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GLUCONEOGENESIS AND COLONIZATION OF THE MOUSE INTESTINE BY E. COLI EDL933 AND *E. COLI* NISSLE 1917

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GLUCONEOGENESIS AND COLONIZATION OF THE MOUSE INTESTINE BY *E. COLI* EDL933 AND *E. COLI* NISSLE 1917

BY

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Abstract

Previous studies in this laboratory have indicated that *E. coli* Nissle 1917 can act as a probiotic and is able to prevent the pathogenic *E. coli* EDL933 from growing to high numbers in the mouse large intestine. This study evaluated the influence of gluconeogenesis in *E. coli* Nissle 1917 and *E. coli* EDL933 to compete against each other and for their maintenance in the intestine. Knockout mutants of both strains were created for the analysis of the importance of gluconeogenesis in colonization experiments in the mouse large intestine. To knock out the ability to use gluconeogenesis and therefore metabolizing substrates such as pyruvate, TCA cycle intermediates and amino acids, simultaneously both genes *pckA*, encoding for phosphoenolpyruvate carboxykinase, and *ppsA*, encoding for phosphoenolpyruvate synthetase, were replaced by a non-functional chloramphenicol cassette.

It was shown that *E. coli* Nissle 1917 uses glycolysis and gluconeogenesis concurrently throughout colonization of the mouse large intestine. *E. coli* EDL933 utilized glycolysis exclusively when it was the only *E. coli* strain present, but switched to gluconeogenesis when it was in the presence of a competing *E. coli* strain. Knocking out the ability to use gluconeogenesis in *E. coli* EDL933, led to a severe disability to colonize the mouse large intestine when it competed against *E. coli* Nissle 1917 which used up most of the glycolytic substrates. By implication *E. coli* Nissle 1917 seems to use up most of the glycolytic substrates and some of the gluconeogenic substrates, *E. coli* EDL933 depends on utilizing gluconeogenic substrates to be able to maintain itself in the mouse large intestine. Phenotypic characterization of the usage of different substrates in *E. coli* Nissle 1917 and *E. coli* EDL933 strains revealed several differences between the strains.

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Preface

This thesis is written using the standard format.

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1 Goals of this Study

Escherichia coli EDL933, an O157:H7 strain, is known to be a noninvasive enteric shiga toxin producing pathogen causing gastrointestinal illnesses including hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) in humans [29, 51] and is ingested by eating undercooked food. Because humans are colonized with different commensal *E. coli* strains, differences in nutrient availability may provide open niches for *E. coli* pathogens in some individuals and a barrier to infection in others.

Nowadays the well-established probiotic *E. coli* strain Nissle 1917 is used to treat different intestinal diseases. Prophylactic clinical colonization trials of *E. coli* Nissle 1917 in newborns have been conducted in Germany and Czech Republic [26]. The colonization trials show that intentional colonization of the newborn's gut with *E. coli* Nissle 1917 or other commensal non-pathogenic *E. coli* strains results in early induction of the immunologic competence of the gut-associated lymphoid tissue (GALT). It also results in prevention of hospital-acquired infections and in prevention of colonization by unwanted bacteria such as multidrug resistant or pathogenic *E. coli* strains [26].

Based on findings like these I decided to focus on the interaction of probiotic *E. coli* Nissle 1917 and the pathogen *E. coli* EDL933 during their colonization of the mouse large intestine to better understand how *E. coli* Nissle 1917 acts as a probiotic.

When competing against *E. coli* EDL933, *E. coli* Nissle 1917 is not yet able to outcompete this pathogen completely from the mouse intestine *in vivo* [33]. Little is known about nutrients that are utilized by *E. coli* Nissle 1917 for growth in the intestine or its metabolic pathways involved in competing against *E. coli* EDL933. If these pathways were defined, it would help in developing more efficient treatments for patients infected with O157:H7 strains. Therefore my focus lies on

generating an *E. coli* Nissle 1917 Δ*pckA* Δ*ppsA* knockout mutant, which grows normally on glycolytic substrates but is unable to utilize tricarboxylic acid (TCA) cycle intermediates and gluconeogenic substrates for growth. In addition ability to colonize the mouse intestine in the presence of *E. coli* Nissle 1917 wild type as well as *E. coli* EDL933 was analyzed. The focus lays also on the importance of the glyoxylate shunt, the shortcut of the TCA cycle, as I analyze an *E. coli* Nissle 1917 Δ*aceA* knockout mutant and its ability to colonize the mouse intestine in the presence of *E. coli* Nissle 1917 wild type.

Finally I define if there are differences in *E. coli* Nissle 1917, *E. coli* Nissle 1917 Δ*pckA* Δ*ppsA*, *E. coli* EDL933 and *E. coli* EDL933 Δ*pckA* Δ*ppsA* regarding their oxidation of 95 different carbon sources to clarify whether or not the presence of specific carbon sources in the intestine might have an impact on colonization abilities.

2 Review of Literature

2.1 General background of Escherichia coli.

Escherichia coli belongs to the family Enterobacteriaceae and is the only member of the genus *Escherichia* [58]. Its appearance is a short gram-negative, non-sporing and fimbriate bacillus [58], which grows as a facultative anaerobe readily on simple culture media and synthetic media with glycerol or glucose as the sole carbon and energy source [58]. The primary habitat of *E. coli* is the gastro-intestinal tract and the bowel of mammals and birds. Natural colonization of commensal *E. coli* strains is known to take place soon after birth [5] and its source is to be found in the mother and in the environment [5]. Once established in the gastro-intestinal tract, *E. coli* still remains a minority member of the fecal flora.

2.2 E. coli O157:H7 pathogenesis.

Commensal *E. coli* strains are not harmful, however there are pathogenic *E. coli* strains, such as enterohemorrhagic *Escherichia coli* (EHEC) serotypes like *E. coli* EDL933 O157:H7 [45], a noninvasive enteric shiga toxin producing pathogen which is known to cause gastrointestinal illnesses like hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) in humans [22, 25, 29, 51] and is ingested by eating undercooked food. Serotype O157:H7 is the major source of reported *E. coli* food poisoning outbreaks in the United States (US) [28]. Shiga toxins produced by EHEC bacteria are thought to damage host endothelial cells in small vessels of the intestine, kidney and brain resulting in thrombotic microangiopathy [28].

Etienne-Mesmin et al., 2012 found new key steps in the pathogenesis of EHEC's infection [13]. They postulated that many bacterial pathogens cross the intestinal barrier through microfold cells which are known to transport organisms and particles from the gut lumen to immune cells across the epithelial barrier, and thus are important in stimulating mucosal immunity. Once

passed through the microfold cells, EHEC cells are captured by mucosal macrophages. Since EHEC belongs to Shiga toxin (Stx) producing bacteria and is able to survive and to produce Stx within the macrophages, this translocation of Stx from the gut lumen to underlying tissues is a decisive step in the development of the infection. Following replication of bacteria in macrophages, their extensive Stx production induces macrophage cell death. Subsequently, released Stx can cross the downstream blood vessels to reach the kidneys, intestine, and the brain. Damage to these organs results in serious life-threatening complications in humans [13].

Njoroge et al. 2012 described a novel mechanism of regulation that links metabolism to pathogenesis [47]. The virulence strategy of EHEC to enter through enterocytes is to form attaching and effacing (AE) lesions on enterocytes (see Figure 1). The expression of most of the genes necessary for this enterocyte effacement requires the regulator Ler. Whereas growth within a glycolytic environment inhibits the expression of *ler*, growth within a gluconeogenic environment activates expression of these genes. This is achieved through two transcription factors calles KdpE and Cra, which directly bind to the *ler* promoter and are sugar-dependently regulated [47]. This means that a gluconeogenic environment is a signal for EHEC to turn on pathogenesis.

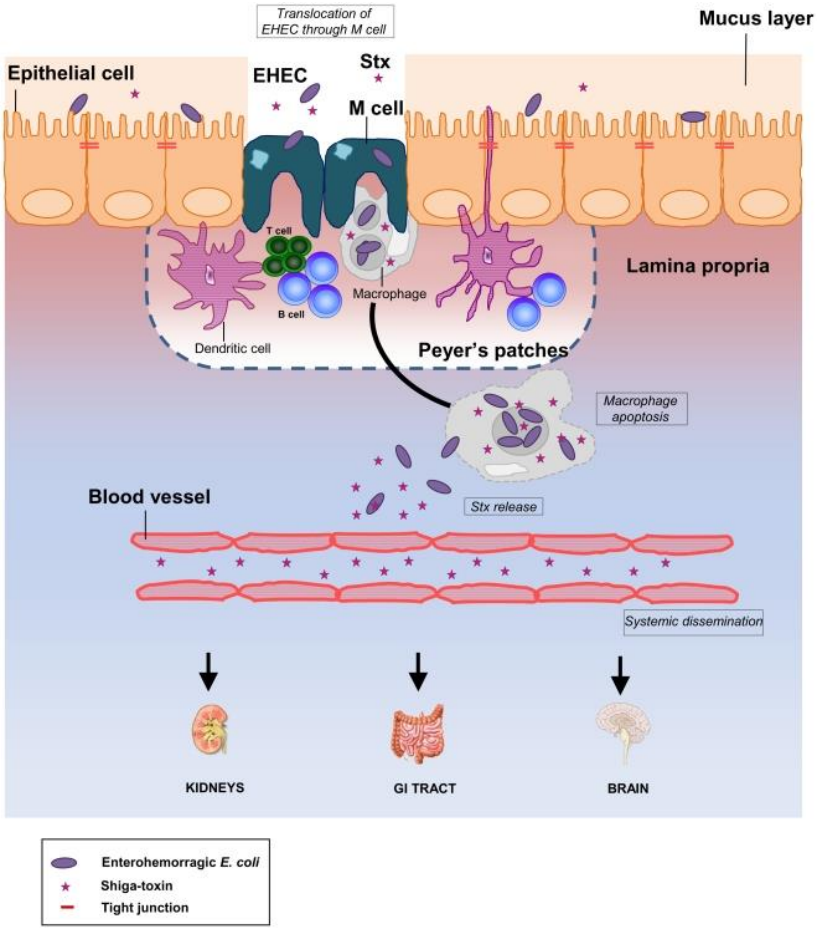

Figure 1: EHEC infection in humans [13]. It is illustrated how EHEC bacteria cross the intestinal epithelial barrier through M cells and reach the lamina propria underneath where they get phagocytosed by macrophages. EHEC's Stx production occurs in the intestine and continues within the macrophages as the bacteria survive their immune response. Stx production within the macrophages leads to apoptosis of macrophages, and thereby releases the Stx toxin which can cross the downstream blood vessels to reach the kidneys, intestine, and brain [13] of the human body.

2.3 Impact of diseases related to E. coli O157:H7 infections

Diseases caused by infection with *E. coli* O157:H7 range from vomiting, stomach cramps, bloody diarrhea to haemolytic-uraemic syndrome (HUS) or haemorrhagic colitis (HC). HUS was first described in 1955 and consists of an acute febrile illness followed by acute renal failure and intravascular haemolysis [58]. HC is characterized by sudden severe abdominal colic and grossly bloody diarrhea and was first described by Riley et al. 1983.

Currently for EHEC infections there is no treatment available [21]. It was found that the use of conventional antibiotics makes Shiga toxin-mediated cytotoxicity worse. In an epidemiology study conducted by the Centers for Disease Control and Prevention, patients treated with antibiotics for EHEC enteritis had a higher risk of developing HUS [56]. Additional studies support the contraindication of antibiotics in EHEC infection. Antibiotics promote Shiga toxin production by enhancing the replication and expression of *stx* genes that are encoded within a chromosomally integrated lambdoid prophage genome [65]. A promising way to treat EHEC infections is the usage of probiotics, such as *E. coli* Nissle 1917. To change the composition of the normal intestinal microbiota from a potentially harmful composition towards a microbiota that would be beneficial for the host has always been the original idea with probiotics [2], [32].

2.4 General background of probiotic Escherichia coli Nissle 1917

E. coli Nissle 1917 belongs to the non-pathogenic *E. coli* strains. It is one of the best analyzed *E. coli* strains so far and is used in the medical field to treat several intestinal or colon related diseases.

Several characteristics make *E. coli* Nissle 1917 such a candidate as a probiotic. It has been shown to produce no toxins like heat-labile or heat-stabile toxins and no Shiga-like toxins, as EDL933 does. *E. coli* Nissle 1917 is non-invasive and doesn't form hemolysins, doesn't possess any *stx* genes [26]. It expresses no CFA I or CFA II fimbriae and has no P-, M-, or S- fimbriae [26].

Focusing on its intestinal colonization ability, *E. coli* Nissle 1917 is generally able to colonize the human gut as well as the mouse intestine [49]. It is able to easily colonize newborns if administered within the first few days after birth, when the colonization resistance is not yet well established. This has been shown in a study in a neonatal unit of a hospital in Hagen as well as in a children's hospital in Prague [11]. Here they postulate that *E. coli* Nissle 1917's

colonization of premature infants stimulates significantly nonspecific natural immunity. To successfully colonize the intestine of adults, it has been shown that this is easily achieved after gut decontamination and/or lavage [32].

2.5 The mammalian large intestine

The large intestine is the terminal part of the digestive system after the stomach and small intestine. The small intestine absorbs most of the nutrients. The large intestine consists of three main sections: the cecum, the colon and the rectum [61]. Its main function is to reabsorb water and inorganic salts [63]. The epithelial surface of the large intestine is unlike the small intestine, rather smooth without intestinal villi and consists of goblet cells and enterocytes [61]. The mucus layer overlies the epithelial surface and is relatively thick. It consists of mucin, a 2-MDa-gelforming glycoprotein and a large number of smaller glycoproteins, proteins, glycolipids, lipids and sugars [3], [50], [60]. The mucus layer is in a dynamic state meaning it is constantly being synthesized and secreted by the mucin-secreting goblet cells and is degraded to a larger extent by the indigenous intestinal microbes [63], [41]. Degraded mucus components are shed into the intestinal lumen, forming a part of the luminal content that is excreted in the feces [41].

To digest and uptake all necessary nutrients the intestine is supported by a huge variety of bacteria colonizing the inner surface and interacting with each other. This is a symbiosis between host intestine and bacteria, since food is presented to bacteria of the digestive system and they in return provide breakdown products of molecules which cannot be broken down by the intestinal enzymes alone. There are about 500 major bacterial strains in the intestine, which interact with each other symbiotically [15]. The microbiota in the intestine of humans and animals consists of a variety of different bacteria including obligate anaerobes such as *Bacteriodes*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Fusobacterium*, *Lactobacillus*,

Peptococcus, *Peptostreptococcus* and *Veillonella* [35, 54]. The obligate anaerobes in the mammalian intestine make up greater than 99.9% of the cultivable bacteria [44]. The predominant facultative anaerobe in the gastrointestinal tract is *Escherichia coli* [15].

According to Freter et al. 1983 there are four microhabitats within each section of the intestine [52]. There is the surface of the epithelial cells, the deep mucus layer of the crypts in the ileum, cecum and colon, the mucus layer that covers the epithelial cells throughout the intestinal tract in the cecum and colon of the mammalian intestine and finally the lumen [52]. Commensal *E. coli* strains colonize the mucus layer covering the epithelial cells [43], whereas especially pathogenic *E. coli* strains colonize the surface of the epithelial cells via attachment to specific receptors [9, 31].

2.6 Mouse models used in colonization studies

The animal model of a conventional laboratory mouse would be ideal to study intestinal colonization of *E. coli*, since its microbiota and biofilm interactions are congruent with those of a conventional human patient. The major problem with this model is to introduce and colonize an invader strain in an established microbiota, meaning to overcome colonization resistance [63]. To prevent this difficulty there are other animal models, such as the germfree animal which doesn't contain an established microbiota, since it has never been exposed to a natural germ-containing environment. This animal model is not only very expensive it also doesn't reflect natural intestinal colonization [63]. The streptomycin-treated mouse model was established in 1954 by Bohnhoff et al. [7]. The advantage of this animal model is that the intestine can be cleared of native facultative anaerobe bacteria [23], such as facultative anaerobic enterobacocci, streptococci, lactobacilli, anaerobic lactobacilli and bifidobacteria purely by adding the antibiotic streptomycin in the drinking water (5g/L). However, besides changes in the concentration of

volatile fatty acids and a decrease of pH (6.42 to 6.73), the numbers of strict anaerobes and populations of the genera *Bacteriodes* and *Eubacterium* remain unchanged [23]. The advantages of this animal model are its relatively low cost and a relatively simple colonization procedure by providing streptomycin in drinking water and feeding streptomycin resistant bacteria to the mice. The streptomycin-treated mouse model is used during this study.

2.7 Nutritional aspects of colonizing the mouse large intestine

The dominant glycoprotein of the intestinal mucus is mucin which consists of about 80% polysaccharide and 20% protein [1]. The five major sugars in mucin are N-acetylgalactosamine, N-acetylglucosamine, N-acetylneuraminic acid, L-fucose and D-galactose [1]. The mucus layer in the mouse intestine also contains smaller proteins, glycoproteins, lipids and glycolipids, and sugars like arabinose, mannose and ribose [1], [50].

As about 500 different cultivable bacteria provide a very diverse microbiota, each organism is best served to cultivate a specific ecological niche in order to be maintained in the intestine. Those ecological niches can be defined by nutrient availability according to Freter et al. 1983 [52], such that the population size of an individual species in the large intestine depends on the available concentration of its preferred nutrient. The population of *E. coli* in the intestine is relatively small, despite its rapid growth rate, which indicates that the concentration of its preferred monosaccharide(s) is low. To survive in the intestine without being physically attached to the host intestinal wall, a bacterial strain would have to out-compete all other organisms for a particular nutrient or nutrient mixture, since failure to do so would lead to displacement from the host. To be able to make predictions about nutritional competition between simultaneously colonizing *E. coli* strains in the mouse large intestine, it is necessary to understand which pathways are utilized by each strain while growing in the intestine.

2.8 The Emden-Meyerhof Parnas pathway (glycolysis)

A major metabolic pathway for glucose utilization is the Entner-Doudoroff pathway, which is present in bacteria but not eukaryotes and is needed to break down gluconate, a sugar also present in the mucus layer of the intestine [59]. The Pentose-Phosphate pathway is needed to produce pentose phosphates and the Emden-Meyerhof Parnas pathway provides glucose for glycolysis into pyruvate.

Glycolysis degrades one molecule of glucose (six-carbon molecule) in a series of enzyme-dependent reactions and yield two molecules of pyruvate (three-carbon molecule) [55]. During several enzyme-catalysed steps, energy is released and conserved in the form of ATP and NADH. Each molecule of pyruvate can then be decarboxylized to yield acetyl-CoA which enters the TCA cycle and serves to conserve more energy [55].

The breakdown of glucose into two molecules of pyruvate occurs during 10 reaction steps [36]. Glucose is first phosphorylated at the hydroxyl group of C-6 (by hexokinase) to D-glucose 6-phosphate. This is further converted into D-fructose 6-phosphate (by phosphohexose isomerase). D-fructose 6-phosphate is phosphorylated at its C-1 leading to D-fructose 1,6-bisphosphate (by phosphofructokinase-1). ATP is for both phosphorylation reactions the phosphoryl group donor. D-fructose 1,6-bisphosphate is further split into dihydroxyacetone phosphate (DHAP) and into glyceraldehyde 3-phosphate (by aldolase). DHAP and glycerol 3-phosphate are isomerized by triose phosphate isomerase. During the next reaction step, two molecules of glyceraldehyde 3-phosphate are phosphorylated by inorganic phosphate to two molecules of 1,3-bisphosphoglycerate (by glyceraldehyde 3-phosphate dehydrogenase), while two molecules of NADH are created. Converting two molecules of 1,3-bisphosphoglycerate by substrate-level phosphorylation into two molecules of 3-phosphoglycerate (by

phosphoglycerate kinase) energy is released in form of two molecules of ATP. The enzyme phosphoglycerate mutase converts two molecules of 3-phosphoglycerate each into 2-phosphoglycerate. Dehydration of two molecules of 2-phosphoglycerate (by enolase) leads to two molecules of phosphoenolpyruvate (PEP). Finally, two molecules of PEP are converted into two molecules of pyruvate via substrate-level phosphorylation (by pyruvate) kinase, releasing energy in the form of two molecules of ATP [46]. The net yield of energy conversion is two molecules of ATP and two molecules of NADH per molecule of glucose. To assure a steady supply of ATP, glycolysis is tightly regulated in coordination with other energy-yielding pathways [55].

2.9 The tricarboxylic acid (TCA) cycle

The TCA cycle can be used both under aerobic and anaerobic conditions. It is the second major stage of respiration which enzymatically further oxidizes the pyruvate produced by glycolysis into H_2O and CO_2 [64]. The energy released during these oxidation reactions is conserved in the reduced coenzymes and electron carriers NADH and $FADH_2$. They themselves undergo oxidation during the third stage of respiration, and transfer their electrons to O_2, producing ATP by the electron flow [46].

The TCA cycle is comprised of eight steps [39]. Pyruvate, the product of glycolysis is converted into acetyl-CoA by pyruvate dehydrogenase, and the acetyl-CoA molecule enters the TCA cycle. First citrate is formed by condensation of the incoming acetyl-CoA with oxaloacetate, catalyzed by citrate synthase. Second isocitrate is formed via cis-aconitate by adding a H_2O molecule catalyzed by aconitase. Third oxidative decarboxylation of isocitrate forms α-ketoglutarate and one molecule of CO_2. This reaction is catalyzed by isocitrate dehydrogenase and also forms a molecule NADH by electron transfer. Fourth another oxidative decarboxylation reaction

produces succinyl-CoA and CO_2. The reaction is catalyzed by α-ketoglutarate dehydrogenase and transfers an electron to NAD^+, leading to NADH. Fifth succinyl-CoA is converted into succinate by succinyl-CoA synthetase and thereby produces GTP. Sixth succinate is oxidized to fumarate catalyzed by succinate dehydrogenase. This reaction also produces $FADH_2$. In a seventh step fumarate is hydrated to malate by fumarase. And in the last eighth step malate dehydrogenase catalyzes the oxidation of malate to oxaloacetate and thereby producing $NADH_2$ [46].

This pathway is cyclic which means that the intermediates of the cycle are not used up. For one molecule of oxaloacetate consumed in the TCA cycle, one is produced. The energy gain per acetyl-CoA oxidized by the TCA cycle are three molecules of NADH, one molecule of $FADH_2$ and one molecule of nucleoside triphosphate (ATP or GTP) [64].

The TCA cycle is not only dependent on entering molecules of acetyl-CoA. Any four or five-carbon intermediate of the TCA cycle can be oxidized further. These intermediates can be products of amino acid breakdowns [36].

2.10 Gluconeogenesis

Gluconeogenesis ("new formation of sugar") is needed as a method for synthesizing glucose from non-carbohydrate precursors by *E. coli* when glucose is depleted. Gluconeogenesis in *E. coli* (see figure 1) starts from simple organic compounds of two or three carbons, such as lactate, acetate and propionate, in their environment or growth medium [46] and it is needed to convert pyruvate and related three- and four-carbon compounds (including TCA cycle intermediates) to glucose [46]. Seven of the reaction steps in gluconeogenesis are catalyzed by the same enzymes as in glycolysis (see glycolysis). Different enzymes are used to reverse three irreversible reaction steps of glycolysis. These reaction steps are first of all the conversion of pyruvate to PEP via oxaloacetate (catalyzed by pyruvate carboxylase and PEP carboxykinase).

Second it is the dephosphorylation of fructose 1,6-bisphosphate by FBPase-1. And third it is the dephosphorylation of glucose 6-phosphate by glucose 6-phosphatase [36]. Gluconeogenesis is energetically expensive as the formation of one molecule of glucose from two molecules of pyruvate requires two molecules of NADH and six high-energy phosphate groups coming from four molecules of ATP and two molecules of GTP [55]. Gluconeogenesis and glycolysis have to be reciprocally regulated to prevent wasteful operation of both pathways at the same time. Some or all of the carbon atoms of most amino acids derived from proteins are ultimately catabolized to pyruvate or to intermediates of the TCA cycle. Such amino acids can therefore undergo net conversion to glucose and are said to be glucogenic [46].

2.11 Glyoxylate shunt

Bacteria like *E. coli* contain the full repertoire of enzymes needed for the glyoxylate cycle as well as those for the TCA cycle in the cytosol. This way they are able to grow on acetate as their sole carbon and energy source [36]. Organisms that lack the glyoxylate shunt cannot synthesize glucose from acetate or fatty acids that give rise to acetyl-CoA. In the glyoxylate cycle , acetyl-CoA is condensed together with oxaloacetate to form citrate. Citrate is further converted into isocitrate, just like in the TCA cycle. Starting from isocitrate both pathway differ. In the glyoxylate cycle isocitrate is cleaved into succinate and glyoxylate by the enzyme isocitrate dehydrogenase. Glyoxylate together with an additional acetyl-CoA forms malate. This reaction is catalyzed by the enzyme called malate synthase. Malate is finally converted into oxaloacetate, which can either condense with another acetyl-CoA and start another turn of the cycle or it can undergo gluconeogenesis and generate glucose. Each turn of the glyoxylate cycle converts two molecules of acetyl-CoA into one molecule of succinate and one molecule of malate. Succinate

can also undergo another turn of the TCA cycle or it can be converted into fumarate, then malate, then oxaloacetate and follow the steps of gluconeogenesis to PEP and glucose [46].

Since the carbon atoms of acetate molecules are converted in 8 steps to oxaloacetate in the TCA cycle, the TCA cycle itself should be able to generate PEP for gluconeogenesis from acetate. But looking at the stoichiometry of the TCA cycle, there is no net conversion of acetate to oxaloacetate. For every two carbons which enter the cycle as acetyl-CoA, two carbons leave as CO_2 which makes it impossible to generate PEP out of it. Looking at the glyoxylate cycle, the two steps of decarboxylation of the TCA cycle are bypassed and with this the net formation of succinate, oxaloactetate and other cycle intermediates from acetyl-CoA becomes possible [46]. As there are many common intermediates being shared between these pathways, they are coordinately regulated. This is accomplished by a covalent but reversible modification of the enzyme isocitrate dehydrogenase. The level of isocitrate dehydrogenase is dependent on its reversible phosphorylation and regulates the partitioning of isocitrate between the TCA cycle and the glyoxylate cycle [39].

2.12 Gene knockout mutants used in colonization experiments

Since the effect of gluconeogenesis during colonization of the mouse large intestine is analyzed and the effect of the glyoxylate shunt is discussed during this study, knockout mutants of the genes which are necessary to utilize both metabolic pathways are generated.

Two required genes involved in gluconeogenesis are *pckA* and *ppsA*. The *pckA* gene encodes for phosphoenolpyruvate (PEP) carboxykinase (EC 4.1.1.49) which is an enzyme catalyzing the first step of gluconeogenesis in *E. coli* [38]. It phosphorylates and decarboxylates the TCA cycle four-carbon intermediate oxaloacetic acid to PEP [53]. PEP carboxykinase is activated by calcium [19] and the pckA gene's expression is regulated by cyclic AMP [20].

The *ppsA* gene encodes for PEP synthetase (EC 2.7.9.2), an enzyme which catalyzes the phosphorylation of pyruvate to PEP, being activated by a Pi-dependent pyrophosphorylation (ATP) and inactivated by an ADP-dependent phosphorylation on a regulatory threonine [8]. During growth on three-carbon substrates that require the gluconeogenesis pathway (such as lactate or pyruvate), phosphoenolpyruvate (PEP) synthetase provides the ability to generate phosphoenolpyruvate, which is required for the synthesis of precursor metabolites for cellular carbon compounds [10].

A Δ*pckA* Δ*ppsA* double mutant does not grow on acetate [48] or succinate [20] as the sole source of carbon, but is able to grow on carbon sources that enter central carbon metabolism "above" PEP such as glycerol.

A required gene involved in the glyoxylate shunt is the gene *aceA*. The *aceA* gene encodes for the enzyme isocitrate lyase (EC 4.1.3.1) which catalyzes the cleavage of isocitrate, forming succinate and glyoxylate, a key step of the glyoxylate shunt. It is activated by phosphorylation on histidine and is inhibited by PEP, 3-phosphoglycerate and succinate [24]. The second reaction of the glyoxylate shunt is catalyzed by an enzyme called malate synthase and catalyses the condensation of glyoxylate with a second molecule of acetyl-CoA, forming malate [34]. The malate is then oxidized to oxaloacetate, which can either start another turn of the cycle or can be converted to PEP by PEP carboxykinase and undergo gluconeogenesis to form glucose [46]. The glyoxylate shunt bypasses two CO_2-evolving steps of the TCA cycle, allowing the net accumulation of carbon from acetyl-CoA [34]. To be able to grow on substrates such as acetate or fatty acids, *E. coli* needs operation of the glyoxylate shunt [30]. This indicates that an *E. coli* Δ*aceA* knockout mutant does not grow on acetate or fatty acids.

In Figure 2 the major metabolic pathways of *E. coli* are shown.

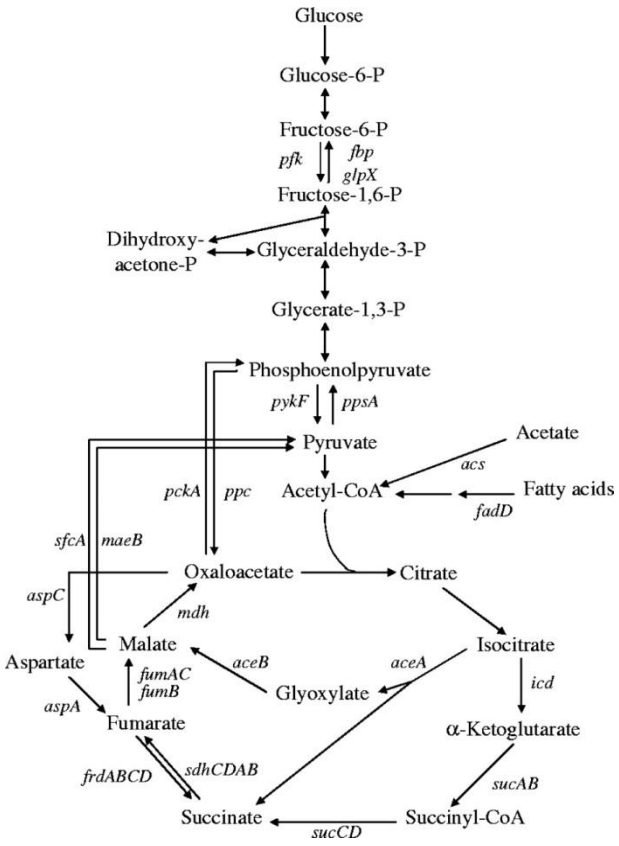

Figure 2: The Embden-Meyerhof-Parnas pathway, gluconeogenic pathway, and TCA cycle. Arrows indicate the physiological directions of the reactions. Genes encoding the enzymes for each reaction are listed beside each reaction [41]. The Embden-Meyerhof-Parnas pathway starts at the six carbon sugar glucose and breaks it down to two molecules of pyruvate (C-3). Pyruvate further enters the TCA cycle and energy in form of ATP, GTP, NADH is released as well as CO_2 as a one carbon molecule. The reverse pathway which is important when there is a depletion of sugar carbon sources in the environment, is the gluconeogenesis pathway. It allows the organism to regenerate glucose out of gluconeogenic substrates such as TCA cycle intermediates, amino acids and other molecules which enter the TCA cycle. Glycolysis yields two high energy phosphate bonds of ATP, gluconeogenesis expends six high energy phosphate bonds of ATP. A futile cycle consisting of both pathways would waste four high energy phosphate bonds of ATP. To prevent this waste glycolysis and gluconeogenesis are reciprocally regulated. To be able to metabolize acetate or fatty acids, the organism has to be able to use the glyoxylate shunt. Both substrates can enter the TCA cycle at acetyl-CoA (C2) and be modified to isocitrate. Starting from isocitrate the TCA cycle is not used anymore, since two molecules of CO_2 would be lost, each during the step leading to alpha-ketoglutarate and the step leading to succinyl-CoA. Since two carbons would be lost, another round of the TCA cycle would be impossible. The only possibilities are to use the glyoxylate shunt avoiding the loss of two molecules of CO_2 as well as using so called anapleurotic reactions such as from PEP to oxaloacetate (C4) which provide two carbons and the TCA can continue further.

3 Methodology

3.1 Agarose gel electrophoresis

This technique is used to separate DNA fragments based on their size and charge. The composition of the gel is 1% [m/v] of multi-purpose agarose (Fisher Scientific, Fair Lawn, NJ) in 100 ml TAE buffer (40 mM Tris-acetate; 1 mM EDTA, Inc., Boulder, CO) with 0.2-0.4 µg/ml of ethidium bromide (Sigma Chemical Co., St. Louis, MO). As the DNA standard marker, a 1 kb DNA Ladder (Promega Corporation, Madison, WI) is chosen.

3.2 Agarose gel photography

To visualize DNA bands, a Fotodyne ultra-violet transilluminator (Fotodyne, Inc., New Berlin, WI) is used and a Kodak DC12 Zoom Digital Camera (Eastman Kodak Company, Rochester, NY) photographs the DNA bands in the gel. Analysis of the digital photographs is done with the help of the Kodak Digital Science 1D software (version 3.0.2; Eastman Kodak Company, Rochester, NY).

3.3 Bacterial strains and plasmids

The bacterial strains as well as the plasmids used in this study are listed in Table 2. *E. coli* Nissle 1917 is a human commensal strain. It has been used as a probiotic agent since the early 1920s. Originally the strain was isolated during World War I from a soldier who escaped a severe outbreak of diarrhea affecting his regiment [57]. Since the 1920s *E. coli* Nissle 1917 has been marketed as a probiotic remedy against intestinal disorders in several European countries. *E. coli* EDL933 is an O157:H7 strain isolated from an outbreak caused by contaminated beef in 1982 [51]. It is known to be a noninvasive enteric shiga toxin producing pathogen causing

gastrointestinal illnesses including hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) in humans [29, 51] and is ingested by eating undercooked food.

3.4 Biolog

The Biolog is a phenotype microarray based on metabolic capabilities. For purposes of this study GN2 MicroPlate™ (Gram negative carbon nutrition) plates are chosen. 95 different nutrients and biochemicals are dried into the wells of each plate (see Table 1).

Table 1: 96 different nutrients of the GN2 MicroPlate ™ [6]. All nutrients and biochemicals are dried into the 96 wells of the plate. The redox dye tetrazolyum violet is used to indicate the utilization of the carbon source. The result is an individual "metabolic fingerprint" of the bacterial strain tested [6].

A1 Water	A2 α-Cyclo-dextrin	A3 Dextrin	A4 Glycogen	A5 Tween 40	A6 Tween 80	A7 N-Acetyl-D-Galacto-samine	A8 N-Acetyl-D-Gluco-samine	A9 Adonitol	A10 L-Arabinose	A11 D-Arabitol	A12 D-Cellobiose
B1 i-Erythritol	B2 D-Fructose	B3 L-Fucose	B4 D-Galactose	B5 Gentiobiose	B6 α-D-Glucose	B7 m-Inositol	B8 α-D-Lactose	B9 Lactulose	B10 Maltose	B11 D-Mannitol	B12 D-Mannose
C1 D-Melibiose	C2 β-Methyl-D-Glucoside	C3 D-Psicose	C4 D-Raffinose	C5 L-Rhamnose	C6 D-Sorbitol	C7 Sucrose	C8 D-Trehalose	C9 Turanose	C10 Xylitol	C11 Pyruvic Acid Methyl Ester	C12 Succinic Acid Mono-Methyl Ester
D1 Acetic Acid	D2 Cis-Aconitic Acid	D3 Citric Acid	D4 Formic Acid	D5 D-Galactonic Acid Lactone	D6 D-Galcturonic Acid	D7 D-Gluconic Acid	D8 D-Gluco-saminic Acid	D9 D-Glucuronic Acid	D10 α-Hydroxy-butyric Acid	D11 β-Hydroxy-butyric Acid	D12 γ-Hydroxy-butyric Acid
E1 p-Hydroxy-phenyl-acetic Acid	E2 Itaconis Acid	E3 α-Keto-butyric Acid	E4 α-Keto-glutaric Acid	E5 α-Keto-valeric Acid	E6 D,L-Lactic Acid	E7 Malonic Acid	E8 Propionic Acid	E9 Quinic Acid	E10 D-Saccharic Acid	E11 Sebacic Acid	E12 Succinic Acid
F1 Bromo-succinic Acid	F2 Succinamic Acid	F3 Glucuron-amide	F4 L-Alaninamide	F5 D-Alanine	F6 L-Alanine	F7 L-Alanyl-Glycine	F8 L-Asparagine	F9 L-Aspartic Acid	F10 L-Glutamic Acid	F11 Glycyl-L-Aspartic Acid	F12 Glycyl-L-Glutamic Acid
G1 L-Histidine	G2 Hydroxy-L-Proline	G3 L-Leucine	G4 L-Ornithine	G5 L-Phenyl-alanine	G6 L-Proline	G7 L-Pyro-glutamic Acid	G8 D-Serine	G9 L-Serine	G10 L-Threonine	G11 D,L-Carnitine	G12 γ-Amino-butyric Acid
H1 Urocanic Acid	H2 Inosine	H3 Uridine	H4 Thymidine	H5 Phenyl-ethylamine	H6 Putrescine	H7 2-Amino-ethanol	H8 2,3-Butanediol	H9 Glycerol	H10 D,L,α-Glycerol-Phosphate	H11 α-D-Glucose-1-Phosphate	H12 D-Glucose-6-Phosphate

The Biolog GN2 MicroPlates are designed to test the ability of gram-negative bacteria to utilize or oxidize compounds from the 96 different carbon sources. This provides a metabolic

fingerprint to each individual strain, which can be compared to other strains to screen for similarities and especially differences in metabolizing these nutrients [6]. Utilizing Biolog GN2 MicroPlates, cells are grown over night in 1x M9 medium containing 0.4 % glycerol and 0.2 M NaCl. Cultures are washed twice in 1x M9 medium containing 0.2 M NaCl without any carbon source and are then diluted to an OD_{600} of 0.06 in 5 ml of 1x M9. The culture is diluted 10 fold before 100 µl/well are delivered into a microtiter 96-well GN2 MicroPlate. The plates are placed into the OmniLog for 48 h at 37 °C. During incubation the cells oxidize the provided carbon sources and simultaneously reduce a tetrazolium violet dye which is measured at 15 min intervals and plotted vs. time using the Biolog's MicroLog computer software. The final outcome is a kinetic curve for each well. The area under the curve is serving as a measurement of oxidation of each carbon source provided in every well. To compare the metabolic fingerprint of two strains (one represented in red the other one in green), the kinetic curves are laid on top of each other, showing overlapping areas (yellow) where the oxidation of carbon sources is the same for both strains or one colored areas indicating utilization of the provided carbon source of only one of the strains [6].

3.5 Construction and characterization of *Escherichia coli* mutants

Gene deletion mutants are constructed by allelic exchange mutagenesis originally described by Datsenko and Wanner [12] and are shown in Table 2.

Table 2: Bacterial strains and plasmids

E. coli strain	Genotype and/or phenotype[a]	Source or reference
E. coli Nissle 1917 Str^r	Spontaneous streptomycin -resistant mutant of Nissle 1917	[4]
E. coli Nissle 1917 Str^r Nal^r	Spontaneous nalidixic acid-resistant mutant of Nissle 1917 Str^r	[4]
E. coli Nissle Str^r Δ*pckA* Δ*ppsA* :: cat	*pckA ppsA* double deletion mutant of Nissle 1917 Str^r, carrying a chloramphenicol resistance cassette in the *ppsA* deletion, unable to metabolize gluconeogenic substrates	This study
E. coli Nissle Str^r Δ*aceA*:: cat	Replacement of *aceA* gene by a chloramphenicol resistance cassette, unable to metabolize fatty acids and actetate	This study
E. coli EDL933 Str^r	Spontaneous streptomycin-resistant mutant of EDL933	[42]
E. coli EDL933 Str^r Rif^r	Spontaneous rifampin-resistant mutant of EDL933 Str^r	[33]
E. coli EDL933 Str^r Δ*pckA* Δ*ppsA*:: cat	*pckA ppsA* double deletion mutant of EDL933 Str^r, carrying a chloramphenicol resistance cassette in the *ppsA* deletion, unable to metabolize gluconeogenic substrates	[42]
pKD3	Template plasmid, contains chloramphenicol resistance cassette flanked by FRT (FLP recombinase) sites	[12]
pKD46	Temperature-sensitive plasmid, contains L-arabinose-inducible λ-red recombinase gene for homologous recombination	[12]
pCP20	Temperature-sensitive plasmid, contains FLP recombinase gene for removal of antibiotic resistance genes	[12]

[a] Str^r, streptomycin resistance; cat, chloramphenicol resistance; Rif^r, rifampin resistance

The complete sequence of *E. coli* Nissle 1917 is not available. Therefore, for constructing the *E. coli* Nissle 1917 mutants, the primers are designed referring to the complete genome of *E. coli* CFT073 (see Table 3), a strain closely related to *E. coli* Nissle 1917. Both forward and reverse primers are designed with flanking FLP Recombinase Site (FRT). The gene knockouts take place via substitution by a cassette construct containing the FRT sites, the homologous regions of the

gene to knock out, and a chloramphenicol sequence as a selection marker. The chloramphenicol cassette is PCR amplified using pKD3 as template.

Table 3: PCR primers used for deletion and confirmation of the deletion. Upper case letters of the primer sequences are *E. coli* gene specific, and bold upper case letters are specific to plasmid pKD3.

PCR primer	Primer sequences in 5'⟶3' direction
E. coli Nissle *pckA* forward primer	5'-GTTAATTATCGCATCCGGGCA-3'
E. coli Nissle *pckA* reverse primer	5'-GTTGTCGATAAACAGTTTCGCCAG-3'
E. coli Nissle *pckA* P1 primer	5'-AGACTTTACTATTCAGGCAATACATATTGGCTA AGGAGCAGTG**GTGTAGGCTGGAGCTGCTTCG**-3'
E. coli Nissle *pckA* P2primer	5'-GCGGGTATCTTTAATCGAGATACGTTTGCC AGTGCCGTTCCAGC**ATATGAATATCCTCCTTAG**-3'
E. coli Nissle *ppsA* forward primer	5'-CCTGTGTGGTTGCAATGTCCG-3'
E. coli Nissle *ppsA* reverse primer	5'-TTGTTCTTCCCGTGATGCAGAC-3'
E. coli Nissle *ppsA* forward2 primer	5'-AACCACCGCTTTCGCCTGA-3'
E. coli Nissle *ppsA* reverse2 primer	5'-CAGAAGATATGCCGGACGCTTCT-3'
Wanner primer towards P1 (start inside Cam-cassette)	5'-CAACAGTACTGCGATGAGTGGCAG-3'
Wanner primer towards P2 (start inside Cam-cassette)	5'-GCGAAGTGATCTTCCGTCACAGGT-3'
E. coli Nissle, EDL *aceA* forward primer	5'-ACGCTTGATGGAACAGATCACC-3'
E. coli Nissle, EDL *aceA* reverse primer	5'-CGAAGTAACCTGTACCCACTTCC-3'
E. coli Nissle, EDL *aceA* P1 primer	5'-TGAAGAATTACAGAAAGAGTGGACTCAACC GCGTTGGGAAGG**CATATGAATATCCTCCTTAGT**-3'
E. coli Nissle, EDL *aceA* P2 primer	5'-GCGAGCAGTTATAAGCCAGCAGTTTGCCCGGA TATTTCGCGTGG**GTGTAGGCTGGAGCTGCTTCG**-3'

Cells to be transformed carry the plasmid pKD46 which is a necessary tool as it is temperature sensitive and contains an ampicillin resistance gene as well as a λ-red recombinase needed for homologous recombination. The cells are grown in 10 ml super optimal broth (SOB) medium [12] at 30 °C to an OD_{600} of 0.6 and are then made electrocompetent by washing three times with ice-cold 10 % glycerol and concentrating the cell concentration 100-fold. 50 µl of the cells mixed with 2 µl of the PCR amplicons are electroporated using the instructions of the Bio-Rad gene pulser at 2.5 V and immediately recovered by adding 950 µl of SOB medium and shaking it at 37 °C with 200 rpm for at least 2 h. After 2 h homologous recombination should have taken place and 300 µl of the culture suspension is plated on LB plates containing chloramphenicol (30 µg/ml) to select chloramphenicol resistant transformants. After 24 h remaining at room temperature, the remainder is plated as well. After primary selection at 37 °C, mutants are colony-purified and additionally tested for ampicillin sensitivity to make sure the mutants have lost the pKD46 plasmid. Since pKD46 is temperature-sensitive, it gets lost by growing cells at 43°C. Using this allelic replacement method the genes *aceA*, *pckA* or *ppsA* are knocked out in *E. coli* Nissle 1917.

Since genes *pckA* and *ppsA* have to be knocked out in the same organism to asure that its ability to use gluconeogenesis is lost completely, the protocol of constructing a second gene deletion is followed. To be able to use the same chloramphenicol cassette with different homologous regions of the corresponding gene, the already inserted chloramphenicol cassette is removed by electroporation with pCP20. This plasmid contains a FLP recombinase to remove the sequence between both FRT sites. The procedure is repeated as already described except for growing and recovering the cells in LB broth instead of SOB medium and for plating transformants on LB plates containing ampicillin (100 µg/ml). To lose pCP20 again, the cell culture is grown in LB broth at 43 °C for a maximum of 5 h and plated on LB plates at 37 °C. The final selection is done

by toothpicking on LB, chloramphenicol and ampicillin plates to select for Amp^S and Cam^S mutants. These mutants have lost the chloramphenicol cassette and are prepared to generate the second mutation. To knockout the second gene, electroporation with the appropriate PCR amplicon is done as previously described for the first gene knockout. Each mutant is verified via PCR and DNA sequencing.

3.6 DNA Sequencing

Sequencing is done at the University of Rhode Island Genomic and Sequencing Center, University of Rhode Island, Kingston, using the Appled Biosystems 3130xl Genetic Analyzer (Applied Biosystems, Foster City CA).

3.7 Growth media and conditions

Bacterial cultures are usually grown in Luria broth (LB) (by Difco Laboratories, Detroit, MI). Routine bacterial growth on plates is on LB agar (Lennox formulation; Difco Laboratories, Detroit, MI) containing appropriate antibiotics. LB agar consists of Luria broth containing 12g of Bacto Agar (Difco) per liter ddH_2O. For Transformation experiments liquid cultures are grown and recovered in super optimal broth (SOB) medium according to the description of Datsenko and Wanner [12]. It consists of 2% [m/v] Bacto™-tryptone (Difco Laboratories, Detroit, MI), 0.5% [m/v] yeast extract (Difco Laboratories, Detroit, MI), 10 mM NaCl (Sigma Chemical Co., St. Louis,MO), 2.5 mM KCl (Sigma Chemical Co., St. Louis,MO), 10 mM $MgCl_2 \cdot 6\ H_2O$ (Sigma Chemical Co., St. Louis, MO), and 10 mM $MgSO_4 \cdot 7\ H_2O$ (Sigma Chemical Co., St. Louis, MO).

M9 minimal broth medium as described by Miller [40] is used to determine bacterial growth and utilization of a manually added carbon source. M9 minimal medium consists of 90% ddH_2O, 10% 10x M9 salts, 1 mM $MgSO_4$ solution and 100 µM $CaCl_2$ solution. The needed 10x M9 salt solution is made of 60 g Na_2HPO_4, 30 g KH_2PO_4, 10 g NH_4Cl and 5 g NaCl dissolved in ddH_2O up to 1 liter.

The carbon sources are added to the M9 minimal broth medium at concentrations of glycerol (0.4% [v/v]), acetate (0.2% [m/v]), and succinate (0.2% [m/v]). These carbon sources were all purchased from Sigma Chemical Co., St. Louis, MO. The M9 medium is used at its original 0.1 M NaCl concentration as well as at a total concentration of 0.2 M NaCl, since this is the molarity found in the mammalian intestine and therefore closely relates to the *in vivo* circumstances in natural colonization environments. For testing the ability of *E. coli* strains to utilize succinate or acetate, the overnight cultures grown in LB are washed twice with M9 minimal medium without any carbon source. 100µl of the washed culture are transferred into 10 ml of M9 minimal medium containing glycerol (0.4% [v/v]) as the only carbon source. The bacterial cultures are incubated overnight at 37°C in 125 ml tissue culture bottles placed in a shaking bath (200 rpm). The following day 100µl of the grown culture are transferred into freshly made M9 minimal medium containing the carbon source of interest. Growth is monitored spectroscopically (OD_{600}) using a Pharmacia Biotech Ultrospec 2000 UV/Visible Spectrometer.

For colonization experiments *E. coli* strains are plated and selected on MacConkey agar plates (Difco Laboratories, Detroit, MI) containing appropriate antibiotics. The plates are prepared as described in the package instructions. Bacteria plated on MacConkey agar are incubated overnight at 37°C unless otherwise indicated. All bacterial culture dilutions for colonization experiments are made in 1% [m/v] Bacto™-tryptone (Fisher Scientific, Fair Lawn, NJ).

The antibiotics streptomycin sulfate, chloramphenicol, and nalidixic acid are purchased from Sigma-Aldrich (St. Louis, MO). Rifampin is purchased from Fisher Scientific (Pittsburgh, PA). Final concentrations of these antibiotics in media unless otherwise indicated are streptomycin sulfate (100 µg/ml), nalidixic acid (50 µg/ml), chloramphenicol (30 µg/ml) and rifampin (50 µg/ml).

3.8 Growth of bacterial cultures

To determine bacterial growth the cell density can be spectrometrically measured using a Pharmacia Biotech Ultrospec 2000 UV/visible spectrometer, since the light beam which passes through the cuvette is scattered depending on the cell density. The optical density (OD) is proportional to the cell density. The OD measurement occurs at a wavelength of 600 nm. The accurate detection range lies between the OD of 0.1 and 0.6 which requires appropriate culture dilutions. A growth curve can be made by plotting the OD_{600} value versus time.

3.9 Isolation, purification and quantification of DNA

Overnight cultures are grown in LB broth at 37 °C. To isolate genomic DNA the Promega Wizard Genomic DNA isolation kit (Catalog number A1120 Promega Corporation, Madison, WI) is used. Storage of isolated genomic DNA is at -20°C. Plasmid DNA is isolated using Eppendorf's Fast Plasmid Mini kit (Catalog number 955150601 Brinkmann Instruments, Inc., Westbury, NY). Overnight cultures for this procedure are grown in LB broth at 30°C and the plasmid DNA is stored at -20°C.

PCR amplified DNA products are concentrated via ethanol precipitation by adding one tenth of its volume of 3 M sodium acetate pH 5.4 (Sigma) and adding 800 µl of 95 % ethanol (Quantum Chemical Co., Tuscula, IL). DNA precipitation is done for 24 h at 4 °C. Afterwards the DNA is pelleted in a microcentrifuge for 10 minutes at 13,000 x g. The supernatant is removed, the pellet washed by centrifugation with 700 µl of 70 % ethanol and air dried for 10 min. The last step is rehydration in nuclease free water (Molecular Biology Grade PCR water, Fisher Scientific, Pittsburg, PA) for several hours. This PCR product is also stored at -20°C.

For sequencing purposes PCR amplified genomic DNA is purified using the instructions by the QIAGEN QIA quick PCR purification kit (Catalog number 28104).

3.10 Streptomycin-treated mouse model used for colonization experiments

The streptomycin-treated mouse model is used to investigate the *in vivo* carbon nutrition of *E. coli* strains and to study the competition in the intestine between streptomycin-resistant *E. coli* strains. Since the numbers of an *E. coli* strain in the mouse large intestine is reflected by their numbers in mouse feces [27], fecal counts are used to judge the relative colonizing abilities of *E. coli* mutants and wild type strains. The streptomycin-treated mouse model has been extensively used by Dr. Cohen's laboratory group [9, 14, 17, 18, 43, 49, 60, 62] and is well established. Treatment of mice with streptomycin during the whole time of colonization is needed to overcome colonization resistance on one hand by the loss of facultative anaerobes from the microbiota and on the other hand by the observed decrease in the concentrations of short-chain fatty acids and hydrogen sulfide [23]. The antibiotic streptomycin is a protein synthesis inhibitor which prevents binding of the $tRNA^{fMet}$ to the bacterial ribosome and therefore leads to codon misreading, protein synthesis inhibition and finally to cell death [66]. *E. coli* Nissle 1917 Str^R, and *E. coli* EDL933 Str^R contain the same point mutation in *rpsL* which makes them resistant to greater than 2 mg/ml of streptomycin sulfate [16]. Some of the introduced *E. coli* strains used in the colonization experiments are, in addition to being streptomycin resistant, also resistant to either chloramphenicol, nalidixic acid, or rifampin, genetic markers that have no effect on their colonization abilities [9, 42, 43].

For colonization experiments mice in the animal facilities in Morrill Hall are given streptomycin sulfate in their drinking water (5 g/liter) over the entire course of these experiments, which selectively removes facultative anaerobic *E. coli*, enterococci, streptococci, lactobacilli, and anaerobic lactobacilli and bifidobacteria [23]. It is important to note that the overall populations of anaerobes, including *Bacteroides* and *Eubacterium*, are unchanged in the cecal contents following streptomycin treatment [23].

The specific procedure of colonization experiments in streptomycin-treated mice has been already described [37, 59, 60]. Briefly, three male CD-1 mice (5 to 8 weeks old) are given drinking water containing streptomycin sulfate (5 g/liter) for 24 h. Following this treatment the mice are starved for food and water for 18h and are then orally fed 1ml of 20% sucrose solution containing either 10^5 CFU (low feed) or 10^{10} CFU (high feed) of the *E. coli* strains of interest. After ingesting the bacterial suspension, both the food (Teklad mouse and rat diet; Harlan, Madison, WI) and streptomycin-water are returned to the mice, and fecal plate counts are determined with 1 g of feces collected after 5 h, 24 h, and on odd-numbered days. All bacterial concentrations are expressed as colony forming units (CFU) of the bacterium per gram of collected feces. Mice are housed individually in cages without bedding and are placed in clean cages at 24-h intervals. Each fecal sample is homogenized and diluted in 1 % Bacto-Tryptone (Difco), and then plated on MacConkey agar plates with appropriate antibiotics. MacConkey agar plates contain either streptomycin sulfate (100 μg/ml); streptomycin sulfate (100 μg/ml) and nalidixic acid (50 μg/ml); streptomycin sulfate (100 μg/ml) and chloramphenicol (30 μg/ml); or streptomycin sulfate (100 μg/ml) and rifampin (50 μg/ml). Streptomycin sulfate, chloramphenicol, and nalidixic acid are purchased from Sigma-Aldrich (St. Louis, MO). Rifampin is purchased from Fisher Scientific (Pittsburgh, PA). All plates are incubated for 18 to 48 h at 37 °C prior to counting.

Statistics. For each strain in the mice the $\log_{10}$ mean number of CFU per gram of feces plus or minus the standard error are calculated for each time point. A difference in all experiments between two strains of about 10-fold CFU/gram of feces (1 order of magnitude) is regarded as being statistically significant, if the *P* value is <0.05 using Student's *t* test (two-tailed with unequal variance).

4 Results

The streptomycin-treated mouse model is well established for colonization experiments of *Escherichia coli* in the mouse large intestine and has been extensively used by Dr. Cohen's laboratory group [9, 14, 17, 18, 43, 49, 59, 62]. The optimal colonization length has been established by Dr. Cohen's laboratory as colonizing bacteria in the mouse large intestine during 15 days for a short colonization experiment and 21 days for a long colonization experiment. Colonization experiments with an *E. coli* strain in the mouse large intestine during 15 days allows the analysis of their initiation and maintenance in the presence of strict anaerobes alone or also in the presence of another *E. coli* strain simultaneously fed to the mice. The rest of the facultative anaerobe members of the natural microflora can't have an impact on the colonization experiment since they are removed from the large intestine by usage of streptomycin (5g/L) in the mice's drinking water. Colonization experiments during a time period of 21 days is needed when an *E. coli* strain is fed ten days after precolonization of the mouse large intestine with a former fed *E. coli* strain. This shows how the late fed *E. coli* strain can compete against an established *E. coli* strain in the intestine. All colonizations are repeated at least once when not indicated differently. In the presence of discordant values for bacterial concentrations in one mouse, the colonization experiment is repeated as many times as needed to be able to recognize a clear behavior of the bacterial concentration throughout the experiment.

4.1 Sequencing and growth of mutants *in vitro*.

All mutants used in this study were sequenced at the University of Rhode Island Genomic and Sequencing Center and all generated deletions were in the expected places in the genome (data not shown). To test the *in vitro* ability to grow on certain substrates, each mutant was tested for

growth in M9 minimal medium containing 0.4% [v/v] glycerol, 0.4% [m/v] succinate, α-ketoglutarate or acetate as the sole carbon and energy source. In glycerol as the only carbon source all mutants grow normally. All mutants having a Δ*pckA* Δ*ppsA* double deletion failed to grow on succinate or on α-ketoglutarate as the sole carbon source. All mutants having a Δ*aceA* deletion failed to grow on acetate as the sole carbon source (data not shown).

4.2 *E. coli* Nissle 1917 uses gluconeogenic substrates as well as glycolytic substrates to colonize the mouse large intestine, when it is the only strain fed to mice

It was determined whether or not the gluconeogenic pathway is important for *E. coli* Nissle 1917 to act as a probiotic strain and to prevent *E. coli* EDL933 from colonizing the mouse large intestine [33]. Gluconeogenesis is generally needed to regenerate the six carbon sugar glucose from gluconeogenic substrates such as pyruvate, TCA cycle intermediates, fatty acids and amino acids being converted into TCA cycle intermediates. To create an *E. coli* mutant which is not able to utilize gluconeogenesis in *E. coli* Nissle 1917 as well as in *E. coli* EDL933, both genes *pckA* (PEP carboxykinase) and *ppsA* (PEP synthetase) were knocked out simultaneously using the allelic recombination method by Wanner and Datsenko [12].

To determine if *E. coli* Nissle 1917 requires gluconeogenic or glycolytic nutrients to colonize the mouse large intestine when it is the only strain fed to mice, *E. coli* Nissle 1917 Str^R Δ*ppsA* Δ*pckA*::*Cam* was co-colonized with its parent strain (Figure 3). *E. coli* Nissle 1917 Str^R Nal^R was simultaneously fed to mice at a low concentration (5.45×10^5 CFU/mouse) together with a low concentration (5.26×10^5 CFU/mouse) of *E. coli* Nissle 1917 Str^R Δ*ppsA* Δ*pckA*::*Cam* (Figure 3). Starting from day one of the colonization experiment both *E. coli* strains show a 10^1 CFU/gram of feces difference. Throughout the colonization in the mouse large intestine the difference between both *E. coli* strains becomes larger (10^2-10^3 CFU/gram of feces) and the number of *E.*

coli Nissle 1917 Str^R Δ*ppsA* Δ*pckA*::*Cam* at day fifteen ends of being 10^2 CFU/gram of feces lower than *E. coli* Nissle 1917 Str^R Nal^R in the intestine. This experiment indicates that *E. coli* Nissle 1917 uses both glycolysis and gluconeogenesis to colonize the mouse large intestine, when it is the only *E. coli* strain present.

4.3 *E. coli* EDL933 utilizes only glycolytic substrates when it is the only strain present during colonization of the mouse large intestine.

Co-colonization of *E. coli* EDL933 Str^R Rif^R and *E. coli* EDL933 Str^R Δ*ppsA* Δ*pckA*::*Cam* in the mouse large intestine shows that the colonization ability of both strains remains the same throughout the colonization when they are the only *E. coli* strain present (Figure 4). The wild type strain colonizes the mouse large intestine beginning from day one with a concentration of 1.9×10^9 CFU/gram of feces, only tenfold higher than its mutant (1.9×10^8 CFU/gram of feces). Towards the end of the colonization both strains colonize the intestine with equal concentrations of 3.2×10^7 CFU/gram of feces (Figure 4). This result confirms the published data by Miranda et al. [42]. Since the gluconeogenic knockout mutant colonizes the intestine about as well as the wild type, it appears that *E. coli* EDL933 uses mainly glycolysis to maintain itself in the intestine and is not dependent on gluconeogenesis, when it is the only *E. coli* strain present.

4.4 In the presence of precolonized *E. coli* Nissle 1917, *E. coli* EDL933 uses both glycolytic substrates and gluconeogenic substrates in order to colonize.

It has been shown [42] that *E. coli* EDL933 switches from glycolytic substrates to gluconeogenic substrates when competing against *E. coli* MG1655 which only uses glycolytic substrates in order to maintain itself in the mouse large intestine.

To answer the question as to whether *E. coli* Nissle 1917 uses up both glycolytic as well as gluconeogenic substrates and therefore is able to limit the colonization of *E. coli* EDL933 in the

intestine, both *E. coli* EDL933 strains, wild type simultaneously together with its gluconeogenic knockout mutant, were fed to mice precolonized with *E. coli* Nissle 1917 wild type (Figure 5), and in another colonization experiment with *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA::Cam*, which is not able to use gluconeogenic substrates (Figure 6). Concentrating on the colonization result of competing against *E. coli* Nissle 1917 wild type (Figure 5), it is interesting that *E. coli* EDL933 wild type grew up to about 1.7 x 10^6 CFU/gram of feces whereas *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* dropped down to numbers of below 10^2 CFU/gram of feces at day twenty-one. The difference of maintenance in the intestine between both *E. coli* EDL933 strains is therefore 10^4-10^5 CFU/gram of feces towards the end of the colonization experiment. Competing against *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA::Cam* only utilizing glycolysis (Figure 6), made it possible for *E. coli* EDL933 wild type to grow up to 1.4 x 10^7 CFU/gram of feces and therefore about an order of magnitude higher than competing against *E. coli* Nissle 1917 wild type. The difference of maintenance in the intestine between both *E. coli* EDL933 strains is 10^5-10^6 CFU/gram of feces towards the end of the colonization experiment. The fact that *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* dropped down in both colonizations shows that *E. coli* Nissle 1917 uses up the majority of glycolytic substrates available in the mouse large intestine. *E. coli* EDL933 can therefore only grow up against *E. coli* Nissle 1917 when it utilizes both glycolysis and gluconeogenesis.

4.5 *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* is not limited by the presence of *E. coli* EDL933, but is outcompeted for nutrients by the presence of *E. coli* Nissle 1917 alone

To determine whether *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* was outcompeted in mice precolonized with either E. coli Nissle 1917 or E. coli Nissle Str^R Δ*pckA* Δ*ppsA::Cam*, because of

the presence of the *E. coli* EDL933 wild type strain, both colonizations were repeated in the absence of *E. coli* EDL933.

E. coli EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* competing against precolonized *E. coli* Nissle 1917 (Figure 7) was fed at day ten (3.8×10^3 CFU/gram of feces) and reached 4×10^3 CFU/gram of feces after one day. Afterwards it gradually decreased until on day fifteen it reached and stayed at about 70 CFU/gram of feces throughout the end of the colonization.

E. coli EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* competing against precolonized *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA::Cam* (Figure 8) was fed at day ten (4.4×10^4 CFU/gram of feces) and dropped gradually from the beginning on until it reached a low of 2×10^2 CFU/gram of feces on day fifteen. After that it came up and leveled off at around 1.5×10^3 CFU/gram of feces.

In both cases, competing against precolonized *E. coli* Nissle wild type or Nissle Str^R Δ*pckA* Δ*ppsA::Cam*, *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* dropped down to 10^2 (Figure 7) and 10^3 CFU/gram of feces (Figure 8) respectively at day twenty-one. This indicates that, while competing against another *E. coli* strain such as *E. coli* Nissle 1917, the presence of E. *coli* EDL933 wild type doesn't have much of an impact on *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* struggle to maintain itself in the intestine.

4.6 In the absence of *E. coli* Nissle 1917, the rest of the microbiota in the mouse large intestine doesn't use up glycolytic substrates, which makes it possible for *E. coli* EDL933 to grow to high numbers

The question now was whether or not *E. coli* Nissle 1917 alone was responsible for using up all glycolytic and much of the gluconeogenic substrates, which keeps *E. coli* EDL933 down or does the anaerobic microbiota contribute? The colonization of *E. coli* EDL933 and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* being fed at day ten without precolonizing another E. strain (Figure 9), but

providing streptomycin containing drinking water throughout the whole colonization, creates clarity. The antibiotic streptomycin in the drinking water (5g/L) of the mice clears out all facultative anaerobes, which leaves a lot of nutrients behind, which can be used for incoming *E. coli* strains to get established in the intestine. The idea of this colonization was to test the effect of the rest of the microbiota in using up all nutrients during the first ten days of the colonization and then analyzing the reaction of both *E. coli* EDL933 and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* without the presence of anther *E. coli* strain.

As shown in Figure 9 both *E. coli* EDL933 strains grew up to high numbers around 10^8-10^9 CFU/gram of feces and remained on top of each other throughout the whole colonization experiment. Since wild type and mutant colonize all mice equally well, the microbiota doesn't use up the nutrients available to *E. coli* in the intestine.

4.7 Preliminary colonization experiments suggest that *E. coli* EDL933 uses gluconeogenic substrates in the presence of *E. coli* Nissle 1917 in order to colonize

E. coli Nissle 1917 is colonized simultaneously with *E. coli* EDL933 and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* (Figure 10). Preliminary results show that all three *E. coli* strains enter the mouse large intestine at the same concentration of 2×10^4 CFU/gram of feces and grow up to high numbers. *E. coli* Nissle 1917 grows up and levels off at a concentration of between 10^9-10^{10} CFU/gram of feces. Both *E. coli* EDL933 strains grow up to a concentration of around 2×10^8 CFU/gram of feces and then gradually decrease until *E. coli* EDL933 wild type reaches a concentration 1.8×10^6 CFU/gram of feces and its gluconeogenic knockout mutant *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* drops down to a concentration of 3.9×10^2 CFU/gram of feces.

The same ratio can also be shown when colonizing *E. coli* Nissle Str^R Δ*pckA* Δ*ppsA::Cam* colonizing simultaneously with *E. coli* EDL933 wild type and *E. coli* EDL933 Str^R Δ*pckA*

Δ*ppsA::Cam* (Figure 11). Preliminary results indicate that all *E. coli* strains enter the mouse large intestine at a similar concentration between 10^4-10^5 CFU/gram of feces. *E. coli* Nissle Str^R Δ*pckA* Δ*ppsA::Cam* grows up to numbers between 10^8-10^9 CFU/gram of feces and remains at these numbers throughout the colonization. *E. coli* EDL933 wild type first grows up even higher than *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA::Cam*, but beginning at day five dramatically drops down in concentrations until it reaches a final concentration 6 x 10^7 CFU/gram of feces. Its gluconeogenic knockout mutant *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* reaches its peak at day one with 5 x 10^7 CFU/gram of feces and like the wild type dramatically drops down to a concentration of 1.6 x 10^4 CFU/gram of feces. It's important to mention that 2 out of three mice died during the experiment, and therefore all data from day 6-15 are based on the fecal samples of only one mouse. Therefore this colonization result can only be interpreted as a preliminary result and needs to be repeated.

Important in both colonizations is the fact that both *E. coli* EDL933 strains decreased in number, but the wild type always remains two or more orders of magnitude higher than the mutant. This suggests that *E. coli* EDL933 has an advantage in surviving in the mouse large intestine while competing against *E. coli* Nissle 1917. This advantage is the usage of gluconeogenesis. Therefore *E. coli* EDL933 definitely switches to using gluconeogenesis in the presence of another *E. coli* strain in the intestine.

4.8 *E. coli* Nissle 1917 relies on the usage of the glyoxylate shunt to colonize the mouse large intestine

Knowing that *E. coli* EDL933 switches to gluconeogenesis in the presence of *E. coli* Nissle 1917, a further focus lies on acetate or fatty acids as gluconeogenic substrates, which are dependent on a functioning glyoxylate shunt. Therefore the *aceA* gene was knocked out using the gene

replacement method by Datsenko and Wanner to generate mutants which are not able to metabolize acetate or fatty acids.

Figure 12 shows *E. coli* Nissle 1917 wild type concurrently fed to mice with *E. coli* Nissle 1917 Str^R Δ*aceA*:Cam^R. Both strains entered the mouse large intestine at a concentration of about 10^5 CFU/gram of feces and grew up to numbers between 10^8-10^9 CFU/gram of feces. Beginning at day five *E. coli* Nissle 1917 Str^R Δ*aceA*:Cam^R dropped down about an order of magnitude and stayed throughout the rest of the colonization about 1.5 orders of magnitude below the wild type. This colonization shows that being able to utilize the glyoxylate shunt seems to be an advantage for *E. coli* Nissle 1917 in order to maintain itself in the intestine.

4.9 Phenotypic characterization of the usage of different substrates in *E. coli* Nissle 1917 and *E. coli* EDL933 strains

The phenotypic metabolic GN2 MicroPlates were used to analyze the ability of a gram-negative bacterium, like *E. coli,* to utilize or oxidize compounds from a preselected panel of different carbon sources. Concomitantly the purple dye tetrazolium violet is reduced and is detected by the OmniLog during a time frame of 48 hours. This assay results in a characteristic pattern of purple wells, called a metabolic fingerprint. Comparing the plates of two strains with each other, usage differences can be shown easily and repeatable. A closer look is taken at the wild type strains *E. coli* Nissle 1917 and *E. coli* EDL933 and their Δ*pckA* Δ*ppsA* double knock-out mutant.

Comparing the metabolic fingerprints of *E. coli* Nissle 1917 with that of *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA::Cam* (Figure 13), it can be seen that the carbon sources in the wells showing more of a green color than red are more rapidly metabolized by the wild type than by the mutant (D5, D-galactonic acid lactone; E6, D,L-lactic acid). Carbon sources which could be metabolized only by the wild type and not by the gluconeogenic knockout mutant are

predominantly gluconeogenic substrates: C11, pyruvic acid methyl ester; C12, succinic acid mono-methyl ester; E12, succinic acid; F1, bromosuccinic acid; F4, L-alaninamide; F5, D-alanine; F6, L-alanine; F7, L-alanyl glycine; F8, L-asparagine; F9, L-aspartic acid; G6, L-proline; G8, D-serine; G9, L-serine; H12, D-glucose 6-phosphate. The mutant doesn't metabolize any of these 95 carbon sources better than its wild type.

Comparing *E. coli* EDL933 with *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* (Figure 14), the carbon sources that are metabolized by only the wild type and not by the mutant are gluconeogenic substrates: C11, pyruvic acid methyl ester; D1, acetic acid; E6, D,L-lactic acid E8, propionic acid; F1, bromosuccinic acid; F5, D-alanine; F6, L-alanine; F7, L-alanyl glycine; G6, L-proline. The mutant metabolizes two carbon sources better than its wild type (B9, lactulose; H10, D,L,α-glycerol phosphate).

Comparing the metabolic fingerprints of *E. coli* Nissle 1917 and of *E. coli* EDL933 (Figure 15), the following carbon sources are metabolized exclusively by *E. coli* Nissle 1917: C6, D-sorbitol; D5, D-galactonic acid lactone; E10, D-saccharic acid; F8, L-asparagine; F9, L-aspartic acid; G8, D-serine; G9, L-serine. *E. coli* Nissle 1917 metabolizes more rapidly but not exclusively the carbon sources: B9, lactulose; C5, L-rhamnose; C12, succinic acid mono-methyl ester; F1, bromosuccinic acid; E12, succinic acid; H10, D,L,α-glycerol phosphate. On the other hand *E. coli* EDL933 does better on carbon sources such as C4, D-raffinose; F3, glucoronamide; H2, inosine; H3, uridine; H4, thymidine. For three carbon sources *E. coli* EDL933 is the only metabolizing strain (C7, sucrose; D1, acetic acid; E8, propionic acid). However growth experiments in 1 x M9 with acetate as the sole carbon source showed that *E. coli* Nissle 1917 is able to metabolize acetate (data not shown).

Last but not least when comparing the metabolic ability of *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA::Cam* and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* (Figure 16) there are differences to detect. *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA::Cam* is the only strain metabolizing C6, D-sorbitol; D5, D-galactonic acid lactone; E6, D,L-lactic acid; E10, D-saccharic acid and metabolizes better the carbon source C5, L-rhamnose. *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* metabolizes more rapidly the carbon sources: A8, N-acetyl-D-glucosamine; B8, α-D-lactose, C4, D-raffinose; F3, glucuronamide; H2, inosine; H3, uridine; H4, thymidine; H12, D-glucose 6-phosphate and is the only strain to metabolize C7, sucrose.

4.10 Summary of the results

According to the sequencing results of the University of Rhode Island Genomic and Sequencing Center all generated deletions were in the expected places in the genome (data not shown). *In vitro* experiments on the mutants' ability to grow on certain carbon sources showed that gluconeogenic knockout mutants failed to grow on gluconeogenic substrates and glyoxylate shunt knockout mutants fail to grow on acetate as the sole carbon source.

Analyzing all colonization experiments of the mouse large intestine, it was found that *E. coli* Nissle 1917 uses glycolytic substrates as well as gluconeogenic substrates to colonize the mouse large intestine, when it is the only strain fed to mice. *E. coli* EDL933 utilizes only glycolytic substrates when it is the only *E. coli* strain present in the intestine. In the presence of any other *E. coli* strain *E. coli* EDL933 switches from using glycolytic substrates to utilizing gluconeogenic substrates. If *E. coli* EDL933 and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* are fed to mice at the same time in the presence of *E. coli* Nissle 1917, *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* seems to be not outcompeted due to the presence of *E. coli* EDL933, but due to the presence of *E. coli* Nissle 1917. This is supported by the colonization showing that in the absence of *E. coli* Nissle

1917, the rest of the microbiota in the mouse large intestine doesn't use up glycolytic substrates.

Focusing on the ability to use the glyoxylate shunt, *E. coli* Nissle 1917 relies on the usage of the glyoxylate shunt to colonize the mouse large intestine successfully. Phenotypic characterization of the usage of different substrates in *E. coli* Nissle 1917 and *E. coli* EDL933 strains shows differences between both strains as well as between their gluconeogenic knockout mutants.

5 Discussion

Analysis of all generated mutants showed that all gene deletions are in the expected places in the genome (data not shown) according to the sequencing results of the University of Rhode Island Genomic and Sequencing Center. Therefore the created *E. coli* mutants are in fact the mutants which they are supposed to be. *In vitro* experiments on the mutants' ability to grow on certain carbon sources show that gluconeogenic knockout mutants fail to grow on gluconeogenic substrates and glyoxylate shunt knockout mutants fail to grow on acetate as the sole carbon source. This proves that the phenotypes of all mutants are correct as expected.

In the gastrointestinal tract of mammals, *Escherichia coli* is the predominant facultative anaerobe [15], but makes up at most only 1 % of the microbiota [44].

Based on colonization experiments of the streptomycin-treated mouse model several conclusions regarding the role of gluconeogenesis for growing in the mouse large intestine can be deduced. *E. coli* Nissle 1917 colonizes the mouse large intestine 2.5 orders of magnitude better than *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA::Cam* (Figure 3). This is an indicator for *E. coli* Nissle 1917 using glycolysis and gluconeogenesis concurrently, when it is the only strain present in the intestine. The wild type has the advantage of using both pathways at the same time and reaches concentrations of 10^8-10^9 CFU/gram of feces, whereas the mutant can only rely on using glycolysis and is therefore able to just grow up to numbers around 10^6 CFU/gram of feces. Co-colonization of *E. coli* EDL933 and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* (Figure 4) shows that both colonize about equally well which indicates that *E. coli* EDL933 only uses glycolytic substrates when it is the only *E. coli* strain present in the mouse large intestine. When co-colonized together with another *E. coli* strain such as *E. coli* Nissle 1917, *E. coli* EDL933 struggles to grow up to high numbers in the intestine, but compared to *E. coli* EDL933 Str^R Δ*pckA*

Δ*ppsA::Cam* it is growing at a significantly higher concentration (Figure 10, Figure 11, Figure 5 and Figure 6). Therefore *E. coli* EDL933 needs the ability to utilize gluconeogenesis in order to be able to maintain itself in the intestine next to *E. coli* Nissle 1917. As described earlier *E. coli* EDL933 doesn't need to use gluconeogenesis when it is the only strain in the intestine, but it needs to use gluconeogenesis in the presence of *E. coli* Nissle 1917.

Further it was assumed that clearing out all facultative anaerobes of the intestine by providing streptomycin treated drinking water to the mice, and not adding *E. coli* during the first ten days would lead to an uptake of all available nutrients by the remaining microbiota in the intestine and when feeding *E. coli* EDL933 and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* at day ten, they would have to overcome the depletion of nutrients. But contrary to expectations both *E. coli* EDL933 strains grow up to high numbers and co-colonized the intestine throughout the colonization experiment at a concentration of around 10^9 CFU/gram of feces. This shows that the remaining microbiota didn't use up the glycolytic nutrients used by *E. coli* Nissle 1917. It is not clear if the microbiota is not able to metabolize the glycolytic substrates. It might also be possible that since this portion of the microbiota is made of strict anaerobes, they need a strict anaerobic environment to be able to survive. Because of the missing facultative anaerobes (*E. coli*) which would usually use up the oxygen, the concentration of strict anaerobes might decrease tremendously and this decrease of the anaerobic concentration could be the reason why the glycolytic substrates are not used up in the intestine. This result may suggest that, since the microbiota itself isn't able to outcompete *E. coli* EDL933 in the mouse large intestine, a commensal *E. coli* strain such as the probiotic *E. coli* Nissle 1917 seems to be strongly needed to be able to nutritionally compete against *E. coli* EDL933.

To conclude the results of all colonization experiments analyzing the effect of gluconeogenesis on colonization, it is suggested that *E. coli* EDL933 strongly relies on the usage of

gluconeogenesis in the presence of another *E. coli* strain in the intestine, since the ability to use glycolytic substrates is not enough for growing up. In case this other *E. coli* strain is able to use glycolysis and gluconeogenesis simultaneously, like *E. coli* Nissle 1917 does, this depletion of glycolytic and gluconeogenic substrates makes *E. coli* EDL933 struggle to be maintained in the intestine.

Concentrating on the role of the glyoxylate shunt in *E. coli* for colonization and maintenance in the mouse large intestine, it was formerly shown that *E. coli* MG 1655 doesn't rely on the usage of this pathway at all since the Δ*aceA* knockout mutant and the wild type colonize the intestine equally well [42]. Interesting here is that *E. coli* Nissle 1917 colonizes the intestine in average two orders of magnitudes higher than its Δ*aceA* mutant (Figure 12). This means that *E. coli* Nissle 1917 seems to rely on the glyoxylate shunt for colonizing the mouse large intestine. To compare the effect of the glyoxylate shunt on colonization of *E. coli* Nissle 1917 with that of *E. coli* EDL933, it might be interesting to generate an *E. coli* EDL933 Δ*aceA* knockout mutant and test its ability to colonize the intestine.

Phenotypic characterization of the usage of different substrates in *E. coli* Nissle 1917 and *E. coli* EDL933 strains shows differences between both strains as well as between their gluconeogenic knockout mutants. All carbon sources which are gluconeogenic substrates, such as TCA cycle intermediates and amino acids, are shown to not be metabolized by the gluconeogenic knockout mutants, but by the wild type, which is expected. It is important to notice that *E. coli* Nissle 1917 uses carbon sources such as D-sorbitol, succinic acid, D-saccharic acid and amino acids like serine, aspartic acid and asparagine. Carbon sources important to mention which are more rapidly metabolized by *E. coli* EDL933 are sucrose, D-raffinose, propionic acid and acetic acid. The result that acetate is more rapidly metabolized by *E. coli* EDL933 is interesting. On the other hand it has been shown that the concentration of acetate in mouse cecal mucus *in vivo* is

just at the limit of detection (0.2 µM) [42], which suggests that acetate is unlikely as a major carbon source in the large intestine.

Comparing the metabolic fingerprints of *E. coli* Nissle 1917 and *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA::Cam* it is interesting to notice that the mutant is not better in metabolizing any of the 95 carbon sources better than the wild type. The situation is different when comparing *E. coli* EDL933 and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam*. The mutant is better than the wild type in metabolizing lactulose and D,L-α—glycerol phosphate. Whether these two carbon sources represent a nutritional niche for the mutant is not clear but the two strains co-colonize equally well.

Most importantly the phenotypic characterization confirms the fact, that in contrast to the wild type, the gluconeogenic knockout mutant is not able to metabolize gluconeogenic substrates. Comparing *E. coli* Nissle 1917 and *E. coli* EDL933, there are no big differences in carbon source preferences of those carbon sources which are known to occur in the mucus layer of the mouse large intestine [9, 14].

6 Outlook

Pathogenic *E. coli* strains, EHEC, of the serotype O157:H7 are nowadays a major cause of foodborne disease, primarily in the United States, Canada, Japan, and Europe [25, 45]. It is important to find a treatment against these pathogenic bacteria. To do so, EHEC strains, such as *E. coli* EDL933 but also probiotic strains such as *E. coli* Nissle 1917 need to be further studied for example in terms of their way of action in the intestine, and their interaction when competing against each other.

Colonization experiments in the streptomycin-treated mouse model of this study support the suggestion that *E. coli* EDL933 relies on the usage of gluconeogenesis in the presence of another *E. coli* strain, like *E. coli* Nissle 1917 (this study) or also the commensal *E. coli* MG1655 [42]. *E. coli* Nissle 1917 by itself being able to use glycolysis and gluconeogenesis simultaneously is able to keep *E. coli* EDL933 at relatively low levels when precolonized in the mouse large intestine. This effect is of even more severe as *E. coli* Nissle 1917 is able to outcompete the gluconeogenic knockout mutant *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* down to a lower concentration or even to a complete loss from the intestine. Very important here is to further conduct a gene complementation of the Δ*pckA* Δ*ppsA* mutation. Only when testing the mutant being restored to wild type will clearify whether or not the phenotypic defects of this mutant are only due to the knockout of the targeted genes.

Based on the fact that *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* is almost eliminated from the mouse large intestine competing against *E. coli* Nissle 1917 (Figure 5, Figure 6, Figure 7, Figure 8), and based on the finding that *E. coli* EDL933 turns on virulence factors in a gluconeogenic environment [47], this leads to an hypothetical approach of defeating *E. coli* EDL933 in the intestine by finding a way to knockout the ability to use gluconeogenesis distictively in *E. coli*

EDL933. If this ability is taken away, *E. coli* EDL933 struggles to maintain in the mouse large intestine and therefore this might become a promising and effective treatment of EHEC related diseases. Based on several studies in which the intestine of newborns are colonized with *E. coli* Nissle 1917 and finding that this colonization supports their immune response to intestinal pathogens [26], this method of treatment right after birth might be a promising way of supporting colonization resistance against incursion of new and harmful microorganisms.

7 List of Abbreviations

Abbreviation	Meaning
aceA	gene coding for isocitrate lyase
ATP	Adenosine triphosphate
Cat^R/Cam^R	chloramphenicol resistance
CFU	Colony forming units
E. coli	*Escherichia coli*
E. coli EDL933	the wild type strain of *E. coli* EDL933
E. coli Nissle 1917	the wild type strain of *E. coli* Nissle 1917
g	gram
GALT	gut-associated lymphoid tissue
L	Liter
LB	Luria broth
m	mass
PEP	phosphoenolpyruvate
pckA	gene coding for phosphoenolpyruvate carboxykinase
ppsA	gene coding for phosphoenolpyruvate synthetase
Rif^R	rifampin resistance
SOB	Super optimal broth
Str^R	streptomycin resistance
v	volume
vs	versus

8 Figures

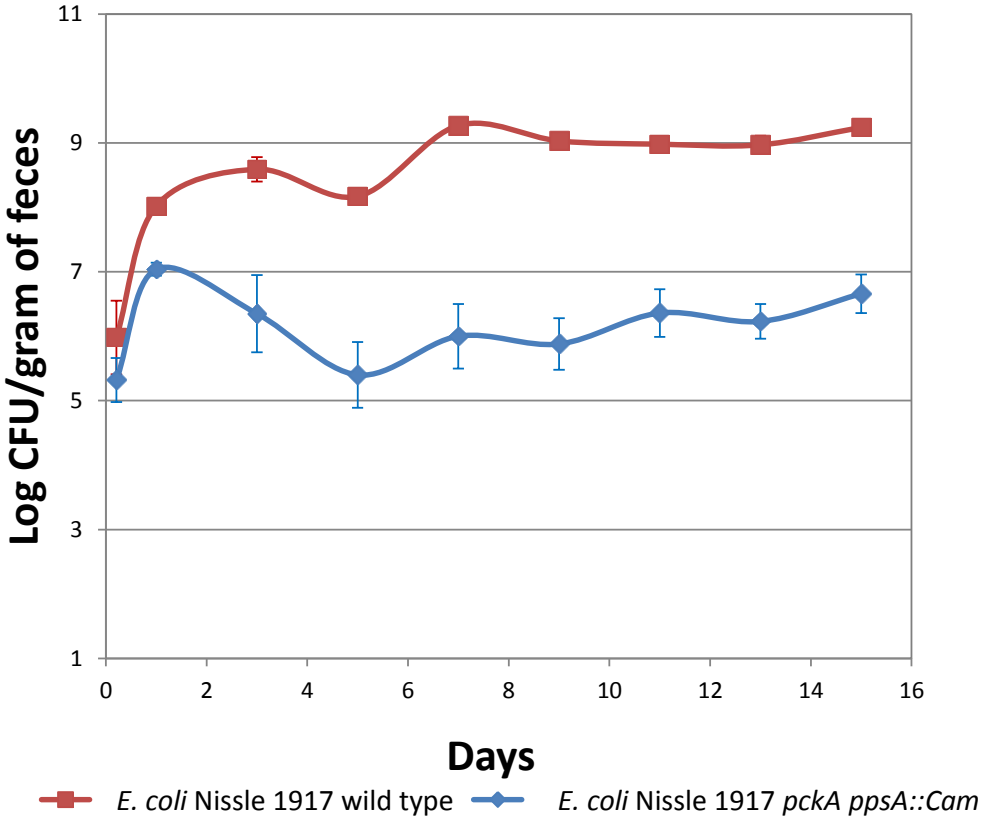

Figure 3: Fifteen day colonization of *E. coli* Nissle 1917 wild type Str^R Nal^R (■) and *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA::Cam* (♦). Two sets of three mice were each simultaneously fed 10^5 CFU of *E. coli* Nissle 1917 wild type Str^R Nal^R and 10^5 CFU of *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA::Cam*. At the indicated times, fecal samples were collected, homogenized, diluted and plated as described in the Materials and Methods. The $\log_{10}$ mean number of CFU per gram of feces plus or minus the standard error is shown for each time point.

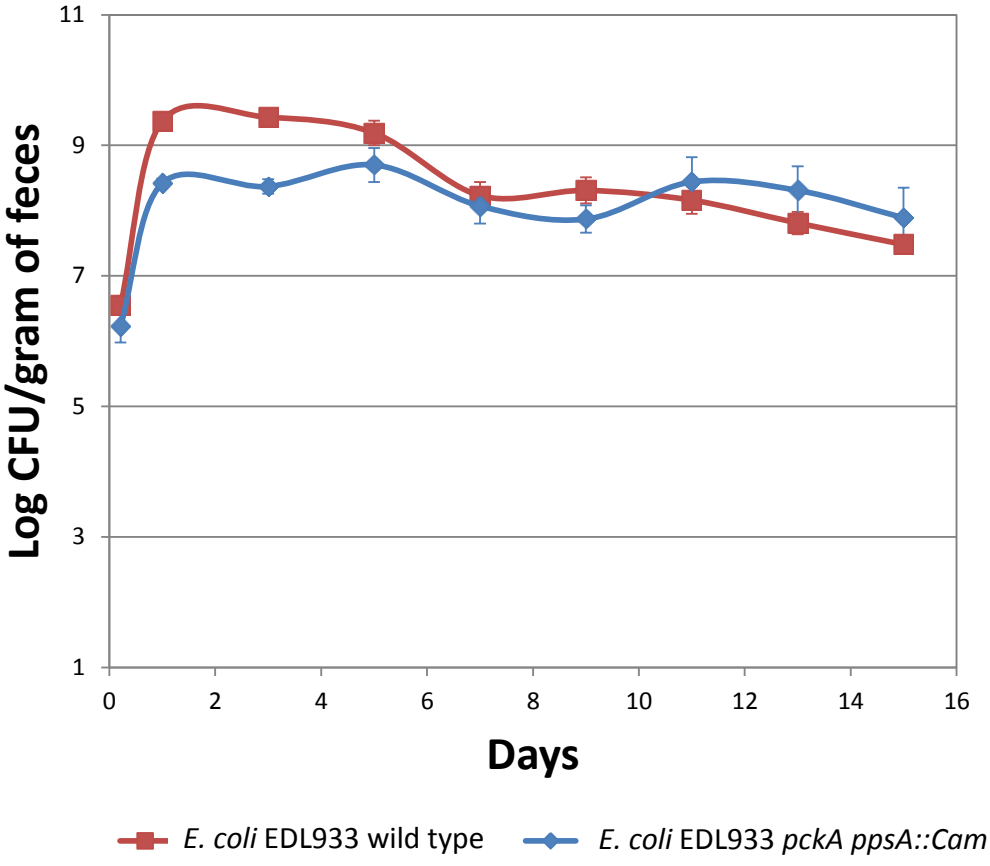

Figure 4: Fifteen day colonization of *E. coli* EDL933 wild type Str^R Rif^R (■) and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* (♦). Two sets of three mice were each fed 10^5 CFU of *E. coli* EDL933 wild type Str^R Rif^R. At day 10 *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* were fed to the same mice. At the indicated times, fecal samples were collected, homogenized, diluted and plated as described in the Materials and Methods. The $\log_{10}$ mean number of CFU per gram of feces plus or minus the standard error is shown for each time point.

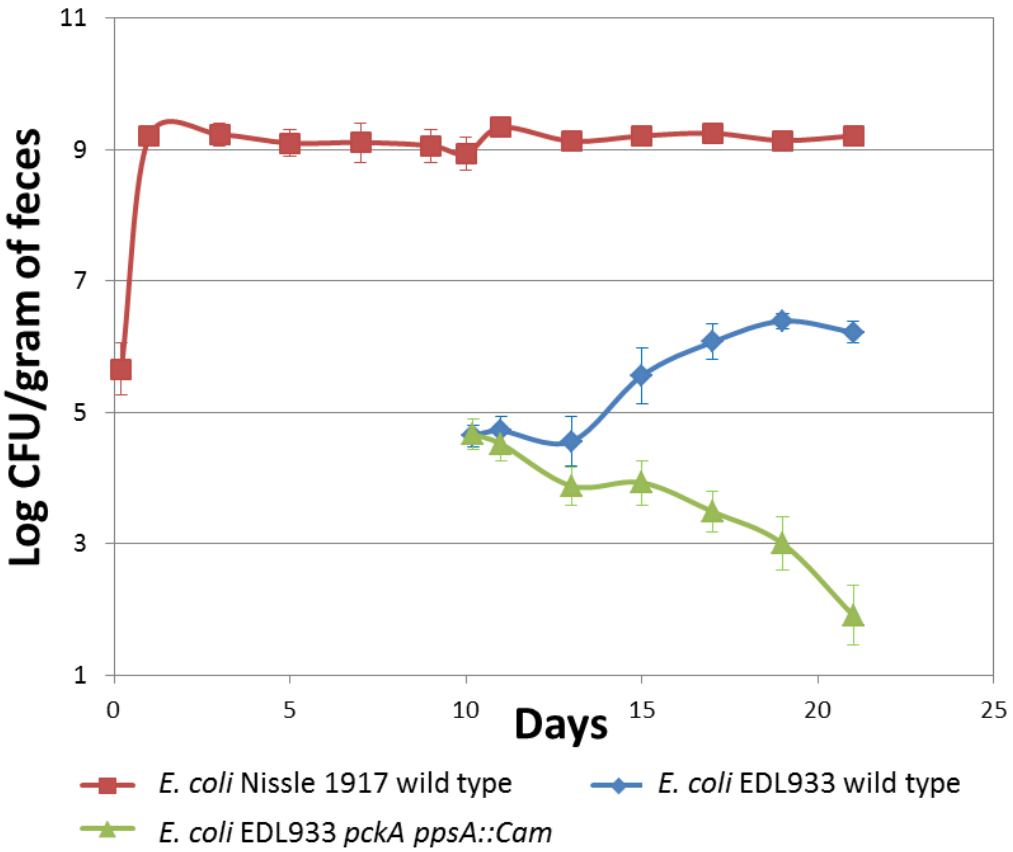

Figure 5: Twenty-one day colonization of *E. coli* Nissle 1917 wild type Str^R Nal^R (■), *E. coli* EDL933 wild type Str^R Rif^R (♦) and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* (▲). Three sets of three mice were each fed 10^5 CFU of *E. coli* Nissle 1917 wild type Str^R Nal^R. At day 10 10^5 CFU of *E. coli* EDL933 wild type Str^R Rif^R and 10^5 CFU of *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* were fed to the same mice. At the indicated times, fecal samples were collected, homogenized, diluted and plated as described in the Materials and Methods. The $\log_{10}$ mean number of CFU per gram of feces plus or minus the standard error is shown for each time point.

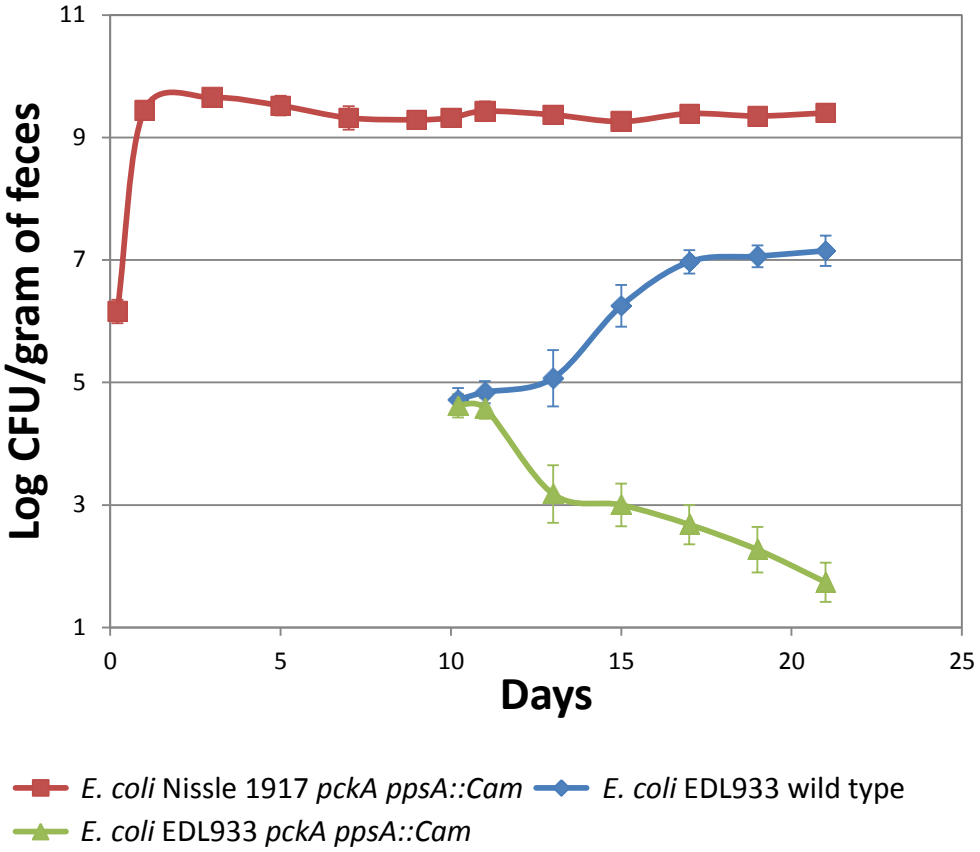

Figure 6: Twenty-one day colonization of *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA::Cam* (■), *E. coli* EDL933 wild type Str^R Rif^R (♦) and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* (▲). Three sets of three mice were each fed 10^5 CFU of *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA::Cam*. At day 10 10^5 CFU of *E. coli* EDL933 wild type Str^R Rif^R and 10^5 CFU of *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* were fed to the same mice. At the indicated times, fecal samples are collected, homogenized, diluted and plated as described in the Materials and Methods. The $\log_{10}$ mean number of CFU per gram of feces plus or minus the standard error is shown for each time point.

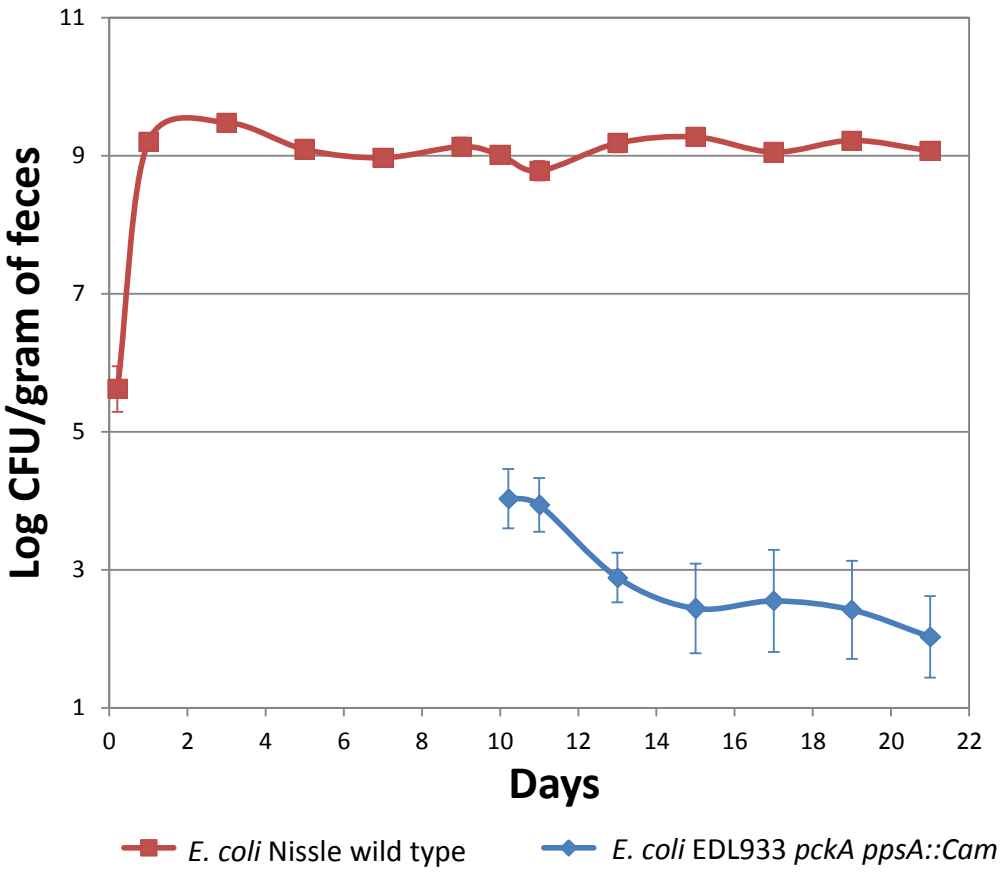

Figure 7: Twenty-one day colonization of *E. coli* Nissle 1917 wild type Str^R Nal^R (■) and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* (♦). Two sets of three mice are each fed 10^5 CFU of *E. coli* Nissle 1917 wild type Str^R Nal^R. At day 10 of the colonization, 10^5 CFU of *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* were fed to the same mice. At the indicated times, fecal samples were collected, homogenized, diluted and plated as described in the Materials and Methods. The $\log_{10}$ mean number of CFU per gram of feces plus or minus the standard error is shown for each time point.

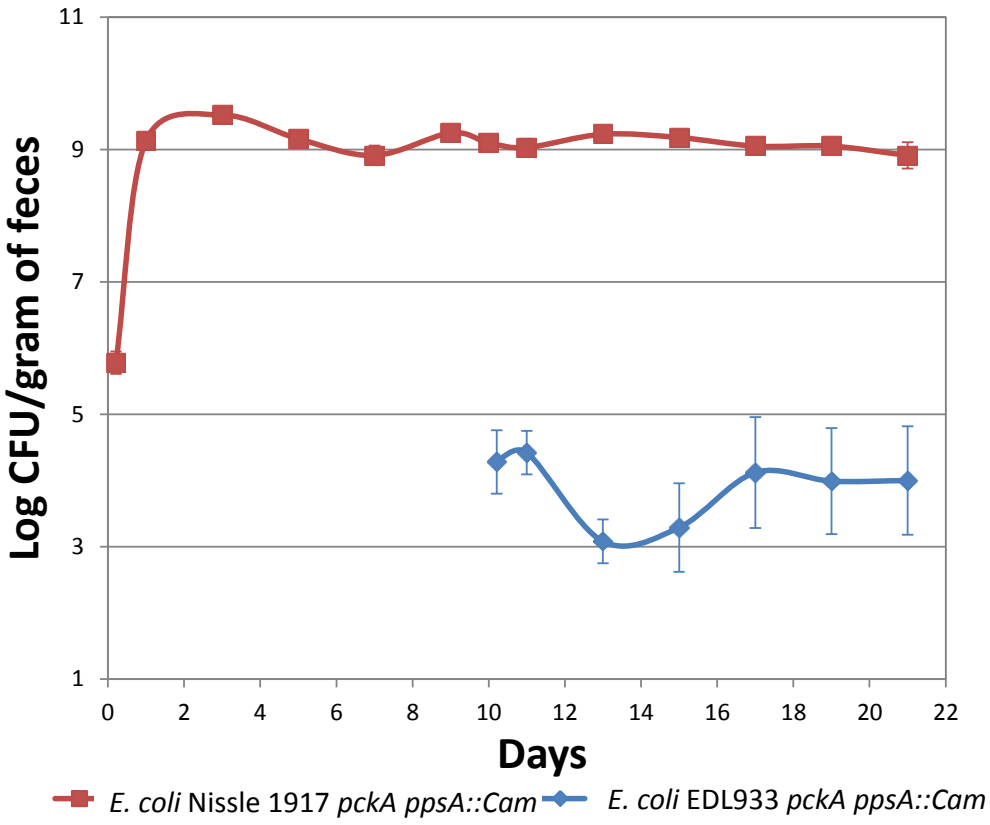

Figure 8: Twenty-one day colonization of *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA::Cam* (■) and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* (♦). Two sets of three mice are each fed 10^5 CFU of *E. coli* Nissle Str^R Δ*pckA* Δ*ppsA::Cam*. At day 10 of the colonization, 10^5 CFU of *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* were fed to the same mice. At the indicated times, fecal samples were collected, homogenized, diluted and plated as described in the Materials and Methods. The $\log_{10}$ mean number of CFU per gram of feces plus or minus the standard error is shown for each time point.

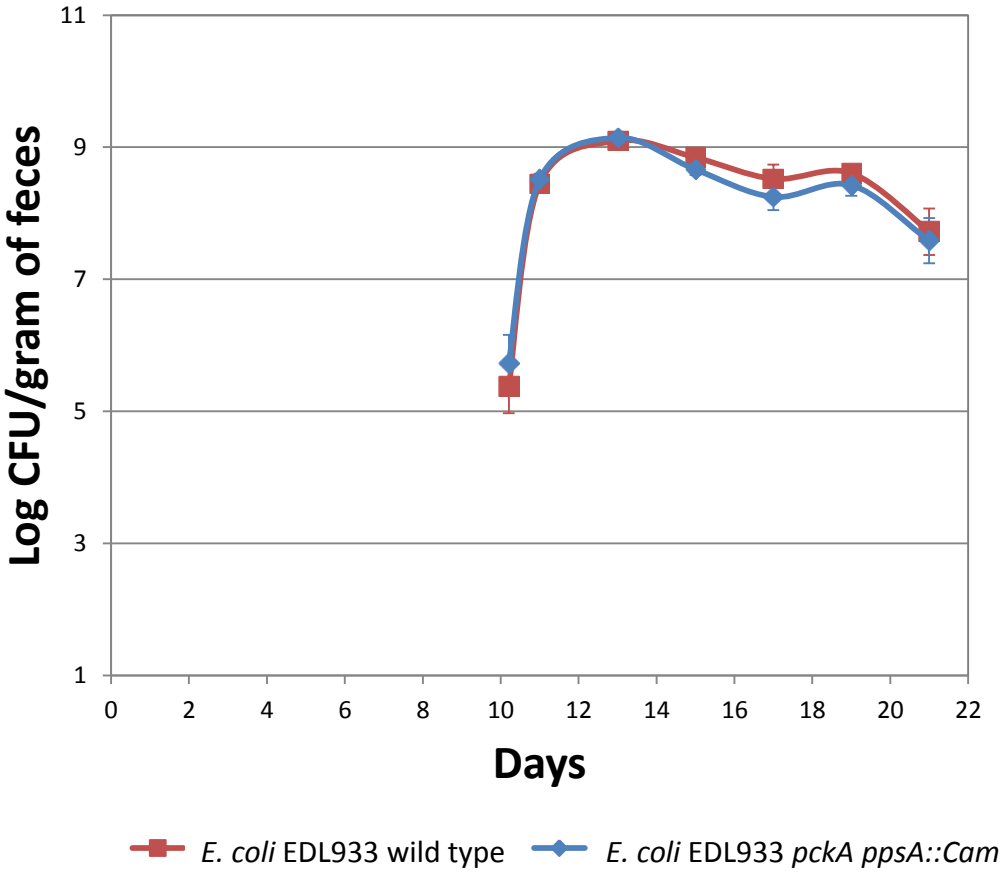

Figure 9: Twenty-one day colonization of *E. coli* EDL933 (■) and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* (♦). At day 10 of the colonization, 10^5 CFU of *E. coli* EDL933 and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* were fed simultaneously to two sets of three mice. At the indicated times, fecal samples were collected, homogenized, diluted and plated as described in the Materials and Methods. The $\log_{10}$ mean number of CFU per gram of feces plus or minus the standard error is shown for each time point.

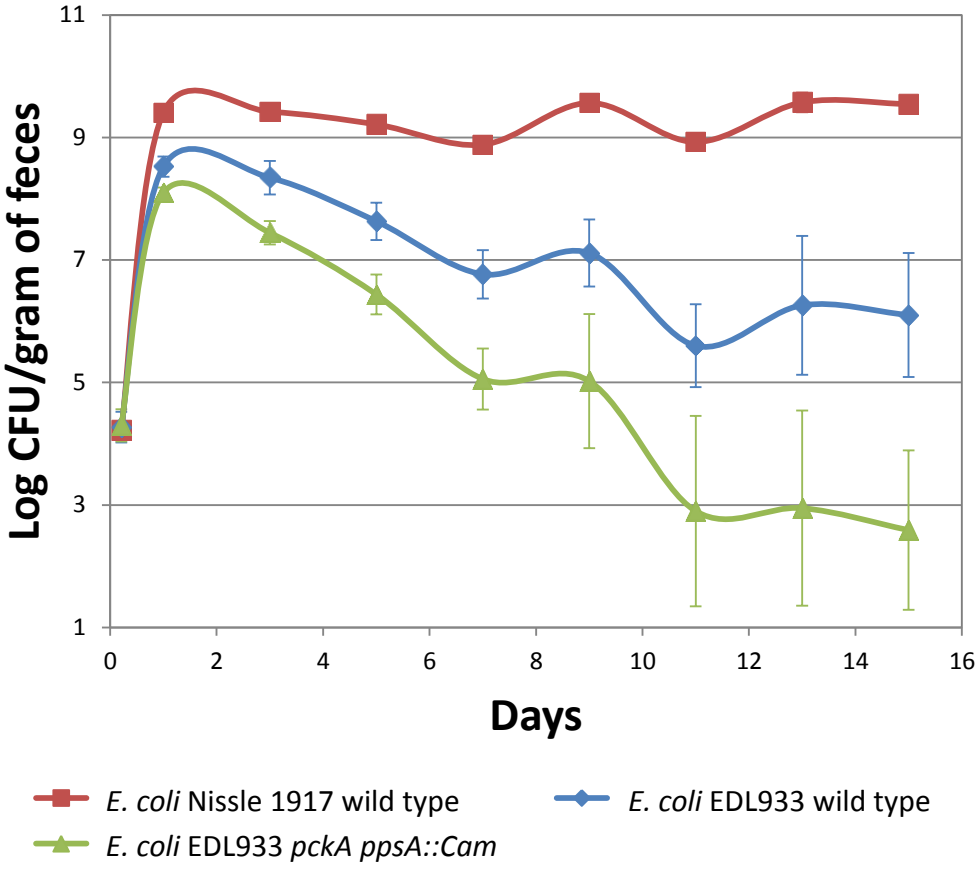

Figure 10: Fifteen day colonization of *E. coli* Nissle 1917 Str^R Nal^R (■), *E. coli* EDL933 (♦) and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* (▲). One set of three mice was simultaneously fed 10^5 CFU of *E. coli* Nissle 1917 Str^R Nal^R, *E. coli* EDL933 and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam*. At the indicated times, fecal samples were collected, homogenized, diluted and plated as described in the Materials and Methods. The $\log_{10}$ mean number of CFU per gram of feces plus or minus the standard error is shown for each time point. On day 5 one mouse died.

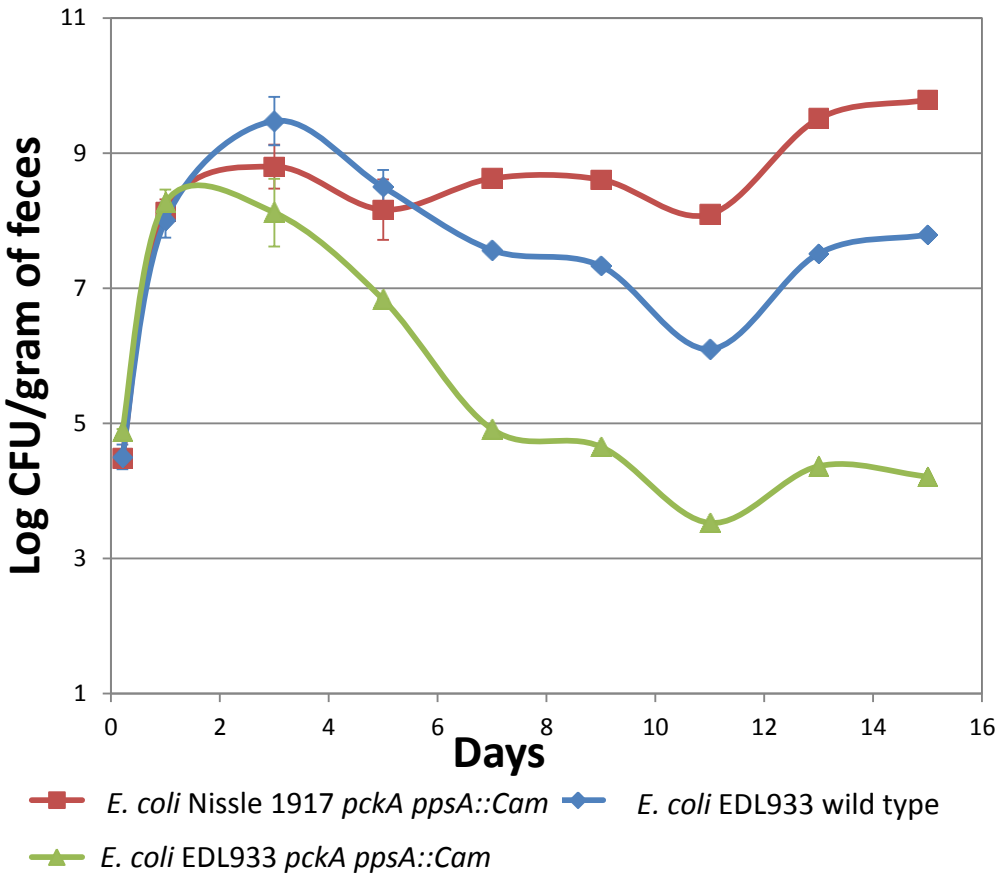

Figure 11: Fifteen day colonization of *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA::Cam* (■), *E. coli* EDL933 (♦) and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam* (▲). One set of three mice were simultaneously fed 10^5 CFU of *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA::Cam*, *E. coli* EDL933 and *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA::Cam*. Two mice died during the experiment each on day five and day six, therefore all data from day 6-15 are based on the fecal samples of only one mouse. At the indicated times, fecal samples were collected, homogenized, diluted and plated as described in the Materials and Methods. The $\log_{10}$ mean number of CFU per gram of feces plus or minus the standard error is shown for each time point.

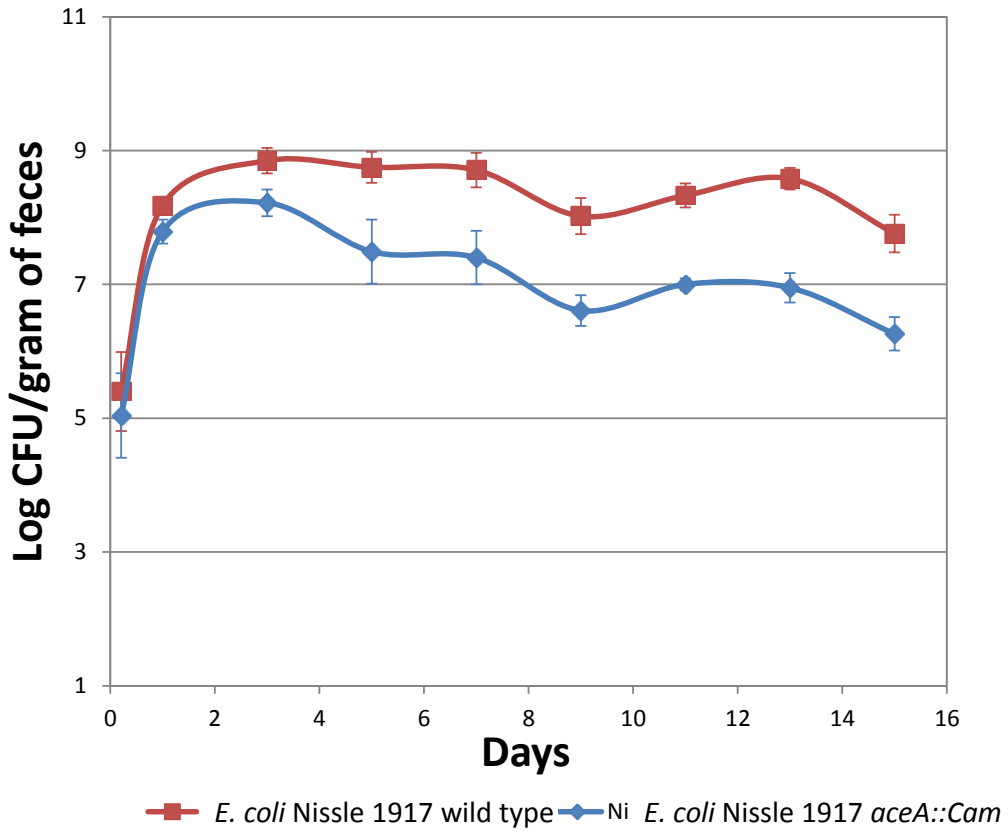

Figure 12: Fifteen day colonization of *E. coli* Nissle 1917 wild type Str^R Nal^R (■) and *E. coli* Nissle 1917 Str^R Δ*aceA*:Cam^R (♦). Two sets of three mice were each simultaneously fed 10^5 CFU of *E. coli* Nissle 1917 wild type Str^R Nal^R and 10^5 CFU of *E. coli* Nissle 1917 Str^R Δ*aceA*:Cam^R. At the indicated times, fecal samples were collected, homogenized, diluted and plated as described in the Materials and Methods. The $\log_{10}$ mean number of CFU per gram of feces plus or minus the standard error is shown for each time point.

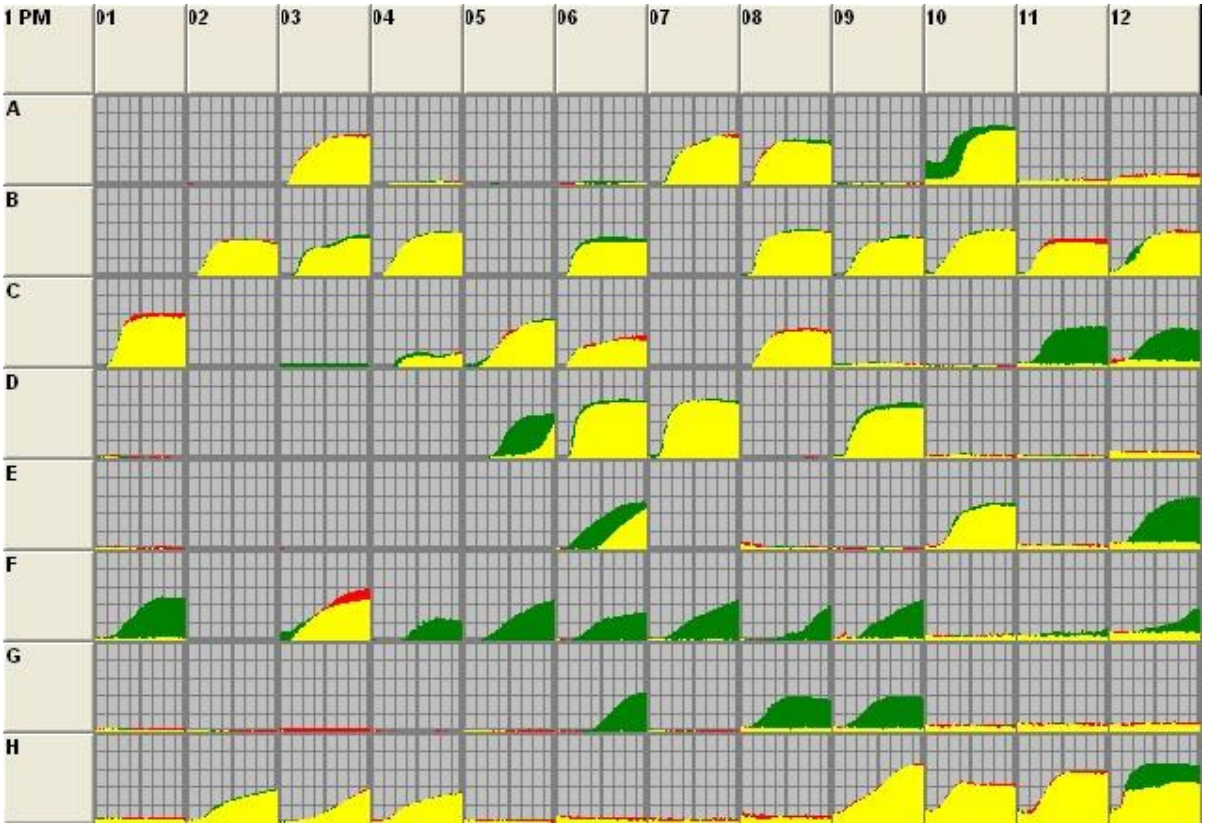

Figure 13: Phenotypic assay results for *E. coli* Nissle 1917 wild type Str^R Nal^R (green) compared to *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA*::Cam (red). Biolog GN2 plates (gram negative carbon nutrition) are utilized to analyze the overall metabolic capacity to consume 95 individual carbon sources (for details see Material and Methods). Each well shows a kinetic curve plotting substrate vs. time (48hours). Green represents carbon source utilization by the *E. coli* Nissle 1917 wild type strain, red represents the mutant strain, and yellow represents carbon utilization by both strains.

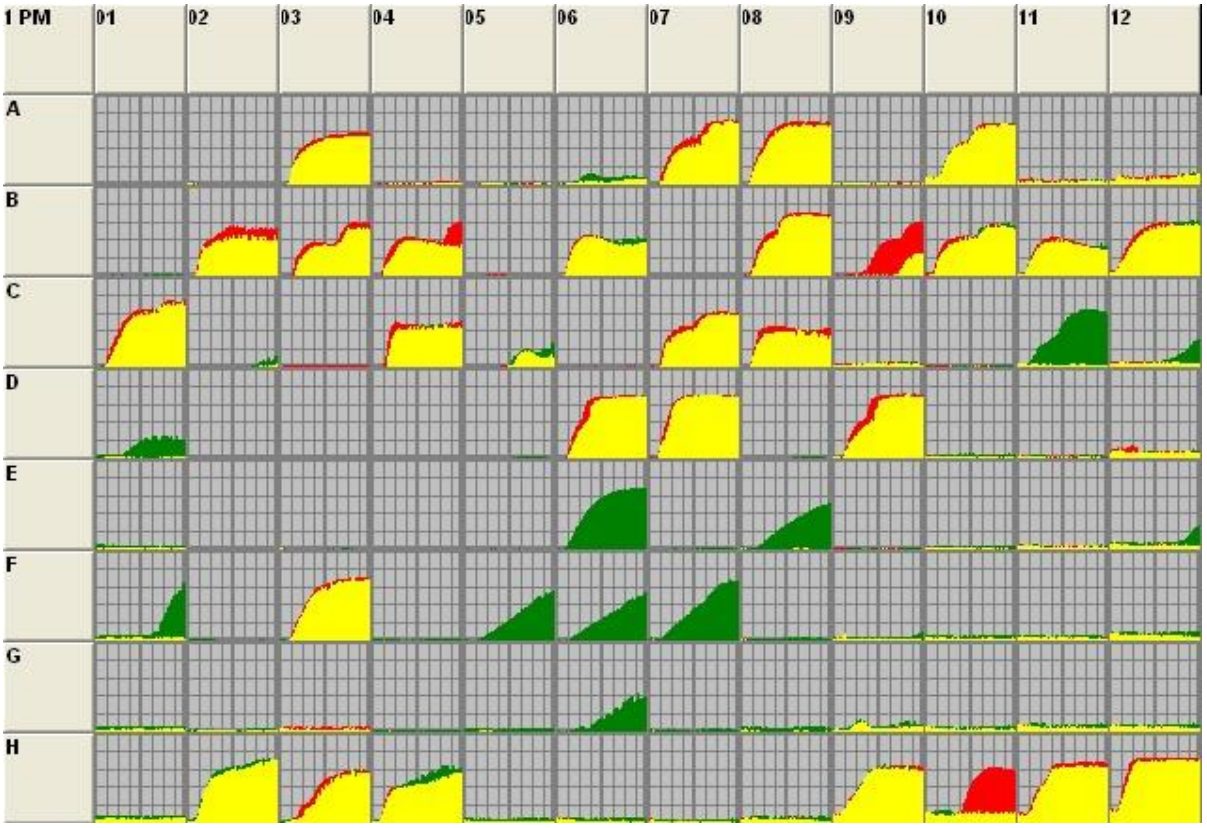

Figure 14: Phenotypic assay results for *E. coli* EDL933 wild type Str^R Rif^R (green) compared to *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA*::Cam (red). Biolog GN2 plates (gram negative carbon nutrition) are utilized to analyze the overall metabolic capacity to consume 95 individual carbon sources (for details see Material and Methods). Each well shows a kinetic curve plotting substrate vs. time (48hours). Green represents carbon source utilization by the *E. coli* EDL933 wild type strain, red represents the mutant strain, and yellow represents carbon utilization by both strains.

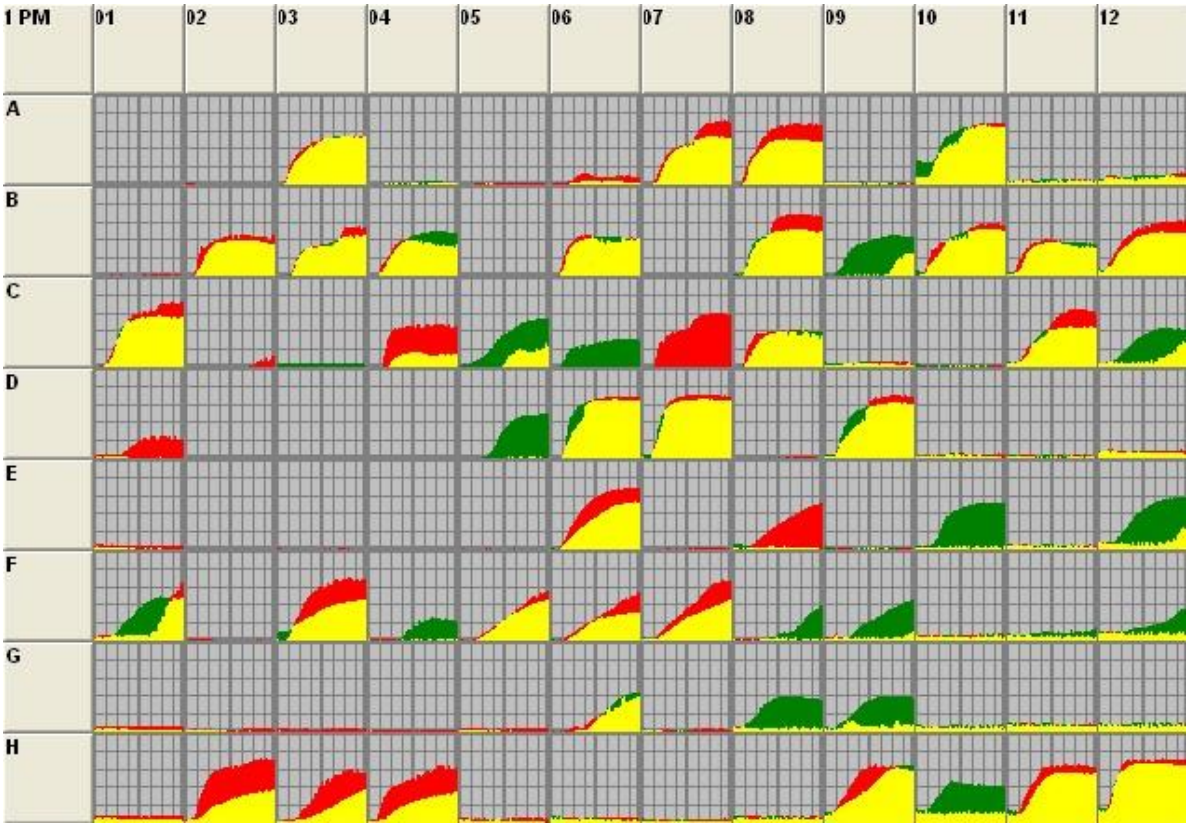

Figure 15: Phenotypic assay results for *E. coli* Nissle 1917 wild type Str^R Nal^R (green) compared to *E. coli* EDL933 wild type Str^R Rif^R (red). Biolog GN2 plates (gram negative carbon nutrition) are utilized to analyze the overall metabolic capacity to consume 95 individual carbon sources (for details see Material and Methods). Each well shows a kinetic curve plotting substrate vs. time (48hours). Green represents carbon source utilization by the *E. coli* Nissle 1917 wild type strain, red represents *E. coli* EDL933 wild type strain, and yellow represents carbon utilization by both strains.

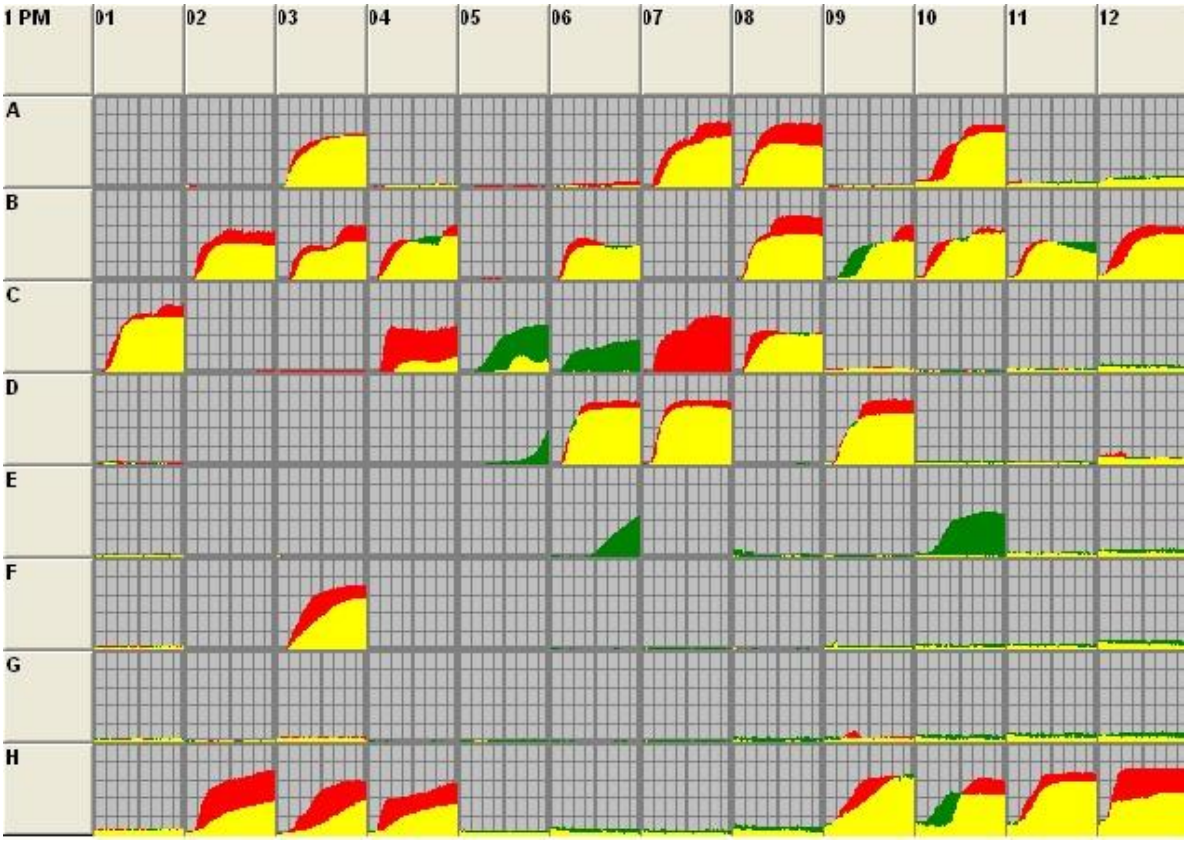

Figure 16: Phenotypic assay results for *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA*::Cam (green) compared to *E. coli* EDL933 Str^R Δ*pckA* Δ*ppsA*::Cam (red). Biolog GN2 plates (gram negative carbon nutrition) are utilized to analyze the overall metabolic capacity to consume 95 individual carbon sources (for details see Material and Methods). Each well shows a kinetic curve plotting substrate vs. time (48hours). Green represents carbon source utilization by the *E. coli* Nissle 1917 Str^R Δ*pckA* Δ*ppsA* :: Cam strain, red represents *E. coli* EDL933 Str^R Δ*pckA* Δ*pps::Cam* strain, and yellow represents carbon utilization by both strains.

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