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MODIFICATION OF THE ESCHERICHIA *coli* NISSLE 1917 *envZ* GENE TO IMPROVE ITS PROBIOTIC POTENTIAL

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BY

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ABSTRACT

The pathogenic *Escherichia coli* EDL933 is known to cause infection in the intestine. *E. coli* Nissle 1917 is used as a probiotic strain to prevent infection (63). When the mouse intestine is pre-colonized with *E. coli* Nissle 1917 it prevents *E. coli* EDL933 from growing up in the intestine. *E. coli* EDL933 is not eliminated from the intestine; it stays at the level in which it was fed to the mice. The P41L *envZ* missense mutation was previously shown to causes an increase in the level of phosporylated OmpR (OmpR~P) produced (67). *E.coli* MG1655 with the P41L missense *envZ* mutation is referred to as *E. coli* MG1655 mot-1. These changes result in an improvement of *E. coli* MG1655 colonization ability in the mouse intestine. The EnvZ/OmpR system positively regulates the small RNAs *omrA* and *omrB* (29). *omrA* and *omrB* were shown to negatively regulate a number of outer membrane genes including *ompT, cirA, fepA,* and *fecA,* (29). *omrA* and *omrB* work by hybridizing to mRNA thereby preventing translation from occurring (28). The mutant *envZ* gene was transferred to *E. coli* Nissle 1917 to create *E. coli* Nissle 1917 *envZ*(mot-1). The mutant *envZ* gene results in *E. coli* Nissle 1917 having improved colonization ability. *E. coli* Nissle 1917 *envZ*(mot-1) grows slower than *E. coli* Nissle 1917 wildtype on many sugars and in cecal mucus. The *E. coli* Nissle 1917 *envZ*(mot-1) mutant grows slower on galactose than *E. coli* Nissle 1917 wildtype. When the ability to utilize galactose is eliminated from *E. coli* Nissle 1917 *envZ*(mot-1) it can no longer grow up in the mouse intestine in the presence of *E. coli* Nissle 1917 wildtype. This displays the importance of galactose utilization in the intestine for *E. coli* Nissle 1917 *envZ*(mot-1). *E. coli* Nissle 1917 *envZ*(mot-1) may have

access to a niche where galactose is being utilized that *E. coli* Nissle 1917 is unable to inhabit. If *E. coli* Nissle 1917 was able to inhabit that niche then it would seem likely that *E. coli* Nissle 1917 would out compete *E. coli* Nissle 1917 *envZ*(mot-1) due to its superior ability to utilize galactose. *E. coli* Nissle 1917 *envZ*(mot-1) was then colonized against *E. coli* EDL933 to see if its improved colonization ability also results in improved probiotic potential. After performing multiple colonization experiments it is evident that *E. coli* Nissle 1917 *envZ*(mot-1) is not a better probiotic than *E. coli* Nissle 1917 wildtype.

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PREFACE

The manuscript format was used in writing this thesis with the style used in the American Society for Microbiology journal, Infection and Immunity.

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INTRODUCTION

When a bacterial species persists indefinitely in the mouse intestine without repeated introduction, the animal is said to be colonized by the bacterium (39). Commensal strains colonize the intestine in the presence of at least 500 cultivable species and 10^{13} to 10^{14} total bacteria (19). *E. coli* colonization cannot be studied in the conventional animal due to the fact that colonization resistance occurs when every niche is filled by the microbiota (15). A model where open niches are available for *E. coli* to colonize in relatively high numbers and also mimic the conventional animal is required to perform such experiments. The streptomycin treated-mouse model fulfills these criteria and is used routinely in the mouse colonization experiments (16, 38).

When streptomycin treated-mice are colonized with motile *E. coli* MG1655 80-90% lack flagella and are non-motile 7 to 9 days after feeding (27, 41). The non-motile 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 (27, 41). FlhD and FlhC form the $FlhD_4C_2$ complex (7, 43, 72), which activates transcription of class II flagellar genes that encode components of the flagellar basal body and export machinery (52). The IS*1* element upstream of the *flhDC* promoter increases expression of the *flhDC* operon which causes *E. coli* MG1655 to be hyper-motile (6, 27). The *flhDC* deletion mutants were found to grow faster than the parent strain in mouse cecal mucus and sugars found in the mucus layer of the intestine (27, 41) which may account for its selection in the intestine. Studies suggest that the *E. coli* MG1655 *flhDC* operon deletion mutants utilize sugars better than their parent strain because a number of metabolic genes are repressed by

the $FlhD_4C_2$ regulatory complex as well as a large number of sugar catabolism operons (22, 53, 54). Also, increased energy is available for other processes due to the absence of hyper flagella synthesis and rotation (27). 10-20% of the *E. coli* MG1655 remained motile over a 15 day period (27) which suggests that they were also better colonizing mutants or that they were residing in an intestinal niche where motility provides an advantage (39). The 10-20% of *E. coli* MG1655 that remained motile were found to be *envZ* missense mutants that display an increased colonization ability (39).

The *envZ* missense mutant that was studied further was the P41L mutant (ccg to tcg), which is a mutation in the N-terminal transmembrane domain of EnvZ (39). The mutant is referred to as *E. coli* MG1655 mot-1. There are two major small molecule porins in the *E. coli* outer membrane, OmpF and OmpC. OmpF is the major porin present at low osmolarity in the outer membrane of *E. coli* and OmpC becomes the predominant porin present at high osmolarity (24). Where the two porins differ are size of the pore and the flow rates, the smaller pore is encoded by *ompC* (24). Regulation of *ompF* and *ompC* is carried out by the two-component EnvZ/OmpR system (24, 25). EnvZ is a sensory histidine kinase that is located on the inner membrane that modulates gene expression in response to osmolarity; it is autophosphorylated via gamma phosphate of cytoplasmic ATP on a histidine residue (24, 71). The phosphoryl group is then transferred from EnvZ to an aspartic acid residue on OmpR, the response regulator, and phosphorylated-OmpR (OmpR~P) alters the expression of *ompF* and *ompC* by binding to sites in their promoters (24) as depicted in Fig 1. The P41L replacement in EnvZ previously was shown to result in

higher than normal levels of OmpR~P (67). OmpR~P regulates several genes (28, 57). The P41L mutation altered several phenotypic traits in the *E. coli* MG1655 mot-1, which resulted in improved mouse intestinal colonization ability (39). *E. coli* MG1655 mot-1 grew faster on several sugars relative to the wildtype and as stated above, also colonized the mouse intestine better than the wildtype (39). The P41L *envZ* missense mutation resulted in *E. coli* MG1655 mot-1 being much more resistant to 5% bile salts than the wildtype parent strain due to the down regulation of the porin that transports bile salts, OmpF (39). The EnvZ/OmpR signal transduction system also regulates *omrA* and *omrB*, two small RNAs that negatively regulate the expression of several outer membrane proteins including CirA, which is the receptor for colicin V (11). Since *E. coli* MG1655 mot-1 appeared to have higher levels of OmpR~P that should result in reduced levels of CirA in the outer membrane, it seemed that they may perhaps be more resistant to the action of colicin V (39). Indeed, *E. coli* F-18, which produces colicin V (44), inhibited the growth of *E. coli* MG1655, to a greater extent than it inhibited the growth of *E. coli* MG1655 mot-1 (39). Furthermore, since *omrA* and *omrB* also negatively regulate expression of a number of other outer membrane proteins (29) such as CsgD (regulator of Curli biosynthesis), it is likely that the outer membrane of *E. coli* MG1655 mot-1 differs markedly from the wildtype (39).

Although *E. coli* MG1655 mot-1 is a better colonizer than the *E. coli* MG1655 Δ*flhD* non-motile mutant selected by the mouse intestine, it grew 15% slower than *E. coli* MG1655 Δ*flhD* in cecal mucus in vitro and on several carbon sources in vitro but grew 30% faster on galactose. In addition, despite its slower growth in mouse cecal

mucus, *E. coli* MG1655 mot-1 appeared to colonize one intestinal niche just as well as *E. coli* MG1655 Δ*flhD* where factors other than nutrition must be responsible and appeared to use galactose to colonize a second intestinal niche in the cecum that is either not colonized or colonized poorly by *E. coli* MG1655 Δ*flhD* (39).

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a pathogenic strain that causes disease in humans when infection occurs and it has virulence factors such as adhesins and toxins (22, 36). Probiotic bacterial strains are used to colonize the intestine and they can prevent a pathogenic strain from colonizing if they are already established in the niche that a pathogen could potentially inhabit. Since the early 1920s *E. coli* Nissle 1917, a human commensal strain, has been utilized as a probiotic (63); however, *E. coli* Nissle 1917 does not completely stop the EHEC strain *E. coli* EDL933 from colonizing the mouse intestine (40). A P41L change in the *envZ* gene of *E. coli* MG1655, a normal human commensal strain, has been shown to modify its nutrition profile, outer surface protein composition, and to improve its mouse intestinal colonizing ability (39). Here we test if replacing the *E. coli* Nissle 1917 wildtype *envZ* gene with the *E. coli* MG1655 mot-1 *envZ* gene will improve its intestinal colonizing ability and its probiotic potential and possibly allow it to stop the pathogen *E. coli* EDL933 from colonizing the intestine and causing infection.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this 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 (4). 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 (4). It has an IS*1* element in the *flhDC* promoter (10). *E. coli* Nissle 1917 has been marketed as a probiotic remedy against intestinal disorders in several European countries since the 1920s (59). The allelic exchange method described by Datsenko and Wanner (18) was used to construct *E. coli* Nissle 1917 *envZ*(mot-1) 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*(mot-1) Δ*galK::cat*, failed to grow in M9 minimal medium containing 0.4% (w/v) galactose as sole carbon and energy source. *E. coli* Nissle 1917 *envZ*(mot-1) Δ*galK::cat* was restored to Gal^+ by allelic replacement (18), selecting for restored growth on M9 minimal galactose agar plates. The restored Gal^+ grew at the same rate as *E. coli* Nissle 1917 *envZ*(mot-1) in M9 galactose minimal medium. The wildtype *envZ* gene in *E. coli* Nissle 1917 was replaced with the *E. coli* MG1655 mot-1 *envZ* gene using an allelic replacement strategy developed by Dr. Barry Wanner and Dr. Kiryl Datsenko at Purdue University as described previously (39) and see sequencing and primes section. All constructions were confirmed by sequencing.

Bacterial Plasmids. Plasmids used in this study are listed in Table 2.

Colicin V sensitivity. *E. coli* F-18 produces colicin V (44). *E. coli* MG1655 strains 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 tested was added to 3 ml of Luria broth Lennox containing 7g/L Difco agar (soft agar), which was then poured onto a 20-ml Luria broth Lennox agar plate. After the soft agar on each plate solidifies, *E. coli* F-18 colonies were toothpicked to each plate, incubated for 18 h at 37°C, and zones of growth inhibition were measured (39).

Motility. Motility of *E. coli* MG1655 strains was assayed by toothpicking colonies from LB agar Lennox onto Luria motility agar (3.5 g/l) containing 200 mM NaCl (5). Plates were incubated for 6 h at 37°C after which spreading was measured from the edge of each colony.

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 (18). For testing carbon and energy source utilization, M9 minimal medium (46) was modified by addition of 120 mM NaCl, to more closely approximate the sodium chloride concentration in the intestine (5). Growth also was 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 (5). Cultures were prepared and growth was monitored spectrophotometrically (A_{600}) with a Pharmacia Biotech Ultrospec 2000 UV/Visible Spectrophotometer (41).

Isolation of cecal mucus and growth. Mouse cecal mucus was isolated as previously described (14). 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/L), the concentration of streptomycin water given to the mice during colonization experiments so the conditions in the intestine were consistent with the conditions that occurred during colonization experiments. 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. The concentration of the mucus was determined using the Bradford Assay and measured spectrophotometrically using the Pharmacia Biotech Ultrospec 2000 Visible Spectrophotometer; the cecal mucus was diluted to 2.5 mg/mL in HEPES-Hanks buffer (pH 7.0). Then growth was measured spectrophotometrically (A_{600}). All animal protocols were approved by the University Committee on Use and Care of Animals at the University of Rhode Island. (14).

Growth in the presence of 5% bile salts. Strains to be tested for bile salts sensitivity were grown overnight in LB broth Lennox, diluted to an A_{600} of about 0.1 into fresh LB broth Lennox containing 5% (w/v) Bacto-Bile Salts No. 3 (Difco Laboratories) and incubated at 37°C with shaking in 125 ml tissue culture bottles (39). Growth was monitored spectrophotometrically (A_{600}) with a Pharmacia Biotech Ultrospec 2000 UV/Visible Spectrophotometer (41).

Sequencing and primers. DNA sequencing was done at the URI Genomics and Sequencing Center, University of Rhode Island, Kingston, using the 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 (39).

The *galK* deletion primers used to make *E. coli* Nissle 1917 envZ(mot-1) Δ*galK::cat* (uppercase letters, *E. coli* Nissle 1917 DNA; lowercase letters, chloramphenicol resistance cassette DNA) were as follows: primer 1, 5′ - AAA CGTAAAAAGTCTCTTTAATACCTGTTTTTGCTTCATATTGTTCAGC gtgtaggctggagctgcttcg-3′; primer 2, 5′- CGACTACAACGACGGTTTCGTTCTG CCCTGCGCGATTGATTATCAAACCcatatgaatatcctccttagt-3′. The primers used for both amplifying the *galK::cat* deletion for allelic exchange into *E. coli* Nissle 1917 envZ(mot-1) and for sequencing to confirm its presence in *E. coli* Nissle 1917 *envZ*(mot-1) Δ*galK::cat* were: forward primer (43 bp upstream of the *galK* coding sequence), 5'-AACAGGCAGCAGAGCGTTTGC-3'; and reverse primer (53 bp downstream of the *galK* coding sequence), 5'-AGTCCATCAGCGTGACTACCATC-3'. The same primers were used for the replacement of the Δ*galK::cat* mutation in *E. coli* Nissle 1917 envZ(mot-1) Δ*galK::cat* with the wildtype *galK* gene and for confirmation of the replacement.

In construction of *E. coli* Nissle 1917 *envZ*(mot-1) the first step replaced the *E. coli* Nissle 1917 wildtype *envZ* with a cassette encoding kanamycin resistance (2) and *parE* under control of the rhamnose promoter (*kan-rha*$_P$*-parE*). The forward primer used was 5' -

TTCATTATCAATCAATTGAAAACAATCTAAAAACGGGAGGCACCTT CACCTCCCGTTTTTTTACCCTTCTTTTGT CTCCCGCTCAGAAGAACTCGTC - 3' (The first 47 basepairs are in the 700 basepair region that occurs upstream of *envZ*). The reverse primer was 5'- GCTTCTCGCCACGAAGTTCATTTGCCCGTACGTTATTGC TCATCGTCACCTTGCTGTTCGGGATCCAATCAGCCCTTGAG - 3' (which is 3 basepairs upstream of *ompR*). These primers have homology to *E. coli* Nissle 1917 and the kanamycin *parE* cassette. 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. The second step replaced the *kan-rha$_P$-parE* cassette with the *E. coli* MG1655 mot-1 *envZ* allele by selecting for growth on M9 minimal agar plates containing 1.0 % w/v rhamnose (which would otherwise kill when rhamnose induces ParE). The forward primer used was 5' - CTTTTGCAAGCGAATCCTTTCATTATCAA TCAATTGAAAACAATCTAAAAACGGGAGGCACCTTCACCTCCCGTTTTTTT ACCCTTCTTTTGT CGTGC - 3' (the 5' end matches Nissle 1917's *envZ* and the 3' end matches MG1655 so the P41L *envZ* can be amplified in *E. coli* MG1655 mot-1). The reverse primer was 5'- TCGACGTGCAGATTTCGCGT -3' (located 105 basepairs downstream of *envZ).* The double recombination event leaves no scars on the genome. All constructions were confirmed by sequencing. The primer 1, 5' - GGTTTACGTCAGCAAGATTTTGC- 3' (which begins 158 basepairs upstream of *envZ* and primer) 2, 5' - TCGACGTGCAGATTTCGCGT – 3' (105 basepairs

downstream of *envZ*) were used to restore the wildtype *E. coli* Nissle 1917 *envZ* gene to *E. coli* Nissle 1917 envZ(mot-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 (44, 49, 64, 65, 70). Briefly, sets of three male CD-1 mice (5 to 8 weeks old) were given drinking water containing streptomycin sulfate (5 g/L) for 24 h to eliminate resident facultative bacteria (45). Following 18 h of starvation for food and water, the mice were fed 1 ml of 20% (w/v) sucrose containing 10^5 CFU or 10^{10} CFU of LB broth Lennox-grown *E. coli* strains. After ingesting 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, 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 gram) 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 µl of 1% Bacto-Tryptone, and plated on a MacConkey agar plate with appropriate antibiotics. This procedure increases the sensitivity of the assay from 10^2 CFU/gram of feces to 10 CFU/per gram of feces. To distinguish the various *E. coli* strains in feces, dilutions were plated on lactose MacConkey agar containing the appropriate antibiotics. All plates were incubated for 18 to 24 h at 37°C prior to counting (39).

Polymerase Chain Reaction. In order to amplify DNA segments PCR was utilized. The Eppendorf Master Cycler 5331 Gradient PCR Thermocycler was used (Westbury, NY). The primers were ordered from Integrated DNA Technologies (Coralville, IA). The DNA polymerase used in the PCR reactions was *Taq* DNA polymerase (New England Biolabs, Ipswich, MA). Conditions used in PCR reactions were, 95^0C for five minutes for the initial denaturation step, then 95^0C for 30 seconds for denaturation, 55^0C for 30 seconds for the annealing step, and 72^0C for three minutes and 30 seconds extension step, with the annealing and extension steps repeating 34 times, and ending with a 72^0C 10 minute final extension for all PCRs except for the primers 5' - CTTTTGCAAGCGAATCCTTTCATTATCAATCAATTGAAAACAATCTAA AAACGGGAGGCAC CTTCACCTCCCGTTTTTTTACCCTTCTTTTGTCGTGC – 3' and 5' – TCGACGTGCAGATTTCGCGT -3' used in amplifying the P41L *envZ* in *E. coli* MG1655 mot-1. With these two primers the 95^0C denaturation step was 15 seconds rather than 30, and the 55^0C annealing step was 45 seconds rather than 30 seconds. All PCR products were visualized on 1% agarose (Fisher Biotec) gels (Wembley, WA).

Preparation of genomic DNA. 10 mL cultures were grown overnight in LB broth at 37^0C. The genomic DNA was isolated using the Promega Wizard Genomic DNA isolation kit (Catalog # A1120 Promega Corporation, Madison, WI). Once isolated the DNA was then stored at -20^0C. In order to isolate plasmids, the culture of bacteria containing the plasmid was grown overnight as stated previously, then the Eppendorf Fast Plasmid Mini kit (#955150601 Brinkman Instruments Inc, Westbury,

NY) was used to isolate the plasmid. Ethanol precipitation (3) was used to concentrate the DNA. First the DNA was divided into equal parts then one tenth the volume of sodium acetate pH 5.4 (Sigma) was added to the DNA. Next 95% ethanol (Quantum Chemical Co., Tuscola, IL) (in the amount of double the total volume of DNA and sodium acetate) was added. The DNA was then allowed to precipitate for 24 hours at 4^0C. After that the precipitated DNA was pelleted in a microcentrifuge for 10 minutes at 13,000 x g. The supernatant was then removed and 500 µL of 70% ethanol was added to the DNA to wash it and it was centrifuged again for 2 minutes at 13,000 x g. Next the supernatant was discarded and the pellet was allowed to air dry for ten minutes. The DNA was then resuspended in 4 µL of Molecular Biology Grade PCR water (Fisher Scientific, Pittsburgh, PA). The precipitated DNA was then stored at -20^0C. In order to determine the concentration of DNA, the NanoDrop 1000 (Thermo Fisher Scientific) spectrophotometer was used. To blank the machine 2 µL of PCR water was used and then 2 µL of DNA sample was loaded onto the machine in order to obtain the concentration. In order to purify DNA for sequencing purposes the QIAGEN QIA quick PCR Purification Kit (# 28104) was used.

Transformation. 10 mL of culture was grown at 37^0C in LB medium to an A_{600} of 0.600 absorbance units. 1.8 mL of cells were then pelleted at 10,000 x g in a microcentrifuge for 2 minutes at 4^0C. The supernatant was then discarded and the cells were washed and resuspended twice in cold 10% glycerol, and the final resuspension was in 100 µL of cold 10% glycerol (competent cells). 2 µL of DNA was mixed with 50 µL of competent cells in an electoporation cuvette (QIAGEN, Inc). The conditions the cells were electroporated at were as follows 2.5 kV, 25 µF,

and 200 Ω using the BI0-RAD Gene Pulser Xcell electroporator (Model #: 165-2662, Hercules, CA). This first transformation was done by adding pKD46 to competent cells. pKD46 is a temperature sensitive plasmid and therefore cells derived from replication above 30^0C will no longer have it (18). The pKD46 plasmid contains a λ Red recombinase gene, which is controlled by an arabinose inducible promoter, and greatly increases recombination events (18). The cells were then allowed to recover adding 1 mL of LB and incubating at 30^0C (shaking 200 rpm) for approximately 2 hours and then 100 µL of cells was plated onto LB plates containing 100 µg/mL of ampicillin, the plates were then incubated at 30^0C. Once a strain had the pKD46 plasmid it was then grown at 30^0C for transformation in 10 mL of SOB medium containing 100 µg/mL of ampicillin and 20 mM L-arabinose. The cells were then transformed in the same manner using a deletion cassette, except the DNA was now recovered in SOB medium as described in (30), with the addition of 20mM glucose (SOC) and cells were plated on LB containing kanamycin (80 µg/mL) or chloramphenicol (30 µg/mL) depending on the resistance genes contained in the cassette used to eliminate the gene of interest. SOB is a rich medium that improves transformation efficiency (30). Next transformation was performed again using pKD46, to improve transformation efficiency. In order to restore a gene using the pKD267 *parE* rhamnose cassette encoding kanamycin resistance, the strain was then recovered in M9 minimal medium with 0.2% glycerol and after 2 hours of recovery it was pelleted in a microcentrifuge at 13,000 x g for 2 minutes. The supernatant was then discarded and it was resuspended in 100 µL of M9 and plated on M9 containing 1% rhamnose (w/v). The *parE* gene, which encodes a DNA gyrase inhibitor; in the

presence of rhamnose the *parE* toxin is present in the absence of its antitoxin thereby killing cells that contain the pKD267 *parE* rhamnose cassette (35). The cells with the restored gene no longer contained the pKD267 plasmid and were thus selected for on M9 1% (w/v) rhamnose plates.

RESULTS

***E. coli* Nissle 1917 *envZ*(mot-1) displays phenotypic traits seen in *E. coli* MG1655 mot-1.** When *E. coli* MG1655 undergoes the *envZ* P41L mutation in the mouse intestine to become *E. coli* MG1655 mot-1, many phenotypic changes occur including reduced motility, reduced sensitivity to colicin V, and reduced sensitivity to bile salts. Several experiments were performed with *E. coli* Nissle 1917 *envZ*(mot-1) in order to determine whether it displayed the same phenotypic traits as *E. coli* MG1655 mot-1. The motility assay was performed with *E. coli* Nissle 1917 *envZ*(mot-1) and *E. coli* Nissle 1917 wildtype (Table 3). When *E. coli* Nissle 1917's *envZ* gene was swapped for the *E. coli* MG1655 mot-1 *envZ* gene, reduced motility occurred. When *E. coli* Nissle 1917 *envZ*(mot-1) was restored to wildtype in terms of the *envZ* gene, its motility increased again to the level of the wildtype, showing the mutated *envZ* gene was responsible for the change in motility (Table 3). Table 4 displays the reduced sensitivity to colicin V that *E. coli* Nissle 1917 *envZ*(mot-1) displays in comparison to the wildtype. When *E. coli* Nissle 1917 *envZ*(mot-1) was restored to wildtype, its sensitivity to colicin V increased and returned to the level of the wildtype showing that the *E. coli* MG1655 mot-1 *envZ* gene was responsible for the change in colicin V sensitivity (Table 4). *E. coli* Nissle 1917 *envZ*(mot-1) also showed a reduced sensitivity to bile salts. In the presence of 5% bile salts *E. coli* Nissle 1917 and *E. coli* Nissle 1917 *envZ*(mot-1) cells began to lyse at the fourth hour in the experiment; however *E. coli* Nissle 1917 *envZ*(mot-1) was able grow to a higher optical density than *E. coli* Nissle 1917 wildtype. By the fourth hour of growth in 5% bile salts, *E. coli* Nissle 1917 *envZ*(mot-1) reached an optical density of

approximately 0.6, whereas *E. coli* Nissle 1917 reached an optical density of 0.3 before lysis initiated. Once restored to wildtype, *E. coli* Nissle 1917 *envZ*(mot1) restored began to lyse at an optical density of 0.3 again. Therefore, the P41L mutation in *envZ* resulted in decreased sensitivity to bile salts in *E. coli* Nissle 1917 *envZ*(mot-1).

***E. coli* Nissle 1917 *envZ*(mot-1) is a better colonizer than *E. coli* Nissle 1917 wildtype.** When 10^5 cfu of *E. coli* Nissle 1917 *envZ*(mot-1) and 10^5 cfu of *E. coli* Nissle 1917 wildtype were fed simultaneously to mice, by day 3 *E. coli* Nissle 1917 *envZ*(mot-1) was 2 orders of magnitudes higher than the wildtype in the mouse intestine, by day 15 it was still an order of magnitude above the wildtype (Fig. 3). As shown in Fig. 4, low numbers of *E. coli* Nissle 1917 *envZ*(mot-1) were able to grow up in the presence of high numbers of *E. coli* Nissle 1917 in the mouse intestine. In addition, low numbers of *E. coli* Nissle 1917 failed to grow up in the presence of high numbers of *E. coli* Nissle 1917 *envZ*(mot-1), i.e. by day 15 *E. coli* Nissle 1917 was almost eliminated from the intestine, at around 10^2 cfu/gram of feces (Fig. 5). Furthermore, *E. coli* Nissle 1917 *envZ*(mot-1) restored to wildtype co-colonized equally well with *E. coli* Nissle 1917 wildtype simultaneously at about 10^5 cfu/gram of feces (Fig. 6). These data suggest that the P41L *envZ* gene was responsible for *E. coli* Nissle 1917 *envZ*(mot-1)'s improved colonization ability.

***E. coli* Nissle 1917 *envZ*(mot-1) utilizes galactose to gain an advantage in the intestine.** As shown in Fig. 7, when *E. coli* Nissle 1917 *envZ*(mot-1) Δ*galK*, a mutant that cannot utilize galactose, was fed to mice in low numbers (10^5 cfu) against high numbers of *E. coli* Nissle 1917 wildtype (10^{10} cfu) it failed to grow up in the

intestine, however it was not washed out from the intestine, *E. coli* Nissle 1917 *envZ*(mot-1) Δ*galK* maintained at 10^5 cfu per gram of feces. This suggests that *E. coli* Nissle 1917 *envZ*(mot-1) used galactose in order to grow up in the intestine in the presence of high numbers of *E. coli* Nissle 1917 wildtype, i.e. when *E. coli* Nissle 1917 *envZ*(mot-1) was able to use galactose, it grew to within 2 orders of magnitude of wildtype *E. coli* Nissle 1917 (Fig. 4). When *E. coli* Nissle 1917 *envZ*(mot-1) Δ*galK* restored to wildtype in respect to Δ*galK* was fed to mice at 10^5 cfu and *E. coli* Nissle 1917 wildtype was fed to mice at 10^{10} cfu, *E. coli* Nissle 1917 *envZ*(mot-1) Δ*galK* restored was able to grow up in the intestine again in the presence of high numbers of *E. coli* Nissle 1917 wildtype (Fig. 8), suggesting that galactose utilization was in fact responsible for *E. coli* Nissle 1917 *envZ*(mot-1) growing up in the intestine in the presence of high numbers of *E. coli* Nissle 1917 wildtype.

Growth of *E. coli* Nissle 1917 *envZ*(mot-1) on a variety of sugars. In Table 5, the generation times for *E. coli* Nissle 1917 and *E. coli* Nissle 1917 *envZ*(mot-1) on several carbon sources are listed (arabinose, fructose, fucose, galactose, gluconate, glucose, maltose, mannose, N-acetylglucosamine, and ribose). *E. coli* Nissle 1917 *envZ*(mot-1) and *E. coli* Nissle 1917 had essentially equal generation times on 5 carbon sources (arabinose, fructose, fucose, N-acetylglucosamine, and ribose). On the other 5 carbon sources (galactose, gluconate, glucose, maltose, and mannose) *E. coli* Nissle 1917 envZ(mot-1) grew slower than the wildtype. Of the 10 carbon sources tested, *E. coli* Nissle 1917 envZ(mot-1) was either equal to or worse than the wildtype, i.e. it did not grow faster than the wildtype on any of the 10 sugars. This was unexpected considering the fact that *E. coli* Nissle 1917 *envZ*(mot-1) was a better

colonizer than the wildtype. It should be noted that galactose provides an advantage in vivo for *E. coli* Nissle 1917 *envZ*(mot-1) (Figs. 4, 7), but in vitro *E. coli* Nissle 1917 *envZ*(mot-1) had a longer generation time on galactose, indicating that factors other than nutrition are important in colonization. Table 5, also depicts the generation times of *E. coli* Nissle 1917 and *E. coli* Nissle 1917 *envZ*(mot-1) on cecal mucus. *E. coli* Nissle 1917 *envZ*(mot-1) grew slower than the wildtype in cecal mucus which further displays the importance of other factors besides nutrition playing a role in the intestine.

Probiotic potential of *E. coli* Nissle 1917 *envZ*(mot-1). Fig. 9 displays a colonization experiment in which *E. coli* Nissle 1917 was pre-colonized in the intestine and on day 10, 10^5 cfu of *E. coli* EDL933 (pathogenic strain of *E. coli*) was fed to the mice. *E. coli* Nissle 1917 wildtype did not completely eliminate *E. coli* EDL933 from the intestine but it stopped it from growing in the intestine and in fact *E. coli* EDL933 dropped two orders of magnitude. As shown in Fig. 10, when *E. coli* Nissle 1917 *envZ*(mot-1) was pre-colonized in the intestine and 10^5 cfu of *E. coli* EDL933 was introduced on day 10, *E. coli* Nissle 1917 *envZ*(mot-1) was able to keep *E. coli* EDL933 from growing up in the intestine, but it also was unable to eliminate *E. coli* EDL933. Moreover, it appeared that *E. coli* Nissle 1917 *envZ*(mot-1) was a slightly worse probiotic than its wildtype counterpart, since *E. coli* EDL933 colonized slightly higher than 10^3 cfu/gram of feces. Fig. 11 shows a colonization experiment in which both *E. coli* Nissle 1917 and *E. coli* Nissle 1917 *envZ*(mot-1) were fed simultaneously to mice and *E. coli* EDL933 was introduced on day 10, this data also suggests that *E. coli* Nissle 1917 *envZ*(mot-1) is a better colonizer than *E. coli* Nissle

1917 wildtype. Even in the presence of both *E. coli* Nissle 1917 strains, *E. coli* EDL933 was still not eliminated from the intestine, i.e. both failed to inhabit all of the niches that *E. coli* EDL933 may reside in.

DISCUSSION

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 *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 (19). 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 (15). Such experiments require an animal model with open niches for *E. coli* to colonize in relatively high 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 since it fulfills these criteria (16, 38).

This study was undertaken to determine whether the *envZ*(mot-1) gene, selected in *E. coli* MG1655 mot-1 by the streptomycin-treated mouse intestine (39), 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 enterohemhorragic 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*(mot-1) is not better than *E. coli* Nissle 1917 at limiting *E. coli* EDL933 growth in the intestine (Fig. 10).

The concept that nutrient availability dictates 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 (26). 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 (26). As a corollary to the hypothesis, when two strains each use one or more major nutrients better than the other strain, low numbers of each strain will grow up in the intestine to higher numbers in the presence of high numbers of the other strain, as observed in mouse colonization studies using a number of human fecal *E. coli* strains isolated from different humans (40).

Despite general acceptance of the nutrient-niche hypothesis, recent data suggests that it is not entirely correct for *E. coli* (39). We recently reported that *E. coli* MG1655 mot-1, 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 bacteria-free mouse cecal mucus in vitro and on several sugars present in cecal mucus (39). 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 (16, 38), 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 (33), but *E. coli* cannot do so (31, 34). It therefore seems likely that *E. coli* obtains the bulk of the mono- and disaccharides it requires for growth in the intestine from degradation by the anaerobes of dietary fiber-derived and mucin-derived oligo- and polysaccharides. 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 (39). 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 raised 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 (22, 40).

The data presented here also are consistent with the Restaurant hypothesis. *E. coli* Nissle 1917 *envZ*(mot-1) did not grow faster than *E. coli* Nissle 1917 on any of the sugars present in mucus that were tested, grew 10%-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 5). 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*(mot-1) colonized the streptomycin-treated mouse intestine 10-fold better in competition with *E. coli* Nissle 1917 (Fig. 3) and grew from low to high numbers in the mouse intestine in the presence of high numbers of *E. coli* Nissle 1917 (Fig. 4). 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 (M. Mokszycki, unpublished), the data can be explained if the lower growth rate of *E. coli* Nissle 1917 *envZ*(mot-1) in those mixed biofilms relative to *E. coli* Nissle 1917 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*(mot-1) has a greater affinity for biofilm binding sites than *E. coli* Nissle 1917. If so, it is not surprising that when *E. coli* Nissle 1917 *envZ*(mot-1) and wildtype *E. coli* Nissle 1917 are fed to mice in equal numbers, *E. coli* Nissle 1917 *envZ*(mot-1) is the better colonizer (Fig. 3). Furthermore, it is not surprising that when mice are fed *E. coli* Nissle 1917 *envZ*(mot-1) and wildtype *E. coli* Nissle 1917 at a ratio of 10^5:1, that ratio would reach 10^6:1 with time (Fig. 5). However, it is surprising that when mice are fed wildtype *E. coli* Nissle and *E. coli* Nissle 1917 *envZ*(mot-1) at a ratio of 10^5:1, that *E. coli* Nissle 1917 *envZ*(mot-1) use galactose to grow to higher numbers (Fig. 4 and Fig. 7), despite wildtype *E. coli* Nissle 1917 being able to grow faster than *E. coli* Nissle 1917 *envZ*(mot-1) on galactose in vitro (Table 5). This can be explained if *E. coli* Nissle 1917 *envZ*(mot-1) uses galactose to grow in a second niche that wildtype *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*(mot-1) has a higher affinity for binding sites than *E. coli* Nissle1917 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 or that inhibits wildtype *E. coli* Nissle 1917 growth more than it inhibits *E. coli* Nissle 1917 *envZ*(mot-1) growth, much in the same way that *E. coli* Nissle 1917 is more sensitive to colicin V than wildtype *E. coli* Nissle 1917 (Table 4).

In support of the view that surface differences between *E. coli* Nissle 1917 *envZ*(mot-1) and *E. coli* Nissle could result in different binding affinities for mixed biofilms, when we replaced the *envZ* gene in *E. coli* Nissle 1917 with *envZ*(mot-1), three phenotypic changes observed in *E. coli* MG1655 mot-1 caused by outer membrane changes resulting from increased OmpR~P were transferred to *E. coli* Nissle 1917 *envZ*(mot-1). These surface changes could contribute to an increased affinity for binding sites in a mixed biofilm, i.e., decreased CirA causing increased resistance to colicin V (11, 39) (Table 4), and decreased motility (Table 3) caused by the negative regulatory effect of increased OmpR~P on the *flhDC* promoter (60). It has been shown that decreased motility favors biofilm formation following the initial adhesion event (8) and that OmpC can serve as an adhesin (55). It should also be mentioned that increased OmpR~P has been shown to stimulate production of curli fibers that also stimulate biofilm formation (51). It therefore seems reasonable that *E. coli* Nissle 1917 *envZ*(mot-1) could have a higher affinity than *E. coli* Nissle 1917 for binding sites on a mixed biofilm.

At the present time we don't know why despite being a better colonizer than *E. coli* Nissle 1917, *E. coli* Nissle 1917 *envZ*(mot-1) is not better at limiting *E. coli* EDL933 colonization than *E. coli* Nissle 1917; however, the possibility that when *E. coli* EDL933 infects the mouse intestine, it initially grows planktonically in mucus and not in mixed biofilms should be considered. If we are correct that *E. coli* Nissle1917 and *E. coli* Nissle1917 *envZ*(mot-1) 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* Nissle1917 or *E. coli* Nissle1917 *envZ*(mot-1) that leave the mixed biofilms. Therefore, it may be that both planktonic *E. coli* Nissle 1917 *envZ*(mot-1) 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*(mot-1) 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*(mot-1) (Fig. 9).

In summary, *E. coli* Nissle 1917 *envZ*(mot-1) is a 10-fold better mouse intestinal colonizer than *E. coli* Nissle 1917 despite growing far slower than *E. coli* Nissle 1917 in bacteria-free, perfectly mixed cecal mucus in vitro and on several sugars present in mucus, yet is not better at limiting *E. coli* EDL933 colonization.

The data presented here are consistent with the Restaurant hypothesis (39) for commensal *E. coli* strains that colonize the intestine as members of mixed biofilms and obtain the sugars they need for growth locally. However, it may be that it is not sessile *E. coli* that compete for sugars with invading *E. coli* pathogens but the small number of planktonic *E. coli* that do so. If so, an efficacious *E. coli* probiotic agent should not only be able to inhabit mixed biofilms better than its parent and as such be a better colonizer, but should also be better at utilizing nutrients for growth to outcompete the invading pathogen.

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APPENDIX

Background of *Escherichia coli.* *Escherichia coli* is a facultatively anaerobic, gram negative rod, that is among the enteric bacterial family *Enterobacteriaceae*; it is an inhabitant of the intestinal tract of animals (66). *E. coli* is a versatile organism that can grow in media containing only glucose as the organic component (66). As Todar states, "Wild-type *E. coli* has no growth factor requirements, and metabolically it can transform glucose into all of the macromolecular components that make up the cell." (66). *E. coli* can grow aerobically when O_2 is available and it can also grow anaerobically in the absence of O_2 (66). The EPA states, "*E. coli* O157:H7 was first recognized as a cause of illness during an outbreak in 1982 traced to contaminated hamburgers." (21). The EPA also states "Since then, most infections are believed to have come from eating undercooked ground beef" (21).

Diseases caused by *E. coli* O157:H7. Outbreaks of *E. coli* O157:H7 occur from time to time (13). They are part of the Shiga toxin-producing *E. coli* (STEC) (13). The CDC states "Around 5–10% of those who are diagnosed with STEC infection develop a potentially life-threatening complication known as hemolytic uremic syndrome (HUS)" (13). The CDC also states "clues that a person is developing HUS include decreased frequency of urination, feeling very tired, and losing pink color in cheeks and inside the lower eyelids" (13). The incubation period for sickness with STEC varies, its typically 3-4 days, but it can range from 1-10 days (13).

When a person is exposed to STEC infection, frequently one of the initial symptoms is belly pain or non-bloody diarrhea (13). The CDC states "The major source for human illnesses is cattle" (13). Modes of transmission according to the CDC are "consumption of contaminated food, consumption of unpasteurized (raw) milk, consumption of water that has not been disinfected, contact with cattle, or contact with the feces of infected people" (13). Methods of prevention from infection include using a food thermometer to ensure food is safe and not eating high risk foods (68). In case of infection staying hydrated and getting rest helps in overcoming infection (68).

Colonization of the intestinal tract. Todar states "*E. coli* is a consistent inhabitant of the human intestinal tract, and it is the predominant facultative organism in the human GI tract; however, it makes up a very small proportion of the total bacterial content" (66). When a bacterium reaches a rate of in vivo growth that is equal to its rate of removal colonization is said to occur (26). Host somatic and germ cells are believed to be at least one order of magnitude less than the amount of microorganisms present in the mucosal surfaces of adult humans (58). Amongst the organisms present in the mucosal surfaces, the most complex population is located in the gastrointestinal tract where at least 400 different species of bacteria reside in the intestine (9, 23, 58). Aerobes and facultative species are exceeded by the anaerobes to a factor of 1 to 1,000 (48). Immediately after birth colonization of the gut initiates (23). Bacteria with high multiplication rates are predominant at first when nutrients and space are not limited in the intestine, but when space and nutrients begin to become limited specialization begins to occur in the intestine and the microbiota

becomes more complex (23). The aerobic and anaerobic bacteria present in the birth canal are the first inhabitants to populate the human gut (23). *Escherichia coli*, *Clostridium* spp., *Streptococcus* spp., *Lactobacillus* spp., *Bacteroides* spp., and *Bifidobacterium* spp are the prevalent species amongst neonates (56). Among breast fed infants, *Bifidobacteria* form the largest portion of the fecal flora (12). Differences in geographic location results in distinct differences in the composition of the fecal flora found between children, displaying the significance of the environment (sanitary conditions) (1). Colonization resistance occurs when all niches in the animal intestine are filled by the microbiota; it refers to the complete microbiota's ability to resist an invading bacterium from colonizing the intestine (69). Administration of an antibiotic results in elimination of Enterobacteriaceae from the intestine, opening up a niche for the introduction of *Escherichia coli* (69).

The intestine of germ-free animals has been studied and it is compared to the intestine of the conventional animal in order to observe the effects that occur with the gastrointestinal system. First observing the behavior of host cells in the absence of bacteria, then examining how the addition of a single or defined population affects host cell activity is a method used in understanding host-microbe interaction (23).

Mouse models used to study colonization. Three in vivo mouse colonization models used commonly to study the colonization of bacterial species are the conventional mouse model, the germ-free mouse model, and the antibiotic-treated mouse model (23, 40). In the conventional mouse, the complete microbiota is present and occupies the intestine (69). In this model, introducing a bacterial strain into the intestine may present difficulty because it must compete with the natural microbiota

present. In the conventional mouse model, introducing a bacterial strain is difficult due to colonization resistance (69). Colonization resistance is not a problem in the germ-free mouse model. The germ-free mouse model differs from the natural intestine where there are many organisms present in the intestine. The data can be used to observe the host's interaction with a specific population but it differs since many other microorganisms that are usually present, are absent in the germ-free mouse model. The antibiotic-treated mouse model is often used with 5 g/L of streptomycin sulfate being the antibiotic chosen (45). A large fraction of the bacteria in the intestine belong to two phyla, the Firmicutes and the Bacteriodetes (20, 42, 62). Extensive microbiological characterization of the microbial community in the streptomycin-treated mouse intestine, demonstrated that streptomycin treatment removes facultative anaerobes with much less impact on the anaerobes (32). Any strategy to overcome colonization resistance will impact community structure (17, 61). The streptomycin-treated mouse model may have an impact on community structure but it also eliminates facultative anaerobes, opening a niche and allowing bacteria to be studied and competition of a variety of *E. coli* strains against a dense and diverse anaerobe population over an extended period of time (39).

Use of Probiotics. Probiotics are live bacteria that may provide health benefits when consumed (50). There is current evidence suggesting that the natural microbiota plays a role in the pathogenesis of inflammatory bowel disease (37). A better understanding of the role the natural microbiota plays could potentially help in altering the natural microbitota or engineering a new probiotic with the intention of alleviating inflammatory bowel disease. Many mechanisms may be responsible for

health benefits involved in probiotic usage (50). The National Center for Complementary and Alternative Medicine states "altering the intestinal "microecology" (e.g., reducing harmful organisms in the intestine), producing antimicrobial compounds (substances that destroy or suppress the growth of microorganisms), and stimulating the body's immune response" are all possible mechanisms that may play a role in the health benefits of probiotics (50). Several gastrointestinal conditions are currently being treated with probiotics including diarrhea and inflammatory bowel disease (50).

Nutrient-niche hypothesis. The concept that nutrient availability dictates the community structure, presented in Freter's nutrient-niche hypothesis, states that species can coexist in the intestine when each is able to grow faster than all others on one or a few limiting nutrients and that the rate of growth is at least equal to the washout rate from the intestine (26). The hypothesis assumes that all nutrients are evenly mixed and are available to all species throughout the intestine (26). 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 (26).

The restaurant hypothesis, a modified version of Freter's nutrient-niche hypothesis, is the concept that anaerobes in the mixed biofilms inhabited by *E. coli* provide them with mono- and di- saccharides locally, rather than from a perfectly mixed pool available to all species, which is an assumption of the nutrient-niche hypothesis (39). It is further hypothesized 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 (39). The restaurant 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 (22, 39, 40).

Tables:

Table 1 Bacterial strains

E. coli strain	Genotype/phenotype	Referred to in text as:	Source/Reference
MG1655 str^R	Spontaneous streptomycin resistant mutant of MG1655	MG1655	(47)
MG1655 str^R mot-1	P41L *envZ* missense mutant of MG1655 str^R	MG1655 mot-1	(39)
MG1655 Δ*flhD*	546-bp deletion beginning immediately downstream of IS1 in the regulatory region of *flhD* and ending in *flhD*, streptomycin and chloramphenicol resistant	MG1655 Δ*flhD*	(41)
Nissle 1917 str^R	Spontaneous streptomycin resistant mutant of Nissle 1917	Nissle 1917	(2)
Nissle 1917 $str^R nal^R$	Spontaneous nalidixic acid resistant mutant of Nissle 1917 str^R	Nissle 1917	This study
Nissle 1917 $str^R rif^{rR}$	Spontaneous rifampin resistant mutant of Nissle 1917 str^R	Nissle 1917	This study
Nissle 1917 str^R *envZ* (P41L)	Nissle 1917 with the *envZ*p41L gene in place of its own str^R	Nissle 1917 envZ(mot-1)	This study
Nissle 1917 $str^R nal^R$ *envZ* (P41L)	Spontaneous nalidixic acid resistant mutant of Nissle 1917 str^R *envZ*(mot-1)	Nissle 1917 envZ(mot-1)	This study
Nissle 1917 str^R *envZ* (P41L) restored	Nissle 1917 *envZ*(mot-1) restored to wildtype in respect to envZ str^R	Nissle 1917 envZ(mot-1) restored	This study
Nissle 1917 str^R *envZ* (P41L) Δ*galk*	912 bp deletion in galactokinase gene replaced by a chloramphenicol resistance cassette	Nissle 1917 envZ(mot-1) Δ*galK*	This study
Nissle 1917 str^R *envZ* (P41L) Δ*galK* restored	Nissle 1917 *envZ*(mot-1) Δ*galK* restored to wildtype in respect to *galK*	Nissle 1917 envZ(mot-1) Δ*galK* restored	This study
Nissle 1917 str^R nal^R *envZ* (P41L) Δ*galK* restored	Spontaneous streptomycin and nalidixic acid resistant mutant of Nissle 1917 str^R *envZ* (P41L) Δ*galK* restored	Nissle 1917 envZ(mot-1) Δ*galK*	This study
F-18 $str^R rif^R$	Spontaneous streptomycin and rifampin resistant mutant of F-18	F-18	(49)
BW37751 (pKD267)*::*Kn^R	The plasmid in this strain contains *parE* under control of the rhamnose promoter and the kanamycin resistance gene	pKD267	Barry Wanner
EDL933	Wild-type O157:H7 strain	EDL933	Dr. Allison O-Brien
EDL933 $str^R rif^R$	Spontaneous streptomycin and rifampin resistant mutant of EDL933	EDL933	This study

Table 2 Plasmids

Plasmid	Function	Source/Reference
pKD3	template plasmid that encodes chloramphenicol resistance, flanked by FRT sites, which contain FLP recognition target sites	(18)
pKD4	template plasmid that encodes kanamycin resistance, flanked by FRT sites, which contain FLP recognition target sites	(18)
pkD46	temperature sensitive, ampicillin resistant, contains L-arabinose inducible red recombinase. Promotes DNA recombination.	(18)
pKD267	a cassette encoding kanamycin resistance (2) and parE under control of the rhamnose promoter (kan-rhaP-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 (35).	Dr. Datsenko and Dr. Wanner

Table 3 Motility Assay

E. coli strain	Spread (mm)	p value
Nissle 1917	1.1 ± 0.3	
Nissle 1917 envZ(mot-1)	0.6 ± 0.2	$p<0.05$
Nissle 1917 envZ(mot-1) restored	1.0 ± 0.1	$p>0.05$

p values calculated with student's t test, values $p<0.05$ are interpreted as significantly different. $p>0.05$ is interpreted as no significant difference. p values are relative to wildtype *E. coli* Nissle 1917. The values are the mean and standard error (n=6). The assay displays the spread on motility agar of each strain in millimeters over 6 hours incubated 37^0 C.

Table 4 Colicin Assay

E. coli strain	Zone of clearance (mm)	p value
Nissle 1917	2.08 ± 0.62	
Nissle 1917 envZ(mot-1)	0.17 ± 0.27	$p<0.05$
Nissle 1917 envZ(mot-1) restored	2.40 ± 0.33	$p>0.05$

p values calculated with the student's t test, values < 0.05 are considered significant different. $p>0.05$ is interpreted as no significant difference. p values are relative to wildtype *E. coli* Nissle 1917. The values are the mean and standard error (n=6). Colicin assays were performed as described in Materials and Methods.

Table 5 Generation times of *E. coli* Nissle 1917 and *E. coli* Nissle 1917 *envZ*(mot-1) on various carbon sources

Carbon Source	*E. coli* Nissle 1917	*E. coli* Nissle 1917 $envZ_{P41L}$
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****

Each p value refers to a comparison between the generation time of *E. coli* Nissle 1917 and *E. coli* Nissle 1917 $envZ_{P41L}$ when growing on a specific sugar: *, $P > 0.05$; **, P >O.10; ***, $P < 0.05$, ****, $P<0.001$, †, $P=0.0036$. The table depicts the mean generation time for several carbon sources with the standard deviation for each strain of *E.coli* (n=3).

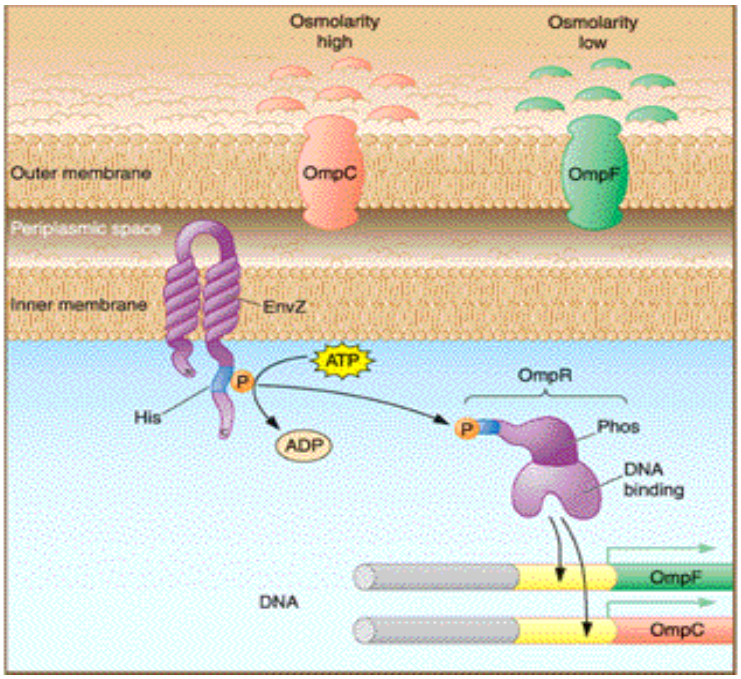

Figure 1. The two-component EnvZ-OmpR system. EnvZ and OmpR are phosphorylated in response to changes in osmotic pressure (24).

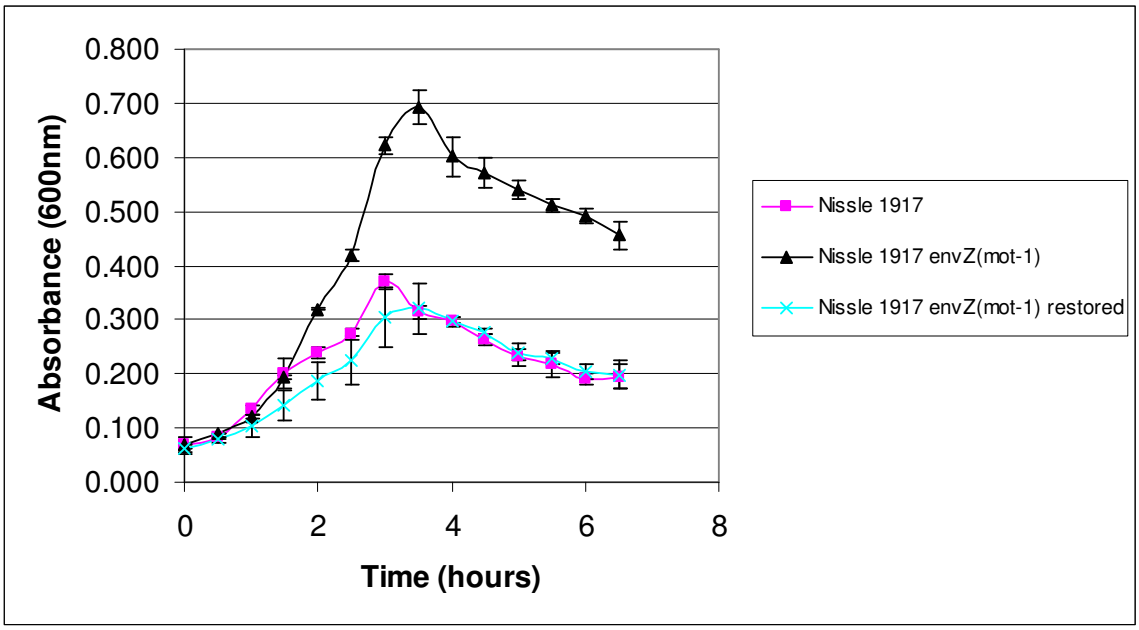

Figure 2. Growth of *E. coli* strains in the presence of 5% bile salts. Growth was performed in LB medium with the addition of 5% bile salts. Half hour time points were collected over 6.5 hours. Bars represent the standard deviation of the A_{600}, for each strain of *E. coli* for each time point (n=3).

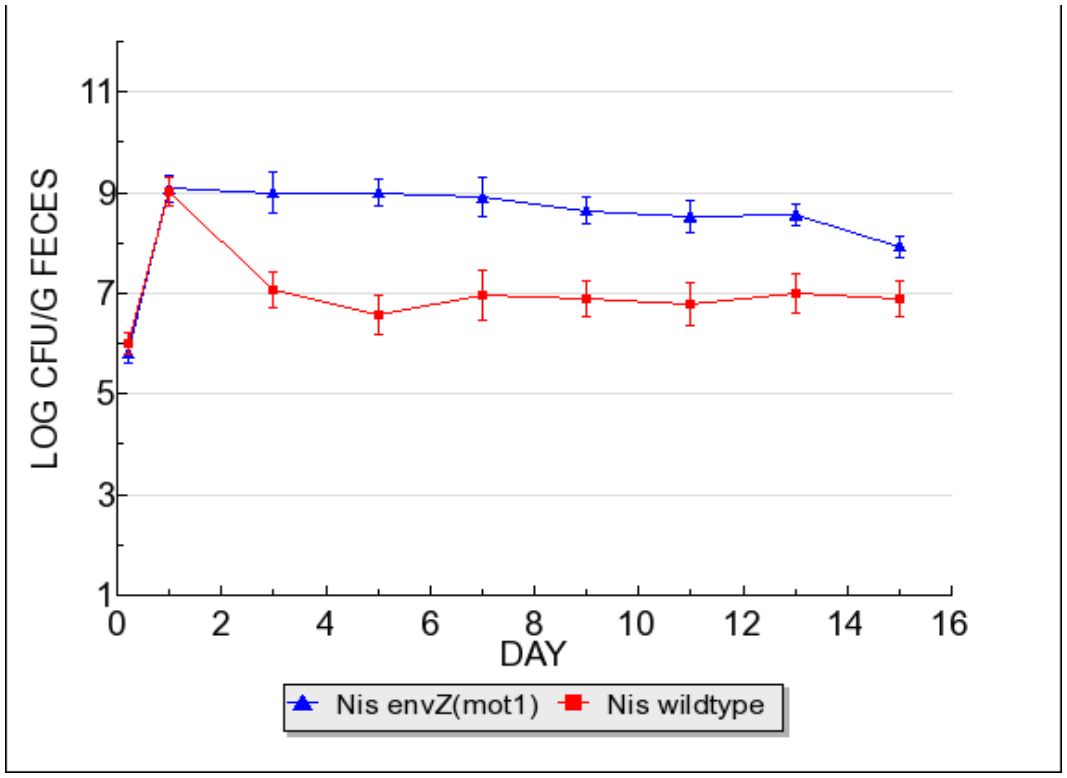

Figure 3. Fifteen day mouse colonization with *E. coli* Nissle 1917 wildtype $Str^{R}Nal^{R}$ (■) and *E. coli* Nissle 1917 envZ(mot-1) Str^{R} (▲). Two sets of three mice were fed 10^{5} cfu of *E. coli* Nissle 1917 envZ(mot-1) and 10^{5} cfu of *E. coli* Nissle 1917. At the indicated times, fecal samples were homogenized, diluted, and plated as described in Materials and Methods. Bars representing standard errors of the $\log_{10}$ mean of CFU per gram of feces are presented for each time point.

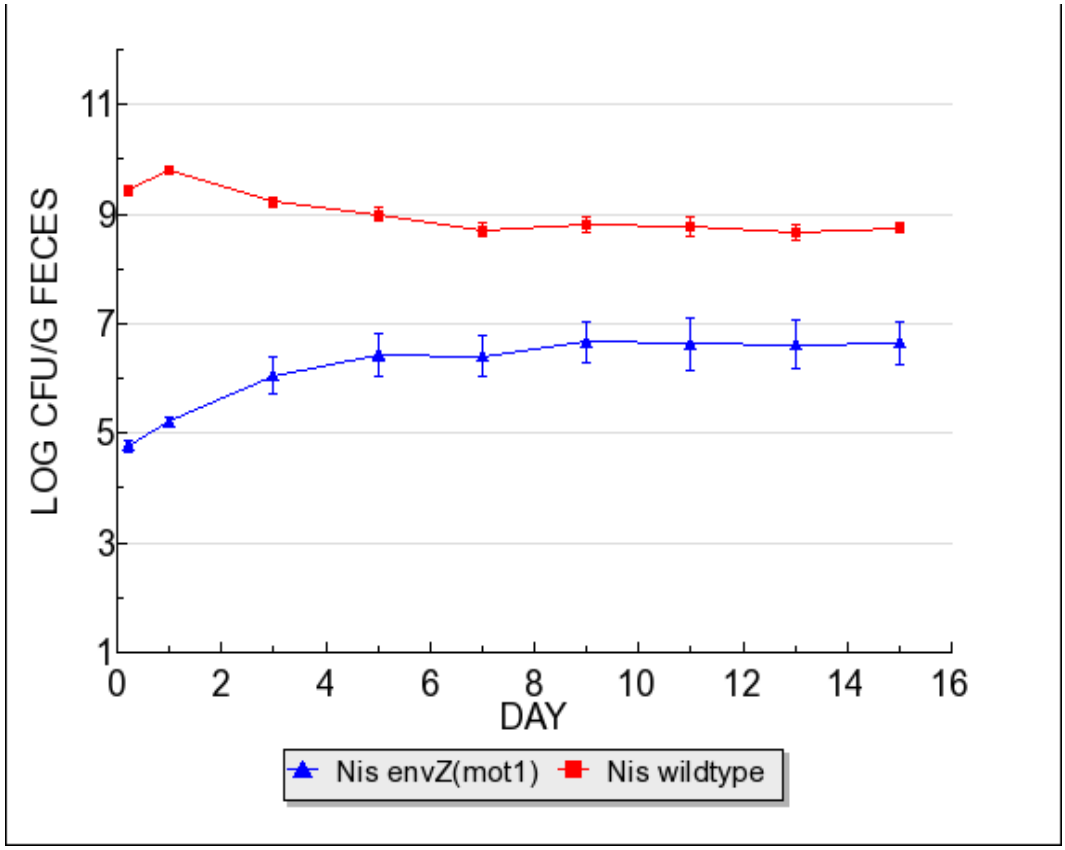

Figure 4. Fifteen day mouse colonization with *E.* coli Nissle 1917 envZ(mot-1) $Str^{R}Nal^{R}$ (▲) and *E.* coli Nissle 1917 wildtype Str^{R} (■). Six sets of three mice were fed 10^{5}cfu of *E. coli* Nissle 1917 envZ(mot-1) and 10^{10} cfu of *E. coli* Nissle 1917 wildtype. At the indicated times, fecal samples were homogenized, diluted, and plated as described in Materials and Methods. Bars representing standard errors of the $\log_{10}$ mean of CFU per gram of feces are presented for each time point.

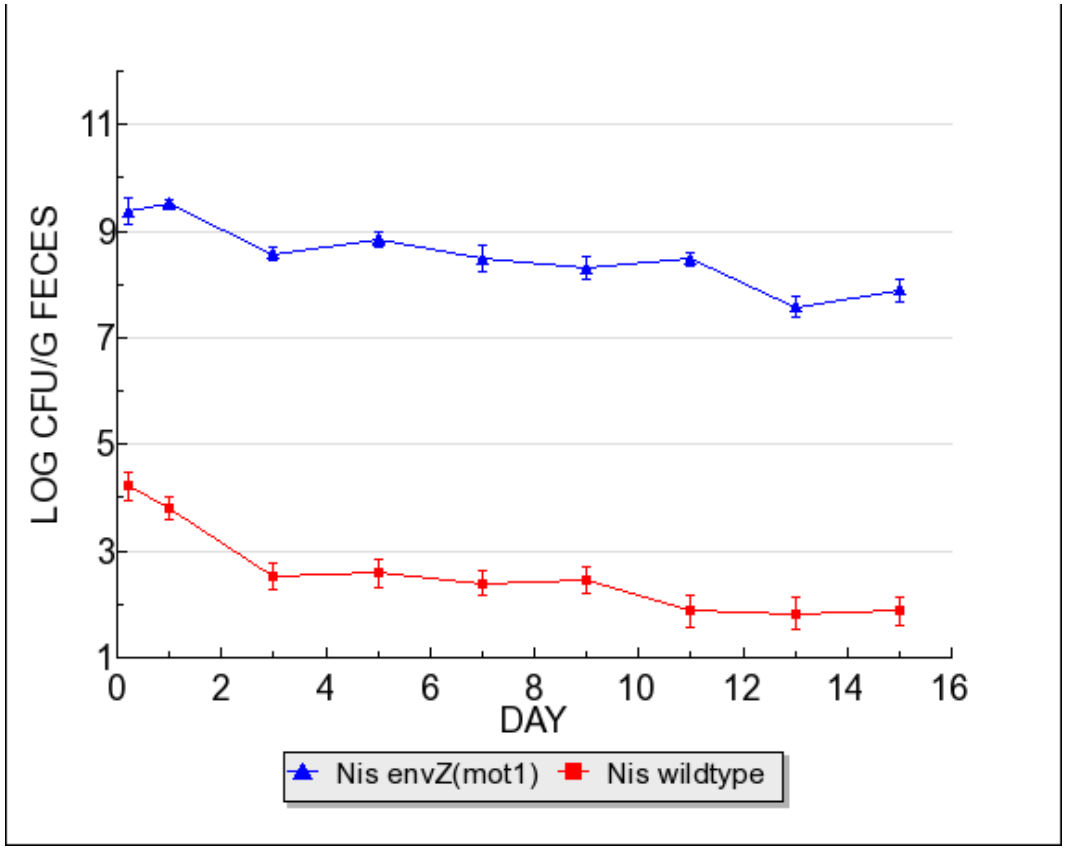

Figure 5. Fifteen day colonization with *E. coli* Nissle 1917 envZ(mot-1) $Str^{R}Nal^{R}$ (▲) and *E. coli* Nissle 1917 wildtype $Str^{R}Rif^{R}$ (■). Three sets of three mice were fed 10^{5} cfu of *E. coli* Nissle 1917 and 10^{10} cfu of *E. coli* Nissle 1917 envZ(mot-1). At the indicated times, fecal samples were homogenized, diluted, and plated as described in Materials and Methods. Bars representing standard errors of the $\log_{10}$ mean of CFU per gram of feces are presented for each time point.

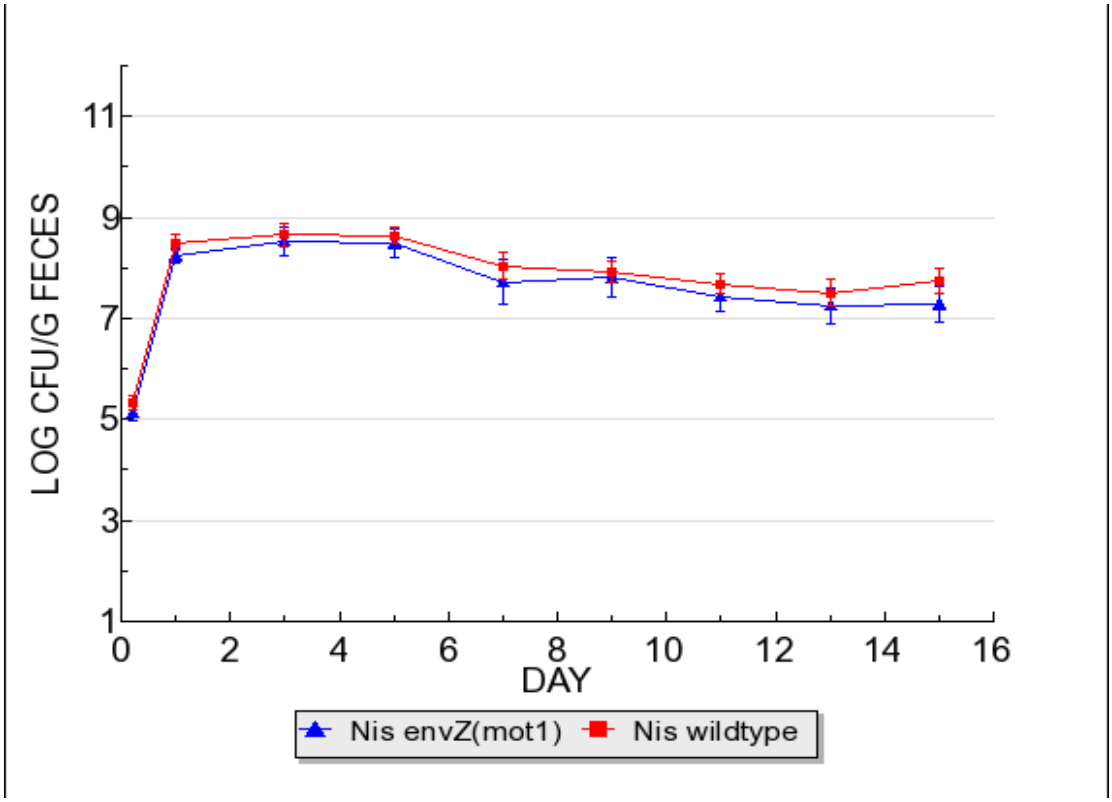

Figure 6. Fifteen day colonization with *E. coli* Nissle 1917 Str^R envZ(mot-1) restored to wildtype, in respect to *envZ*, (▲) and *E. coli* Nissle 1917 wildtype Str^RNal^R (■). Two sets of three mice were fed 10^5 cfu of *E. coli* Nissle 1917 envZ(mot-1) restored to wildtype and 10^5 cfu of *E. coli* Nissle 1917 wildtype. At the indicated times, fecal samples were homogenized, diluted, and plated as described in Materials and Methods. Bars representing standard errors of the $\log_{10}$ mean of CFU per gram of feces are presented for each time point.

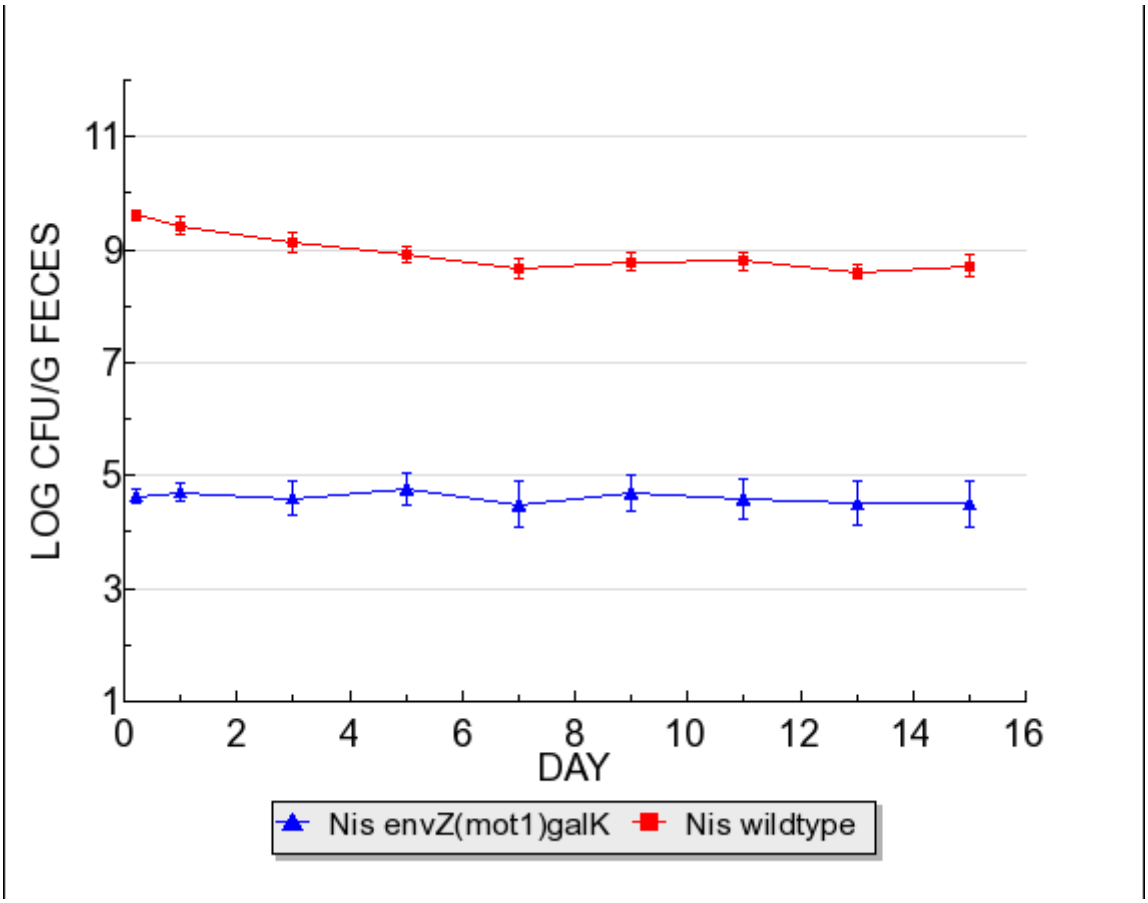

Figure 7. Fifteen day colonization with *E. coli* Nissle 1917 envZ(mot-1) Δ*galK* $Str^{R}Cam^{R}$ (▲) and *E. coli* Nissle 1917 wildtype Str^{R} (■). Four sets of three mice were fed 10^{5} cfu of *E. coli* Nissle 1917 envZ(mot-1) Δ*galK* and 10^{10} cfu *E. coli* Nissle 1917 wildtype. At the indicated times, fecal samples were homogenized, diluted, and plated as described in Materials and Methods. Bars representing standard errors of the $\log_{10}$ mean of CFU per gram of feces are presented for each time point.

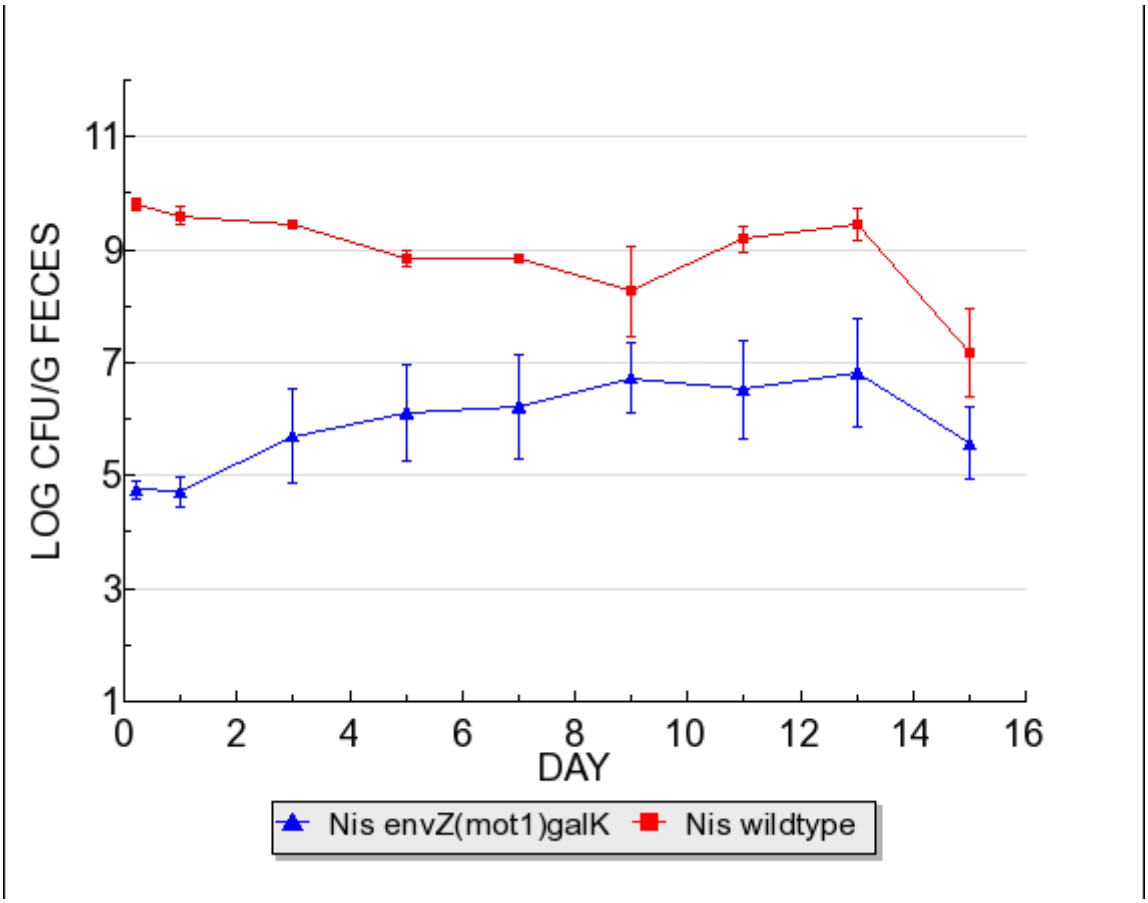

Figure 8. Fifteen day colonization with *E. coli* Nissle 1917 envZ(mot-1) Δ*galK* restored, in respect to Δ*galK*, $Str^{R}Nal^{R}$ (▲) and *E. coli* Nissle 1917 wildtype $Str^{R}Rif^{R}$ (■). Two sets of three mice were fed 10^{5} cfu of *E. coli* Nissle 1917 envZ(mot-1) Δ*galK* and 10^{10} cfu of *E. coli* Nissle 1917 wildtype. At the indicated times, fecal samples were homogenized, diluted, and plated as described in Materials and Methods. Bars representing standard errors of the $\log_{10}$ mean of CFU per gram of feces are presented for each time point.

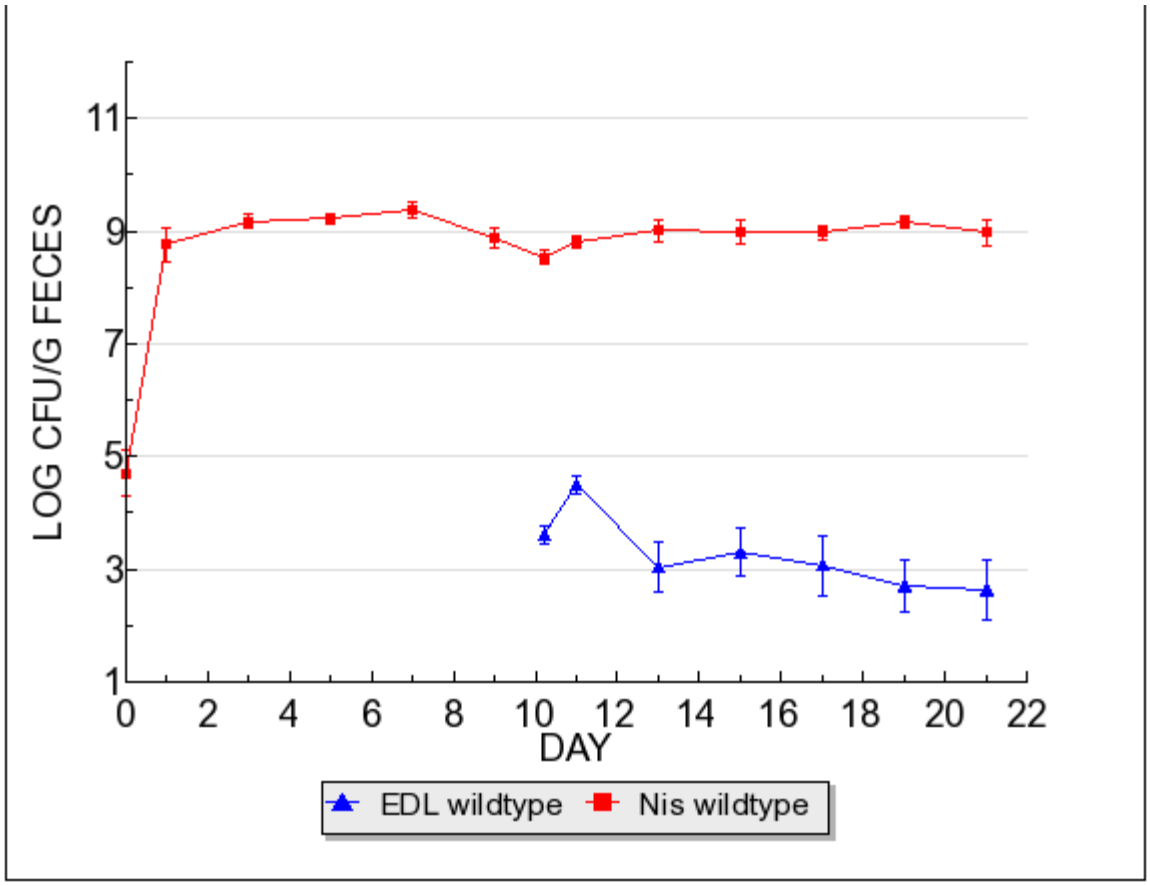

Figure 9. Twenty one day colonization with *E. coli* EDL933 wildtype $Str^{R}Rif^{R}$ (▲) and *E. coli* Nissle 1917 wildtype $Str^{R}Nal^{R}$ (■). Three sets of three mice were fed 10^5 cfu of *E. coli* Nissle 1917 wildtype. On day 10 three sets of three mice were fed 10^5 cfu of *E. coli* EDL933. At the indicated times, fecal samples were homogenized, diluted, and plated as described in Materials and Methods. Bars representing standard errors of the $\log_{10}$ mean of CFU per gram of feces are presented for each time point.

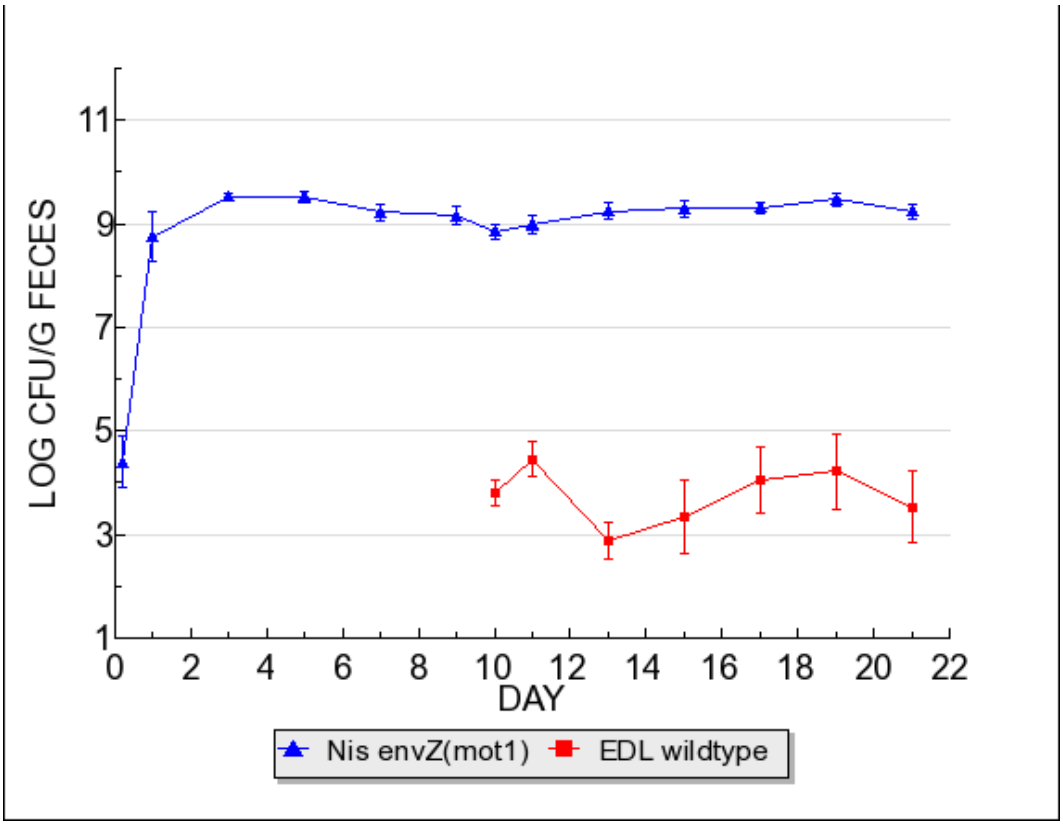

Figure 10. Twenty one day colonization with *E. coli* Nissle 1917 envZ(mot-1) $Str^{R}Nal^{R}$ (▲) and *E. coli* EDL933 wildtype $Str^{R}Rif^{R}$ (■). Three sets of three mice were fed 10^{5} cfu of *E. coli* Nissle 1917 envZ(mot-1). On day 10 three sets of three mice were fed 10^{5} cfu of *E. coli* EDL933. At the indicated times, fecal samples were homogenized, diluted, and plated as described in Materials and Methods. Bars representing standard errors of the $\log_{10}$ mean of CFU per gram of feces are presented for each time point.

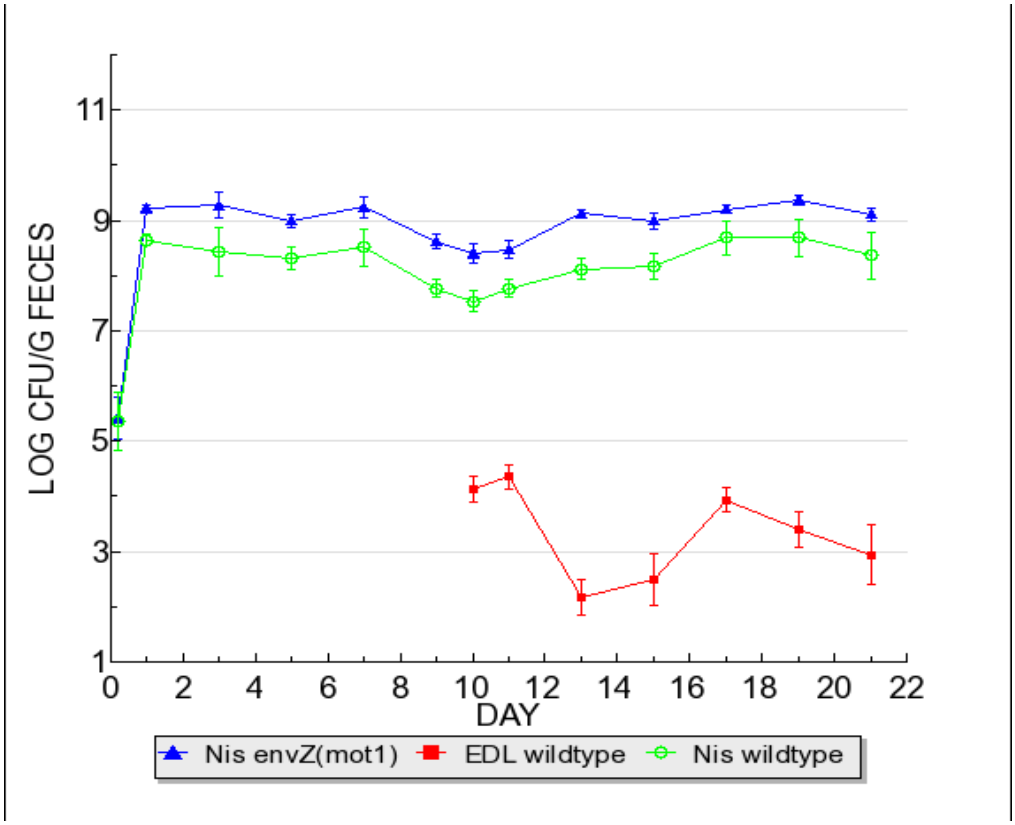

Figure 11. Twenty one day colonization with *E. coli* Nissle 1917 envZ(mot-1) Str^R (▲) and *E. coli* EDL933 wildtype Str^RRif^R (■) and *E. coli* Nissle 1917 wildtype Str^RNal^R (●). Two sets of three mice were fed 10^5 cfu of *E. coli* Nissle 1917 envZ(mot-1) and *E. coli* Nissle 1917 wildtype. On day 10 two sets of three mice were fed 10^5 cfu of *E. coli* EDL933. At the indicated times, fecal samples were homogenized, diluted, and plated as described in Materials and Methods. Bars representing standard errors of the $\log_{10}$ mean of CFU per gram of feces are presented for each time point.