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Escherichia coli EDL933 Requires Gluconeogenic Nutrients To Successfully Colonize the Intestines of Streptomycin-Treated Mice Precolonized with *E. coli* Nissle 1917

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***Escherichia coli* MG1655, a K-12 strain, uses glycolytic nutrients exclusively to colonize the intestines of streptomycin-treated mice when it is the only *E. coli* strain present or when it is confronted with *E. coli* EDL933, an O157:H7 strain. In contrast, *E. coli* EDL933 uses glycolytic nutrients exclusively when it is the only *E. coli* strain in the intestine but switches in part to gluconeogenic nutrients when it colonizes mice precolonized with *E. coli* MG1655 (R. L. Miranda et al., Infect Immun 72:1666–1676, 2004, http://dx.doi.org/10.1128/IAI.72.3.1666-1676.2004). Recently, J. W. Njoroge et al. (mBio 3:e00280-12, 2012, http://dx.doi.org/10.1128/mBio.00280-12) reported that *E. coli* 86-24, an O157:H7 strain, activates the expression of virulence genes under gluconeogenic conditions, suggesting that colonization of the intestine with a probiotic *E. coli* strain that outcompetes O157:H7 strains for gluconeogenic nutrients could render them nonpathogenic. Here we report that *E. coli* Nissle 1917, a probiotic strain, uses both glycolytic and gluconeogenic nutrients to colonize the mouse intestine between 1 and 5 days postfeeding, appears to stop using gluconeogenic nutrients thereafter in a large, long-term colonization niche, but continues to use them in a smaller niche to compete with invading *E. coli* EDL933. Evidence is also presented suggesting that invading *E. coli* EDL933 uses both glycolytic and gluconeogenic nutrients and needs the ability to perform gluconeogenesis in order to colonize mice precolonized with *E. coli* Nissle 1917. The data presented here therefore rule out the possibility that *E. coli* Nissle 1917 can starve the O157:H7 *E. coli* strain EDL933 of gluconeogenic nutrients, even though *E. coli* Nissle 1917 uses such nutrients to compete with *E. coli* EDL933 in the mouse intestine.**

While much attention has been paid to the role of specific virulence factors in bacterial pathogenesis, until recently little attention has been paid to the roles of specific metabolic pathways in the ability of bacterial pathogens to initiate the pathogenic process. Yet clearly, if a bacterial pathogen is unable to initiate pathogenesis because nutrients essential for its growth are unavailable in the host, it will be unable to establish itself and cause disease. Therefore, it is important to determine whether pathogens require glycolytic nutrients, gluconeogenic nutrients, or both for success in invading and subsequently colonizing the host. Indeed, recent studies have suggested that some pathogens use glycolytic nutrients, some use gluconeogenic nutrients, and some use both to adapt to diverse host habitats (1–7).

We are interested in the nutritional basis of *Escherichia coli* colonization of the intestine and particularly in whether precolonization by commensal *E. coli* strains can be used to prevent invading *E. coli* intestinal pathogens from colonizing. Commensal *E. coli* strains colonize the human intestine in the presence of a dense and diverse intestinal microbiota comprising at least 500 cultivable species and 10^{13} to 10^{14} total bacteria (8). Unfortunately, *E. coli* colonization cannot be studied experimentally in conventional animals due to colonization resistance, which occurs when all niches are filled by the microbiota (9). Such experiments require an animal model with open niches for *E. coli* to colonize the intestine in relatively high numbers, but to be authentic, the model intestine must also have a dense and diverse anaerobic community that matches the native microbiota of the conventional animal as closely as possible. The streptomycin-treated mouse model is used routinely for this purpose, since streptomycin-treated mice fulfill these criteria (10, 11). Using this model, we have shown that for *E. coli* to colonize, it must obtain nutrients in the mucus layer, which overlies the epithelium (10, 11), that it resides in the mucus layer as a member of mixed biofilms (12, 13), and that each *E. coli* strain displays a unique nutritional program in the intestine (14).

E. coli strains can utilize both glycolytic and gluconeogenic nutrients for growth *in vitro*, and we reported previously that both *E. coli* MG1655, a commensal strain, and *E. coli* EDL933, an O157:H7 pathogenic strain, use glycolytic but not gluconeogenic nutrients to colonize the intestines of streptomycin-treated mice when each is the only *E. coli* strain fed to mice (15). However, we also reported that in the presence of *E. coli* MG1655, *E. coli* EDL933 switches, at least in part, to gluconeogenesis in order to maintain itself in the intestine, while *E. coli* MG1655 continues to use glycolytic nutrients exclusively (15). These findings are of extreme interest in view of recent reports showing that *E. coli* 86-24, also an O157:H7 strain, activates the expression of virulence genes

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TABLE 1 Bacterial strains

E. coli strain	Genotype or phenotype	Designation in the text	Source or reference
Nissle 1917 Str^r	Spontaneous streptomycin-resistant mutant of *E. coli* Nissle 1917	*E. coli* Nissle 1917	42
Nissle 1917 Str^r Nal^r	Spontaneous nalidixic acid-resistant mutant of *E. coli* Nissle 1917 Str^r	*E. coli* Nissle 1917	42
Nissle 1917 Str^r Δ*ppsA* Δ*pckA*::*cat*	*ppsA pckA* double deletion mutant of *E. coli* Nissle 1917 Str^r carrying a chloramphenicol resistance cassette in the *ppsA* deletion; unable to metabolize gluconeogenic substrates	*E. coli* Nissle 1917 Δ*ppsA* Δ*pckA*	This study
MG1655 Str^r Nal^r	Spontaneous streptomycin- and nalidixic acid-resistant mutant of *E. coli* MG1655	*E. coli* MG1655	15
EDL933 Str^r	Spontaneous streptomycin-resistant mutant of *E. coli* EDL933	*E. coli* EDL933	15
EDL933 Str^r Rif^r	Spontaneous rifampin-resistant mutant of *E. coli* EDL933 Str^r	*E. coli* EDL933	36
EDL933 Str^r Δ*ppsA* Δ*pckA*::*cat*	*ppsA pckA* double deletion mutant of *E. coli* EDL933 Str^r carrying a chloramphenicol resistance cassette in the *ppsA* deletion; unable to metabolize gluconeogenic substrates	*E. coli* EDL933 Δ*ppsA* Δ*pckA*	15

under gluconeogenic conditions (16, 17), because this suggests the possibility that precolonization of the intestine with a probiotic *E. coli* strain that starves *E. coli* O157:H7 strains of gluconeogenic nutrients might render them nonpathogenic.

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 (18). Several features of *E. coli* Nissle 1917 have been proposed to be responsible for its probiotic nature, including its ability to express two microcins (19), the absence of known protein toxins, its semirough lipopolysaccharide and hence its serum sensitivity (20, 21), and the presence of six iron uptake systems (22). More recently, it has been shown that *E. coli* Nissle 1917 inhibits the adherence of *E. coli* EDL933 to human epithelial cells *in vitro* and simultaneously inhibits its growth and Shiga toxin 2 (Stx2) production (23). It has also been shown that in the intestines of streptomycin-treated mice, *E. coli* Nissle 1917 is better than a number of other commensal *E. coli* strains at limiting the growth of *E. coli* EDL933 (12, 13). In this study, we examine whether *E. coli* Nissle 1917 uses both glycolytic and gluconeogenic nutrients to colonize the intestines of streptomycin-treated mice and whether *E. coli* EDL933 switches to gluconeogenic nutrients in order to maintain itself in the intestines in the presence of *E. coli* Nissle 1917.

MATERIALS AND METHODS

Bacterial strains. The 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 (24). 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 (24). It has an IS*1* element in the *flhDC* promoter (25). *E. coli* EDL933 is the prototype strain of enterohemorrhagic *E. coli* (EHEC) O157:H7 (26). *E. coli* Nissle 1917 was originally isolated during World War I from a soldier who escaped a severe outbreak of diarrhea (18). 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 (18).

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 (27). For testing carbon and energy source utilization in the Biolog system, M9 minimal medium (28) was modified by the addition of 120 mM NaCl to more closely approximate the sodium chloride concentration in the intestine (29).

Mutant construction. The allelic exchange method described by Datsenko and Wanner (27) was used to construct *E. coli* Nissle 1917 Str^r Δ*pckA* Δ*ppsA*::*cat* (Table 1). The method allows the sequential construction of double deletion mutants by use of a removable chloramphenicol cassette (27). The primers used to construct the deletion mutants were designed according to the genome sequence of *E. coli* CFT073 (30), which is closely related to that of *E. coli* Nissle 1917 (22). Deletions of 1,327 bp and 2,367 bp were made in the *pckA* gene and the *ppsA* gene, respectively. The *pckA* deletion primers used to make *E. coli* Nissle 1917 Str^r Δ*pckA*::*cat* were as follows: primer 1, 5′-AGACTTTACTATTCAGGCAATACATAT TGGCTAAGGAGCAGTGgtgtaggctggagctgcttcg-3′; primer 2, 5′-GCGG GTATCTTTAATCGAGATACGTTTGCCAGTGCCGTTCCAgcatatgaat atcctccttag-3′ (capital letters, *E. coli* Nissle 1917 DNA; lowercase letters, chloramphenicol resistance cassette DNA). The *ppsA* deletion primers used to make *E. coli* Nissle 1917 Str^r Δ*pckA* Δ*ppsA*::*cat* were as follows: primer 1, 5′-GCGACTAAACGCCGCCGGGGATTTATTT TATTTCTTCA GTgtgtaggctggagctgcttcg-3′; primer 2, 5′-AATGTGTTTCTCAAAC CGTTC ATTTATCACAAAAGGATTGTTCgcatatgaatatcctccttag-3′ (capital letters, *E. coli* Nissle 1917 DNA; lowercase letters, chloramphenicol resistance cassette DNA). Gene knockouts were confirmed by PCR and sequencing using the following upstream and downstream primers: Δ*pckA* forward, 5′-GTTAATTATCGCATCCGGGCA-3′; Δ*pckA* reverse, 5′-GTTGTCGA TAAACAG TTTCGCCAG-3′; Δ*ppsA* forward, 5′-CCTGTGTGGTTGCA ATGTCCG-3′; Δ*ppsA* reverse, 5′-TTGTTCTTCCCGTGATGCAGAC-3′. DNA sequencing was carried out 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 cycle sequencing kit (version 3.1; Applied Biosystems) was used for the sequencing reactions.

Biolog phenotypic analysis. For confirmation that *E. coli* Nissle 1917 Str^r Δ*pckA* Δ*ppsA*::*cat* was able to grow on glycolytic substrates but unable to grow on gluconeogenic substrates, this strain and its wild-type parent were grown overnight in M9 minimal medium containing 0.4% (vol/vol) glycerol and 0.2 M NaCl at 37°C. Cultures were washed twice in M9 minimal medium containing 0.2 M NaCl without any carbon source and were then diluted to an optical density at 600 nm (OD_{600}) of 0.06 in 5 ml of M9 minimal medium without any carbon source. Each culture was diluted an additional 10-fold, and 100 μl/well was added to a 96-well microtiter plate (Biolog GN2 MicroPlate). Plates were incubated at 37°C for 48 h in the OmniLog system. Levels of reduction of tetrazolium violet dye were measured at 15-min intervals and were plotted against time, resulting in a kinetic curve. *E. coli* Nissle 1917 and *E. coli* Nissle 1917 Δ*pckA* Δ*ppsA* each displayed the highest final level of tetrazolium reduction when either gluconate or glycerol was used as the sole carbon source. Final levels of tetrazolium reduction on all other carbon sources were normalized to that on glycerol.

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 (12). 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 (31). Following 18 h of starvation for food and water, the mice were fed 1 ml of 20% (wt/vol) sucrose containing 10^5 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 the streptomycin-containing 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 times indicated in the figures. 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 large debris 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/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) either alone or together with nalidixic acid (50 μg/ml), chloramphenicol (30 μg/ml), or 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. 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. All animal protocols were approved by the University Committee on Use and Care of Animals at the University of Rhode Island.

FISH. Fluorescence *in situ* hybridization (FISH) was performed as described previously (12, 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, the 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 (Integrated DNA Technologies [IDT], Coralville, IA) 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 Cy3-conjugated probe (5′-Cy3-CAC CGT AGT GCC TCG TCA TCA-3′) (red) and the eubacterial 23S rRNA-specific FAM (6-carboxyfluorescein) probe (5′-GCT GCC TCC CGT AGG AGT-FAM-3′) (green) were used at concentrations of 5 ηg/μl and 25 ηg/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 was 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 were allowed to soak for 20 min at 50°C. Slides were then rinsed with distilled water and were 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 anti-bleaching agent that helps prolong fluorescence for imaging.

Statistics. The colonization data for *E. coli* EDL933 from day 17 through day 21 in Fig. 3A were combined, as were the corresponding data in Fig. 6A, and these Fig. 3A data and Fig. 6A data were compared by a two-tailed Student *t* test. Also, the colonization data for *E. coli* MG1655 from day 17 through day 21 in Fig. 2A and B were combined, as were those for *E. coli* Nissle 1917 from day 17 through day 21 in Fig. 3A and B, and these data for the two strains were compared by a two-tailed Student *t* test. *P* values of <0.05 were interpreted as indicating a significant difference.

TABLE 2 Biolog phenotypic analysis of *E. coli* Nissle 1917 strains grown on glycolytic and gluconeogenic carbon sources

Carbon source	Relative final tetrazolium dye reduction level[a]	
	E. coli Nissle 1917	*E. coli* Nissle 1917 Δ*ppsA* Δ*pckA*
Galactose	0.81	0.81
Gluconate	1.00	1.00
Glucose	0.65	0.65
Glycerol	1.00	1.00
Mannose	0.81	0.81
Alanine	0.52	<0.05
Aspartate	0.68	<0.05
Proline	0.65	<0.05
Pyruvic acid methyl ester	0.68	<0.05
Serine	0.65	<0.05
Succinate	0.97	<0.05

[a] *E. coli* Nissle 1917 Str^r Nal^r and *E. coli* Nissle 1917 Str^r Δ*pckA* Δ*ppsA*::*cat* each displayed the highest final level of tetrazolium reduction when either gluconate or glycerol was used as the sole carbon source (see Materials and Methods). Final levels of tetrazolium reduction on all other carbon sources were normalized to that on glycerol. Results for only 11 of 96 carbon sources tested are shown.

RESULTS

***E. coli* Nissle 1917 uses glycolytic and gluconeogenic nutrients for growth in the mouse intestine.** We reported previously that both *E. coli* MG1655, a commensal strain, and *E. coli* EDL933, an O157:H7 strain, use glycolytic but not gluconeogenic nutrients to colonize the intestines of streptomycin-treated mice when each is the only *E. coli* strain fed to mice (15). It was of interest to determine whether the same is true of *E. coli* Nissle 1917, an effective probiotic strain (18). Phosphoenolpyruvate carboxykinase converts oxaloacetate to phosphoenolpyruvate and is encoded by the *E. coli pckA* gene (33). Phosphoenolpyruvate synthase is encoded by the *E. coli ppsA* gene and converts pyruvate to phosphoenolpyruvate (34). Since the conversion of tricarboxylic acid (TCA) cycle intermediates to phosphoenolpyruvate is completely blocked in a *ppsA pckA* double mutant, this mutant is unable to grow on gluconeogenic nutrients, such as acetate, pyruvate, amino acids that feed into the TCA cycle, fatty acids, and TCA cycle intermediates, as sole carbon and energy sources but should be able to grow on phosphoenolpyruvate and amino acids that feed into the central carbon metabolism "above" pyruvate, as long as the wild-type strain can do so (35). Also, since glycolytic pathways are not affected in a *ppsA pckA* double mutant, this mutant should be able to grow as well as the wild type on sugars as sole carbon and energy sources (35). An *E. coli* Nissle 1917 Δ*ppsA* Δ*pckA* mutant was constructed and, as expected, failed to grow on gluconeogenic nutrients but was still able to grow as well as wild-type *E. coli* Nissle 1917 on glycolytic nutrients (Table 2). Next, we tested the abilities of these strains to colonize mouse intestines.

When 10^5 CFU of *E. coli* Nissle 1917 and 10^5 CFU of *E. coli* Nissle 1917 Δ*ppsA* Δ*pckA* were fed simultaneously to mice, by day 1 postfeeding, each had grown 2 orders of magnitude, suggesting that *E. coli* Nissle 1917 did not use gluconeogenic nutrients for growth in the intestine during that initial period, presumably because glycolytic nutrients were plentiful (Fig. 1). However, from

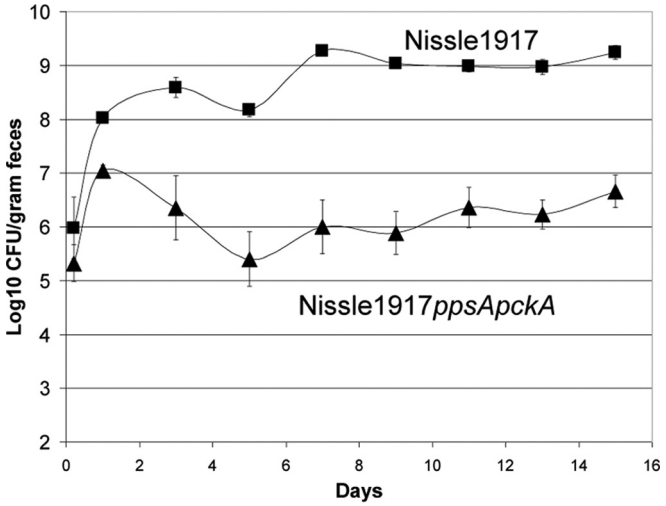

FIG 1 Colonization of the intestines of streptomycin-treated mice by *E. coli* Nissle 1917 and *E. coli* Nissle 1917 Δ*ppsA* Δ*pckA*. Two sets of 3 mice each were fed 10^5 CFU of *E. coli* Nissle 1917 Str^r Nal^r (■) and 10^5 CFU of *E. coli* Nissle 1917 Str^r Δ*ppsA* Δ*pckA*::*cat* (▲). At the indicated times, fecal samples were homogenized, diluted, and plated as described in Materials and Methods. Data are mean $\log_{10}$ CFU per gram of feces for each time point; error bars represent standard errors.

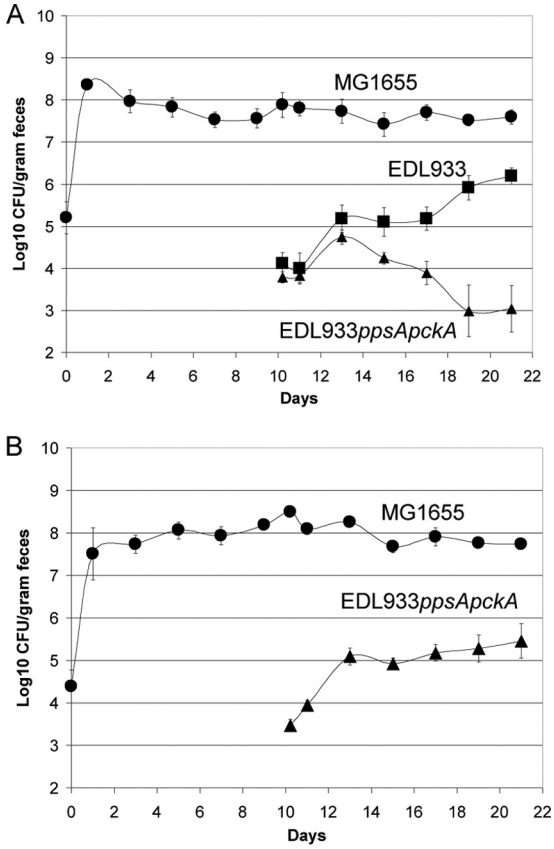

FIG 2 Colonization of the intestines of streptomycin-treated mice by preadministered *E. coli* MG1655, *E. coli* EDL933, and *E. coli* EDL933 Δ*ppsA* Δ*pckA*. (A) Three sets of 3 mice were fed 10^5 CFU of *E. coli* MG1655 Str^r Nal^r on day 0 (●) and 10^5 CFU each of *E. coli* EDL933 Str^r Rif^r (■) and *E. coli* EDL933 Str^r Δ*ppsA* Δ*pckA*::*cat* (▲) on day 10 postfeeding. (B) Three sets of 3 mice were fed 10^5 CFU of *E. coli* MG1655 Str^r Nal^r on day 0 (●) and 10^5 CFU of *E. coli* EDL933 Str^r Δ*ppsA* Δ*pckA*::*cat* (▲) on day 10 postfeeding. At the indicated times, fecal samples were homogenized, diluted, and plated as described in Materials and Methods. Data are mean $\log_{10}$ CFU per gram of feces for each time point; error bars represent standard errors.

day 1 to day 5 postfeeding, *E. coli* Nissle 1917 remained in the intestine in relatively constant numbers, whereas the numbers of *E. coli* Nissle 1917 Δ*ppsA* Δ*pckA* bacteria dropped 2 orders of magnitude, suggesting that during that period, glycolytic nutrients were in short supply, forcing *E. coli* Nissle 1917 to switch, at least in part, to gluconeogenic nutrients, which *E. coli* Nissle 1917 Δ*ppsA* Δ*pckA* was unable to do (Fig. 1). Since we already have established that *E. coli* Nissle 1917 uses glycolytic nutrients for colonization (14), these results also suggest that *E. coli* Nissle 1917 uses both glycolytic and gluconeogenic nutrients for growth in the mouse intestine between day 1 and day 5 postfeeding. Beyond day 5 postfeeding, the ratio of *E. coli* Nissle 1917 to *E. coli* Nissle 1917 Δ*ppsA* Δ*pckA* remained fairly constant (Fig. 1), suggesting either that *E. coli* Nissle 1917 stopped using gluconeogenic nutrients for growth in the intestine or that *E. coli* Nissle 1917 Δ*ppsA* Δ*pckA* maintained itself in constant numbers by switching to the use of one or more glycolytic nutrients not being used by *E. coli* Nissle 1917. Since there is no obvious reason why *E. coli* Nissle 1917 Δ*ppsA* Δ*pckA* would use glycolytic nutrients not being used by *E. coli* Nissle 1917, we favor the hypothesis that beyond day 5 postfeeding, *E. coli* Nissle 1917 and *E. coli* Nissle 1917 Δ*ppsA* Δ*pckA* colonized an intestinal niche in which glycolytic nutrients were in ample supply, so that the use of gluconeogenic nutrients was not necessary, and therefore, *E. coli* Nissle 1917 stopped using them for growth. For discussion purposes, we will assume that this is the case. In contrast to the 2- to 3-order-of-magnitude advantage that *E. coli* Nissle 1917 displayed in the intestine when 10^5 CFU of wild-type *E. coli* Nissle 1917 and 10^5 CFU of *E. coli* Nissle 1917 Δ*ppsA* Δ*pckA* were fed simultaneously to mice, the colonization abilities of these strains were identical when they were fed separately, each colonizing at about 10^9 CFU/g of feces (compare Fig. 3 and 6). Parenthetically, these data make it unlikely that growth or survival in feces has a differential effect on either strain and thereby validate the use of fecal counts to measure their relative colonizing abilities when both strains are fed to mice simultaneously.

***E. coli* EDL933 Δ*ppsA* Δ*pckA* is nearly eliminated from the intestines of mice precolonized with *E. coli* Nissle 1917, but not with *E. coli* MG1655.** Humans infected with *E. coli* O157:H7 strains are presumably colonized with at least one commensal *E. coli* strain prior to infection. To mimic that situation, streptomycin-treated mice can be precolonized with a commensal *E. coli* strain for 10 days and then fed *E. coli* EDL933 (14, 36). When mice were precolonized with the commensal *E. coli* strain MG1655 for 10 days and were then fed 10^5 CFU each of wild-type *E. coli* EDL933 and *E. coli* EDL933 Δ*ppsA* Δ*pckA*, wild-type *E. coli* EDL933 grew to a level between 10^6 and 10^7 CFU/g of feces, whereas *E. coli* EDL933 Δ*ppsA* Δ*pckA* dropped to about 10^3 CFU/g of feces (Fig. 2A), suggesting, as reported previously (15), that under these conditions, *E. coli* EDL933 uses both glycolytic and gluconeogenic nutrients for colonization. In contrast, in mice precolonized with *E. coli* MG1655 for 10 days and then fed 10^5 CFU of *E. coli* EDL933 Δ*ppsA* Δ*pckA* but no wild-type *E. coli* EDL933, the gluconeogenesis mutant maintained itself at a level between 10^5

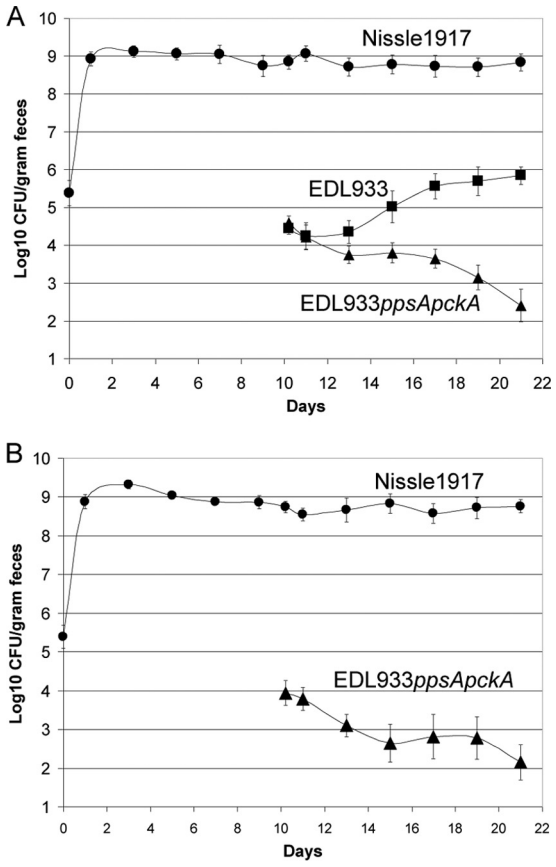

FIG 3 Colonization of the intestines of streptomycin-treated mice by preadministered *E. coli* Nissle 1917, *E. coli* EDL933, and *E. coli* EDL933 Δ*ppsA* Δ*pckA*. (A) Four sets of 3 mice were fed 10^5 CFU of *E. coli* Nissle 1917 Str^r Nal^r on day 0 (●) and 10^5 CFU each of *E. coli* EDL933 Str^r Rif^r (■) and *E. coli* EDL933 Str^r Δ*ppsA* Δ*pckA*::*cat* (▲) on day 10 postfeeding. (B) Four sets of 3 mice were fed 10^5 CFU of *E. coli* Nissle 1917 Str^r Nal^r on day 0 (●) and 10^5 CFU of *E. coli* EDL933 Str^r Δ*ppsA* Δ*pckA*::*cat* (▲) on day 10 postfeeding. At the indicated times, fecal samples were homogenized, diluted, and plated as described in Materials and Methods. Data are mean $\log_{10}$ CFU per gram of feces for each time point; error bars represent standard errors.

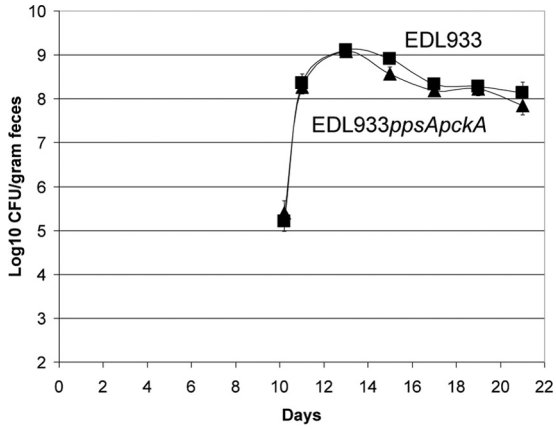

FIG 4 Colonization of the intestines of streptomycin-treated mice by *E. coli* EDL933 and *E. coli* EDL933 Δ*ppsA* Δ*pckA* 10 days after streptomycin treatment. Two sets of 3 mice were fed 10^5 CFU each of *E. coli* EDL933 Str^r Rif^r (■) and *E. coli* EDL933 Str^r Δ*ppsA* Δ*pckA*::*cat* (▲) 10 days after streptomycin treatment had begun. Streptomycin treatment was continued for the duration of the experiment. At the indicated times, fecal samples were homogenized, diluted, and plated as described in Materials and Methods. Data are mean $\log_{10}$ CFU per gram of feces for each time point; error bars represent standard errors.

and 10^6 CFU/g of feces, 100-fold higher than the level in the presence of wild-type *E. coli* EDL933 (Fig. 2B), suggesting that in the absence of wild-type *E. coli* EDL933, enough glycolytic nutrients were available for *E. coli* EDL933 Δ*ppsA* Δ*pckA* to sustain that level in the intestine. In similar experiments, when mice were precolonized with *E. coli* Nissle 1917 for 10 days and were then fed 10^5 CFU each of wild-type *E. coli* EDL933 and *E. coli* EDL933 Δ*ppsA* Δ*pckA*, wild-type *E. coli* EDL933 grew to a level between 10^5 and 10^6 CFU/g of feces, whereas *E. coli* EDL933 Δ*ppsA* Δ*pckA* was nearly eliminated, dropping to about 10^2 CFU/g of feces (Fig. 3A). Moreover, in the absence of wild-type *E. coli* EDL933, *E. coli* EDL933 Δ*ppsA* Δ*pckA* levels still dropped to about 10^2 CFU/g of feces in mice precolonized with *E. coli* Nissle 1917 for 10 days (Fig. 3B), suggesting that in mice precolonized with *E. coli* Nissle 1917, invading *E. coli* EDL933 must use both the available glycolytic and gluconeogenic nutrients to maintain itself in the intestine; i.e., the available concentration of glycolytic nutrients alone is not sufficient. Furthermore, since in the absence of wild-type *E. coli* EDL933, *E. coli* EDL933 Δ*ppsA* Δ*pckA* colonized mice precolonized with *E. coli* Nissle 1917 for 10 days at a level 1,000-fold lower than that for mice precolonized with *E. coli* MG1655 for 10 days (compare Fig. 2B and 3B), it appears that far fewer glycolytic nutrients were available to *E. coli* EDL933 in *E. coli* Nissle 1917-precolonized mice than in *E. coli* MG1655-precolonized mice.

In the absence of precolonization with any commensal *E. coli* strain, *E. coli* EDL933 and *E. coli* EDL933 Δ*ppsA* Δ*pckA* fed to mice treated with streptomycin for 10 days colonize mouse intestines equally well. The data to this point suggested that precolonization of mouse intestines with *E. coli* Nissle 1917 leaves far fewer glycolytic nutrients available to invading *E. coli* EDL933 than does precolonization with *E. coli* MG1655, raising the possibility that *E. coli* EDL933 competes for glycolytic nutrients only/mainly with the preadministered *E. coli* strain and not with the rest of the microbiota. To determine whether *E. coli* EDL933 does or does not compete with the microbiota to colonize, mice were treated with streptomycin but were not precolonized with any *E. coli* strain. At day 10, the mice were fed 10^5 CFU each of *E. coli* EDL933 and *E. coli* EDL933 Δ*ppsA* Δ*pckA*. Indeed, the two strains grew at the same rate in mouse intestines for 3 days, to a level of about 10^9 CFU/g of feces, and cocolonized intestines at a level of about 10^8 CFU/g of feces thereafter (Fig. 4). These results suggest that in the absence of *E. coli* MG1655, *E. coli* Nissle 1917, or any other *E. coli* strain, glycolytic nutrients were in sufficient supply in the intestine such that a switchover to gluconeogenic nutrients by *E. coli* EDL933 was not necessary. Parenthetically, the results show that *E. coli* EDL933 and *E. coli* EDL933 Δ*ppsA* Δ*pckA* grow/survive equally well in feces, thus validating the use of fecal plate counts in experiments comparing the two strains in mice.

Visualization of a dense and diverse microbiota in the cecal mucus of mice treated with streptomycin for 10 days. *E. coli* strains colonize the large intestines of streptomycin-treated mice by growing in the mucus layer as members of mixed biofilms (12, 13), and the glycolytic nutrients they use for growth appear to be mono- and disaccharides produced by the anaerobes as extracellular degradation products of oligo- and polysaccharides (14, 37,

38). Clearly, there were sufficient mono- and disaccharides in the intestines of mice fed streptomycin for 10 days in the absence of any precolonizing *E. coli* strain to allow *E. coli* EDL933 to grow to high numbers without using gluconeogenic nutrients (Fig. 4), suggesting that if a robust anaerobe population was present, it was producing mono- and disaccharides but not using them for growth. Indeed, the cecal mucus layer in mice fed streptomycin for 10 days in the absence of any precolonizing *E. coli* strain did contain a large and diverse microbiota consisting of members of various sizes and shapes, but no *E. coli* (Fig. 5A). As expected, *E. coli* was present in the cecal mucus of mice precolonized with *E. coli* Nissle 1917 for 10 days (Fig. 5B).

Growth of *E. coli* EDL933 and *E. coli* EDL933 Δ*ppsA* Δ*pckA* in the intestines of mice precolonized with *E. coli* Nissle 1917 Δ*ppsA* Δ*pckA*. The data presented in Fig. 1 suggested that *E. coli* Nissle 1917 uses gluconeogenic nutrients for growth in the mouse intestine between day 1 and day 5 postfeeding but then stops using them. However, when mice were precolonized with *E. coli* Nissle 1917 Δ*ppsA* Δ*pckA* for 10 days and were then fed 10^5 CFU each of wild-type *E. coli* EDL933 and *E. coli* EDL933 Δ*ppsA* Δ*pckA*, wild-type *E. coli* EDL933 grew to about 10^7 CFU/g of feces (Fig. 6A), a level >10-fold higher than that in mice precolonized with wild-type *E. coli* Nissle 1917 ($P < 0.00001$) (compare Fig. 3A and 6A, and see Materials and Methods for an explanation of *P* values), suggesting that *E. coli* Nissle 1917 does use gluconeogenic nutrients to limit the growth of *E. coli* EDL933 (Fig. 1). Moreover, *E. coli* EDL933 Δ*ppsA* Δ*pckA* was nearly eliminated, dropping to about 10^2 CFU/g of feces (Fig. 6A), suggesting that *E. coli* EDL933 uses both glycolytic and gluconeogenic nutrients for growth in mice precolonized with *E. coli* Nissle 1917 Δ*ppsA* Δ*pckA*.

We wanted to test further whether *E. coli* Nissle 1917 uses gluconeogenic nutrients to limit the growth of *E. coli* EDL933 in the mouse intestine, as suggested by the preceding experiment. If *E. coli* Nissle 1917 was not using gluconeogenesis to limit the growth of *E. coli* EDL933, precolonization with *E. coli* Nissle 1917 Δ*ppsA* Δ*pckA* should limit colonization by *E. coli* EDL933 Δ*ppsA* Δ*pckA* as well as precolonization with wild-type *E. coli* Nissle 1917 does. However, we found that *E. coli* EDL933 Δ*ppsA* Δ*pckA* colonized the intestines of mice precolonized with *E. coli* Nissle 1917 Δ*ppsA* Δ*pckA* at a level between 10^5 and 10^6 CFU/g of feces (Fig. 6B), >1,000-fold higher than the level in mice precolonized with *E. coli* Nissle 1917 (compare Fig. 3B and 6B). Therefore, it appears that *E. coli* Nissle 1917 does use gluconeogenic nutrients to nearly eliminate *E. coli* EDL933 Δ*ppsA* Δ*pckA* from the mouse intestine, despite appearing to stop using gluconeogenic nutrients 5 days postfeeding (Fig. 1).

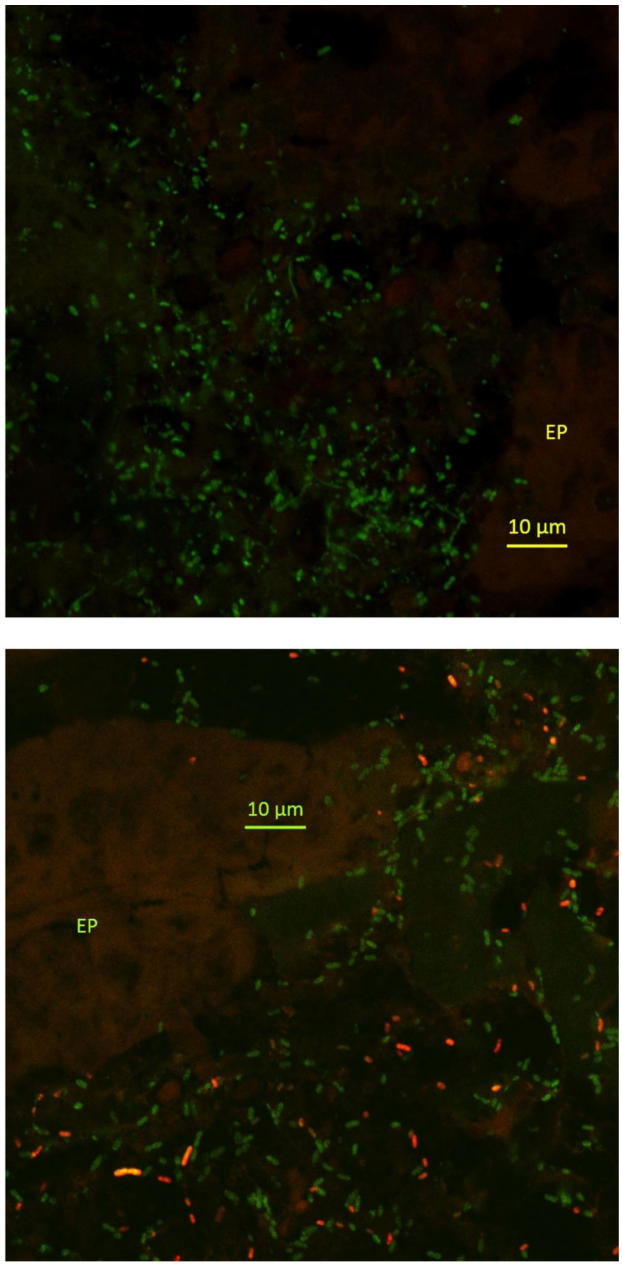

FIG 5 *In situ* hybridization with fluorescence-labeled oligonucleotide probes. Ten days after mice were sham fed without *E. coli* (A), and 10 days after mice were fed *E. coli* Nissle 1917 Str^r Nal^r (B), cecal mucosal sections were hybridized with an *E. coli*-specific oligonucleotide probe (red) and a eubacterium-specific oligonucleotide probe (green). EP, epithelium. *E. coli* cells appear red, while all other bacteria appear green.

DISCUSSION

It was reported recently that *E. coli* 86-24, an O157:H7 strain, activates the expression of virulence genes under gluconeogenic conditions (16, 17). The present study was undertaken to determine whether *E. coli* Nissle 1917, routinely used as a probiotic agent, depletes gluconeogenic nutrients by using them for growth in the mouse intestine, thereby preventing *E. coli* EDL933, also an O157:H7 strain, from activating virulence gene expression. We found that *E. coli* Nissle 1917 does indeed use gluconeogenic nutrients to colonize the mouse intestine (Fig. 1). Yet we also found that *E. coli* EDL933 relies heavily on gluconeogenesis to colonize mice precolonized with *E. coli* Nissle 1917 for 10 days; i.e., under these conditions, wild-type *E. coli* EDL933 colonized at a level almost 10,000-fold higher than that of *E. coli* EDL933 Δ*ppsA* Δ*pckA* at day 21 (Fig. 3A). These data eliminate the possibility that *E. coli* Nissle 1917 depletes the intestine of gluconeogenic nutrients and therefore show that *E. coli* Nissle 1917 would not prevent the activation of virulence gene expression by *E. coli* EDL933, assuming that virulence gene expression is activated in *E. coli* EDL933 as it is in *E. coli* 86-24. We also showed that only under conditions in which *E. coli* EDL933 competes for nutrients with precolonizing *E. coli* strains does it utilize gluconeogenic nutrients

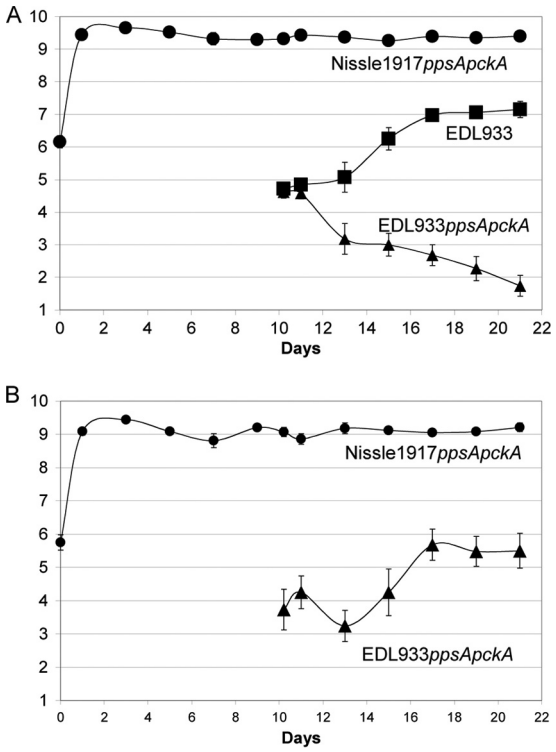

FIG 6 Colonization of the intestines of streptomycin-treated mice by preadministered *E. coli* Nissle 1917 Δ*ppsA* Δ*pckA*, *E. coli* EDL933, and *E. coli* EDL933 Δ*ppsA* Δ*pckA*. (A) Three sets of 3 mice were fed 10^5 CFU of *E. coli* Nissle 1917 Str^r Δ*ppsA* Δ*pckA* on day 0 (●) and 10^5 CFU each of *E. coli* EDL933 Str^r Rif^r (■) and *E. coli* EDL933 Str^r Δ*ppsA* Δ*pckA* (▲) on day 10 postfeeding. (B) Two sets of 3 mice were fed 10^5 CFU of *E. coli* MG1655 Str^r Δ*ppsA* Δ*pckA* on day 0 (●) and 10^5 CFU of *E. coli* EDL933 Str^r Δ*ppsA* Δ*pckA* (▲) on day 10 postfeeding. At the indicated times, fecal samples were homogenized, diluted, and plated as described in Materials and Methods. Data are mean $\log_{10}$ CFU per gram of feces for each time point; error bars represent standard errors.

for growth in the mouse intestine; i.e., in mice that had not been precolonized with any *E. coli* strain, but that contained a dense and apparently diverse microbiota (Fig. 5A), wild-type *E. coli* EDL933 and *E. coli* EDL933 Δ*ppsA* Δ*pckA* grew at the same rate, to about 10^9 CFU/g of feces, and cocolonized mice equally well thereafter, at about 10^8 CFU/g of feces (Fig. 4).

Despite the finding that *E. coli* EDL933 switches in part to gluconeogenesis in both *E. coli* MG1655- and *E. coli* Nissle 1917-precolonized mice (Fig. 2 and 3), differences in the competition of *E. coli* EDL933 Δ*ppsA* Δ*pckA* against *E. coli* MG1655 and against *E. coli* Nissle 1917 were observed. That is, in mice precolonized for 10 days with *E. coli* Nissle 1917 and fed 10^5 CFU of *E. coli* EDL933 Δ*ppsA* Δ*pckA*, the gluconeogenesis mutant was nearly eliminated, colonizing at a level of only 10^2 CFU/g of feces (Fig. 3B). In contrast, in mice precolonized with *E. coli* MG1655 and fed 10^5 CFU of *E. coli* EDL933 Δ*ppsA* Δ*pckA*, the gluconeogenesis mutant colonized at a level of 10^5 CFU/g of feces, 1,000-fold higher than the level in mice precolonized with *E. coli* Nissle 1917 (Fig. 2B). Therefore, it appears that glycolytic nutrients were less available to *E. coli* EDL933 in mice precolonized with *E. coli* Nissle 1917 than in mice precolonized with *E. coli* MG1655. In support of this view, *E. coli* Nissle 1917 colonized the intestine at a level about 10-fold higher than *E. coli* MG1655 ($P < 0.00001$) (compare Fig. 2 and 3) and presumably used more glycolytic nutrients in the process, leaving fewer glycolytic nutrients for *E. coli* EDL933.

We also found that *E. coli* Nissle 1917 uses gluconeogenic nutrients for growth in the intestines of streptomycin-treated mice between day 1 and day 5 postfeeding but appears to stop using them thereafter (Fig. 1). Paradoxically, we found that gluconeogenic nutrients are used by precolonizing *E. coli* Nissle 1917 at and beyond 10 days postfeeding to limit the growth of invading *E. coli* EDL933 (Fig. 3A and B). How, then, does precolonizing *E. coli* Nissle 1917 appear to stop using gluconeogenic nutrients that are available in the mouse intestine beyond 5 days postfeeding and yet use them to limit the growth of invading *E. coli* EDL933 at 10 days postfeeding? This paradox can be explained if beyond 5 days postfeeding, *E. coli* Nissle 1917 resides both in a large niche in the mouse intestine, in which it uses only glycolytic nutrients, and in a much smaller niche, where it competes with invading *E. coli* EDL933 for both glycolytic and gluconeogenic nutrients. This explanation fits well with our "restaurant" hypothesis, which we summarize immediately below and which we recently proposed to account for the fact that *E. coli* mutants that grow poorly relative to their parents in bacterium-free cecal mucus *in vitro* can colonize the mouse intestine better than their parents (12, 13).

It is known that to grow in and colonize the mouse large intestine, *E. coli* strains must penetrate into the mucus layer (10, 11). It is also known that *E. coli* strains reside in mucus as members of mixed biofilms (12, 13). Furthermore, *E. coli* strains use mono- and disaccharides as glycolytic nutrients for growth in the mouse large intestine (14, 37), yet mono- and disaccharides are absorbed in the small intestine. Since most *E. coli* strains do not secrete extracellular polysaccharide hydrolases (38–40) and therefore cannot degrade dietary-fiber-derived and mucin-derived oligo- and polysaccharides, it is likely that during long-term colonization, *E. coli* depends on the anaerobes in mucus that can degrade oligo- and polysaccharides, e.g., *Bacteroides thetaiotaomicron* (41), to provide them with the mono- and disaccharides they use for growth. We hypothesized (13) that during the initiation phase of large-intestine colonization, a commensal *E. coli* strain enters an initiation niche in mucus in which it competes for nutrients from a perfectly mixed pool with any other *E. coli* strain present in that niche. We propose that it is in this niche that *E. coli* Nissle 1917 is a better colonizer than *E. coli* Nissle 1917 Δ*ppsA* Δ*pckA* between day 1 and day 5 postfeeding, because *E. coli* Nissle 1917 is able to use both glycolytic and gluconeogenic nutrients for growth, and it is in this niche that *E. coli* Nissle 1917 competes with invading *E. coli* EDL933 for both glycolytic and gluconeogenic nutrients. We also hypothesized that in addition to continuously colonizing the initiation niche during long-term colonization, each commensal *E. coli* strain also resides on a long-term basis as a member of the mixed biofilms we see in mucus (12, 13), together with specific anaerobes. We called the mixed biofilms "restaurants," and we hypothesized that each commensal *E. coli* strain receives mono- and disaccharides locally from specific anaerobes with which it interacts physically and metabolically rather than from a perfectly mixed pool of nutrients and as such does not compete directly with any other *E. coli* strain within its "restaurant." We hypothesize that these biofilms, presumably formed by 5 days postfeeding, constitute the larger niche, which is colonized equally well by *E. coli* Nissle 1917 and *E. coli* Nissle 1917 Δ*ppsA* Δ*pckA*, because sufficient glycolytic nutrients are made available locally by the anaerobes residing there such that any gluconeogenic nutrients

produced are not needed by *E. coli* Nissle 1917 for growth and therefore diffuse to the smaller initiation niche.

Different *E. coli* strains use different glycolytic nutrients to colonize the intestines of streptomycin-treated mice (14, 37). We recently reported that when mice were precolonized with *E. coli* HS, a human commensal strain, and *E. coli* Nissle 1917, which together use 5 of 7 known glycolytic nutrients used by *E. coli* EDL933 to colonize (14), invading *E. coli* EDL933 bacteria were completely eliminated from the mouse intestine (14). However, precolonizing *E. coli* HS and *E. coli* Nissle 1917 were unable to eliminate *E. coli* CFT073, a uropathogenic *E. coli* (UPEC) strain, or *E. coli* E2348/69, an enteropathogenic *E. coli* (EPEC) strain (14). In fact, both pathogens grew to levels of about 10^8 CFU/g of feces under these conditions (14). Although the possibility exists that *E. coli* CFT073 and *E. coli* E2348/69 use several glycolytic nutrients that *E. coli* HS and *E. coli* Nissle 1917 do not use to colonize the mouse intestine, thus allowing their growth, the data presented here raise the possibility that different *E. coli* pathogens may also use different gluconeogenic nutrients that contribute to their ability to colonize in the face of competition from commensal strains. Further research is required to test this possibility.

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