

Nutrition of *Escherichia coli* within the intestinal microbiome

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ABSTRACT In this chapter, we update our 2004 review of “The Life of Commensal *Escherichia coli* in the Mammalian Intestine” (<https://doi.org/10.1128/ecosalplus.8.3.1.2>), with a change of title that reflects the current focus on “Nutrition of *E. coli* within the Intestinal Microbiome.” The earlier part of the previous two decades saw incremental improvements in understanding the carbon and energy sources that *E. coli* and *Salmonella* use to support intestinal colonization. Along with these investigations of electron donors came a better understanding of the electron acceptors that support the respiration of these facultative anaerobes in the gastrointestinal tract. Hundreds of recent papers add to what was known about the nutrition of commensal and pathogenic enteric bacteria. The fact that each biotype or pathotype grows on a different subset of the available nutrients suggested a mechanism for succession of commensal colonizers and invasion by enteric pathogens. Competition for nutrients in the intestine has also come to be recognized as one basis for colonization resistance, in which colonized strain(s) prevent colonization by a challenger. In the past decade, detailed investigations of fiber- and mucin-degrading anaerobes added greatly to our understanding of how complex polysaccharides support the hundreds of intestinal microbiome species. It is now clear that facultative anaerobes, which usually cannot degrade complex polysaccharides, live in symbiosis with the anaerobic degraders. This concept led to the “restaurant hypothesis,” which emphasizes that facultative bacteria, such as *E. coli*, colonize the intestine as members of mixed biofilms and obtain the sugars they need for growth locally through cross-feeding from polysaccharide-degrading anaerobes. Each restaurant represents an intestinal niche. Competition for those niches determines whether or not invaders are able to overcome colonization resistance and become established. Topics centered on the nutritional basis of intestinal colonization and gastrointestinal health are explored here in detail.

KEYWORDS microbiome, *E. coli*, colonization, nutrition, intestine, colonization resistance

Escherichia coli is arguably the best understood of model organisms; the species comprised commensal and pathogenic strains (1). As a commensal, *E. coli* is a persistent colonizer of the vertebrate gut (2). It is estimated there are 10^{21} *E. coli* cells among the human population (3). As a pathogen, *E. coli* strains can cause both intestinal and extra-intestinal infections (2). *E. coli* infections are a significant health problem which is associated with increased mortality, morbidity, and health-care-related costs around the world (4). *E. coli* is responsible for a million deaths per year around the globe (5). Colonized commensal *E. coli* acts as a barrier to prevent colonization by invading pathogens (6). Furthermore, invading pathogens almost always need to colonize the intestine before initiating a successful infection (3, 7, 8). However, how *E. coli* colonizes the mammalian intestine is not completely understood. A better understanding of colonization mechanisms can be exploited to prevent pathogens from causing gastrointestinal diseases. The colonic mucus layer is the primary site of *E. coli* colonization, where it competes with other organisms for limiting nutrients (3). The mucus

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layer covering the intestinal epithelium not only provides nutrients but also provides attachment sites (9, 10). Innate and adaptive immunity, bacterial cell-to-cell communication, etc., also play important roles in the colonization of the intestine; these topics are discussed elsewhere (11–14). In this article, we focus discussion on the nutrients available in the mucus layer, competition for nutrients that support *E. coli* colonization, colonization resistance, and how invaders overcome colonization resistance.

MUCOSAL HABITAT OF THE LARGE INTESTINE

Since commensal Enterobacteriaceae generally colonize the mucus layer of the mammalian intestine, we begin by considering the mucus layer as a microbial habitat. Very few microorganisms can survive the drastic acidic pH of gastric juices in the stomach. Analysis of stomach mucosal biopsies and gastric juices revealed there are 10^2 – 10^4 cultivable bacteria per gram of stomach contents. Isolates from the stomach include *Propionibacterium*, *Lactobacillus*, *Streptococcus*, and *Staphylococcus* (15). In the small intestine, the bacterial population in the duodenum increases from 10^4 – 10^5 CFU/mL to 10^7 – 10^8 CFU/mL in the distal ileum. Bacteria in the small intestine must respond to changing conditions such as rapid transit of the luminal contents and influx of digestive enzymes and bile. Representative genera in the small intestine include *Bacteroides*, *Clostridium*, *Lactobacillus*, *Staphylococcus*, *Streptococcus*, etc. (16).

In contrast to the small intestine, the large intestine is colonized by 10^{11} – 10^{12} CFU/mL (17). The mammalian large intestine is divided into three sections—the cecum, the colon, and the rectum. The epithelium of the large intestine, which consists of goblet cells and enterocytes, is renewable, and in humans, as many as 2 – 5×10^6 epithelial cells are shed per minute (17).

The epithelium of the large intestine is covered by a mucus layer secreted by goblet cells lining the epithelium (10, 18). The mucus coat protects the epithelial cells from bacteria, digestive enzymes, and toxic substances (10). The major component of the mucus layer is the gel-forming glycoprotein, MUC2 mucin, which forms a large net-like polymer (18, 19). Structurally, mucin domains have a central protein core characterized by abundant proline, threonine, and serine, with glycans projecting in all directions (20). Sugars commonly found in the glycan chains include galactose, N-acetylglucosamine (NAG), N-acetylgalactosamine (GalNAc), fucose, and sialic acid [N-acetylneuraminic acid (NANA)] (21). The O-glycan repertoire on MUC2 mucin varies between host species, and the glycosylation of mucin makes it non-degradable by host digestive enzymes; however, the glycans can be degraded by bacterial species (20). Study of the O-glycosylation of MUC2 mucin from the human colon showed more than 100 MUC2 O-glycans of chain lengths between 2 and 12 residues, including some that were identified for the first time (21). Mouse colonic mucus consists of two layers totaling about 150 μm in depth compared to about 800 μm in humans (18, 22). The tightly packed inner layer is firmly attached to the epithelial lining and contains few, if any bacteria (18, 19). The firm inner mucus layer is renewed constantly and is continuously converted into the soft outer layer. The outer mucus layer proximal to the intestinal lumen is looser because of the proteolytic degradation of mucin (18, 19). The thickness of the outer layer is highly variable depending on what bacteria are present (23). The outer layer of mucus and the digesta in the lumen is colonized by over 500 species of microorganisms, mostly obligate anaerobes. Representative bacteria present in the colon include *Bacteroides* spp., *Bifidobacterium* spp., *Clostridium* spp., *Peptococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Lactobacillus* spp., members of the family Enterobacteriaceae, etc. (24–27).

Mucosal glycans, besides serving as attachment sites for bacterial adhesins (28), also serve as a source of nutrients for the gastrointestinal microbiota (29). Since the glycans on MUC2 mucin vary between host species, bacteria colonizing a specific host carry a distinct set of enzymes to degrade the glycans (20, 29). However, not all bacteria in the gut have the required glycan-degrading enzymes, and therefore, they

rely on cross-feeding by other bacteria in the community (20). Cross-feeding of mucin non-degraders by mucin-degrading species contributes to the stability of the microbial community (30). Several members of Verrucomicrobia, Bacteroidetes, and Firmicutes harbor one or multiple copies of mucin-degrading glycoside hydrolases (31). Commensal *E. coli* is dispersed in the mucus layer but is not attached to the epithelium (32, 33). However, *E. coli* and other enteric bacteria do not usually produce extracellular enzymes for degrading mucin oligosaccharides (34). *E. coli* grows primarily on simple monosaccharides and disaccharides, which are released by anaerobes in the gut (35, 36). Henderson and colleagues (37) identified and characterized a secreted serine protease protein, Pic (for “protein involved in intestinal colonization”), in enteroaggregative *E. coli* (EAEC), which had mucinolytic activity. Homologs of Pic have been reported in some isolates of uropathogenic *E. coli* (UPEC), enteroinvasive *E. coli*, and enteropathogenic *E. coli* (EPEC) (38–42). However, none of the isolates of non-pathogenic *E. coli* tested had Pic (42). Pic promotes intestinal colonization of EAEC in streptomycin-treated mice and its growth in cecal mucus, cecal contents, and in minimal media containing mucin but not in the absence of mucin (43). Recently, using a human colonic organoid model, it was shown that purified Pic degrades the mucin layer, and EAEC producing Pic showed increased colonization of the colonic epithelium compared to a Pic deletion mutant (44). Thus, Pic plays an important role in MUC2 barrier disruption in the intestine (44), promoting mucus layer penetration and attachment to the epithelium, and hence, is a virulence factor; however, whether Pic plays a role in increasing the availability of any nutrients from mucus is not known.

Since the population of *E. coli* in the large intestine remains constant, its growth rate in the mucus layer must exceed the rate it is sloughed into the lumen to prevent being washed out of the mucus layer (45). It was estimated that *E. coli* needs to divide roughly every 2 hours to maintain a stable population of 10^8 CFU/g of feces, which was consistent with the generation time calculated for *E. coli* BJ4, a rat isolate (45–47). In these studies, growth rates were determined by 23S rRNA-targeted fluorescent *in situ* hybridization to estimate ribosomal counts, which correlate with the growth rate. *E. coli* BJ4 grows in the mucus layer of the mouse large intestine with a generation time of 40–80 min (46). The growth rates were similar in streptomycin-treated and germ-free mice (45). In conventionalized streptomycin-treated mice and conventionalized germ-free mice, the estimated generation time increased to 116–130 min following 9 days of conventionalization. This contrasts with the rapid growth (30-min generation time) of *E. coli* BJ4 in intestinal mucus *in vitro* (48, 49). Since the generation time of *E. coli* was higher in conventionalized mice as compared to streptomycin-treated and germ-free mice, it is thought that conventionalization possibly leads to a decrease in the rate of mucus sloughing (45). *E. coli*, however, does not grow on intestinal luminal contents *in vitro* (23, 48, 49), and the mucus layer hosts a distinct intestinal microbial niche compared to the luminal contents (23, 48, 49). This suggests that nutrients secreted by the host in the form of mucus are more important than the nutrients ingested by the host (47), or some inhibitors present in the luminal contents inhibit the growth of *E. coli* in the contents (48).

NUTRIENT SOURCES IN THE INTESTINE

The mammalian intestine is a highly complex ecosystem that is shaped by multiple factors: the host, complex microbial community, and interactions with the environment such as food intake and pathogens (50). Limiting nutrients are major determinants of species composition in an ecosystem, including the mammalian intestine (3, 51). The microbial community in the intestine competes for carbon and energy sources, terminal electron acceptors, nitrogen sources, and iron (52–56). Dietary nutrients, endogenously secreted nutrients, shed epithelial cells, microbial metabolites, and components of mucus are the nutrient sources for bacteria in the intestine (3, 17, 57–62). Absorption of nutrients by the host determines the availability of dietary nutrients to the gut microbiota (57). Animals have developed numerous physiological strategies to absorb nutrients in the small intestine, so most digestible nutrients are absorbed there and

are not available to the microbiota of the large intestine (55). However, undigested carbohydrates such as dietary fiber and some of the escaped digestible carbohydrates enter the colon and are available to the microbiota (63). In addition to the dietary nutrients entering the gastrointestinal tract, other substances from endogenous sources, such as saliva, bile, urea, glutathione, etc., simultaneously enter into the lumen where they can be metabolized by the microbiota (59). Human saliva, for example, contains approximately 3,000 mg/L of total proteins in the form of amylases, mucins MUC5B (MG1) and MUC7 (MG2), cystatin, histatins, statherin, etc., the majority of which are glycosylated (64). In pigs weighing 30–40 kg, 500 g of saliva flows per day which has been calculated to contribute 400 mg of nitrogen daily (59). Since the epithelium of the colon is renewable and cells are constantly shed, the dead epithelial cells are also an important source of nutrition for the microbiota (17, 65).

Goblet cells in the intestine continuously synthesize and secrete mucin which is the dominant glycoprotein component of mucus in the intestine (66). Mucin comprised 80% polysaccharide and 20% protein and is highly viscous (65). In addition to mucin, mucus also contains many other glycoproteins, proteins, sugars, lipids, and glycolipids, most of which can serve as nutrient sources for the microbiota (65). Indigenous intestinal microbes encode enzymes required to degrade the mucus layer and utilize it. As the mucus is degraded, the components are also shed into the lumen which forms part of the luminal contents that are excreted in the feces (65).

NUTRIENT CONSUMPTION AND CROSS-FEEDING OF THE INTESTINAL MICROBIOTA

Dietary carbohydrates and proteins are primarily absorbed in the proximal part of the small intestine (67). Mass spectrometric analysis of free amino acids from the proximal small intestine and colon revealed that >90% of amino acids are absorbed in the small intestine (68). Since facultative anaerobes generally cannot degrade oligosaccharides or polysaccharides [rare exceptions include dextrin (3) by *E. coli* and L-arabinose liberated from plant polysaccharides by *Salmonella* Typhimurium (69)], they must rely on sugars that are liberated by obligate anaerobes upon hydrolysis of complex polysaccharides during their expansion in the gut (70, 71) (Fig. 1). Ng and co-workers (70) demonstrated

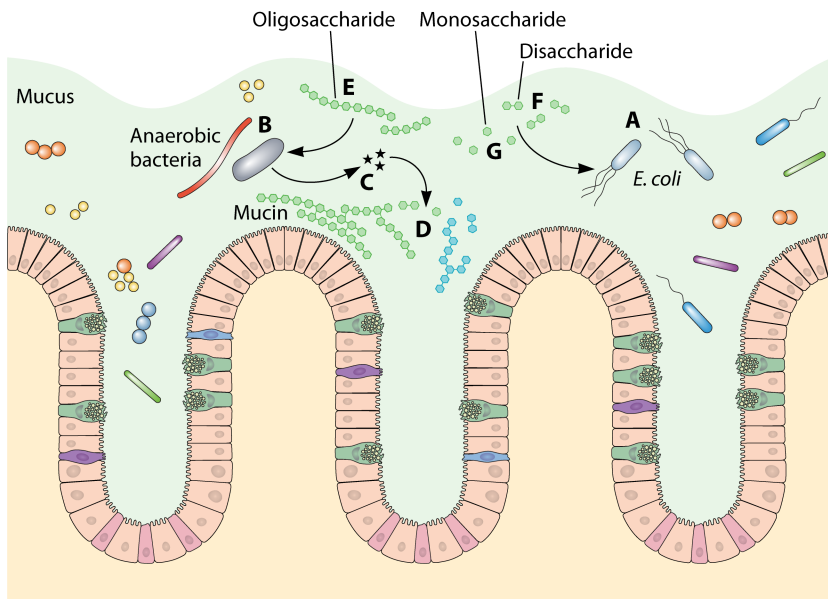


FIG 1 Illustration of how *E. coli* (A) obtains nutrients in the mucosal layer of the large intestine. Anaerobes (B) produce glycosyl hydrolases (C) which degrade complex polysaccharides in dietary fibers and mucin (D) to release oligosaccharides (E), disaccharides (F), and monosaccharides (G). Oligosaccharides are preferred by anaerobes, while monosaccharides and disaccharides are preferred by *E. coli*.

that *Bacteroides thetaiotamicron* (*B. thetaiotamicron*) enhanced the levels of free sialic acids in the ceca of *B. thetaiotamicron*-mono-associated mice as compared to germ-free mice. While *B. thetaiotamicron* prefers dietary fiber, it can switch to mucus-derived glycans in the cecum when dietary polysaccharides are absent (72). Under conditions of chronic or intermittent dietary fiber deficiency, mucus-eroding microbiota promote access to the epithelium and increase susceptibility to pathogens (73). *S. Typhimurium* utilizes the monosaccharides, fucose and sialic acid, liberated by the microbiota; when those catabolic pathways were knocked out, competitive fitness of *S. Typhimurium* was reduced. *Clostridium difficile* also utilized sialic acid liberated by *B. thetaiotamicron*, and reduction of free sialic acid impaired *C. difficile* expansion in the gut (70). It is known that *B. thetaiotamicron* lacks the catabolic pathway required to consume sialic acid but has enzymes that release sialic acid from the mucus (70, 74). Jimenez and co-workers showed that *B. thetaiotamicron* digests dietary pectin and makes galacturonic acid available in the gut (75). Pectin, which is rich in D-galacturonic acid, is present in plant cell walls and can be digested by saccharolytic members of the microbiota such as *B. thetaiotamicron* (75, 76). Galacturonic acid released by the gut microbiota in the lumen is used by enterohemorrhagic *E. coli* (EHEC) and *Citrobacter rodentium* as a carbon source and as a signal to aid in the colonization and initiate infection (75).

B. thetaiotamicron has polysaccharide utilization loci (PULs) which encode glycolytic enzymes needed to utilize host as well as plant glycans (74). During colonization of germ-free mice with *B. thetaiotamicron* and *Eubacterium rectale*, *B. thetaiotamicron* upregulated several PULs and signaled the host to produce mucosal glycans which it can utilize. *E. rectale* responded to *B. thetaiotamicron* by downregulating glycan-hydrolyzing enzymes while upregulating sugar and amino acid transporters (77). In response to the signal from *B. thetaiotamicron*, the colonic epithelium upregulated genes involved in the synthesis of mucosal glycans (glycosphingolipids and O-glycans), suggesting the possibility that *B. thetaiotamicron* increases host production of glycans and benefits by utilizing those glycans. However, no follow-up studies of this possibility have been reported.

Studies of microbial interactions demonstrate functional redundancy among members of different phyla, and this metabolic flexibility helps to ensure the stability of the intestinal ecosystem (3, 77). More than 50% of its glycan hydrolase enzymes are predicted to be secreted by *B. thetaiotamicron* into the periplasm or to the cell exterior, thus liberating monosaccharides and oligosaccharides from complex polysaccharides (72). *B. thetaiotamicron* appears to utilize liberated monosaccharides, such as arabinose, xylose, fucose, galacturonate, β -hexosamines, fructose, glucose, galactose, etc., as genes involved in their transport and utilization are fitness determinants (78). Previously, it was also demonstrated that the colonization of germ-free mice by *B. thetaiotamicron* significantly reduced the concentrations of abundant monosaccharides present in the cecum (72). Many bacteria other than *E. coli* also rely on simple sugars released by anaerobic digestion (79, 80). Li and co-workers (23) provided evidence that genes for maltose utilization were upregulated in *E. coli* when colonizing germ-free mice together with *B. thetaiotamicron* as compared to *E. coli* colonizing germ-free mice alone, which is consistent with cross-feeding.

E. coli is usually grown in the laboratory in batch cultures on a high concentration of a single carbon source. When a mixture of two carbon sources at high concentration is present in a batch culture, the carbon source that supports the highest growth rate is typically utilized by *E. coli* first, resulting in diauxic growth (81). However, natural environments such as the intestine may contain mixtures of many carbon sources present at low concentrations (82). When *E. coli* was fed a mixture of six sugars—glucose, galactose, maltose, ribose, arabinose, and fructose, all six sugars were utilized simultaneously (81). When commensal *E. coli* MG1655 was grown *in vitro* in a minimal medium containing 13 different sugars which are known to be present in mucus, gluconate, NANA, galactose, mannose, N-acetylglucosamine, N-acetylgalactosamine, glucosamine, glucuronate, galacturonate, fucose, ribose, L-arabinose, and maltose, all nutrients except

GalNAc were used (36). Catabolite-repressing sugars, i.e., NAG, gluconate, and galactose, were used first, and before those sugars were completely exhausted, the remaining sugars were metabolized simultaneously (36). Interestingly, the *in vitro* order of nutrient preference of commensal *E. coli* MG1655 and pathogenic EHEC EDL933 was almost identical except that EHEC EDL933 also used GalNAc (36). To determine which of those carbon sources supported colonization, a series of knockout mutants defective in carbon catabolism pathways were constructed and competed against the wild-type *E. coli* (36) using the streptomycin-treated mouse model (49, 83–86). Commensal *E. coli* MG1655 mutants defective in the utilization of arabinose, gluconate, fucose, N-acetylglucosamine, and N-acetylneuraminic acid showed colonization defects when competed against the wild-type commensal *E. coli* MG1655 (36, 87), indicating these carbon sources support colonization of the mouse intestine.

Gluconate, glucuronate, and galacturonate feed into the Entner-Doudoroff pathway and from there into the bottom half of glycolysis. It was known since the late 1990s that the Entner-Doudoroff pathway is critical for *E. coli* to colonize the mouse intestine (47). Eriksson and colleagues implicated the Entner-Doudoroff pathway as being important for the growth of *Salmonella* inside macrophages (88). The Thompson group went on to prove that glycolysis is required by *Salmonella* to replicate in macrophages and infect mice (89).

NUTRIENT LIMITATION IN THE INTESTINE

In nature, nutrients for bacterial growth are almost always limiting and are rarely available continuously. Since *E. coli* MG1655 uses up to seven different sugars during colonization (36, 87), this suggests that *E. coli* leads a scavenger lifestyle. It is likely these preferred nutrients are limiting in the mammalian intestine. In such nutrient-limiting conditions, *E. coli* relies on intracellularly stored carbon and energy (90). Glycogen is the primary carbon and energy storage molecule for enteric bacteria, which is synthesized when carbon is abundant and mobilized when needed to offset nutrient limitation (90–92). Knockout mutants unable to synthesize or degrade glycogen had significant colonization defects when competed against the wild type, suggesting that *E. coli* encounters famine conditions in the mouse intestine, and therefore, glycogen reserves are required for efficient colonization (90). The dependence of *E. coli* on glycogen during colonization was confirmed by providing an excess of gluconate (2% wt/vol) in the drinking water, which rescued the colonization defects of the glycogen metabolism mutants. Gluconate is not absorbed by animals and when it is provided to mice in excess, it therefore provides energy for *E. coli* to overcome mutational loss of glycogen stores. The conclusion is *E. coli* leads a feast and famine existence in the intestine that is mitigated by glycogen as a readily mobilizable energy source during intermittent famine.

NUTRIENT CONSUMPTION BY *E. COLI* PATHOGENS

Research on nutrient use by *E. coli* was not limited to commensal *E. coli* MG1655; nutrient use by other commensal strains *E. coli* Nissle 1917 and *E. coli* HS and some pathogenic strains EHEC EDL933, UPEC strain CFT073, and EPEC strain E2348/69 has also been explored. *E. coli* Nissle 1917 was isolated during World War I from a soldier who resisted infection during a *Shigella* outbreak (93). Since the early 1920s, Nissle 1917 has been used for the treatment of gastrointestinal diseases and is marketed under the trade name “Mutaflor” (94). Mutaflor is known to be effective in causing remission of ulcerative colitis without any side effects (94–96). Studies showed that *E. coli* Nissle 1917 uses seven different carbon sources during colonization of the intestine, including arabinose, fucose, galactose, gluconate, N-acetylgalactosamine, N-acetylneuraminic acid, and mannose (6). *E. coli* HS was isolated from a healthy human in 1958 and is considered a true human commensal as it colonizes humans at 10^{10} CFU/g of feces without causing disease (6, 97, 98). Although *E. coli* HS can use many carbon sources *in vitro*, it uses only six of those for colonizing the intestine, including galactose, arabinose, gluconate, N-acetylglucosamine, lactose, and ribose (6).

EHEC EDL933 caused the first major hamburger-borne outbreak of hemorrhagic colitis and hemolytic uremic syndrome in the United States (99). As described above, laboratory cultures of *E. coli* MG1655 and *E. coli* EDL933 utilize 13 monosaccharides equally well and in the same order (36). However, *E. coli* EDL933 occupies a distinct niche in the intestine which is defined by the utilization of seven different sugars: arabinose, galactose, hexuronates, mannose, N-acetylglucosamine, ribose, and sucrose (36). This indicates that *E. coli* strains with nearly identical catabolic potential vary significantly in the sugars that support their colonization. Studies of bovine intestinal contents (BIC) showed that *E. coli* EDL933 uses mannose, N-acetylglucosamine, N-acetylneuraminic acid, and galactose as its preferred carbohydrates during growth in bovine small intestinal contents with live endogenous microbiota (100). Although N-acetylneuraminic acid was not a preferred nutrient source used by *E. coli* EDL933 during colonization of the mouse large intestine (36), its utilization during colonization of bovine small intestinal contents (100) suggests that the nutritional requirements of *E. coli* strains may vary when it colonizes the small intestine as compared to the large intestine.

UPEC CFT073, a human urinary tract pathogen isolated from a patient with pyelonephritis, uses arabinose, galactose, mannose, N-acetylglucosamine, and ribose while colonizing the intestine, whereas it needs to import peptides and relies on gluconeogenesis for fitness during urinary tract infection (3, 101). Carbon sources utilized by EPEC E2348/69, the first diarrheal strain to be tested in humans (102), are also known to some extent (3). EPEC E2348/69 utilizes at least arabinose, galactose, mannose, N-acetylglucosamine, and ribose to colonize the intestine (3). In murine models of non-infectious colitis, the release of lactate by intestinal epithelial cells increased lactate availability in the gut lumen, and commensal *E. coli* and pathogenic *Salmonella* utilized lactate to increase their fitness (103). Garzetti and co-workers demonstrated that *E. coli* Mt1B1, which was isolated from a conventional mouse (104), utilizes galactitol while colonizing the mouse intestine. Galactitol is contained in several plants and is generated by galactose oxidation in the intestine; ~50% of *E. coli* strains can utilize it (71). Thus, the pathogens that have been investigated each utilize different carbon and energy sources to colonize.

NITROGEN ASSIMILATION IN THE INTESTINE

Until now, most research into the nutrition of enteric bacteria focused on carbon and energy sources and electron acceptors. Recently, nutrient sources other than sugars used by *E. coli* during colonization of the intestine have gained attention. Schubert and co-workers (68) showed that aspartate serves as the source of fumarate used for anaerobic respiration by *E. coli* in the intestine. Aspartase (L-aspartate ammonia-lyase) deaminates aspartate to produce fumarate and ammonia (105). The Schubert group also showed that *E. coli* assimilates the ammonia released by aspartase enzyme, which serves as the sole source of nitrogen under aerobic and anaerobic conditions in laboratory cultures (68, 106). It has been shown that, in an inflamed gut, pathogenic *E. coli* LF82 reprograms its metabolism to utilize L-serine, while the genes involved in the catabolism of simple sugars were significantly downregulated (107). When L-serine utilization genes were knocked out, it reduced the competitive fitness of the strain during inflammation. When L-serine was removed from the diet, its availability in the lumen was reduced, and this diminished the bloom of *E. coli* LF82 in the inflamed gut (107). However, it is important to note that serine use by commensal *E. coli* has not been studied in the intestine. The *eut* (ethanolamine utilization) gene cluster was upregulated in EHEC O157:H7 grown in bovine intestinal contents, and ethanolamine present in BIC was shown to be utilized by *E. coli* (108). EHEC can use ethanolamine as a nitrogen source *in vitro* but not as a carbon source (108). Taken together, these findings suggest that EHEC might use ethanolamine as a nitrogen source in the intestine. Interestingly, ethanolamine utilization genes are also upregulated in commensal *E. coli* MG1655 when grown in cecal mucus *in vitro* (87) and when colonizing the mouse intestine (109). However, an ethanolamine utilization pathway mutant (*E. coli* MG1655 Δ eutBC) was not defective in colonization (87), suggesting that ethanolamine is not used by commensal *E. coli* in

the intestine. One conclusion from these findings is that different *E. coli* commensal and pathogenic strains utilize diverse nutrients while colonizing the mammalian intestine (Table 1), and nutrient choice may vary according to niche occupation, diet, and host physiology.

COMPETITION FOR NUTRIENTS IN THE MOUSE INTESTINE

The source of nutrients in the large intestine and some of the specific nutrients used by some of the resident microorganisms are known. However, not much is known about the mechanisms used by resident microbes to compete for the available nutrients. There are at least four different ways by which *E. coli* can compete for nutrients in the intestine (Fig. 2).

First, as discussed above, since different *E. coli* strains have different *in vivo* nutritional preferences (3, 6, 36, 87), they can utilize nutrients not used by competitors in the intestine. To identify the nutrients used by each strain in the intestine, mutations in genes involved in the utilization of 13 different nutrients known to be present in the mucus were constructed in each strain, and the mutants were competed against the isogenic wild type using the streptomycin-treated mouse model. Based on competitive colonization experiments, if the mutant in a particular metabolic pathway had a significant defect in colonization when competing against its isogenic parent strain ($\geq 0.8 \log_{10}$ fold difference and P -value < 0.05), it was concluded that the strain uses that nutrient for colonization. It was found that *E. coli* MG1655 uses five nutrients present in mucus: arabinose, fucose, gluconate, N-acetylglucosamine, and N-acetylneuraminic acid (sialic acid) (36, 87). *E. coli* Nissle 1917 used a different set of seven carbon sources to colonize the intestine: arabinose, fucose, galactose, gluconate, N-acetylgalactosamine, N-acetylneuraminic acid, and mannose. Another commensal strain, *E. coli* HS, used six different sugars, including arabinose, galactose, gluconate, N-acetylglucosamine, lactose, and ribose (6). Each of the three commensal strains, *E. coli* MG1655, *E. coli* Nissle 1917, and *E. coli* HS, utilized at least one sugar not used by the other strains in the intestine (6, 36, 87). These results suggest that each commensal can colonize the intestine in the presence of other strains by occupying distinct nutrient-defined niches. Thus, it is not surprising that mice pre-colonized with one of the human commensal *E. coli* strains allowed subsequent colonization by another human commensal *E. coli* strain (110). For example, when mice were pre-colonized for 10 days with *E. coli* MG1655 and subsequently fed *E. coli* HS or *E. coli* Nissle 1917, either competing strain grew from low to higher numbers, suggesting they occupied distinct niches in the intestine (110). Similar results were obtained when mice were pre-colonized with *E. coli* HS or *E. coli* Nissle 1917 and then challenged with the other commensals (110). These results are discussed in more detail below in the section entitled "Overcoming Colonization Resistance."

The nutrient niches occupied by *E. coli* EDL933 differed from *E. coli* MG1655, in that the former strain used galactose, hexuronates, mannose, and ribose which were not used by the latter strain. Conversely, *E. coli* MG1655 used N-acetylneuraminic acid and gluconate which were not used by *E. coli* EDL933 (36). *E. coli* EDL933 also appeared to utilize ethanolamine, possibly as a nitrogen and carbon source, whereas *E. coli* MG1655 did not appear to utilize it (87, 108, 111). Similarly, *E. coli* EDL933 displayed a different

TABLE 1 Nutrients used by different *E. coli* bio- and pathotypes to colonize the mammalian intestine

Strain	Limiting nutrients ^a in the intestine known so far
<i>E. coli</i> MG1655	Arabinose, fucose, gluconate, N-acetylglucosamine, N-acetylneuraminic acid, maltose, glycogen
<i>E. coli</i> Nissle 1917	Arabinose, fucose, galactose, gluconate, N-acetylgalactosamine, N-acetylneuraminic acid, and mannose
<i>E. coli</i> HS	Arabinose, galactose, gluconate, lactose, N-acetylglucosamine, and ribose
<i>E. coli</i> CFT073	Arabinose, galactose, mannose, N-acetylglucosamine, and ribose
<i>E. coli</i> E2348/69	Arabinose, galactose, mannose, N-acetylglucosamine, and ribose
<i>E. coli</i> EDL933	Arabinose, fucose, galactose, hexuronates, mannose, N-acetylglucosamine, ribose, maltose, glycogen, and ethanolamine

^a"Limiting nutrients" indicates mutants in the pathways involved in the utilization of the compound are defective in competitive colonization against the wild type by $> 0.8 \log_{10}$ fold and Student's t -test value of < 0.05 .

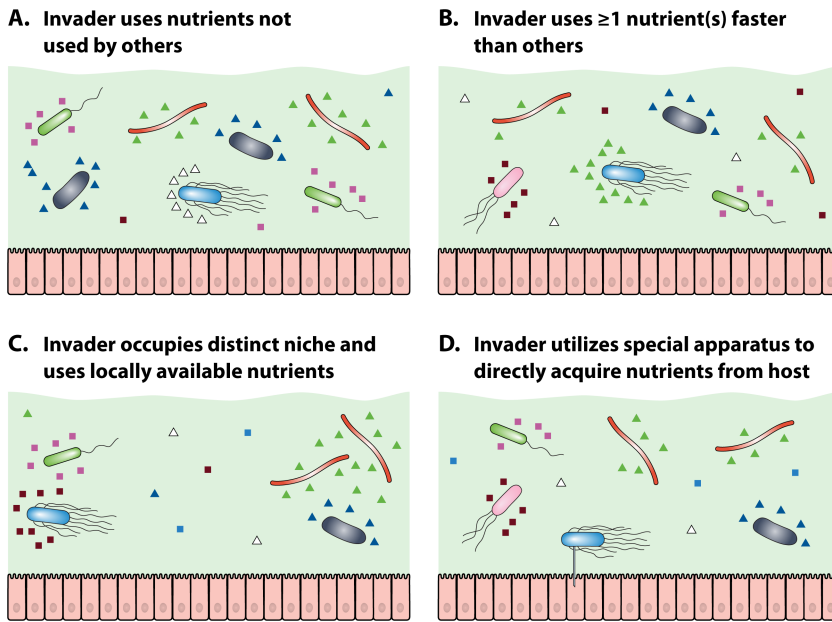


FIG 2 Four strategies used by an invading bacterium (blue) to compete for nutrients (represented by colored triangles and squares) with resident microbiota; anaerobes are shown in green and black, and facultative anaerobes are shown in red and pink. (A) Invader utilizes nutrients (white squares) not used by other members of the microbiota, and hence, does not have to compete for the nutrients. (B) Invader utilizes at least one nutrient (green triangles) faster than other members of the microbiota [in this case, another facultative anaerobe (red)]; this provides an advantage over the competing microbiota. (C) Invader occupies a distinct niche in the intestine where it uses a nutrient (magenta squares) that is locally available. Although the invader may fail to compete with the microbiota in major niches, it will thrive on a locally available nutrient. (D) Invader acquires some nutrients directly from the host cell using a nanotube, so it does not have to compete with other bacteria for nutrients (created with BioRender.com).

nutritional program compared to *E. coli* Nissle 1917 or *E. coli* HS while colonizing the mouse intestine (6, 36). Furthermore, *E. coli* EDL933 demonstrated metabolic flexibility by switching to gluconeogenic substrates from glycolytic substrates when competing against commensal *E. coli* strains (112, 113). Bertin et al. (114), also demonstrated the importance of gluconeogenesis for maintaining colonization of enterohemorrhagic *E. coli* in the bovine intestine.

Since none of the commensal strains alone can saturate all the niches occupied by *E. coli* EDL933 in the intestine, they cannot prevent *E. coli* EDL933 from colonizing. In mice pre-colonized with *E. coli* MG1655 and *E. coli* HS individually, *E. coli* EDL933 grew from 10^5 CFU fed to 10^6 – 10^7 CFU/g of feces, showing that these commensal strains cannot prevent *E. coli* EDL933 from colonizing (110). In the case of *E. coli* Nissle 1917 pre-colonized mice, colonization of *E. coli* EDL933 was limited and reduced to 10^3 CFU/g of feces, but the pathogen was not eliminated (110). However, when *E. coli* EDL933 was fed to mice pre-colonized with three commensal strains, *E. coli* MG1655, *E. coli* Nissle 1917, and *E. coli* HS, it was eliminated from the intestine (110). Since together these commensal strains consumed all the nutrients utilized by *E. coli* EDL933 to colonize the intestine, these strains could exert colonization resistance based on nutrient limitation (110). In fact, just two pre-colonized strains fed together, *E. coli* Nissle 1917 and *E. coli* HS, utilized five sugars which are important for *E. coli* EDL933 colonization and thus prevented colonization of *E. coli* EDL933 (6).

While it seems reasonable to find a set of commensal *E. coli* strains which could be used to prevent colonization by any single pathogenic *E. coli* strain, it is unlikely to find a small set of commensal *E. coli* strains that will be effective in preventing colonization of all *E. coli* pathogens. This is understandable since *E. coli* pathotypes each occupy

distinct intestinal niches (86). Uropathogenic *E. coli* CFT073 and enteropathogenic *E. coli* E2348/69 occupy niches distinct from those occupied by *E. coli* EDL933, as *E. coli* CFT073 and *E. coli* E2348/69 grew from low to higher numbers in *E. coli* EDL933 pre-colonized mice (86). When uropathogenic *E. coli* CFT073 pre-colonized mice were challenged with *E. coli* EDL933, *E. coli* EDL933 failed to grow to higher numbers but was not eliminated, suggesting that *E. coli* CFT073 outcompetes *E. coli* EDL933 in its preferred niche(s) (86). As expected, when *E. coli* Nissle 1917 and *E. coli* HS were pre-colonized together, a combination that conferred colonization resistance against *E. coli* EDL933, they failed to prevent colonization of *E. coli* CFT073 or *E. coli* E2348/69, indicating these pathotypes occupy intestinal niches not occupied by commensals *E. coli* Nissle 1917 and *E. coli* HS (86). The examples described above in this section indicate the six strains examined each use different nutrients to compete in distinct intestinal niches.

The second way in which *E. coli* can compete for nutrients is by growing faster on nutrients that are used by other strains, thereby outcompeting them (115). Spontaneous mutants of *E. coli* MG1655 with deletions in the *flhDC* operon were obtained during passage through the streptomycin-treated mouse intestine. These mutants grew 15%–30% faster than the wild type on several carbon sources (115). The *flhDC* operon encodes FlhD₄C₂ transcriptional regulator which controls expression of flagellar genes in *E. coli* (116). Those mutants were non-motile and were better colonizers of the mouse intestine than the wild type (115). During intestinal colonization by *E. coli* MG1655, it was found that 90% became non-motile by day 15 after feeding; all the non-motile mutants had 4–500 bp deletions in the *flhDC* promoter region (117). Colonization of germ-free mice with *E. coli* also led to the rapid selection of non-motile mutants having deletions in the *flhDC* operon (118). *E. coli* M1655 *flhDC* mutants had a competitive advantage in two ways: (i) genes involved in carbon and energy metabolism were upregulated, leading to more rapid growth, and (ii) energy conserved by being non-motile was directed to other cellular activities (115, 117). Thus, *E. coli* can gain a competitive advantage by outcompeting other microorganisms for their preferred nutrients.

The third strategy employed by *E. coli* to compete for nutrients is to occupy a distinct niche by entering a symbiotic association with anaerobes that release its preferred nutrients (83). When streptomycin-treated mice were fed *E. coli* MG1655 wild type, 90% of the cells became non-motile as a result of deletions in *flhDC* operon (117). The remaining 10% retained motility yet were better colonizers than the wild type during competition experiments (83). Those motile strains were *envZ* missense mutants; one of them (*envZ*_{P41L}) was more resistant to colicin V and bile salts and grew faster than the wild type on several sugars (83). The *E. coli* MG1655 *envZ*_{P41L} mutant isolated from mice had higher levels of OmpR~P and displayed different outer membrane protein profiles (higher OmpC and lower OmpF) than the wild type (84). When germ-free mice were fed *E. coli* MG1655, the germ-free mouse intestine also selected for mutants in the EnvZ/OmpR two-component system that had lower OmpF production and increased OmpC production compared to the wild type (119). In response to the osmolarity of the environment, EnvZ, the histidine kinase of the EnvZ/OmpR two-component system, phosphorylates response regulator OmpR which then regulates over 100 genes including those encoding outer membrane proteins OmpC and OmpF (120). As compared to *flhDC* deletion mutants, *E. coli* MG1655 *envZ*_{P41L} grew 15% slower *in vitro* in mouse cecal mucus and several sugars present in the mucus. However, the mutant colonized the mouse intestine better than the wild-type *E. coli* MG1655 and equally well as the *E. coli* MG1655 *flhDC* deletion mutant (83). Furthermore, *E. coli* MG1655 *envZ*_{P41L} appeared to occupy a minor galactose-defined intestinal niche that was not colonized or poorly colonized by an *E. coli* MG1655 *flhDC* mutant. When the *envZ*_{P41L} gene was transferred from the *E. coli* MG1655 background into *E. coli* Nissle 1917, the newly constructed *E. coli* Nissle 1917 *envZ*_{P41L} also produced higher levels of phosphorylated OmpR, more OmpC, and less OmpF than the parent strain *E. coli* Nissle 1917 (84). Like *E. coli* MG1655 *envZ*_{P41L}, it was more resistant to bile salts and colicin V and also grew slower than the parent strain in cecal mucus and on several sugars present in mucus, yet

it colonized the mouse intestine better than the parent strain by 10-fold (84). Although *E. coli* Nissle 1917 *envZ*_{P41L} did not grow as fast as the parent strain on galactose as the sole carbon source *in vitro*, it utilized galactose to colonize a minor intestinal niche not colonized or poorly colonized by *E. coli* Nissle 1917 (84). These results suggest that hyper-activation of OmpR caused the *envZ*_{P41L} mutants to occupy a niche that is distinct from those occupied by the parent strains.

Since *E. coli envZ*_{P41L} colonized better by occupying a galactose-defined niche and had higher levels of OmpC and lower levels of OmpF, we hypothesized that outer membrane proteins played a role in colonizing distinct nutritional niches in the intestine. In a recent study (109), we found that changes in outer membrane protein profiles contributed to bile tolerance in the intestine and conferred better colonizing ability. An *ompC* deletion mutant was outcompeted by the wild type because it took time to adapt to the bile salt concentration in the intestine. Furthermore, we found an *ompF* deletion mutant outcompeted the wild type during colonization. Higher production of OmpC in the *ompF* mutant made it a better colonizer. However, OmpC overproduction via a constitutive promoter alone was not sufficient for better colonization except when *ompF* was deleted. A strain which overproduced OmpC and lacked OmpF was a better colonizer than the parent strain, suggesting that fine-tuning of OmpC and OmpF is crucial for colonization. We provided evidence that OmpC is important for *E. coli* to colonize the intestine because its smaller pore size excludes bile salts, while OmpF is deleterious because its larger pore size allows bile salts to enter the periplasm. However, the *ompF* mutant competed against the wild-type *E. coli* MG1655 in the same niche and did not grow faster than the parent strain with galactose as the sole carbon source. This suggests the galactose niche occupied by *E. coli* MG1655 *envZ*_{P41L} was not due to the alteration in outer membrane protein composition, and some other functions regulated by EnvZ/OmpR must be responsible for better galactose utilization. Interestingly, there is a putative OmpR-binding site upstream of *galR* promoter between *galR* and *omrB* (83, 121). Galactose transport and metabolism in *E. coli* are under the control of the GalR repressor (122). However, it is not yet known whether OmpR regulates *galR* transcription. Negative regulation of GalR by phosphorylated OmpR could explain why *E. coli* MG1655 *envZ*_{P41L} occupies a galactose-specific niche not occupied by wild-type *E. coli* MG1655.

Fourth, recently, a novel strategy used by EPEC to acquire nutrients from infected host cells was described by Pal and co-workers (123). In a process termed host-nutrient extraction, the authors showed that EPEC uses the CORE complex composed of integral inner membrane proteins to form tubular outer membrane extensions connecting the bacteria to the infected host cells. The nanotubes serve as a channel to import nutrients from the host cell cytoplasm (123). The authors showed that the infected bacteria acquired amino acids through the nanotubes, which suggests the possibility that other nutrient sources also could be acquired by the pathogens using this strategy (123). Since the attached pathogen is getting nutrients directly from the host cells without releasing nutrients to the competing microbiota, this could be a furtive strategy employed by pathogens to compete for nutrients and colonize the intestine.

NON-NUTRITIONAL ASPECTS OF COLONIZATION RESISTANCE

When intestinal pathogens enter the intestine, they must compete with the host microbiota to colonize and successfully establish an infection. Thus, the intestinal microbiota serves as a barrier to colonization by many invading pathogens (17, 50, 124, 125). Potential mechanisms of colonization resistance are not fully understood but are thought to include nutritional and non-nutritional aspects (126). Regarding non-nutritional causes, susceptibility to invading pathogens can be enhanced by perturbations of the microbiota caused by diverse factors such as diet, inflammation, or antibiotics (127–131). For instance, Sekirov and co-workers (130) demonstrated that clinically relevant doses of streptomycin and vancomycin in drinking water had a dose-dependent alteration on intestinal bacteria composition of C57BL/6 mice. They went on to show that greater alterations in the intestinal microbiota resulted in increased susceptibility

to *Salmonella* Typhimurium colonization and more severe intestinal pathology. Thus, colonization resistance describes direct and indirect interactions between the microbiota, host, and pathogens that prevent pathogens from invading (132, 133). However, the mechanisms by which the resident microbiota confers colonization resistance are not completely understood. In addition to direct competition between the microbiota and pathogens for essential nutrients (6, 70, 71, 134), several factors are known to contribute to colonization resistance, including inhibition of pathogen growth by microbiota-derived substances including short-chain fatty acids (SCFA) and bacteriocins (135–138), ability to tolerate environmental stressors (109, 127), type VI secretion system-mediated killing of invading bacteria (139), and microbiota-induced stimulation of innate and adaptive immune responses (50, 140).

Although exploring the nutritional basis of colonization resistance is the focus of this review, it is worth discussing, in brief, some other non-nutritional mechanisms of colonization resistance. Osbelt et al. (135) demonstrated that the presence of SCFA (especially butyrate)-producing bacteria belonging to the phylum Firmicutes inhibited the growth of *C. rodentium* in mice. Supplementation of mice with butyrate was sufficient to reduce *C. rodentium* growth *in vivo*, suggesting the role of microbiota-derived metabolites in colonization resistance (135). SCFAs are known to exert diverse effects on pathogenic bacteria (141). For instance, SCFAs inhibit the growth of Enterobacteriaceae by acidifying the proximal colon and triggering intracellular acidification (136). Bacteriocins produced by many bacterial species, including lactic acid bacteria, have potent antimicrobial activity (142). *Lactobacillus salivarius* UCC118 has been shown to produce a bacteriocin Abp118 which significantly enhances resistance to infection by food-borne pathogen *Listeria monocytogenes* (137). *Clostridium scindens*, which converts primary bile acids to secondary bile acids, inhibits *C. difficile* infection in secondary bile acid-dependent fashion (143). *E. coli* Nissle 1917, which is an excellent biofilm former, outcompetes pathogenic *E. coli* strains during biofilm formation (144). *E. coli* Nissle 1917 has been shown to secrete a bifunctional (protease and chaperone) periplasmic protein DegP extracellularly and control biofilm formation by pathogenic strains (138). However, whether DegP has any role in colonization resistance conferred by *E. coli* Nissle 1917 is not yet known. Besides the microbiota-derived metabolites, environmental stressors like bile acids can also play a key role in colonization resistance. *E. coli* and *Salmonella* demonstrate enhanced bile resistance compared to other members of the microbiota when switched from plant-based maintenance diet to a high-fat diet (western diet), which promoted colonization by *E. coli* and *Salmonella* (127). Since a high-fat diet requires bile salts to be released into the gut lumen, high fat elevates the level of bile salts which inhibits other bacterial species but not *E. coli* or *Salmonella* spp., leading to a loss of colonization resistance against these species (127). While *S. Typhimurium* colonization was boosted by diet shifts and fat-elicited bile in mice lacking *E. coli* in their microbiota, competitive *E. coli* strains (mixture of three strains) provided protection against *S. Typhimurium* in the fat-challenged gut, suggesting that *E. coli* might limit the blooms of pathogens promoted by fat (127).

We recently showed that mice pre-colonized with *E. coli* MG1655 $\Delta ompC$, which is sensitive to physiological bile salt concentration in the intestine, failed to prevent colonization of *E. coli* MG1655, further supporting the finding that environmental stressors play important roles in colonization resistance (109). Hecht et al. (139) demonstrated the role of the type VI secretion system in colonization resistance in *Bacteroides fragilis*. When mice were colonized with a symbiotic non-toxigenic *B. fragilis*, it limited the colonization of pathogenic enterotoxigenic *B. fragilis*. The strain-specific competition was attributed to the type VI secretion system of non-toxigenic *B. fragilis*, and the colonization resistance conferred by non-toxigenic *B. fragilis* protected the host from enterotoxigenic *B. fragilis*-induced colitis (139). *E. coli* has also been shown to utilize a type VI secretion system to fight against the invading competitor *C. rodentium* and limit its colonization. Interestingly, *C. rodentium* also utilizes the same strategy of contact-dependent inhibition to compete for intestinal niches with other bacteria

(145). The resident microbiota can also stimulate host immune responses resulting in the production of antimicrobial peptides. These antimicrobial peptides can effectively inhibit intestinal pathogens and confer colonization resistance (50). For instance, the colonization of germ-free mice with intestinal microbiota triggers the expression of RegIII from intestinal epithelial cells (140). C-type lectin RegIII is an antimicrobial peptide which targets the peptidoglycan of Gram-positive bacterial cell walls, inhibiting pathogens such as Enterococci and *Listeria monocytogenes* in the intestine (140, 146).

NUTRIENT-DEPENDENT COLONIZATION RESISTANCE

Competition for available nutrients in the gut is profound, and the complexity of the gut microbiota ensures maximum utilization of the available substrates (147). Therefore, in a healthy, fully developed microbiota, it is unlikely that an incoming species finds an uncontested niche because it has to outcompete the established microbiota for nutrients to become established (148). This apparently explains why only a fraction of persons exposed to an enteric pathogen get infected during an outbreak. For instance, of the total 47,643 Japanese school children exposed to white radish sprout-associated *E. coli* O157:H7 in Sakai City, Osaka, Japan, only 398 were hospitalized, and 8,355 were symptomatic (149). Experiments with conventional mice have also demonstrated that the attack rate of different *E. coli* strains was less than 20% (7). In conventional mice, the feces contains 10^5 – 10^6 CFU of *E. coli* per gram of feces (32), which might exert colonization resistance against introduced *E. coli* strains depending on their nutritional programs. It has been shown that antibiotic-treated mice and germ-free mice have increased amounts of nutrients in the gut, including carbohydrates and amino acids, which lead to reduction in colonization resistance (150, 151). Thus, susceptibility to an invading pathogen can be determined by the availability to invaders of nutrients that are not consumed by the resident microbiota.

The fact that different *E. coli* strains have different nutritional programs in the intestine could explain why some humans are susceptible to infection, while in others, the microbiota exerts colonization resistance (149, 152). Previous studies in our laboratory revealed the nutrients used by commensal and pathogenic *E. coli* to colonize. Different *E. coli* strains demonstrated distinct preferences in the utilization of sugars present in mucus, and a defect in utilizing one or more of those sugars led to a competitive disadvantage against the wild type (6, 36, 86, 87, 90, 110, 112, 113). When mice pre-colonized with any of three commensal *E. coli* strains (*E. coli* MG1655, *E. coli* HS, or *E. coli* Nissle 1917) were challenged with isogenic strains, the pre-colonized strain eliminated the isogenic challenge strain (110). Since isogenic strains compete equally well for the same nutrients, the pre-colonized strain conferred colonization resistance to the isogenic challenge strain by consuming nutrients (110). This supports the previous finding that when *E. coli* strains isolated from the feces of healthy human volunteers were fed to the same individual, they could not colonize (153). This is also true for pathogenic *E. coli*. It was demonstrated that two closely related O157:H7 EHEC strains, *E. coli* EDL933 and *E. coli* Sakai, exert colonization resistance against each other and compete in the same niches (86). These two strains have similar physiology, including an identical nutrient utilization spectrum, as they have >99.9% sequence identity in their orthologous protein-coding genes (154). The minor genomic differences reflect a difference in the annotation strategies that were used for the two genomes (155). The nearly identical physiology and metabolism of these two O157:H7 strains explain how they are able to exert colonization resistance against each other.

OVERCOMING COLONIZATION RESISTANCE

Having discussed many potential mechanisms of colonization resistance, we turn our attention to how invading pathogens overcome colonization resistance. When mice pre-colonized with one human *E. coli* commensal strain (*E. coli* MG1655, *E. coli* HS, or *E. coli* Nissle 1917) were challenged with a different commensal strain, the challenge strains were able to overcome colonization resistance, and the two strains co-colonized

the intestine (110). Since each of the three human *E. coli* commensal strains utilizes at least one sugar not used by the other strain, these commensal strains are able to occupy distinct nutrient-defined niches not occupied by the other commensal strains (36, 87, 110). Prior colonization of any one of three commensal *E. coli* strains cannot prevent pathogenic *E. coli* EDL933 from colonizing the mouse intestine, although the population of the *E. coli* EDL933 challenge strain varied depending on the pre-colonized commensal strain. For example, in mice pre-colonized with *E. coli* Nissle 1917, *E. coli* EDL933 dropped from 10^5 CFU/g of feces to 5×10^3 CFU/g of feces (110), whereas in mice pre-colonized with *E. coli* MG1655 or *E. coli* HS, *E. coli* EDL933 grew to higher populations. Likely, the varying responses to *E. coli* EDL933 challenge are due to the different nutritional programs of the commensal strains (6, 36, 87). *E. coli* Nissle 1917 is a superior colonizer compared to *E. coli* MG1655 and *E. coli* HS and most likely fills more niches than the other commensal strains (110). Although *E. coli* EDL933 could overcome colonization resistance to any individual commensal strain, when mice were pre-colonized with mixtures containing *E. coli* Nissle 1917, *E. coli* HS, and *E. coli* MG1655 the commensal strains conferred colonization resistance (110). Thus, it appears that an approach to prevent or treat enteric infections would be to pre-colonize humans with strains that would occupy all nutritional niches such that the pathogens would be eliminated from the intestine. This strategy could be one mechanism by which Mutaflor mitigates traveler's diarrhea. This approach, however, would not work if the invading species grows faster on the same nutrients and outcompetes the members of the microbiota. For instance, mice pre-colonized with *E. coli* MG1655 could not exert colonization resistance to *E. coli* MG1655 *flhDC* mutants as the mutants grew 15%–30% faster than the wild type on several carbon sources and, therefore, colonized better than the wild type (115).

E. coli competes for niches not only with other *E. coli* but also with other bacteria which utilize identical nutrients in the intestine. In germ-free mice infected and stably colonized with *C. rodentium*, challenge with *E. coli* decreased the *C. rodentium* population by almost 500-fold within 14 days of challenge (134). Both *C. rodentium* and *E. coli* grew well on monosaccharides, suggesting that the depletion of simple sugars by *E. coli* allowed it to outcompete the pathogen (134). Utilization of the limiting nutrient galactitol by *E. coli* prevented niche invasion by *Salmonella* Typhimurium which could otherwise use galactitol for colonization (71). *Klebsiella michiganensis* conferred colonization resistance against *E. coli* via superior sugar utilization; provision of a carbon source (galactitol) that is accessible to *E. coli* but not to *K. michiganensis* mitigated colonization resistance (156). This suggests that strategies can be developed where commensal strains selectively prevent colonization of pathogenic *E. coli* and other enteric pathogens. To this end, scientists have created a minimal bacterial community that provides colonization resistance to enteric pathogens. A bacterial consortium comprised 12 bacterial isolates also known as Oligo-Mouse-Consortia (Oligo-OMM¹²), providing partial protection against *S. Typhimurium* (157). When three more facultative anaerobes including *E. coli* Mt1B1 were added to Oligo-OMM¹², the mixture conferred complete protection against *S. Typhimurium* (157). Microbial strains in the Oligo-MM¹² could be tailored (expanded or reduced) to provide colonization resistance to specific pathogens (157). Although the focus of this article is on the nutritional basis of colonization resistance by different *E. coli* strains, this concept should hold true for other members of the resident microbiota and invading pathogens (133, 158).

RESTAURANT HYPOTHESIS

The microbial community structure within the intestine is determined by competition for resources. According to Rolf Freter, competition for nutrients is the most important factor for success in the intestinal ecosystem, although several other factors could contribute to an organism's ability to colonize (159). Freter and colleagues developed an anaerobic continuous-flow culture which demonstrated that stable multispecies communities similar to those in the large intestine of mice could be established (160).

Freter postulated that, for many species to co-colonize, each species must use at least one limiting nutrient better than all others (7, 159). Thus, if the resident microbiota in the intestine consumes all the nutrients that an invading species needs to colonize, the invading species will not find the nutrients it requires and will fail to become established (62). While Freter's nutrient-niche hypothesis has been widely accepted and is consistent with ecological succession during conventionalization of mice and microbiota-generated nutrient utilization by pathogens (161–163), its validity has been challenged by findings which demonstrate mixed-substrate utilization, metabolic flexibility, and heterogeneity of nutrients in time and space (reviewed extensively by Pereira and Berry)(148).

Some of the findings in studies conducted in our laboratory are also inconsistent with Freter's nutrient niche hypothesis (83, 84, 117). For example, while Freter's hypothesis can easily explain why the streptomycin-treated mouse intestine selects *E. coli* MG1655 *flhDC* mutants (115, 117), it cannot explain the selection of motile *E. coli* MG1655 *envZ* missense mutants by the streptomycin-treated mouse intestine (83, 117). *E. coli* MG1655 *flhDC* mutants grow 15%–30% faster on several sugars present in cecal mucus than *E. coli* MG1655 wild type, which explains why they were selected, consistent with the nutrient-niche hypothesis (115, 117). However, *E. coli* MG1655 *envZ*_{P41L} grew ~15% slower *in vitro* than the *E. coli* MG1655 *flhDC* mutant on mouse cecal mucus and several sugars present in mucus but was a much better colonizer than the wild-type *E. coli* MG1655 like the *E. coli* MG1655 *flhDC* mutant (83). Slower growth of *envZ*_{P41L} mutants that colonize equally well as the *E. coli* MG1655 *flhDC* mutants during competitive colonization is inconsistent with the nutrient-niche hypothesis (83). In follow-up studies in our laboratory, *E. coli* Nissle 1917 *envZ*_{P41L}, like *E. coli* MG1655 *envZ*_{P41L}, appeared to use galactose to colonize a second intestinal niche not colonized or poorly colonized by *E. coli* Nissle 1917 wild type (84). However, *E. coli* Nissle 1917 *envZ*_{P41L} grows slower than *E. coli* Nissle 1917 on galactose as the sole carbon source (84). Interestingly, *E. coli* Nissle 1917 *envZ*_{P41L} appeared to be worse than its parent strain at preventing EHEC EDL933 colonization despite being a better colonizer (84). These results, which are inconsistent with the nutrient-niche hypothesis, led to the development of the "restaurant hypothesis," which emphasizes that organisms colonize the intestine as members of mixed biofilms and obtain the sugars they need for growth locally through cross-feeding from polysaccharide-degrading anaerobes (3, 83, 84). This contrasts with the nutrient-niche hypothesis, which assumes that nutrients are perfectly mixed in the intestine and are equally available to all species present (7).

Biofilms form in the mucus layers of the mammalian large intestine (164, 165) and our previous research demonstrated that commensal *E. coli* resides in mixed biofilms in the mouse intestine (83, 84). As discussed previously, *E. coli* growth in the intestine depends on the anaerobes, which degrade polysaccharides releasing monosaccharides and disaccharides (70, 71). Thus, anaerobes in the mixed biofilms release sugars that *E. coli* uses, in contrast to using nutrients from a perfectly mixed pool as assumed by the nutrient-niche hypothesis (3, 7). Those mixed biofilms feed *E. coli*, and the restaurant hypothesis proposes that different *E. coli* strains reside in different restaurants and interact with different anaerobes physically and metabolically. Hence, different *E. coli* strains may enter different restaurants which serve different nutrients. This hypothesis explains why different *E. coli* strains, despite utilizing the same nutrients in identical order *in vitro*, display different nutritional programs in the mouse intestine (3, 6, 36, 87).

The restaurant hypothesis can explain why *E. coli* MG1655 *envZ*_{P41L} and *E. coli* Nissle 1917 *envZ*_{P41L} are better colonizers than their parent strains, although they grow more slowly in cecal mucus and on several sugars present in mucus (83, 84). First, the mutants appear to enter a different biofilm (niche) consisting of different groups of anaerobes, which serve galactose in a niche that is not colonized or poorly colonized by the parent strains. Second, *E. coli* MG1655 *envZ*_{P41L} and *E. coli* Nissle 1917 *envZ*_{P41L} have decreased motility and different outer membrane protein profiles such as higher *OmpC* and lower *OmpF* than the parent strains, which could increase affinities to mixed biofilms. The higher phosphorylated *OmpR* levels present in *envZ* missense mutants also differentially

regulate the expression of several other outer membrane protein genes *cirA*, *fecA*, *fepA*, and *ompT* via *omrA* and *omrB* small RNA expression (121). OmpC has been shown to function as an adhesin (166) and is highly expressed in biofilms compared to planktonic cells (167), suggesting that OmpC might contribute to biofilm formation. Interestingly, in our recently published study (109), we showed that an *E. coli* MG1655 *ompC* mutant colonizes the mouse intestine poorly compared to *E. coli* MG1655 wild type primarily for two reasons. First, the strain lacking *ompC* is more sensitive to physiological bile salt concentrations than the parent strain and is outcompeted by the parent strain *in vitro* and *in vivo* in the presence of bile salts. The poor colonization of *E. coli* MG1655 *ompC* is attributed to the mutant taking time to adapt to bile salts in the intestine. Second, we showed that mice pre-colonized with *E. coli* MG1655 wild type exerted colonization resistance to an *E. coli* MG1655 *ompC* mutant and eliminated it from the intestine. However, in pre-colonized mice, an *E. coli* MG1655 *ompC* mutant does not exert complete colonization resistance against *E. coli* MG1655 wild type, and the wild type persists at 10^2 – 10^3 CFU/g of feces in the intestine. Although the *E. coli* MG1655 *ompC* mutant and *E. coli* MG1655 wild type compete primarily in the same niche, there is a minor intestinal niche not saturated by the *E. coli* MG1655 *ompC* mutant in which *E. coli* MG1655 wild type thrives. This can also be explained by the restaurant hypothesis: *E. coli* MG1655 wild type enters a minor “restaurant” not colonized or poorly colonized by *E. coli* MG1655 *ompC*, in addition to a major “restaurant” in which *E. coli* MG1655 wild type and *E. coli* MG1655 *ompC* compete and differ in their tolerance to an environmental stressor, i.e., bile salts, and have different binding affinities in the mixed biofilms.

The restaurant hypothesis also explains why *E. coli* Nissle 1917 *envZ*_{p41L}, despite being a better colonizer than *E. coli* Nissle 1917 wild type, was worse than its parent strain at preventing EHEC EDL933 colonization (84). This result suggests that when EHEC EDL933 enters the mouse intestine, it grows in mucus planktonically before entering a biofilm. According to the restaurant hypothesis, when *E. coli* Nissle 1917 wild type and *E. coli* Nissle 1917 *envZ*_{p41L} grow in different biofilms utilizing different sugars, some sugars might escape the biofilms and become available to planktonic *E. coli* cells. Some planktonic anaerobic bacteria may also release sugars that become available to planktonic *E. coli*. EHEC EDL933 may then compete planktonically with escaped planktonic *E. coli* Nissle 1917 wild type or *E. coli* Nissle 1917 *envZ*_{p41L} for the available nutrients, and the outcome of this competition depends on the nature of available nutrients and the nutritional program of the two competitors. This could explain why a better grower in cecal mucus *in vitro*, i.e., *E. coli* Nissle 1917, appears to prevent colonization of EHEC EDL933 in the intestine more than *E. coli* Nissle 1917 *envZ*_{p41L} does (3, 84).

OVERCOMING COLONIZATION RESISTANCE AS A MECHANISM FOR INVASION

Under steady-state conditions, the intestinal microbiota can successfully exert colonization resistance to many invading pathogens (17, 50, 124, 125). When the microbiota is perturbed by different factors such as diet, inflammation, or antibiotics, expansion of invading pathogens can occur (127–131). Besides the external factors that perturb the microbiota, the invaders may also utilize strategies to exploit gaps in colonization resistance and promote their expansion in the gut (147).

Some invaders utilize their metabolic versatility to overcome colonization resistance in the gut. When EHEC EDL933 is the only *E. coli* strain in the intestine, it does not use gluconeogenic substrates and relies on glycolytic substrates; only when it is competing against *E. coli* MG1655 or *E. coli* Nissle 1917 does EHEC appear to switch to gluconeogenic substrates (112). This metabolic flexibility and its different nutritional programs from commensal *E. coli* allows EHEC EDL933 to overcome colonization resistance exerted by *E. coli* MG1655 or *E. coli* Nissle 1917 (6, 36, 110, 112). *S. Typhimurium* overcomes the inhibitory effects of propionate produced by gut microbiota by using it as a carbon source (168). This short-chain fatty acid, which is produced by anaerobic fermentation of sugars, appears to exert colonization resistance against *S. Typhimurium* by acidifying

the intracellular space of *S. Typhimurium* and producing toxic by-products during its metabolism (169–172). *Klebsiella pneumoniae* also demonstrated metabolic flexibility, utilizing L-fucose to bypass nutritional competition with the resident microbiota and overcome colonization resistance (173). *C. rodentium* turned on the biosynthesis of amino acids to overcome colonization resistance exerted by the microbiota. Biosynthesis of amino acids was required for the colonization of specific pathogen-free mice, which have an intact microbiota, but not in germ-free mice (150). This is understandable since the concentrations of several amino acids were reduced in the intestinal contents of specific pathogen free (SPF) mice compared to those in germ free (GF) mice (150). Thus, metabolic versatility appears to be a key mechanism used by pathogens to overcome colonization resistance in the gut.

The other widely employed mechanism to overcome colonization resistance by invading pathogens is by inducing host-driven inflammation and utilizing nutrients uniquely present in those conditions (147). Stecher and colleagues (128) showed that inflammation induced by *S. Typhimurium* is sufficient to overcome colonization resistance by altering the microbiota composition. Winter et al. (174) showed, using models of colitis and intestinal injury, that nitrate generated by the host during inflammation conferred a growth advantage to *E. coli* in the inflamed gut of mice. We had previously shown that, in addition to aerobic respiration, *E. coli* utilizes fumarate and nitrate to respire anaerobically. However, fumarate is more important to *E. coli* than nitrate since nitrate is limiting in the intestine (53, 54), while aspartate is abundant and readily converted to fumarate (68). Similarly, during inflammation, the release of lactate by colonocytes increases the level of lactate in the gut lumen and bacteria like *E. coli* and *Salmonella* utilize the lactate to promote colonization (103). In addition to nitrate and lactate, further research demonstrated that the inflammatory host response induced by *S. Typhimurium* causes release of exudates that contain additional energy sources (electron donors) and anaerobic electron acceptors that promote pathogenic blooms in the gut (175, 176).

Some enteric pathogens also utilize “counter-attack” mechanisms such as bacteriocin production and use of secretion systems to antagonize the normal microbiota and replicate in the gut. For example, *S. Typhimurium* produces colicin Ib, which conferred a competitive advantage against commensal *E. coli* strains in the inflamed gut (177). *Vibrio cholerae* uses a type VI secretion system to kill commensal bacteria (178, 179). Other members of Enterobacteriaceae family, such as *C. rodentium* and *S. Typhimurium*, also utilize type VI secretion systems that provide a competitive advantage against the resident microbiota and promote their replication in the gut (145, 180). *C. rodentium* directly targeted and outcompeted commensal *E. coli* using its type VI secretion system to successfully establish itself within the murine gut (145). However, normally, enteric pathogens enter stationary phase upon excretion by a host and are typically dormant or viable but not culturable in the presence of various stressors in the environment (181, 182). Therefore, once an invading pathogen enters the intestine, it must exit stationary phase and enter the growth phase, utilizing the nutrients that provide energy, before inducing inflammation or antagonizing the microbiota. Thus, it appears that metabolic flexibility may be more important in overcoming colonization resistance than other mechanisms used by pathogens.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

In this review, we focused on the nutritional aspects of intestinal colonization by *E. coli*. It is increasingly clear that colonized *E. coli* grows on free monosaccharides and disaccharides liberated by anaerobes in the intestine. Furthermore, different *E. coli* strains have different nutritional programs in the intestine despite using identical carbon and energy sources *in vitro*. There is fierce competition for nutrients in the intestine between invading species and the resident microbiota such that invaders can colonize only if they can outcompete the resident microbiota. While multiple factors can contribute to colonization and subsequent infection by invaders, it appears

that competition for nutrients and the ability of pathogens to demonstrate metabolic flexibility are key. If the invading species can utilize a different nutrient not utilized by commensals or if the invading species can grow faster than the resident microbiota on a particular nutrient, it can grow, first planktonically, and then enter a restaurant where it can thrive. However, too little is known about the mechanisms used by those microbes to compete for nutrients. Direct evidence for the availability of nutrients in the intestine and the use of those nutrients by competing *E. coli* strains in the intestine is lacking. A metabolomic approach to determine the nutrients available and used by commensals and nutrients available post-colonization for invading pathogens can provide more information regarding the nutritional basis for overcoming colonization resistance. Metabolomic analysis using high-throughput LC-MS has revealed that there is an abundance of sugars, gluconeogenic substrates, amino acids, and their derivatives in streptomycin-treated mouse cecal mucus, which explains why *E. coli* strains can grow to $\sim 10^9$ CFU/g of feces in streptomycin-treated mice (unpublished work). Further work in this area is indicated to better understand the mechanisms used by microbes to colonize the intestine. Understanding the nutritional program of pathogens in the intestine and developing strategies to prevent pathogens from acquiring essential nutrients in the intestine could prove valuable in the fight to prevent gastrointestinal diseases.

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