42). 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 (42). As a corollary to the hypothesis, when two strains each use one or more major nutrients better than the other strain, small numbers of each strain will grow up in the intestine to larger numbers in the presence of large numbers of the other strain, as observed in mouse colonization studies using a number of human fecal *E. coli* strains isolated from different humans (39).

Despite general acceptance of the nutrient-niche hypothesis, recent data suggest that it is not entirely correct for *E. coli* (3). We recently reported that *E. coli* MG1655 $envZ_{P41L}$, selected by the mouse intestine as a better colonizer than *E. coli* MG1655, colonizes as well as *E. coli* MG1655 Δ*flhD*, also a better colonizer selected by the mouse intestine, but grows less well than *E. coli* MG1655 Δ*flhD* in bacterium-free mouse cecal mucus *in vitro* and on several sugars present in cecal mucus (3). These results were in contrast to what would be expected if the nutrient-niche hypothesis were entirely correct as discussed above. Furthermore, *E. coli* requires mono- and disaccharides for growth in the intestine (35, 36), but mono- and disaccharides present in the diet are unlikely to be available to *E. coli* because they are absorbed in the small intestine. The anaerobes in the intestine secrete polysaccharide hydrolases (43), but *E. coli* cannot do so (44, 45). Indeed, *Salmonella enterica* serovar Typhimurium, which is in the same family as *E. coli* and has a very similar metabolism, catabolizes fucose and sialic acid liberated from mucosal polysaccharides by *Bacteroides thetaiotaomicron* (46). It therefore is clear that *E. coli* obtains the bulk of the mono- and disaccharides it requires for growth in the intestine from degradation of dietary fiber-derived and mucin-derived polysaccharides by anaerobes. In view of this information and since we found that *E. coli* MG1655 resides in mixed biofilms in the mucus layer of the intestine but does not appear to adhere to the intestinal wall, to explain our findings we suggested the "restaurant hypothesis" as a modification to the nutrient-niche hypothesis (3). According to the restaurant hypothesis, the mono- and disaccharides that *E. coli* requires for growth are not derived from the diet and are not perfectly mixed in the intestine but are served locally to *E. coli* by the anaerobes within the mixed biofilms that *E. coli* inhabits. The restaurant hypothesis also raises the possibility that *E. coli* strains with different surfaces (e.g., fimbriae, capsule, O, K, and H antigens, outer membrane protein profiles, etc.) might reside in mixed 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 despite using the same sugars when cultured *in vitro* (38, 39).

The data presented here are also consistent with the restaurant hypothesis. *E. coli* Nissle 1917 $envZ_{P41L}$ did not grow faster than *E. coli* Nissle 1917 on any of the sugars present in mucus that were tested, grew 10% to 30% slower than *E. coli* Nissle 1917 on several of the sugars tested, and grew 50% slower in cecal mucus *in vitro* in which all nutrients were perfectly mixed, i.e., in UV-sterilized cecal mucus that had been centrifuged free of bacteria (Table 3). Yet in contrast to what would be expected solely on the basis of competition for nutrients from a mixture that is equally available to all, *E. coli* Nissle 1917 $envZ_{P41L}$ colonized the streptomycin-treated mouse intestine 10-fold better in competition with *E. coli* Nissle 1917 (Fig. 5A) and grew from small to large numbers in the mouse intestine in the presence of large numbers of *E. coli* Nissle 1917 (Fig. 5D).

Although these data cannot be explained solely by the nutrient-

niche hypothesis, since *E. coli* Nissle 1917 strains appear to reside in mixed biofilms in cecal mucus *in vivo* (Fig. 7A), the data can be explained if the lower growth rate of *E. coli* Nissle 1917 $envZ_{P41L}$ than that of *E. coli* Nissle 1917 in those mixed biofilms is more than compensated for by its ability to occupy most of the binding sites in those biofilms, i.e., if *E. coli* Nissle 1917 $envZ_{P41L}$ has a greater affinity for biofilm binding sites than *E. coli* Nissle 1917. If so, then it is not surprising that when *E. coli* Nissle 1917 $envZ_{P41L}$ and wild-type *E. coli* Nissle 1917 are fed to mice in equal numbers, *E. coli* Nissle 1917 $envZ_{P41L}$ is the better colonizer (Fig. 5A). Furthermore, it is not surprising that when mice are fed *E. coli* Nissle 1917 $envZ_{P41L}$ and wild-type *E. coli* Nissle 1917 at a ratio of 10^5:1, the ratio reaches 10^6:1 with time (Fig. 5C). However, it is surprising that when mice are fed wild-type *E. coli* Nissle 1917 and *E. coli* Nissle 1917 $envZ_{P41L}$ at a ratio of 10^5:1, *E. coli* Nissle 1917 $envZ_{P41L}$ uses galactose to grow to larger numbers (Fig. 5D and 6A), despite wild-type *E. coli* Nissle 1917 being able to grow faster than *E. coli* Nissle 1917 $envZ_{P41L}$ on galactose *in vitro* (Table 3). This can be explained if *E. coli* Nissle 1917 $envZ_{P41L}$ uses galactose to grow in a second niche that wild-type *E. coli* Nissle 1917 cannot colonize. That second niche could be another mixed biofilm consisting of a different group of anaerobes in which *E. coli* Nissle 1917 $envZ_{P41L}$ has a higher affinity for binding sites than *E. coli* Nissle 1917 and thereby prevents it from occupying that niche. Alternatively, that second niche could be another mixed biofilm that contains an anaerobe that makes a bacteriocin that inhibits wild-type *E. coli* Nissle 1917 growth more than it inhibits *E. coli* Nissle 1917 $envZ_{P41L}$ growth, much in the same way that *E. coli* Nissle 1917 is more sensitive than wild-type *E. coli* Nissle 1917 to colicin V (Table 2). To reiterate, we emphasize that the $envZ_{P41L}$ mutation, when transferred from *E. coli* MG1655 $envZ_{P41L}$ into *E. coli* Nissle 1917, directs *E. coli* Nissle 1917 $envZ_{P41L}$ to occupy a distinct niche in which it is served galactose (Fig. 5D), just as it does in *E. coli* MG1655 $envZ_{P41L}$ (3). Moreover, occupation of the galactose niche gives *E. coli* Nissle 1917 $envZ_{P41L}$ a colonization advantage, despite its growing more slowly on galactose than the wild type *in vitro*. We consider this to be strong evidence for the restaurant hypothesis.

In support of the view that surface differences between *E. coli* Nissle 1917 $envZ_{P41L}$ and *E. coli* Nissle 1917 could result in different binding affinities for mixed biofilms, when we replaced the $envZ_{Nis}$ gene in *E. coli* Nissle 1917 with $envZ_{P41L}$, three phenotypic changes observed in *E. coli* MG1655 $envZ_{P41L}$, caused by outer membrane changes resulting from increased OmpR~P, were transferred to *E. coli* Nissle 1917 $envZ_{P41L}$. These surface changes, i.e., decreased OmpF and increased OmpC (Fig. 3), decreased CirA causing increased resistance to colicin V (3, 13) (Table 2), and decreased motility (Table 2) caused by the negative regulatory effect of increased OmpR~P on the *flhDC* promoter (12), could contribute to an increased affinity for binding sites in a mixed biofilm. It has been shown that decreased motility favors biofilm formation following the initial adhesion event (47) and that OmpC can serve as an adhesin (48). It should also be mentioned that increased OmpR~P (Fig. 1) has been shown to stimulate production of curli fibers that also stimulate biofilm formation (49). It therefore seems reasonable that *E. coli* Nissle 1917 $envZ_{P41L}$ could have a higher affinity than *E. coli* Nissle 1917 for binding sites on a mixed biofilm.

At this time, we do not know why despite being a better colonizer than *E. coli* Nissle 1917, *E. coli* Nissle 1917 $envZ_{P41L}$ is not better at limiting *E. coli* EDL933 colonization than *E. coli* Nissle 1917; however, the possibility should be considered that when *E. coli* EDL933 infects the mouse intestine, it initially grows planktonically in mucus and not in mixed biofilms. If we are correct that *E. coli* Nissle 1917 and *E. coli* Nissle 1917 $envZ_{P41L}$ colonize the mouse intestine by being served specific sugars by the anaerobes in the mixed biofilms they inhabit, then small amounts of these sugars that escape the mixed biofilms might be available to invading *E. coli* EDL933 as well as to the small numbers of planktonic *E. coli* Nissle 1917 or *E. coli* Nissle 1917 $envZ_{P41L}$ that leave the mixed biofilms. Therefore, it may be that both planktonic *E. coli* Nissle 1917 $envZ_{P41L}$ and *E. coli* Nissle 1917 compete directly with planktonic *E. coli* EDL933 for the sugars that escape the biofilms or that are produced by small numbers of planktonic members of the rest of the microbiota that leave the biofilms. This scenario would allow planktonic *E. coli* EDL933 to colonize to the extent allowed by the available concentrations of those sugars in competition with planktonic *E. coli* Nissle 1917 $envZ_{P41L}$ or *E. coli* Nissle 1917, which could explain why *E. coli* Nissle 1917, the faster grower in perfectly mixed mucus, appears to limit *E. coli* EDL933 growth in the intestine more than *E. coli* Nissle 1917 $envZ_{P41L}$ does (Fig. 8).

In summary, *E. coli* Nissle 1917 $envZ_{P41L}$ is a 10-fold better mouse intestinal colonizer than *E. coli* Nissle 1917, despite growing far slower than *E. coli* Nissle 1917 in bacterium-free, perfectly mixed cecal mucus *in vitro* and on several sugars present in mucus, yet it is not better at limiting *E. coli* EDL933 colonization. The data presented here are consistent with the restaurant hypothesis for commensal *E. coli* strains that colonize the intestine as members of mixed biofilms and obtain the sugars they need for growth locally (3). Indeed, introduction of an $envZ_{P41L}$ mutation into either of two *E. coli* commensal strains directs the mutants to a niche that is defined at least in part by the availability of galactose and cannot be occupied by the parent strains. However, it may be that it is not sessile *E. coli* but the small number of planktonic *E. coli* cells that compete for sugars with invading *E. coli* pathogens. If so, then an efficacious *E. coli* probiotic agent not only should be able to inhabit mixed biofilms better than its parent and, as such, be a better colonizer but also should be better at utilizing nutrients for growth to outcompete the invading pathogen.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant GM095370 to T.C. and P.S.C. and grant VA 1IOBX-000372 to L.J.K.

We thank Kaylin Terhune for excellent technical assistance.

REFERENCES

1. **Leatham MP, Stevenson SJ, Gauger EJ, Krogfelt KA, Lins JJ, Haddock TL, Autieri SM, Conway T, Cohen PS.** 2005. The mouse intestine selects nonmotile *flhDC* mutants of *Escherichia coli* MG1655 with increased colonizing ability and better utilization of carbon sources. Infect. Immun. **73:**8039–8049. http://dx.doi.org/10.1128/IAI.73.12.8039-8049.2005.
2. **Gauger EJ, Leatham MP, Mercado-Lubo R, Laux DC, Conway T, Cohen PS.** 2007. Role of motility and the *flhDC* operon in *Escherichia coli* MG165 colonization of the mouse intestine. Infect. Immun. **75:**3315–3324. http://dx.doi.org/10.1128/IAI.00052-07.
3. **Leatham-Jensen MP, Frimodt-Moller J, Adediran J, Mokszycki ME, Banner ME, Caughron JE, Krogfelt KA, Conway T, Cohen PS.**2012. The streptomycin-treated mouse intestine selects *Escherichia coli envZ* missense mutants that interact with dense and diverse intestinal microbiota. Infect. Immun. **80:**1716–1727. http://dx.doi.org/10.1128/IAI.06193-11.
4. **Wang S, Fleming RT, Westbrook EM, Matsumura P, McKay DB.** 2006. Structure of the *Escherichia coli* FlhDC complex, a prokaryotic hetero-

meric regulator of transcription. J. Mol. Biol. **355:**798–808. http://dx.doi.org/10.1016/j.jmb.2005.11.020.
5. **Prüss BM.** 2000. FlhD, a transcriptional regulator in bacteria. Recent Res. Dev. Microbiol. **4:**31–42.
6. **Barker CS, Prüss BM, Matsumura P.** 2004. Increased motility of *Escherichia coli* by insertion sequence element integration into the regulatory region of the *flhD* operon. J. Bacteriol. **186:**7529–7537. http://dx.doi.org/10.1128/JB.186.22.7529-7537.2004.
7. **Fabich AJ, Leatham MP, Grissom JE, Wiley G, Lai H, Najar F, Roe BBA, Cohen PS, Conway T.** 2011. Genotype and phenotypes of an intestine-adapted *Escherichia coli* K-12 mutant selected by animal passage for superior colonization. Infect. Immun. **79:**2430–2439. http://dx.doi.org/10.1128/IAI.01199-10.
8. **Prüss BM, Campbell JW, Van Dyk TK, Zhu C, Kogan Y, Matsumura P.** 2003. FlhD/FlhC is a regulator of anaerobic respiration and the Entner-Doudoroff pathway through induction of the methyl-accepting chemotaxis protein Aer. J. Bacteriol. **185:**534–543. http://dx.doi.org/10.1128/JB.185.2.534-543.2003.
9. **Prüss BM, Liu X, Hendrickson W, Matsumura P.** 2001. FlhD/FlhC-regulated promoters analyzed by gene array and lacZ gene fusions. FEMS Microbiol. Lett. **197:**91–97.
10. **Walthers D, Go A, Kenney LJ.** 2004. Regulation of porin gene expression by the two-component regulatory system EnvZ/OmpR, p 1–24. *In* Benz R (ed), Bacterial and eukaryotic porins. Wiley-VCH Verlag, Weinheim, Germany.
11. **Aiba H, Mizuno T.** 1990. Phosphorylation of a bacterial activator protein, OmpR, by a protein kinase, EnvZ, stimulates the transcription of the *ompF* and *ompC* genes in *Escherichia coli*. FEBS Lett. **261:**19–22. http://dx.doi.org/10.1016/0014-5793(90)80626-T.
12. **Shin S, Park C.** 1995. Modulation of flagellar expression in *Escherichia coli* by acetyl phosphate and the osmoregulator OmpR. J. Bacteriol. **177:**4696–4702.
13. **Braun V, Patzer SI, Hantke K.** 2002. Ton-dependent colicins and microcins: modular design and evolution. Biochimie **84:**365–380. http://dx.doi.org/10.1016/S0300-9084(02)01427-X.
14. **Schulze J, Sonnenborn U.** 1995. Oral administration of a certain strain of live *Escherichia coli* for intestinal disorders? Infection **23:**184–188. http://dx.doi.org/10.1007/BF01793863.
15. **Patzer SI, Baquero MR, Bravo D, Moreno F, Hantke K.** 2003. The colicin G, H and X determinants encode microcins M and H47, which might utilize the catecholate siderophore receptors FepA, Cir, Fiu and IroN. Microbiology **149:**2557–2570. http://dx.doi.org/10.1099/mic.0.26396-0.
16. **Blum G, Marre R, Hacker J.** 1995. Properties of *Escherichia coli* strains of serotype O6. Infection **23:**234–236. http://dx.doi.org/10.1007/BF01781204.
17. **Grozdanov L, Zähringer U, Blum-Oehler G, Brade L, Henne A, Knirel YA, Schombel U, Schulze J, Sonnenborn U, Gottschalk G, Hacker J, Rietschel ET, Dobrindt U.** 2002. A single nucleotide exchange in the wzy gene is responsible for the semirough O6 lipopolysaccharide phenotype and serum sensitivity of *Escherichia coli* strain Nissle 1917. J. Bacteriol. **184:**5912–5925. http://dx.doi.org/10.1128/JB.184.21.5912-5925.2002.
18. **Grozdanov L, Raasch C, Schulze J, Sonnenborn U, Gottschalk G, Hacker J, Dobrindt U.** 2004. Analysis of the genome structure of the nonpathogenic probiotic *Escherichia coli* strain Nissle 1917. J. Bacteriol. **186:**5432–5441. http://dx.doi.org/10.1128/JB.186.16.5432-5441.2004.
19. **Bachmann BJ.** 1996. Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12, p 2460–2488. *In* Neidhardt FC, Ingraham JI, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE (ed), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, 2nd ed, vol 2. American Society for Microbiology, Washington, DC.
20. **Blattner FR, Plunkett G, III, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y.** 1997. The complete genome sequence of *Escherichia coli* K-12. Science **277:**1453–1474. http://dx.doi.org/10.1126/science.277.5331.1453.
21. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. **97:**6640–6645. http://dx.doi.org/10.1073/pnas.120163297.
22. **Miller JH.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
23. **Guillier M, Gottesman S.** 2006. Remodeling of the *Escherichia coli* outer membrane by two small regulatory RNAs. Mol. Microbiol. **59:**231–247. http://dx.doi.org/10.1111/j.1365-2958.2005.04929.x.
24. **Wayne KJ, Li S, Kazmierczak KM, Tsui HC, Winkler ME.** 2012. Involvement of WalK (VicK) phosphatase activity in setting WalR (VicR) response regulator phosphorylation level and limiting cross-talk in *Streptococcus pneumoniae* D39 cells. Mol. Microbiol. **86:**645–660. http://dx.doi.org/10.1111/mmi.12006.
25. **Morona R, Reeves P.** 1982. The *tolC* locus of *Escherichia coli* affects the expression of three major outer membrane proteins. J. Bacteriol. **150:**1016–1023.
26. **Kawaji H, Mizuno T, Mizushima S.** 1979. Influence of molecular size and osmolarity of sugars and dextrans on the synthesis of outer membrane proteins O-8 and O-9 of *Escherichia coli* K-12. J. Bacteriol. **140:**843–847.
27. **Banwell JG, Gorbach SL, Pierce NF, Mitra R, Mondal A.** 1971. Acute undifferentiated human diarrhea in the tropics. II. Alterations in intestinal fluid and electrolyte movements. J. Clin. Invest. **50:**890–900.
28. **Cohen PS, Laux DC.** 1995. Bacterial adhesion to and penetration of intestinal mucus *in vitro*. Methods Enzymol. **253:**309–315. http://dx.doi.org/10.1016/S0076-6879(95)53026-6.
29. **Miller CP, Bohnhoff M.** 1963. Changes in the mouse's enteric microbiota associated with enhanced susceptibility to *Salmonella* infection following streptomycin-treatment. J. Infect. Dis. **113:**59–66. http://dx.doi.org/10.1093/infdis/113.1.59.
30. **Welch RA, Burland V, Plunkett G, III, Redford P, Roesch P, Rasko D, Buckles EL, Liou SR, Boutin A, Hackett J, Stroud D, Mayhew GF, Rose DJ, Zhou S, Schwartz DC, Perna NT, Mobley HL, Donnenberg MS, Blattner FR.** 2002. Extensive mosaic structure revealed by the complete genome sequence of uropathogenic *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **99:**17020–17024. http://dx.doi.org/10.1073/pnas.252529799.
31. **McCormick BA, Franklin DP, Laux DC, Cohen PS.** 1989. Type 1 pili are not necessary for colonization of the streptomycin-treated mouse large intestine by type 1-piliated *Escherichia coli* F-18 and *E. coli* K-12. Infect. Immun. **57:**3022–3029.
32. **Møller AK, Leatham MP, Conway T, Nuijten PJM, de Haan LAM, Krogfelt KA, Cohen PS.** 2003. An *Escherichia coli* MG1655 lipopolysaccharide deep-rough core mutant grows and survives in mouse cecal mucus but fails to colonize the mouse large intestine. Infect. Immun. **71:**2142–2152. http://dx.doi.org/10.1128/IAI.71.4.2142-2152.2003.
33. **Russo FD, Silhavy TJ.** 1991. EnvZ controls the concentration of phosphorylated OmpR to mediate osmoregulation of the porin genes. J. Mol. Biol. **222:**567–580. http://dx.doi.org/10.1016/0022-2836(91)90497-T.
34. **Maltby R, Leatham-Jensen MP, Gibson T, Cohen PS, Conway T.** 2013. Nutritional basis for colonization resistance by human commensal *Escherichia coli* strains HS and Nissle 1917 against *E. coli* O157:H7 in the mouse intestine. PLoS One **8:**e53957. http://dx.doi.org/10.1371/journal.pone.0053957.
35. **Conway T, Krogfelt KA, Cohen PS.** 29 December 2004, posting date. Chapter 8.3.1.2, The life of commensal *Escherichia coli* in the mammalian intestine. *In* Böck A, Curtiss R, III, Kaper JB, Karp PD, Neidhardt FC, Nyström T, Slauch JM, Squires CL, Ussery D (ed), EcoSal—*Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, DC.
36. **Laux DC, Cohen PS, Conway T.** 2005. Role of the mucus layer in bacterial colonization of the intestine, p 199–212. *In* Nataro JP, Cohen PS, Mobley HLT, Weiser JN (ed), Colonization of mucosal surfaces. ASM Press, Washington, DC.
37. **Chang DE, Smalley DJ, Tucker DL, Leatham MP, Norris WE, Stevenson SJ, Anderson AB, Grissom JE, Laux DC, Cohen PS, Conway T.** 2004. Carbon nutrition of *Escherichia coli* in the mouse intestine. Proc. Natl. Acad. Sci. U. S. A. **101:**7427–7432. http://dx.doi.org/10.1073/pnas.0307888101.
38. **Fabich AJ, Jones SA, Chowdhury FZ, Cernosek A, Anderson A, Smalley D, McHargue DJW, Hightower GA, Smith JT, Autieri SM, Leatham MP, Lins JJ, Allen JH, Laux DC, Cohen PS, Conway T.** 2008. Comparison of carbon nutrition for pathogenic and commensal *Escherichia coli* strains in the mouse intestine. Infect. Immun. **76:**1143–1152. http://dx.doi.org/10.1128/IAI.01386-07.
39. **Leatham MP, Banerjee S, Autieri SM, Mercado-Lubo R, Conway T, Cohen PS.** 2009. Precolonized human commensal *Escherichia coli* strains serve as a barrier to *E. coli* O157:H7 growth in the streptomycin-treated mouse intestine. Infect. Immun. **77:**2876–2886. http://dx.doi.org/10.1128/IAI.00059-09.

40. **Dethlefsen L, Huse S, Sogin ML, Relman DA.** 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol. **6:**2383–2400. http://dx.doi.org/10.1371/journal.pbio.0060280.
41. **Conlan JW, Bardy SL, KuoLee R, Webb A, Perry MB.** 2001. Ability of *Escherichia coli* O157:H7 isolates to colonize the intestinal tract of conventional adult CD1 mice is transient. Can. J. Microbiol. **47:**91–95.
42. **Freter R, Brickner H, Fekete J, Vickerman MM, Carey KE.** 1983. Survival and implantation of *Escherichia coli* in the intestinal tract. Infect. Immun. **39:**686–703.
43. **Hoskins L.** 1984. Mucin degradation by enteric bacteria: ecological aspects and implications for bacterial attachment to gut mucosa, p 51–65. *In* Boedecker EC (ed), Attachment of organisms to the gut mucosa, vol II. CRC Press, Inc, Boca Raton, FL.
44. **Henrissat B, Davies G.** 1997. Structural and sequence-based classification of glycoside hydrolases. Curr. Opin. Struct. Biol. **7:**637–644. http://dx.doi.org/10.1016/S0959-440X(97)80072-3.
45. **Hoskins LC, Agustines M, McKee WB, Boulding ET, Kriaris M, Niedermeyer G.** 1985. Mucin degradation in human colon ecosystems. Isolation and properties of fecal strains that degrade ABH blood group antigens and oligosaccharides from mucin glycoproteins. J. Clin. Invest. **75:**944–953.
46. **Ng KM, Ferreyra JA, Higginbottom SK, Lynch JB, Kashyap PC, Gopinath S, Naidu N, Choudhury B, Weimer BC, Monack DM, Sonnenburg JL.** 2013. Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. Nature **502:**96–99. http://dx.doi.org/10.1038/nature12503.
47. **Beloin C, Roux A, Ghigo JM.** 2008. Escherichia coli biofilms. Curr. Top. Microbiol. Immunol. **322:**249–289.
48. **Rolhion N, Carvalho FA, Darfeuille-Michaud A.** 2007. OmpC and the sigma(E) regulatory pathway are involved in adhesion and invasion of the Crohn's disease-associated *Escherichia coli* strain LF82. Mol. Microbiol. **63:**1684–1700. http://dx.doi.org/10.1111/j.1365-2958.2007.05638.x.
49. **Prigent-Combaret C, Brombacher E, Vidal O, Ambert A, Lejeune P, Landini P, Dorel C.** 2001. Complex regulatory network controls initial adhesion and biofilm formation in *Escherichia coli* via regulation of the *csgD* gene. J. Bacteriol. **183:**7213–7223. http://dx.doi.org/10.1128/JB.183.24.7213-7223.2001.