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RESPIRATION AND ENERGY METABOLISM OF *ESCHERICHIA COLI* IN THE INTESTINE

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degree of

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By

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RESPIRATION AND ENERGY METABOLISM OF *ESCHERICHIA COLI* IN THE INTESTINE

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY

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DEDICATION

This dissertation is dedicated to my two loving parents, Ali and Zhila Jones, whose constant support and sacrifice has allowed me to develop into the person I am today and pursue my love for science. Their encouragement and guidance not only allowed me to further my academic career but also gave me the strength to endure life's many challenges.

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ABSTRACT

Colonization of the gastrointestinal tract by *Escherichia coli* is dependent upon its ability to compete with the commensal microflora. We have established that nutrient acquisition is important for competition and fitness of *E. coli* strains in the mammalian intestine. However, the importance of respiration and the respiratory pathways used by *E. coli* for colonization was unknown prior to this study. We used a systematic mutational approach to test the hypothesis that respiratory metabolism is important for *E. coli* to colonize the mouse intestine. We found that a strain lacking cytochrome bo_3 oxidase (*cyoAB*) co-colonized with the wild-type. A mutant lacking the high oxygen affinity terminal cytochrome *bd* oxidase (*cydAB* and *cydDC*) failed to initiate and could not colonize. Of the four anaerobic respiratory pathways, dimethylsulfoxide (DMSO) reductase (*dmsAB*), trimethylamine-N-oxide (TMAO) reductase (*torCA*), and nitrite reductase (*nirBD*) were found to be unimportant, while nitrate reductase (*narG*, *narZ*, and *napDA*) and fumarate reductase (*frdA*) mutants were able to initiate colonization but showed major defects in maintenance. Our findings lead us to conclude that respiration of oxygen provides a competitive advantage to *E. coli in vivo* despite the low availability of its preferred carbon sources. *E. coli* also requires use of alternative anaerobic electron acceptors, such as nitrate and fumarate, to be competitive in the intestine. Furthermore, respiratory flexibility allows *E. coli* to maximize its colonization efficiency to ensure its success in the mammalian gastrointestinal tract. The experiments described in this study suggest that facultative anaerobes may make the intestinal environment more anaerobic, thereby promoting the stability of the predominantly anaerobic microflora. Indeed, the intestinal environment is likely microaerobic, oxygen tension almost certainly fluctuates in the intestine. These experiments provide the first evidence for an aerobic microhabitat in the intestine and suggest a hierarchy of alternative electron acceptors support colonization. Additionally, the results show that energy storage in the form of glycogen may also be important during times of nutrient limitation to enhance persistence of *E. coli* in the intestine.

Chapter 1

Literature Review

1.1 Introduction

Humans live in close association with a large number of microorganisms that are found on the skin, in the mouth, and in the intestinal tract. Many different bacterial species can colonize the large intestines of humans and animals. The human gastrointestinal (GI) tract is host to a complex and dense microbial ecosystem. There are approximately 10^{12} microorganisms per milliliter of luminal contents consisting of an estimated 800 different bacterial species: the intestinal microbial population outnumbers human cells by approximately 10:1 (30, 36, 78, 117, 234). *Escherichia coli* is the predominant facultative anaerobe found in the mammalian GI tract (85). However, despite being one of the best understood microorganisms, precisely how *E. coli* colonizes the mammalian large intestine still remains unknown (218).

The gut microflora is dependent on the availability of both nutrients and electron acceptors to establish colonization. The intestinal microbiota is important for many metabolic functions that support the digestive system and also provides resistance to bacterial infection. Control and manipulation of the intestinal microflora may prove to be beneficial for both therapeutic and preventive medicine that specifically relate to gastroenteritis.

1.2 Gastrointestinal tract

The human gastrointestinal tract includes the small and large intestine. The small intestine is responsible for chemical digestion and nutrient absorption and is divided into three sections: the duodenum, the jejunum, and the ileum. Unlike the small intestine in which only few bacteria inhabit (10^3 to 10^4 microorganisms per milliliter of intestinal contents), the large intestine contains the majority of intestinal microorganisms (approximately 10^{12} microorganisms per milliliter of intestinal contents) (157). The large intestine is the terminal part of the digestive tract and consists of three parts: the cecum, the colon, and the rectum. The primary function of the large intestine is to reabsorb water and inorganic salts where the intestinal contents are compacted and eliminated as feces. Unlike the small intestine, the mucosal surface of the large intestine is smooth and contains no intestinal villi. The colonic epithelial lining is estimated to shed 2 x 10^6 to 5 x 10^6 cells per minute (61). A thick layer of mucus overlies the epithelium and is largely made up of mucin, glycoproteins, proteins, glycolipids, lipids, and sugars (49, 157). The mucus layer is in a constant state of synthesis and secretion, the contents of which are degraded by the intestinal microflora. The mucus layer of a conventional mouse turns over approximately every two hours (157, 193). *E. coli* commensal strains have been shown to colonize the cecum and colon of the mammalian intestine, specifically in the mucus layer (231).

To colonize the intestine, bacteria ingested in relatively low numbers must acquire nutrients to grow and persist in high numbers. In general, the nutrients that support persistence of the intestinal microflora are thought to be fermentable carbohydrates (203). Little attention has been given to the possible role of bacterial respiration for coupling ATP generation to carbohydrate oxidation. If it is found that respiratory metabolism is important for bacteria *in vivo*, then electron acceptors need to be added to the list of nutrients that support colonization. The nutrient-niche hypothesis states: to coexist, each species in the intestine must grow better on one or a few limiting nutrients than all other species. Thus, ecological niches within the intestine are defined by nutrient availability (88). Our recently completed systematic analysis of bacterial carbon nutrition in the intestine calls into question the number of carbon sources that might support growth of a single species because we showed that at least seven monosaccharides contribute to a larger or lesser extent to colonization of *E. coli* (39). This finding suggests the nutritional diversity of *E. coli* in the intestine is a reflection of both its biochemical flexibility and the availability of carbon sources. *E. coli* is nearly equally flexible in its energy yielding metabolism. This dissertation addresses whether bioenergetic flexibility and the availability of electron acceptors in the intestine is important for colonization of a facultative anaerobe.

1.3 Intestinal microflora

The intestinal microbiota has been the subject of many studies since it was first discovered to be important to maintain gut stability, and therefore, a healthy condition

for human hosts. In 1965, Dubos et al. described the basic principles of the microbial ecology of the gastrointestinal tract (73). They hypothesized that the gastrointestinal microflora, which they termed the "indigenous flora" of any animal species is made up of the microbes present during the evolution of the animal (nonindigenous microbes), the ubiquitous microbes found in the animal's community established in all its members (normal microbiota), and the pathogenic microorganisms that have been accidentally acquired and are capable of persisting in the system (73). A microbial ecosystem is defined as a complex community of microorganisms in a particular environment and the surroundings with which the organisms are associated. An ecosystem contains habitats and niches for the residing microorganisms. Habitats are the physical spaces in the system, and the way an organism makes its living in its habitat defines its niche in the ecosystem (234).

The human colon is a dynamic habitat where individual bacteria exist in different microhabitats and metabolic niches. Information regarding the composition of the gut microbiota has largely arisen from fecal studies. The composition of the microbiota of human feces has been used to determine the composition of the microbial communities in the colonic lumen (234). A diverse bacterial population has been demonstrated to colonize the GI tracts of healthy individuals. The intestine is a challenging area to study due to immense species diversity, the vast range in abundance of the resident flora, and the inability to culture many of these species. Of the cultivable microorganisms, the dominant species found in the GI tract are limited to obligate anaerobes. In humans, the obligate anaerobes comprise more than 99.9 %

of the cultivable microorganisms (234). The colonic bacteria include *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Fusobacterium*, *Lactobacillus*, *Peptococcus*, *Peptostreptococcus*, *Streptococcus*, *Enterococcus*, *Ruminococcus*, and *Veillonella* (35, 64, 74, 233, 235, 237). These bacteria, and many others, form the complex and stable community of the GI tract. The presence of these microorganisms in the murine gastrointestinal tract will be considered in the next section.

1.4 Streptomycin-treated mouse model

There are both advantages and disadvantages in using *in vivo* models for intestinal colonization. There are three colonization models used to study colonization of the intestinal tract. The first of these models is the conventional mouse model. This mouse contains a complete microflora that allows for the examination of the interactions between the already established microbial populations and invading microbes. However, this model is not always practical because colonization is difficult or impossible for many invading microorganisms, due to colonization resistance. Colonization resistance refers to the ability of the indigenous intestinal microbiota to prevent colonization of or provide a barrier to newly introduced pathogenic bacteria (157). The second model for intestinal colonization studies is the gnotobiotic animal, or germfree animal. This model is advantageous in that there is an absence of the indigenous microflora and colonization is readily achieved, even for organisms with limited fitness for colonization, because there is no competing microflora in gnotobiotic animals. A major drawback to this model is that handling

gnotobiotic animals is more complicated and rather expensive. Furthermore, the intestinal physiology of the gnotobiotic mouse varies from that of conventional animals in that there is a higher oxygen tension found in the intestine as well as a thin and villous mucosa in the cecum (231, 234). The third *in vivo* model for colonization is the streptomycin-treated mouse model. This model was first developed in 1954 when mice were given an oral administration of streptomycin and infected with streptomycin resistant strains of *Salmonella enteritidis* (23). Subsequently, the microecology of the cecal contents was determined to be altered following streptomycin treatment (92, 116, 236). A decrease in the facultative anaerobic enterococci, streptococci, and lactobacilli was observed (236). Nonetheless, the Bacteroides and Eubacterium populations were unaltered in the cecal contents of streptomycin-treated mice (116). In addition, the anaerobic population was found to be equivalent in numbers in both streptomycin-treated and conventional mice, approximately 1 x 10^9 to 2 x 10^9 CFU/g of contents (116). Thus, the streptomycin-treated mouse model allows for the establishment and examination of invading microbes in the presence of high numbers of strict anaerobes in the mammalian GI tract (212). Also, the streptomycin-treated mouse model is inexpensive and has been widely used to study *E. coli* and *Salmonella Typhimurium* colonization of the mouse intestine (309). The use of this model is simple, demanding only that streptomycin sulfate be administered in the drinking water (5g/liter), and that the strains of bacteria fed to the animals be resistant to streptomycin (295).

1.5 *E. coli* colonization of the mouse intestine

Colonization of the gastrointestinal tract by commensal and pathogenic organisms is dependent on the ability of these organisms to compete with the existing microflora. Colonization is the first step in gastrointestinal infections and is defined as the stable persistence of a microbial population without the reintroduction of the bacterium to the animal (232, 294). *E. coli* O157:H7 and K-12 colonize the mammalian intestinal tract in the presence of the existing indigenous intestinal microbes. Strains of *E. coli* fed to streptomycin-treated mice grow from low numbers (10^5 CFU/g of feces) at 5 hours postfeeding to high numbers (10^8 CFU/ g of feces) by 24 hours postfeeding (39, 157). There are two defined stages of intestinal colonization: initiation and maintenance (157). The first stage of colonization, initiation, continues after the introduction of a bacterial strain up until day 3 postfeeding (157). Following initiation, wild-type *E. coli* strains normally reach a stable population of about 10^8 CFU/g of feces. This phase of colonization is known as maintenance and occurs after day 7, presumably when nutrients become limiting (157).

Using the streptomycin-treated mouse model, mice are simultaneously fed two competing strains of *E. coli*. The relative ability of each strain to colonize the intestine is then monitored over a period of 15 days by conducting fecal plate counts. Genetic manipulation of *E. coli* strains allows for the role of particular genes of interest in their colonization ability to be examined during competition with wild-type parent strains. Therefore, the relative fitness of the mutant strains can be measured

during colonization (160). The degree to which mutant strains are defective in colonization during competition with their wild-type parent is categorized as: major, significant, minor, or none. Mutant strains that do not compete effectively with wild-type strains during initiation or maintenance are classified as having a colonization defect based on the degree to which the mutant strain is able or unable to colonize. The level of colonization is described by the log difference in CFU/g of feces. A major colonization defect is marked by a 3 log difference or greater between that of the mutant and wild-type strain. A significant colonization defect is a 1.5 to 3 log difference, whereas a minor defect is characterized by a 1 to 1.5 log difference. Mutant strains that are able to co-colonize with their wild-type parent strains are recognized as having no colonization defect. The physiological factors involved in *E. coli* colonization of the murine gastrointestinal tract, with respect to respiratory metabolism, will be the focus of this dissertation. The remainder of this chapter is a discussion of metabolic capabilities of the *E. coli* cell.

1.6 Respiratory oxidoreductases

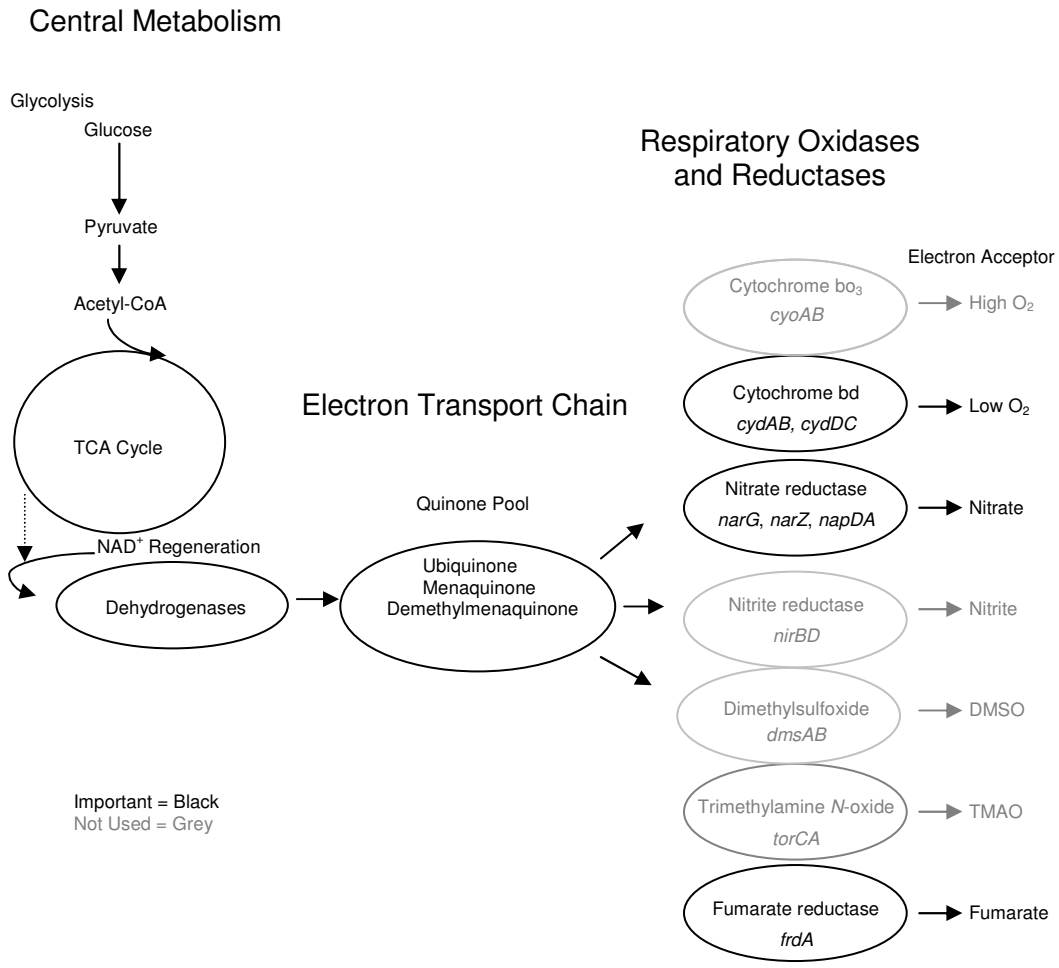
The respiratory metabolism of *E. coli* in its natural habitat, the GI tract, has been completely ignored. However, recently more interest has developed regarding the metabolic pathways used by *E. coli* and other microorganisms to produce energy for growth and survival during colonization of mucosal surfaces (39, 248). These topics are addressed in Chapter 2 of this dissertation. In order to survive changing environmental conditions and the challenges faced in the flourishing

microenvironment of the GI tract, *E. coli* must be able to adapt to a variety of growth conditions including electron acceptor availability. *E. coli* is able to grow aerobically by respiration, and in the absence of oxygen by anaerobic respiration using alternative electron acceptors (93). If oxygen, nitrate, nitrite, dimethylsulfoxide (DMSO), trimethylamine *N*-oxide (TMAO), and fumarate are unavailable, then *E. coli* can also produce energy by fermentation (93). The ability of facultatively anaerobic bacteria, such as *E. coli*, to alter their respiratory metabolism suggests a type of respiratory flexibility that the organism displays according to its environment. However, the terminal oxidoreductases used by the organism to colonize the intestine *in vivo* have remained unknown, warranting further investigation. The respiratory metabolism of *E. coli in vivo* is the main focus of this dissertation and is further addressed in Chapters 2 and 3. A model of *E. coli's* respiratory pathways examined in this dissertation is shown in figure 1.1.

The electron transport system is the major source for ATP production for many microorganisms. The electron transport chain functions to regenerate NAD^+, nicotinamide adenine dinucleotide, and provide ATP to support cellular processes. The process by which ATP is formed is a result of the transfer of electrons from reduced nicotinamide adenine dinucleotide (NADH) to an electron acceptor, such as oxygen, through a series of electron carriers located in the cell membrane. An important function of respiration is the generation of an electrochemical gradient, or proton motive force, across the cytoplasmic membrane. Proton motive force is generated by coupled oxidation-reduction reactions, catalyzed by the components of

Figure 1.1 Model of respiratory pathways used by *E. coli* during intestinal colonization.

Figure 1.1 Model of *E. coli* respiratory pathways used *in vivo*.



the respiratory chain, which pumps protons to the outside of the cell. Furthermore, the respiratory chain maintains redox balance by regenerating NAD^+ from NADH. Aerobic and anaerobic respiratory pathways allow *E. coli* to oxidize a wide array of substrates, while passing the electrons on to a number of different oxidants. There are three types of respiratory components: substrate-specific dehydrogenases, responsible for the oxidation of organic substrates and feeding electrons to the electron transport chain; quinones, small membrane soluble molecules which serve as electron carriers; and the terminal oxidoreductases, responsible for the reduction of the terminal electron acceptors being used. The amount of each of these respiratory components is carefully regulated in order to ensure the optimal use of the electron transport chain according to the substrates and electron acceptors available and the physiological needs of the cell.

E. coli has the ability to combine its various respiratory components to alter its bioenergetic efficiency of NADH oxidation (measured by the H^+/e^- ratio) over a considerable range. This suggests that the optimized electron transport chain does not always have the highest value of H^+/e^-. Instead, one principal function of the electron transport chain, independent of its bioenergetic efficiency, is to maintain redox balance and regenerate NAD^+ from NADH. The cells have to simultaneously balance the challenging needs for bioenergetic efficiency, NAD^+ regeneration and net electron flux, as well as oxygen scavenging. This balance is accommodated by *E. coli's* rapid adjustment of the electron transport chain components in response to availability of substrates and electron acceptors in its microenvironments.

Some indigenous microbes of the GI tract, such as *E. coli*, have the capability to generate energy by aerobic oxidative phosphorylation. Therefore, these microorganisms may occupy habitats *in vivo* where oxygen is available. These aerobic habitats could be in close proximity to epithelial cells where O_2 molecules might pass from the blood through the epithelium to the adjacent bacteria (164). *E. coli* may be responsible for creating and maintaining an anaerobic, or oxygen free, environment and conditions of a low oxidation-reduction potential that favor the strict anaerobes present in the large bowel (234). The existence of such niches has not been established and is considered in Chapter 2 of this dissertation.

Since *E. coli* has both a diverse and flexible respiratory metabolism capable of respiring favorable electron acceptors, such as oxygen, this organism is, therefore, able to maintain a stable population in changing microhabitats (123). *E. coli* has two respiratory oxidases, cytochrome *bd* and cytochrome bo_3. The cytochrome *bd* ubiquinol oxidase (encoded by *cydAB* and *cydDC*), which is synthesized under low oxygen or microaerophilic conditions, has an extremely high affinity for oxygen and is used when oxygen concentrations are low. Alternatively, *E. coli* can use a low affinity cytochrome bo_3 oxidase (*cyoAB*) that is expressed under high oxygen tensions to respire oxygen. Both of these enzymes are quinol oxidases. Cytochrome bo_3 is a member of a large family of proton pumping heme-copper respiratory oxidases (38, 92). Cytochrome *bd* is not a member of the heme-copper oxidase family and is found in only a small number of bacterial species, namely the enterics (100, 148). Regulation of aerobic respiration is primarily mediated by a two-component

regulator, ArcA and ArcB (51, 126, 128, 131, 133, 134). ArcA acts to repress *cyoA* operon expression and activates *cydAB* expression under microaerobic conditions (50, 53, 91, 126, 129, 130, 285). Control of respiratory metabolism and its role in colonization is discussed further in Chapter 2 of this dissertation.

Nitrate (NO_3^-) respiration is widespread amongst bacterial species, including the enterobacteria (290). Nitrate is the most energetically favorable terminal electron acceptor of the anaerobic electron acceptors used by *E. coli*. Additionally, nitrate has been demonstrated to inhibit the synthesis of other reductases, which eliminates the use of many of the other terminal electron acceptors (93). This particular role sets the basis for a hierarchy of electron acceptors that is used by *E. coli* and is discussed in Chapter 3 of this dissertation. *E. coli* O157:H7 and K-12 have multiple respiratory nitrate reductases (185, 203). The primary *E. coli* nitrate reductase (encoded by *narGHI*) is a cytoplasmic membrane-bound enzyme that is synthesized under nitrate concentrations greater than 2.5 mM *in vitro* (263, 300). Regulation of nitrate reductase synthesis, the primary and periplasmic reductase, is provided by the regulatory control proteins, Fnr and NarL (26, 27, 41, 105, 300). Fnr is a global regulator that mediates anaerobic respiration for both nitrate and fumarate respiration in response to anaerobiosis (168, 259, 260). However, another level of transcriptional control in response to nitrate availability is supplied by the regulatory proteins, NarL and NarP (124, 125, 214, 262, 264, 268, 270). Together, these regulators act to control anaerobic respiration in *E. coli*. A secondary cytoplasmic reductase (encoded by *narZYWV*) has not been extensively characterized and the physiological role of the

enzyme is unclear (22, 25, 93, 124, 125). However, it is known that the homologous enzyme is not regulated by nitrate (40). Instead, NarZ is RpoS dependent and induced during stationary phase (40). Various bacteria also contain a nitrate reductase in their periplasm. *E. coli* contains a third nitrate reductase (encoded by *napFDA*) located in its periplasmic space (102, 300). Synthesis of the periplasmic nitrate reductase is induced by a low concentration of nitrate during anaerobic growth, approximately 1 mM (300). The expression of the nitrate reductases under varying concentrations of oxygen or nitrate has been extensively studied *in vitro* and will be discussed in more detail in Chapter 2 and 3 when nitrate measurements are made *in vivo*.

E. coli can also use fumarate as an alternative terminal electron acceptor during anaerobic respiration (46, 162, 163). Fumarate reductase (encoded by *frdABCD*) expression requires anaerobic growth conditions; however, its synthesis is repressed by the presence of nitrate (118, 127, 155, 258, 265). Expression of fumarate reductase, controlled by anaerobic and nitrate regulation, is mediated by Fnr and NarL, respectively (105, 106, 136, 137, 291). Fumarate is the least favorable of the terminal electron acceptors that *E. coli* can use during anaerobic respiration. Alternative electron acceptors that are more energetically favorable than fumarate are discussed below.

E. coli can use multiple alternative electron acceptors for anaerobic respiration, the primary reductases for the remaining anaerobic reductase systems including nitrite, DMSO, and TMAO. The most favorable of these electron acceptors is nitrite.

Nitrite, (NO_2), which is the product of nitrate reduction, can also serve as an anaerobic terminal electron acceptor in *E. coli* (1). The anaerobic nitrite reductase, encoded by *nirBD*, is the primary reductase that mainly functions to regenerate NAD^+ and detoxify nitrite that accumulates from nitrate respiration (93, 199, 226). Additionally, enzyme synthesis of the primary nitrite reductase is induced by nitrate or nitrite (199). *E. coli* has a second nitrite reductase, encoded by *nrfAB*, located in its periplasm (299). Synthesis of this enzyme is induced only by nitrite and is repressed by nitrate (199, 299). Regulation of both nitrite reductase operons is mediated by Fnr and NarL (261). Alternatively, *E. coli* can use *S*- and *N*- oxides as terminal electron acceptors during anaerobic respiration (63). The primary DMSO reductase, encoded by *dmsAB*, in *E. coli* requires anaerobic conditions for synthesis (21, 228, 229). However, enzyme synthesis is not induced by DMSO but instead is repressed by nitrate, mediated by Fnr and NarL (52, 289, 302). DMSO reductase has a broad specificity that can support anaerobic respiration with either DMSO or TMAO (20, 302, 303). Conversely, the primary TMAO reductase (encoded by *torCA*) in *E. coli* also requires anaerobic growth conditions for synthesis (10, 254, 277). However, synthesis of TMAO reductase is not repressed by nitrate (132, 277). Instead, enzyme synthesis is strongly induced by TMAO (253, 254). Furthermore, anaerobic regulation of TMAO reductase is not mediated by Fnr and the mechanism of regulation by oxygen is unknown (93, 201). *E. coli* has two other alternative reductases for DMSO and TMAO. The *ynfEFGHI* operon encodes a paralog of DmsABC, and the *torYZ* operon encodes a paralog of TorAC. The DmsABC, YnfEFGH, and TorYZ systems all exhibit broad substrate specificity for S-oxides and

N-oxides, and therefore overlap substantially. The respiratory oxidoreductases used by *E. coli in vivo* and their contribution to colonization are discussed in Chapter 2 of this dissertation.

1.7 Hierarchy of aerobic and anaerobic electron acceptors

The ability to use multiple electron acceptors provides the respiratory flexibility and diversity that *E. coli* is thought to need in order to compete in a densely populated habitat such as the mammalian large intestine (44). In addition, the ability to regulate electron transport chain components allows *E. coli* to preferentially use the terminal electron acceptor that yields the maximum amount of energy (105). This establishes a hierarchy of the available electron acceptors that can be used to ensure energy is produced efficiently. The use of these electron acceptors *in vitro* has been extensively studied (132, 269). However, the hierarchy of these electron acceptors *in vivo* has never been examined and is addressed in Chapter 3 (97), which focuses mainly on the terminal electron acceptors oxygen, nitrate, and fumarate since they were found to be used by *E. coli* in the mouse intestine.

The regulation of respiratory gene expression allows *E. coli* to take advantage of the compound that will provide the highest redox potential. Indeed, the synthesis of respiratory enzymes is under hierarchical control to make certain that respiratory terminal electron acceptors are consumed preferentially. Oxygen ($E^{\circ\prime} = +0.82$ V) has the highest energy yield of all electron acceptors, is preferred over all other terminal electron acceptors, and is consumed first (132, 170, 288). The preferred order of

electron acceptors follows the redox potential for the given acceptor where nitrate ($E^{\circ\prime} = + 0.42$ V) produces the maximum amount of energy in the absence of oxygen, and fumarate ($E^{\circ\prime} = + 0.031$ V) produces the least amount of energy among all of the alternative terminal electron acceptors (5, 101, 115, 142, 144).

The hierarchical order of preference operates under aerobic and anaerobic conditions (283). The first level of hierarchical control involves the cell's response to oxygen (287, 289). Aerobically grown cultures only synthesize basal levels of anaerobic respiratory enzymes (289). However, the oxygen sensitive Fnr transcription factor activates transcription of anaerobic reductases in anaerobic cultures (144, 198, 243). The second level of hierarchical control involves the cell's response to nitrate that is mediated by paralogous two-component sensor regulatory systems, NarX-NarL and NarQ-NarP (45, 86, 167, 293, 310). The NarX-NarL system broadly controls gene expression under nitrate respiration, whereas the NarQ-NarP system plays a more restricted role in control when electron acceptors, such as nitrate, are limiting (97, 140, 141, 298). Nitrate reductase synthesis is induced and simultaneously represses the synthesis of enzymes used for respiring the alternative anaerobic electron acceptors, such as fumarate reductase (84, 86, 136, 213, 250, 278, 279). In addition, nitrate reduction results in the accumulation of nitrite (227). This then induces the synthesis of nitrite reductase once nitrate is depleted which is further mediated by NarX-NarL and NarQ-NarP (53, 214, 220, 221).

1.8 Role of quinone pool

The electron transport system in *E. coli* has the ability to oxidize a variety of substrates and to use different terminal electron acceptors (169). There are three types of electron driven proton pumps. The first of these is the substrate specific dehydrogenases. This complex carries out the oxidation of organic substrates that feeds electrons into a pool of quinones. NADH dehydrogenase (complex I), composed of NDH-1 (*nuo*) and NDH-II (*ndh*), and succinate dehydrogenase (complex II) make up this first complex in aerobic respiration (93). Electrons are carried from complex I to a pool of quinones (complex III) (81). Quinones then transport electrons to the appropriate terminal oxidoreductases as described above (206).

Quinones are lipid-soluble molecules that mediate the transfer of electrons between the protein components of the electron transport chain (57, 75). *E. coli* synthesizes three types of quinones: a benzoquinone, ubiquinone (Q), and two napthoquinones, menaquinone (MK) and demethylmenaquinone (DMK) (16-18, 55, 57-60, 176, 314-316). The functions of the different quinones have mainly been studied through mutant analysis and are discussed below (55, 57-60, 103, 104, 179-182, 313). Ubiquinone (Q) is primarily involved in aerobic respiration while menaquinone (MK) is involved in nitrate and anaerobic respiration (58, 107, 150, 151, 180, 192). A third quinone produced by *E. coli*, demethylmenaquinone (DMK), is mainly involved in anaerobic respiration with acceptors other than nitrate (103,

183). Mutants defective in Q (*ubiCA* and *ubiE*) and MK/DMK (*menA* and *ubiE*) biosynthesis have been isolated and characterized (257, 272, 314).

4-Hydroxybenzoate formation from chorismate is the first committed step in Q biosynthesis (176). The reaction is catalyzed by the enzyme chorismate lyase, which is encoded by the *ubiC* gene (16). In addition, prenylation of 4-hydroxybenzoate is carried out by the enzyme 4-hydroxybenzoate octaprenyltransferase, which is encoded by the *ubiA* gene (58, 154, 158). *ubiCA* mutants only lack Q and still possess both MK and DMK (59). 2-octaprenyl-6-methoxy-1,4-benzoquinol methyltransferase (*C*-methyltransferase), encoded by *ubiE*, is required for the biosynthesis of both ubiquinone and menaquinone (161, 207). *ubiE* catalyzes the formation of ubiquinone from 4-hydroxybenzoate and menaquinone from demethylmenaquinone (180). Ubiquinone and menaquinone serve as the redox mediator in aerobic and nitrate respiration, where *ubiE* mutants lack both Q and MK but retain DMK (296). Demethylmenaquinone is the major electron carrier during anaerobic respiration using terminal electron acceptors other than oxygen and nitrate (181, 192, 206). Biosynthesis of demethylmenaquinone from 2-succinylbenzoate involves the enzyme 4-dihydroxy-2-naphthoate octaprenyltransferase, encoded by *menA* (249). Additionally, deletion of the *menA* gene not only prevents DMK biosynthesis but also prevents synthesis of MK. Prior studies have shown that fumarate respiration and DMSO respiration specifically require MK (103, 181, 311, 312), whereas growth studies and measurements of hydrogen-dependent enzyme

activities with a *ubiE* (Q^- MK^- DMK^+) mutant show that DMK can support both fumarate and DMSO respiration (286, 312).

The relative concentrations of Q, MK, and DMK have been shown to change with varying growth conditions (55, 75, 81, 266, 306, 315). Studies have previously shown that aerated cells contain about four or five times more Q than MK and DMK, whereas anaerobic cells contain about one-third as much Q as MK and DMK (158). The ability of the three quinones to shuttle electrons under different respiratory conditions in the electron transport chain can be best explained by their relative affinities for the oxidoreductases as discussed in Chapter 4. The regulation of quinone levels is unknown. However, it is established that an active *fnr* gene is not necessary for the synthesis of MK and DMK (286).

Given that the specific quinones used by *E. coli in vivo* have never been determined, the quinone pool was examined to confirm the results obtained from the terminal oxidoreductase studies. Previous studies have shown all three quinones to be present during both aerobic and anaerobic respiration. Therefore, the specific quinones that couple NADH oxidation to aerobic and anaerobic electron acceptors during colonization of the mouse intestine requires further investigation so that electron flow through the quinone pool can be better understood as addressed in Chapter 4 of this dissertation.

1.9 Maltose and glycogen metabolism

Successful colonization of a host relies on a bacterium's ability to utilize available nutrients to generate energy and grow. Furthermore, many microorganisms oxidize carbohydrates for their main source of cellular energy. Recently, studies have shown carbohydrate metabolism to contribute to microbial pathogenesis for several different human pathogens (121, 135, 156, 177, 189, 190, 244, 245, 282). In addition, prior studies have found that acquisition of complex sugars is essential for mucosal pathogens to successfully colonize and infect their hosts (156, 244, 246, 247). Together, these findings suggest that utilization of carbohydrates *in vivo* is essential for pathogenic bacteria to cause disease in humans and is further discussed in Chapter 5 of this dissertation. In addition, since we had already studied several carbohydrates utilized by *E. coli in vivo*, we decided to turn our attention to maltose and glycogen metabolism, where an exogenous supply of complex sugar and endogenous energy storage were examined for their contribution to colonization.

The role of maltose and glycogen utilization during *E. coli* colonization of the mammalian large intestine is currently not known. However, regulation of the *mal* regulon has been studied extensively (22, 60, 61). The maltodextrin system in *E. coli* is a major paradigm for understanding complex sugar-utilizing systems in bacteria (71, 72). *E. coli* has several genes that are involved in maltose and maltodextrin utilization and transport (28, 29, 37, 69, 240-242). MalT is the activator of all the *mal* genes (47, 62, 67, 68, 79, 215, 219, 220). Maltose and maltodextrins are actively transported across the cytoplasmic membrane of *E. coli* via high affinity periplasmic binding ABC transport systems (108-110). Transport of maltose is accomplished by

the ABC transporter, which is comprised of a maltose binding protein, MalE (54, 76, 80, 143, 251, 252), and two membrane bound subunits that make up the transmembrane pore, MalF and MalG (120, 241, 284). *E. coli* also has another maltose transporter, *malX,* that encodes a typical phosphotransferase (PTS) enzyme IIBC complex (80, 217). Maltodextrin transport requires a specific outer membrane diffusion porin, LamB (19, 90, 119, 145, 216). This maltoporin serves also as the λ phage receptor and is located in the outer membrane (82, 83, 139, 275, 276). *malP* and *malQ* encode enzymes for maltodextrin and maltose metabolism, respectively (239, 307, 308). MalQ, amylomaltase, specifically cleaves maltodextrins, and thus releases the reducing-end glucose or dextrin (175, 187, 200, 211). MalP, maltodextrin phosphorylase, is responsible for removing the nonreducing glucosyl residue to yield α-glucose-1-phosphate (29, 175, 195, 239). The degradation of glycogen produces maltodextrins which are channeled into metabolism by MalQ and MalP, where MalQ will release glucose from maltodextrin and MalP will yield glucose-1-phosphate from maltodextrins by phosphorolysis. Uptake and utilization of maltose and maltodextrins by *E. coli* is a model for understanding how microorganisms transport and use complex sugars and is the focus of Chapter 5 (189, 247).

Glycogen is the major form of stored carbon for *E. coli* as well as many other organisms (149, 223, 224). It serves as a readily metabolized substrate for maintaining energy levels in the cell when nutrients become limiting. The synthesis of glycogen occurs when carbon is abundant and a nutrient that is required for growth

is limiting (66). The biosynthesis of glycogen involves two enzymes, *glgA* and *glgS* (7, 8, 31, 99, 196, 197, 208-210). Glycogen synthase (encoded by *glgA*) is involved in ADP-dependent synthesis of glycogen and is the only recognized synthetase responsible for synthesis of glycogen (29, 87, 112, 153, 208). Glycogen synthesis is affected by overexpression of a growth-phase-regulated, RpoS-dependent gene, *glgS* (28, 29, 32, 70, 95, 114). However, the role and contribution of GlgS in glycogen biosynthesis has not been identified and has only been found to stimulate synthesis of glycogen when overexpressed (114, 147). Glycogen degradation involves glycogen phosphorylase (encoded by *glgP*) to form glucose-1-phosphate (29, 317).

Glycogen is a major energy reserve for the cell and more than likely contributes significantly to cell survival (55, 148). Glycogen synthesis and accumulation varies with nutrient abundance, whereas glycogen degradation depends on nutrient depletion (9). During periods when the availability of exogenous carbon sources is intermittent, glycogen can supply carbon (178). This feast and famine lifestyle and the importance of maltose and glycogen metabolism during colonization are discussed further in Chapter 5. Increasing our understanding of carbohydrate metabolism and transport pathways may, therefore, create new insights into similar mechanisms by which other pathogens are able to infect hosts.

1.10 Rationale and significance

Both pathogenic and commensal strains of *E. coli*, strains O157:H7 and K-12, are able to colonize the mammalian intestinal tract. *E. coli* is the predominant facultative anaerobe found in the large intestine amongst a population largely dominated by obligate anaerobes. Commensal bacteria present in the intestine, such as *E. coli* K-12, play a significant role in that they provide a defensive barrier to invading pathogens (157). *E. coli* O157:H7, a common pathogen of the large intestine, causes both hemorrhagic colitis and hemolytic-uremic syndrome (HUS) that can ultimately result in death (42, 43, 101, 221, 281). Infection with *E. coli* O157:H7 causes an estimated 70,000 diarrheal illnesses per year in the United States (15, 159, 280, 292). It is also the most common cause of renal failure in children (14, 166, 222). Transmission of *E. coli* O157:H7 can be through contaminated food, water, or in direct contact with infected people or animals (77, 98, 122, 191, 194, 273, 305). The infectious dose is very low, approximately 10 organisms, thus contributing to virulence (142). Since O157:H7 can colonize the intestine, it must be able to compete successfully with the existing commensal flora in order to cause disease. The respiratory pathways used by *E. coli* during colonization of the intestine are currently unknown. Understanding how this organism is able to survive and grow in the intestine will add to our knowledge of the GI tract as well as provide insight as to how to treat and prevent infection and disease that occur in the microbial rich habitat of the gut.

It has been assumed that strictly anaerobic GI bacteria would not require respiratory cytochrome oxidases. However, recent studies have shown that strict

anaerobes, such as *Bacteroides fragilis*, *Desulfovibrio gigas* and *Moorella thermoacetica*, contain a cytochrome *bd* oxidase that is advantageous under conditions of nanomolar concentrations of oxygen (11, 64, 172). Furthermore, He et al. found oxygen present in low concentrations in the intestine (113). Together, these findings confirm that oxygen is available in the intestine, and furthermore suggest that the intestine is indeed microaerobic. Specifically, that the anaerobic population in the intestine can tolerate and even use very low levels of oxygen. The facultative anaerobes may be responsible for lowering oxygen in the intestine to levels that are tolerated by the anaerobes. This possibility has not been tested *in vivo*.

Since most mucosal pathogens are facultative anaerobes, our conclusions may be extended to include other mucosal pathogens. In support of this idea, cytochrome *bd* oxidase and nitrate transport were both found to be required for adaptation of *Mycobacterium tuberculosis* to host immunity during chronic infection in mouse lung (248). Likewise, cytochrome *bd* expression was also found to be required for the intracellular survival and virulence of *Shigella flexneri in vitro* and in mouse infections (301). These examples demonstrate the importance of respiration during infection by these mucosal pathogens and support the idea that oxygen stimulates infectious disease by providing a competitive advantage for pathogens.

The ability of pathogens to generate energy is essential to their success, growth, and persistence in the host. Understanding the respiratory metabolism of pathogenic microorganisms opens a new field of pathogenesis: how energy metabolism

contributes to colonization of mucosal surfaces. These studies may yield novel and broadly applicable insights into microbial host-pathogen interactions.

1.11 Hypothesis

Although it has never been tested, the availability of different electron acceptors in the gut should enable *E. coli* to respire either aerobically or anaerobically. Respiration plays an important role in energy generation in *E. coli*. Therefore, I hypothesize that respiratory metabolism is important for *E. coli* to colonize the mammalian gastrointestinal tract. A systematic mutational approach was used to examine which respiratory oxidoreductases are important for colonization. In addition, to complete our understanding of the respiratory pathways used *in vivo*, we determined the hierarchy of electron acceptors used by *E. coli* to colonize the mouse intestine as well as the quinones needed to support intestinal colonization. Ultimately, the identification of the respiratory pathways used by *E. coli in vivo* will contribute to and improve our understanding of the mammalian GI tract which was once presumed to be anaerobic. Furthermore, comparison of the behavior of pathogenic and commensal strains in the mouse intestine will increase and enhance our knowledge of the intestinal microenvironment and how to treat gastrointestinal infections.

Chapter 2

Respiration of *Escherichia coli* in the Mouse Intestine

2.1 Introduction

The intestinal microflora is dominated by diverse anaerobes, providing both a health benefit to the host (78) and a barrier to infection (89, 115). Despite being present in substantially lower numbers, facultative anaerobes, primarily *Escherichia coli* and *Enterococcus faecalis*, are ubiquitous in mammalian intestines (234). While the intestine is commonly thought to be anaerobic (6), the tissues surrounding the lumen are oxygen-rich and oxygen diffuses into the intestine at appreciable levels (113). Furthermore, oxygen from swallowed air is present in flatus (165). Oxygen in the intestine apparently has minimal impact on persistence of anaerobes and it was recently shown that at least one predominant anaerobe, *Bacteroides fragilis*, respires oxygen at low concentrations (11). In contrast to obligate anaerobes, facultative anaerobes (e.g., *E. coli*) grow most rapidly when respiring oxygen and switch to anaerobic respiration in the absence of oxygen or to fermentation in the absence of alternative electron acceptors (93). However, the extent to which facultative anaerobes utilize oxygen to maximize their growth rate in the intestine is not known.

Nutrients consumed for growth of the microflora are thought primarily to be fermentable carbohydrates, the bulk of which are in the form of polysaccharides (203). *E. coli* colonizes the mouse intestine by growing within the polysaccharide-rich mucus layer covering the epithelium, but is unable to degrade polysaccharides.

Apparently, *E. coli* consumes the mono- and disaccharides released during degradation of mucosal polysaccharides and dietary fiber (39) by polysaccharide hydrolase enzymes secreted by members of the anaerobic microflora (48) and perhaps host colonic epithelial cells (12). Recent studies from our laboratory demonstrate that seven mucus-derived sugars contribute to *E. coli* colonization of the mouse intestine, suggesting that biochemical flexibility is key to its competitiveness *in vivo* (39). *E. coli* is nearly equally flexible in its respiratory metabolism (93), but nothing is known about the role of bacterial respiration for coupling ATP generation to carbohydrate oxidation *in vivo*. Thus, it is important to test the hypothesis that oxygen respiration confers a competitive advantage to *E. coli* in the intestine.

Enterohemorrhagic *E. coli* (EHEC) has an infectious dose for humans as low as 10 microorganisms and, following ingestion, grows rapidly to a population approaching a billion bacteria per gram of feces (142). Since colonization is the first step in the infection process, it is crucial to understand how EHEC colonizes the intestine because low numbers can survive transport to consumers in foodstuffs such as leafy vegetables, which have caused recent outbreaks in the US (202). It is not known how EHEC acquires nutrients and generates energy for growth *in vivo*. While respiration is not a virulence factor *per se*, our experiments seek to establish the fundamental importance of house keeping functions, such as energy metabolism, for pathogenesis. Since most mucosal pathogens are facultative anaerobes, these studies of *E. coli* may be extended to include many diseases.

Here we report the results of a systematic mutational analysis designed to identify which respiratory pathways contribute to the ability of commensal and pathogenic *E. coli* to colonize the streptomycin-treated mouse intestine. Our findings lead us to conclude that respiration provides an enormous competitive advantage to *E. coli in vivo*. The results challenge the traditional view that the intestine is strictly anaerobic (6). Instead, we provide evidence that *E. coli* colonization of the mouse intestine is maximized by the ability to respire oxygen.

2.2 Materials and Methods

Bacterial Strains and Growth Conditions. The bacterial strains used in this study were derived from *E. coli* MG1655 Str^r (streptomycin-resistant), a K-12 strain (186), and *E. coli* EDL933 Str^r, the prototypical O157:H7 strain (184). Cultures were grown at 37°C in Luria-Bertani (LB), with gyratory shaking at 250 rpm. Null alleles were constructed by using the allelic replacement method of Datsenko and Wanner (65), as described previously (39), such that target genes were deleted and replaced with kanamycin- or chloramphenicol-resistance cassettes (used as selectable markers in mouse colonization assays, as described below). The null allele strains are identified in the text by the genes that were deleted; single gene deletions began with the start codon and ended with the stop codon, and multiple gene deletions began with the start codon of the first gene deleted and ended with the stop codon of the last gene deleted. Strains containing multiple mutations were constructed by sequential allelic replacement; the first inserted cassette was removed with FLP recombinase (65),

followed by subsequent allelic replacement(s) and removal of the insertion as necessary, leaving the selected marker in the last mutation made. Mutant strains were verified by phenotype analysis and DNA sequencing. The bacterial strains and plasmids used in this study are listed in Table A.2.1.

Phenotypic analysis. MOPS (3-(*N*-morpholino) propanesulfonic acid) defined medium was used to grow cultures for growth curves, as described previously (39). Anaerobic cultures were grown in culture tubes filled to the top with N_2-sparged media, sealed and incubated in Balch tubes. To test for nitrate or fumarate reductase activity, mutant strains were grown anaerobically overnight in MOPS medium with glycerol (1.6%) as the carbon source and either 50 mM nitrate or fumarate as the electron acceptor. Cell growth was monitored spectrophotometrically at 600 nm (OD_{600}).

HPLC Analysis. A Dionex DX-500-Microbore system was used with an IonPac AS11 column for anion analysis of mucus. Standards and blanks were analyzed and standard curves were developed for a 1 mg sample of cecal mucus. Mouse cecal mucus was isolated from the cecum of CD-1 male mice and lyophilized as described previously (295). Regression coefficients for standard curves were calculated and used to demonstrate the linearity of the peak area with respect to concentration.

Mouse Colonization Experiments. The streptomycin-treated mouse model has been used extensively to study colonization of the mouse large intestine by *E. coli* and

Salmonella enterica serovar Typhimurium (39, 49, 116, 295). Briefly, three CD-1 male mice, six weeks of age, were given drinking water containing streptomycin sulfate (5 g/L) for 24 h to remove the existing resident facultative microflora, and then starved for food and water for 18 to 24 h. The mice were then fed approximately 10^5 colony-forming units (CFU) of both the wild-type and mutant strains in 1 ml of 20% sucrose. The wild-type strains were *E. coli* MG1655 Str^r Nal^r (nalidixic acid resistance) (176) and *E. coli* EDL933 Str^r Nal^r (184); Nal^r was used to distinguish the wild-type (reference strain) from the null allele mutants in fecal plate counts. After the bacterial suspension was ingested, food and streptomycin-water were restored and fecal plate counts were determined at 5 h, 24 h, and on every other day thereafter for 15 days. Fecal samples were homogenized and diluted in 1% tryptone broth and plated on MacConkey agar containing either streptomycin (100 μg/ml) and nalidixic acid (50 μg/ml) to count the wild-type or streptomycin and kanamycin (40 μg/ml) or chloramphenicol (30 μg/ml) to count the null allele mutants. Each colonization experiment was repeated, on separate occasions, and the plotted values (in figures) represent the average for 6 mice. The $\log_{10}$ mean number of CFU per gram of feces ± the standard error for each strain in the mice was calculated for each time point. In all experiments, a difference between two strains $\geq$10 CFU/g feces was statistically significant, i.e., $P<0.005$ in student t-test (two-tailed with unequal variance). The limit of detection in fecal plate counts was 10^2 CFU/g feces. To determine the role of strains during the maintenance stage of colonization, mice were colonized for 10 days with a mutant strain of *E. coli* EDL933, starved for food and streptomycin-water

overnight, and fed 10^{10} CFU of the wild-type parent strain *E. coli* EDL933, after which food and streptomycin-water were replaced.

2.3 Results

Colonization assays. The preferred animal model for measuring the relative fitness of two bacterial strains for intestinal colonization is the streptomycin-treated mouse. Streptomycin treatment selectively removes facultative anaerobes while leaving the anaerobic microflora essentially intact; this opens a previously unavailable niche, which can then be colonized by newly introduced microorganisms such as *E. coli* (45, 149, 279). In this model, competing wild-type and mutant strains are fed together to mice and their populations are monitored in fecal plate counts. Previously we showed that colonization involves an initiation stage (5 h to 3 days post-feeding) in which nutrients are not limiting and the population increases from low to high numbers, and a maintenance stage (7 days post-feeding and beyond) in which nutrients are limiting and the population persists at a level correlated with the mutant strain's relative fitness for colonization (39). For this reason the colonization data shown in Table 2.1 are given for day 1 (initiation) and day 9 (maintenance) of the 15 day long experiments. To compare the bioenergetics of a commensal strain to that of a pathogenic strain, each of the respiratory mutations described in this report were constructed in *E. coli* MG1655 (5), derived from the human isolate, *E. coli* K-12, and *E. coli* EDL933 (194), the prototypical strain of *E. coli* O157:H7. Please note that the streptomycin-treated mouse serves as a colonization model for EHEC strains, which

do not cause disease in CD-1 mice (278). We found that each of the mutations tested in the commensal and pathogenic strains had a nearly identical impact on colonization. For this reason, colonization curves are shown for *E. coli* EDL933 experiments only.

ATP synthase is necessary for colonization. Since respiratory energy conservation requires ATP synthase (185), we tested mutants that lack ATPase for their ability to compete with the wild-type in the streptomycin-treated mouse colonization model. The ATPase mutant constructions deleted the *atpA* and *atpG* genes, which encode the F1 alpha and gamma subunits, respectively, resulting in strains capable only of fermentative energy metabolism (56). In competition with their respective wild-types, *E. coli* EDL933 Δ(*atpA-atpG*)::*cat* and *E. coli* MG1655 Δ(*atpA-atpG*)::*cat* were eliminated from mice within 5 days (Table 2.1, Fig. 2.1A, Fig. A.2.1A). It is formally possible that mutations inadvertently introduced elsewhere on the genome caused the observed colonization phenotype. However, we would argue that this was not the case, for the following reasons. First, each of the allelic replacements described here and elsewhere (35, 152, 174, 176) were obtained with a frequency that varied less than one order of magnitude. Second, half of the mutants tested here and in similar studies had no colonization defects. Third, the results were essentially identical in two different genetic backgrounds (EDL933 and MG1655). The failure of the Δ(*atpA-atpG*)::*cat* mutants to initiate colonization could be due to a general inability to grow in the intestine or inability to compete with the wild-type. To distinguish these possibilities, the mutants were fed alone to mice and found to

Table 2.1. Competitive colonization between respiratory mutants and wild-type *E. coli* strains. [a]

		E. coli EDL933		*E. coli* MG1655	
Respiratory Enzyme	**Mutant**	**Day 1**	**Day 9**	**Day 1**	**Day 9**
ATP synthetase	Δ(*atpA-atpG*)	**2.7 ± 0.1**	**5.6 ± 0.1**	**5.3 ± 0.5**	**5.9 ± 0.1**
Regulator of aerobic genes	Δ*arcA*	**4.7 ± 0.1**	**5.6 ± 0.2**	**3.0 ± 0.2**	**6.5 ± 0.3**
Regulator of anaerobic genes	Δ*fnr*	**1.4 ± 0.2**	**5.6 ± 0.2**	**1.3 ± 0.1**	**6.8 ± 0.1**
Cytochrome *bo* oxidase	Δ(*cyoA-cyoB)*	0.1 ± 0.1	0.4 ± 0.3	0.4 ± 0.1	0.8 ± 0.1
Cytochrome *bd* oxidase	Δ(*cydA-cydB*)	**2.5 ± 0.1**	**5.2 ± 0.1**	**3.8 ± 0.2**	**5.0 ± 0.1**
Cytochrome *bd* oxidase (assembly)	Δ(*cydD-cydC)*	**2.4 ± 0.1**	**5.0 ± 0.2**	**3.3 ± 0.1**	**6.0 ± 0.2**
Nitrate reductase	Δ*narG*	0.3 ± 0.1	**2.0 ± 0.1**	0.4 ± 0.2	**2.2 ± 0.1**
Nitrate reductase	Δ*narZ*	0.6 ± 0.1	0.1 ± 0.1	0.3 ± 0.1	0.1 ± 0.2
Periplasmic nitrate reductase	Δ(*napD-napA*)	0.4 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.6 ± 0.1
Nitrate reductases	Δ*narG,* Δ*narZ*	0.9 ± 0.1	**2.3 ± 0.1**	0.1 ± 0.1	**2.9 ± 0.2**
Nitrate reductases	Δ*narG*, Δ*narZ*, Δ(*napD-napA*)	**1.3 ± 0.1**	**3.1 ± 0.1**	0.9 ± 0.1	**3.8 ± 0.1**
Fumarate reductase	Δ*frdA*	0.8 ± 0.1	**3.1 ± 0.2**	0.1 ± 0.1	**2.8 ± 0.2**

[a] Mice were fed 10^5 CFU each of a mutant and its wild-type parent. Mice were transferred to fresh cages every day and feces no older than 24h were assayed every other day for 15 days. At each time point, for each mouse the Log_{10} CFU/gram of feces for the mutant was subtracted from the Log_{10} CFU/gram of feces for the wild-type. The average ± the standard error of the mean of Day 1 and Day 9 data from 6 mice are shown. Differences of at least one order of magnitude (10-fold) are in bold type; all values shown in bold are statistically significant: $P < 0.005$ (student's t-test).

Figure 2.1. Respiratory mutants exhibited colonization defects in competitive colonization assays. *E. coli* EDL933 Δ(*atpA-atpG*) (A) and *E. coli* EDL933 Δ(*cydA-cydB*) (C) mutants were eliminated during competition with wild-type *E. coli* EDL933, but were able to colonize when fed alone to mice (B and D, respectively).

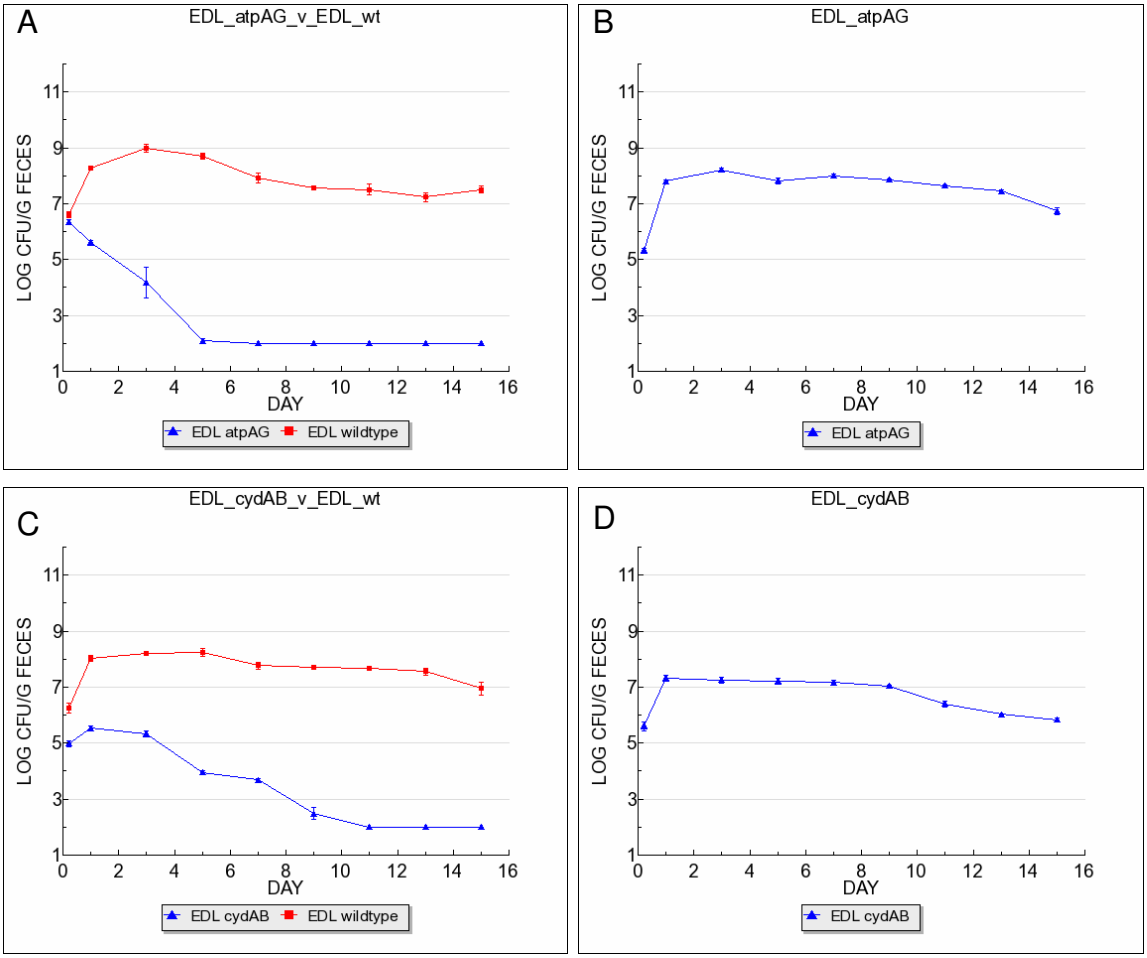


Figure 2.1. ATPase and cytochrome *bd* oxidase mutants of *E. coli* EDL933 exhibit colonization defects.

colonize at wild-type levels (Fig. 2.1B, Fig. A.2.1B). The ability of the respiratory-defective mutants to colonize alone indicates that fermentation is sufficient for growth of *E. coli* in the mouse intestine, but oxidative phosphorylation is essential for competition with respiratory-competent strains. This finding led us to consider whether one or more modes of respiration are crucial to occupation of intestinal niches formed by electron acceptor availability.

Respiration of oxygen is necessary for colonization. Proof that aerobic respiration is essential for colonization by *E. coli* was obtained by competing mutants lacking the high-affinity cytochrome *bd* oxidase with their respective wild-types. The Δ(*cydA-cydB*)::*cat* mutant constructions deleted genes encoding both subunits (I and II) of cytochrome *bd* oxidase, as shown previously (100). The *E. coli* EDL933 Δ(*cydA-cydB*)::*cat* and *E. coli* MG1655 Δ(*cydA-cydB*)::*cat* strains were eliminated by day 11 during competition with their wild-type parents (Table 2.1, Fig. 2.1C, Fig. A.2.1C). The fitness defect observed for the Δ(*cydA-cydB*)::*cat* strains was not merely a growth defect, since the mutants colonized when fed alone to mice (Fig. 2.1D, Fig. A.2.1D). To confirm the requirement for high-affinity oxygen respiration, Δ(*cydD-cydC*)::*cat* mutants, which cannot assemble cytochrome *bd* oxidase in the membrane (205), were tested and found to have similar colonization defects (Table 2.1 and Fig. 2.2A, Fig. A.2.2A). The Δ(*cydD-cydC*)::*cat* mutants were able to colonize when fed to mice alone (Fig. 2.2B, Fig. A.2.2B). We note that in addition to being required for cytochrome *bd* oxidase assembly, *cydDC* encodes a glutathione transport system (204), making it possible that the colonization defect of the *cydDC* mutant resulted

Figure 2.2. Cytochrome *bd* oxidase assembly mutants of *E. coli* EDL933 exhibited colonization defects in competitive colonization assays. (A) *E. coli* EDL933 Δ(*cydD-cydC*) was defective in competition with wild-type *E. coli* EDL933 (B) but colonized at wildtype levels when fed alone.

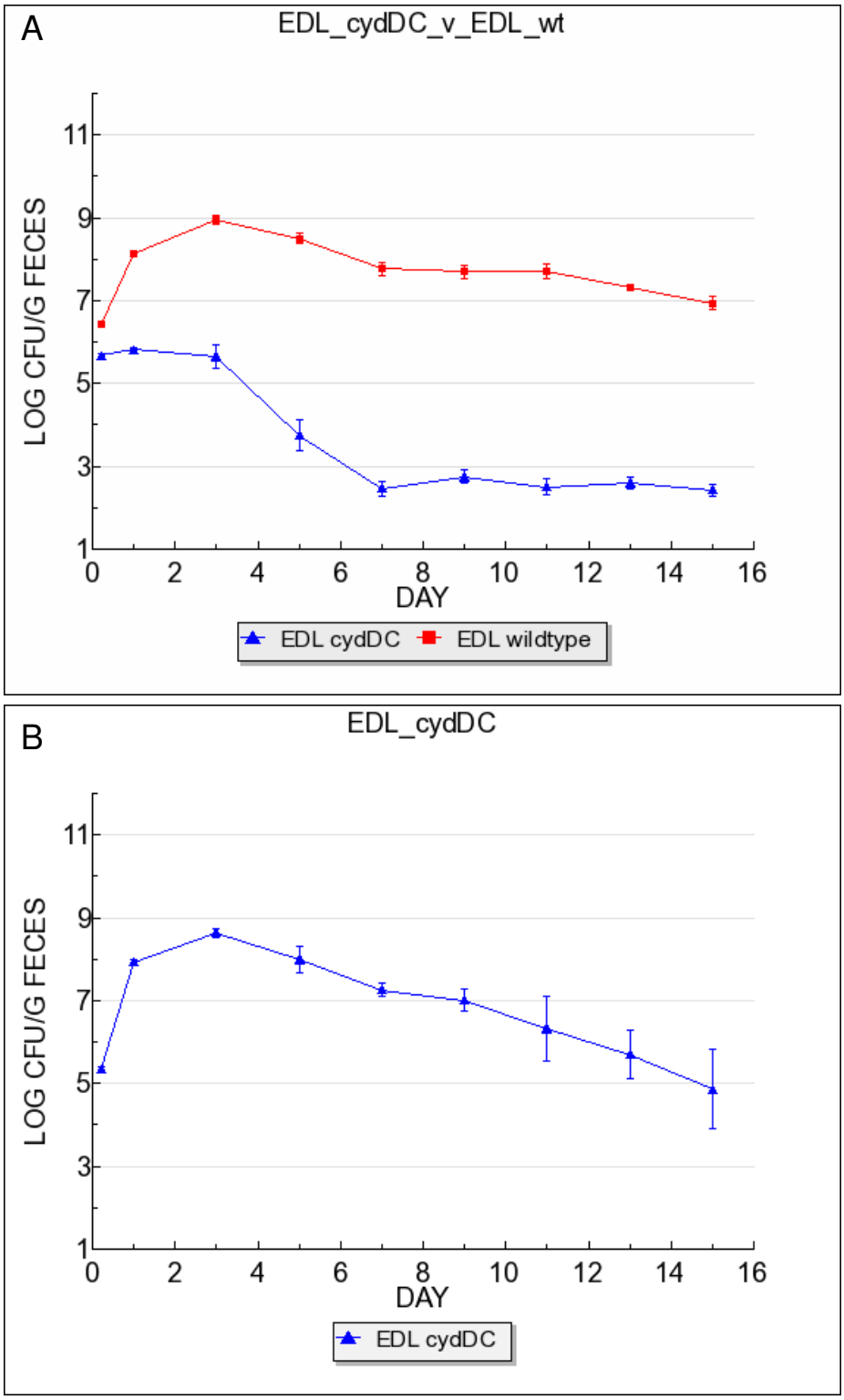


Figure 2.2. Cytochrome *bd* oxidase assembly mutants of *E. coli* EDL933 exhibit colonization defects.

from one of the other phenotypes associated with loss of glutathione uptake. Since the Δ(*cydA-cydB*)::*cat* and Δ(*cydD-cydC*)::*cat* mutants failed to initiate colonization (i.e., 24 h), it was necessary to determine if they were also defective in the maintenance stage (beyond 7 days). Therefore, mice were pre-colonized (for 10 days) with *E. coli* EDL933 Δ(*cydD-cydC*)::*cat* and then challenged with *E. coli* EDL933 wild-type; the mutant was eliminated by the wild-type in 7 days, confirming that functional cytochrome *bd* was important for maintenance of colonization (Fig. 2.3).

We examined also the importance of the low-affinity cytochrome bo_3 oxidase for colonization. The construction of Δ(*cyoA-cyoB*)::*cat* mutants deleted the genes encoding subunits I and II of the cytochrome bo_3 oxidase, as shown previously (284). The results showed that *E. coli* EDL933 Δ(*cyoA-cyoB*)::*cat* and *E. coli* MG1655 Δ(*cyoA-cyoB*)::*cat* strains co-colonized with their respective wild-type parents, indicating that respiration of high oxygen levels was not necessary for colonization (Table 2.1 and Fig. A.2.3). In summary, the colonization defects of the cytochrome *bd* oxidase mutants challenge the traditional view that the intestine is anaerobic (6). Instead, the results support the hypothesis that a microaerobic niche is critical for both establishing and maintaining *E. coli* in the intestine. Thus, a competitive advantage *in vivo* is conferred on strains that respire oxygen.

Aerobic respiratory control is necessary for colonization. *E. coli* governs respiratory flexibility via the global regulators ArcA and Fnr. ArcA is a two-component regulator of several hundred genes (171) that responds to the oxidation state of the quinone pool, which is sensed by ArcB (94). Under high oxygen tension *E. coli* expresses the low-

Figure 2.3. The Δ(*cydD-cydC*) mutant exhibited a maintenance defect when mice were pre-colonized with *E. coli* EDL933 Δ(*cydD-cydC*) and challenged with wild-type *E. coli* EDL933 at day 10.

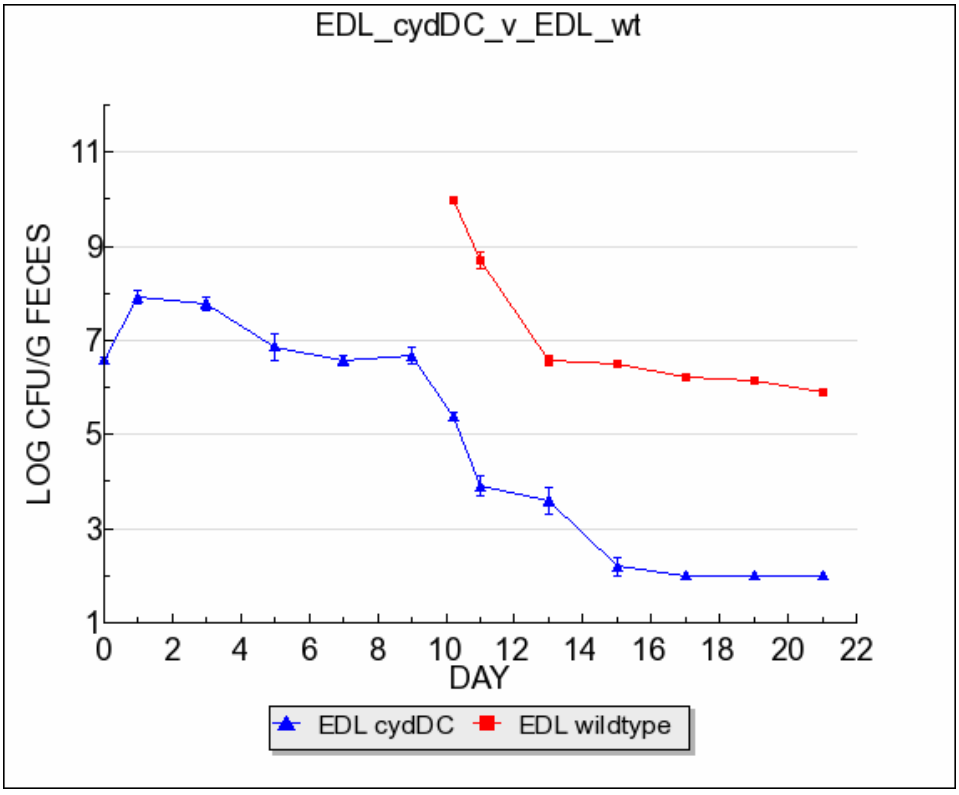


Figure 2.3. Cytochrome *bd* oxidase assembly mutants of *E. coli* EDL933 exhibit colonization defects.

affinity oxidase, cytochrome bo_3 (encoded by *cyoABCDE*) and the high-affinity oxidase, cytochrome *bd* (encoded by *cydAB*) is repressed. Under microaerobic conditions, where oxygen scavenging may play an important role for growth and survival, ArcB phosphorylates ArcA, which represses the *cyoABCDE* operon and activates the *cydAB* and *cydDC* operons (2). Since the experiments described above indicated the importance of cytochrome *bd*, we reasoned that *arcA* mutants would have colonization defects. The construction of *E. coli* EDL933 Δ*arcA*::*cat* and *E. coli* MG1655 Δ*arcA*::*kan* deleted the gene encoding the response regulator of the ArcAB two component system, as previously described (130). Although Δ*arcA* mutants colonized when fed alone (Fig. 2.4B and A.2.4B), they could not compete with their respective wild-types and were eliminated from mice within 3 days (Table 2.1, Fig. 2.4A, A.2.4A). While it is tempting to speculate that the colonization defect of the Δ*arcA* mutants resulted solely from failure to induce *cydAB*, the pleiotropic phenotype of the Δ*arcA* strain makes a number of alternative explanations possible. What is clear from this experiment is that appropriate regulation of aerobic respiratory genes is necessary for *E. coli* to be competitive *in vivo*.

Anaerobic control is necessary for colonization. Induction of anaerobic processes in *E. coli* is controlled by Fnr, an oxygen-labile transcription factor which activates the transcription of hundreds of genes, including genes that encode respiratory pathways for nitrate and fumarate (137, 186). Since induction of anaerobic respiratory pathways requires Fnr, we constructed Δ*fnr*::*kan* mutants to test the contribution of this global regulator during colonization. The *E. coli* EDL933 Δ*fnr*::*kan* and *E. coli* MG1655 Δ*fnr*::*kan* mutants were fed together with the respective wild-types and found to initiate

Figure 2.4. Aerobic respiratory and anaerobic global regulatory mutants exhibited colonization defects in competitive colonization assays. (A) *E. coli* EDL933 Δ*arcA* was eliminated during competition with wild-type *E. coli* EDL933, (B) but was able to colonize when fed alone. (C) *E. coli* EDL933 Δ*fnr* was eliminated during competition with wild-type *E. coli* EDL933, (D) but was able to colonize when fed alone.

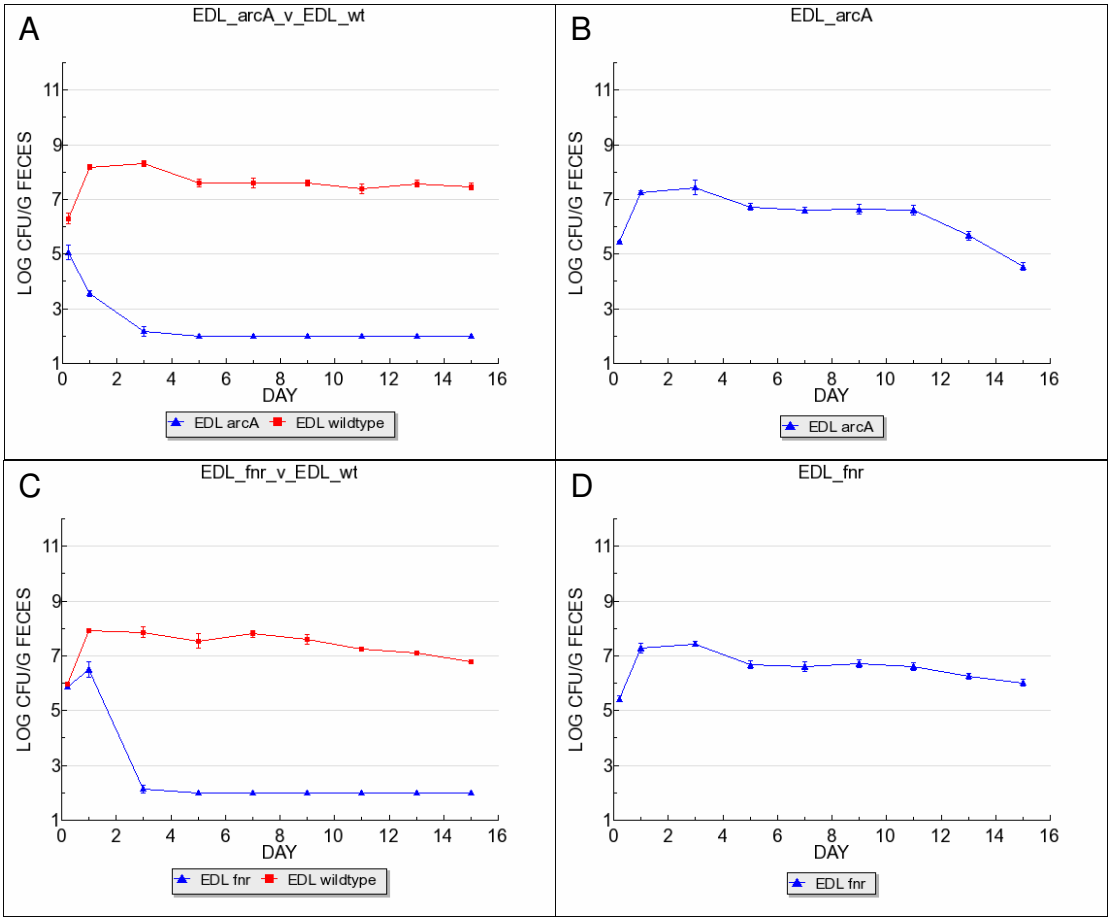


Figure 2.4. ArcA and Fnr mutants of *E. coli* EDL933 exhibit colonization defects.

colonization (although not as well as the wild-type), and then were eliminated by day 5 (Table 2.1, Fig. 2.4C, A.2.4C). The defect was not an inability to grow in the intestine, since the Δ*fnr*::*kan* mutants colonized when fed alone to mice (Fig. 2.4D and Fig. A. 2.4D). Since the Δ*fnr*::*kan* mutant exhibits a colonization defect in competition with the wild-type, this implies that *E. coli* experiences conditions in the intestine that are required for Fnr function (13), i.e., anaerobic or nearly anaerobic conditions. Thus, we conclude that Fnr-dependent genes contribute to colonization success. While these results indicate that appropriate regulation of anaerobic respiration is important for colonization, the specific roles of the alternative pathways were unclear.

Nitrate reductase is necessary for colonization. To determine which anaerobic respiratory pathways were used during colonization, we considered the *in vivo* role of nitrate reduction. Since *E. coli* has three systems for nitrate respiration, it was necessary to consider strains with mutations that eliminated each one individually and in combination (93). Mutation of the primary nitrate reductase was accomplished by deleting *narG* (264) to create *E. coli* EDL933 Δ*narG*::*kan* and *E. coli* MG1655 Δ*narG*::*kan.* When Δ*narG*::*kan* strains were fed together with their respective parent strains, they initiated colonization, but then declined numerically (2.3 logs, $P<0.003$) (Table 2.1, Fig. 2.5A, Fig. A.2.5). To test the involvement in colonization of the secondary nitrate reductase, mutants were constructed which deleted *narZ*, the phenotype of which was described previously (267). Likewise, the involvement of the periplasmic nitrate reductase was tested with a construction that deleted *napD* and *napA*, which encode the assembly protein and large subunit of the reductase, respectively (267). In

Figure 2.5. Anaerobic respiration mutants exhibited colonization defects in competitive colonization assays. (A) *E. coli* EDL933 Δ*narG*, (B) *E. coli* EDL933 Δ*narG* Δ*narZ*, (C) *E. coli* EDL933 Δ*narG* Δ*narZ* Δ*napDA*, (D) and *E. coli* EDL933 Δ*frdA* mutants were defective in competition with wild-type *E. coli* EDL933.

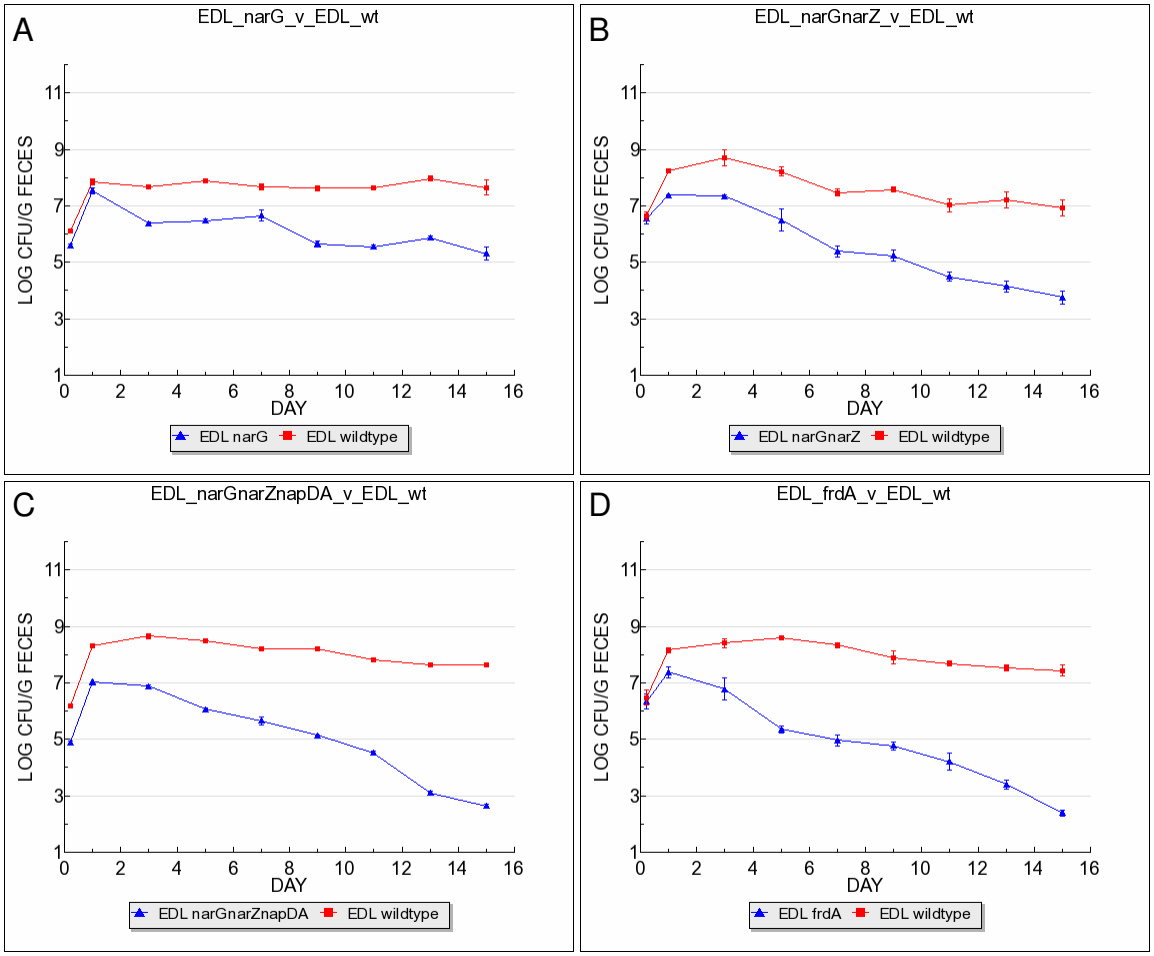


Figure 2.5. Alternative terminal reductase mutants of *E. coli* EDL933 exhibit colonization defects.

colonization assays, *E. coli* EDL933 Δ*narZ*::*cat*, *E. coli* MG1655 Δ*narZ*::*cat*, *E. coli* EDL933 Δ(*napD-napA*)::*cat* and *E. coli* MG1655 Δ(*napD-napA*)::*cat* co-colonized with their wild-type parents, indicating that strains with these individual mutations had no phenotype in the intestine (Table 2.1 and Fig. A.2.8).

Although the individual Δ*narZ*::*cat* and Δ(*napD-napA*)::*cat* mutants did not exhibit colonization defects, there is reason to believe these gene systems should be expressed in the wild-types under the conditions present in the intestine. First, the intestine contains regions that are microaerobic and others that are anaerobic (113), yet apparently is sufficiently anaerobic overall for Fnr control to be a factor in success of the entire *E. coli* population (Fig. 2.4). Second, we measured a concentration of 2.62 ± 0.21 mM nitrate in mouse cecal mucus (Table 2.2), a concentration which is physiologically relevant for controlling expression of the two nitrate-inducible systems (284). Third, *E. coli* cells isolated from the intestine show both logarithmic and stationary phase characteristics (152). The *narZYWV* operon that encodes the secondary nitrate reductase is not regulated by anaerobiosis or nitrate, but instead is RpoS-dependent and induced in stationary phase (40). The *napFDAGHBC* operon that encodes the periplasmic nitrate reductase is maximally induced at 1 mM nitrate and is expressed at one-half of the maximal level at 2.5 mM (284). Thus, it is reasonable to expect that both the periplasmic and secondary nitrate reductases are expressed in the intestine. To investigate the phenotypes of these nitrate reductase mutations in the absence of the primary nitrate reductase, we constructed Δ*narG*

Table 2.2. HPLC analysis of biologically relevant anions present in mouse cecal mucus.

Anions	Concentration in Mucus (mM)
Nitrate	2.62 ± 0.21
Nitrite	ND
Chloride	140 ± 0.56
Sulfate	14.5 ± 0.39
Fumarate	ND

ND = not detected

Δ*narZ*::*cat* and Δ*narG* Δ*narZ* Δ(*napD-napA*)::*cat* mutants. In colonization assays *E. coli* EDL933 Δ*narG* Δ*narZ*::*cat* and *E. coli* MG1655 Δ*narG* Δ*narZ*::*cat* mutants declined 3.2 log units (P<0.000006) relative to the wild-type strain by day 15 (Table 2.1, Fig. 2.5B, Fig. A.2.6). Also, the *E. coli* EDL933 Δ*narG* Δ*narZ* Δ(*napD-napA*)::*cat* and *E. coli* MG1655 Δ*narG* Δ*narZ* Δ(*napD-napA*)::*cat* mutants declined by 5 log units (P<0.000006) to populations just above the limit of detection (<10^3 CFU/g feces) by the conclusion of experiments (Table 2.1, Fig. 2.5D, Fig. A.2.7). The additive effect of the sequential nitrate reductase mutations in competition with the wild-type *E. coli* EDL933 in these experiments is statistically significant: between Δ*narG*::*kan* and Δ*narG* Δ*narZ*::*cat* (P<0.003) and between Δ*narG* Δ*narZ*::*cat* and Δ*narG* Δ*narZ* Δ(*napD-napA*)::*cat* (P<0.004). From these results, we conclude the primary nitrate reductase plays the larger role of the three systems in the mouse intestine. In contrast, the Δ*narZ* and Δ(*napD-napA*) mutations affected colonization only in the Δ*narG* background, suggesting a synergy, rather than mere redundancy, between the primary nitrate reductase and the other two systems (Fig. A.2.9). Apparently, conditions in the intestine signal induction of the three nitrate reductase gene systems, which together confer a competitive advantage to *E. coli.*

Fumarate reductase is necessary for colonization. To test the importance of fumarate as an alternative electron acceptor *in vivo*, we constructed mutants deleted for *frdA*, which are known to inactivate fumarate reductase (258). *E. coli* EDL933 Δ*frdA*::*kan* and *E. coli* MG1655 Δ*frdA*::*kan* mutants were fed together with their respective parent strains and found to initiate colonization, but then declined by approximately 4 logs relative to

the wild-type (Table 2.1, Fig. 2.5D, Fig. A.2.10). Thus, fumarate reductase mutants competed poorly, indicating that fumarate is used in the intestine as an alternative electron acceptor. Since fumarate was not detected in intestinal mucus (Table 2.2), it most likely was generated endogenously, giving rise to succinate as a fermentation product during anaerobiosis (238).

Alternative respiratory pathways are not used during colonization. Other alternative electron acceptors reduced by *E. coli* include dimethyl sulfoxide, trimethylamine monoxide, and nitrite (93). The results showed that Δ*dmsAB*, Δ*torCA*, and Δ*nirBD* mutants did not have colonization defects in mice (Table A.2.2, Fig. A.2.11, Fig. A.2.12, Fig. A.2.13). These results served to reinforce our previous observation that not all mutations cause colonization defects, lending confidence that the observed defects in colonization can be attributed specifically to the metabolic lesions being tested (39). We conclude that DMSO and TMAO are not used for colonization, suggesting they are unavailable in the intestine. Nitrite should be present in the intestine as a result of nitrate reduction, but nitrite was not detected in intestinal mucus (Table 2.3). Since *nirBD* and the wild-type strains co-colonized, we conclude that nitrite is not present at levels sufficient to provide a growth advantage. Since *E. coli* has redundant reductases for nitrite, DMSO, and TMAO, we can only conclude that the primary reductases examined in this study are not important for intestinal colonization. Further examination of the alternative reductases will be required.

2.4 Discussion

Figure 2.6 shows a model of the respiratory pathways that are critical for successful colonization by both EHEC and commensal *E. coli*. The results indicate that the gut is not strictly anaerobic because the high-affinity cytochrome *bd* oxidase is required to successfully compete with the wild-type for colonization (Figs. 2.1 and 2.2). Also, anaerobic respiration of nitrate and fumarate is essential for *E. coli in vivo* (Fig. 2.5). We therefore conclude that success of *E. coli* in the gastrointestinal tract demands respiratory flexibility and use of the best available electron acceptor. The results allow us to deduce the intestinal environment as it is perceived by *E. coli*. Accordingly, the niches defined by mutational analysis of respiratory pathways should correspond with *in vivo* availability of exogenous electron acceptors, i.e., oxygen and nitrate, but not necessarily fumarate, which is generated endogenously by sugar degradation via central metabolism. These respiratory niches could be open to the entire population, wherein each bacterium simultaneously uses both oxygen and nitrate, or individual cells and micro-colonies could each use different electron acceptors. If distinct microaerobic and anaerobic niches were to exist in the intestine, then mutants in the respective respiratory pathways would be maintained at populations corresponding to availability of the cogent electron acceptor when in competition with respiratory-competent wild-types. However, since the loss of the high affinity oxygen, nitrate, or fumarate respiratory pathways leads to the near or complete elimination of the entire population in competition with respiratory-competent strains (Fig. 2.1C, 2.5C and 2.5D), the data give us reason to think that both aerobic and anaerobic niches are equally crucial. Thus, the behaviour of the *E. coli* respiratory

Figure 2.6. Model of *E. coli* respiratory pathways. Respiratory oxidases and reductases used by *E. coli in vivo* are shown in black. The oxidase not affecting colonization is shown in grey. The environmental conditions affecting the key regulators of the genes encoding the oxido-reductases are shown. Activation is shown with arrowheads and repression is shown with diamond heads.

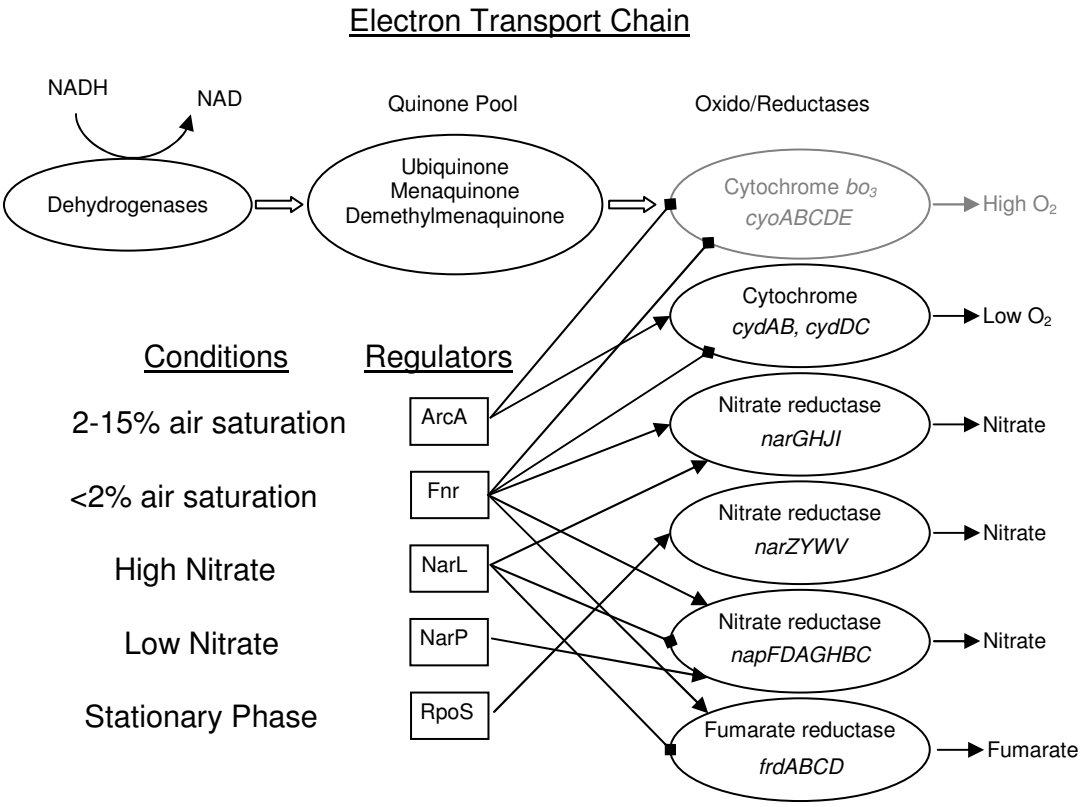


Figure 2.6. Model of *E. coli* respiratory pathways used *in vivo*.

mutants implies that the intestinal habitat is at one time microaerobic and at another time anaerobic. Indeed, oxygen tension in the intestine may fluctuate due to dynamic cycles of oxygen diffusion and respiratory consumption by facultative anaerobes.

Cole (44) postulated that the redundancy of respiratory systems and complexities of their regulation readies bacteria for changes in electron acceptor availability: when respiring nitrate *E. coli* can respond rapidly to the "arrival" of oxygen or cope when nitrate is exhausted. Thus, the physiology, biochemistry, and genetic control of respiratory pathways provides flexibility that is thought to be essential for survival in a changing environment (44). It would appear this strategy is singularly important during animal colonization. We summarize these factors in Figure 2.5. For *E. coli* to colonize the intestine, appropriate aerobic respiratory control (ArcA) and anaerobic control (Fnr) is required (Fig. 2.4). ArcA is most active under microaerobic conditions, i.e., oxygen tension 2-15% of air saturation (2), and Fnr is most active during transition to anaerobic conditions, i.e., oxygen tension less than 2% of air saturation (13). Whole-animal measurements indicated oxygen tensions in the 2-7% air saturation range for the mouse colon (113). The measured nitrate concentration in intestinal mucus (2.6 mM, see results) is near that which results in maximal expression of both nitrate reductase systems, i.e., 1 mM for the periplasmic nitrate reductase and 7 mM for the primary nitrate reductase (284). Regulation of the periplasmic nitrate reductase genes requires both NarL and NarP (216), while regulation of the primary reductase genes requires NarL only (262). Since NarP

exerts its control at low nitrate levels (<4 mM) and NarL control dominates at higher nitrate levels (282), and since both the primary and periplasmic nitrate reductases are necessary for efficient colonization, this suggests that nitrate availability might fluctuate in the intestine. Thus, if the intestinal oxygen tension fluctuates in the anaerobic to microaerobic range and the nitrate concentration fluctuates in the 1-7 mM range, then cytochrome *bd* oxidase, the primary nitrate reductase, the periplasmic nitrate reductase, and fumarate reductase all will be expressed *in vivo*. Indeed, regulation of these gene systems is poised to be most responsive to changes in oxygen and nitrate availability in these concentration ranges (269).

The inference that oxygen availability fluctuates because it is consumed by bacterial respiration suggests the interesting possibility that facultative anaerobes may make the intestinal environment more anaerobic. Indeed, this conclusion is supported by previous studies of the effect of streptomycin-treatment on the mouse anaerobic microflora, which selectively removes facultative anaerobes, i.e., *E. coli*, enterococci, streptococci, and lactobacilli. Following administration of streptomycin, populations of strict anaerobes, e.g., bifidobacteria and clostridia decreased, while populations of so-called "nanaerobes", e.g., *Bacteroides fragilis*, which respires oxygen when available in low concentrations (11), were unchanged (116). Thus, comparisons of the populations of anaerobes in mice with or without facultative anaerobes present supports the hypothesis that oxygen-scavenging facultative anaerobes, e.g., *E. coli*,

promote the stability of the predominantly anaerobic microflora, exemplifying how a minor member can have a large impact on an ecosystem.

Despite the apparent competitive advantage gained by oxygen respiration, the *E. coli* population is limited to between 10^8 and 10^9 CFU/g feces, i.e., *E. coli* represents between 1 in 1,000 and 1 in 10,000 bacteria in the intestine. The nutrient-niche hypothesis states that to be successful each species of the intestinal microflora must use at least one carbon source better than all other species (89). Corollary to this hypothesis, the population size of any member of the microflora is determined by the concentration of its preferred nutrient(s). The available concentrations of the seven sugars that contribute to colonization by *E. coli* MG1655 are quite low (45, 191). Since *E. coli* does not secrete polysaccharide-degrading enzymes, its preferred substrates are most likely provided by anaerobes, which degrade mucosal polysaccharides and dietary fiber, and are thought to release the breakdown products for use by the host and other microbes (48). These facts lead to the conclusion that *E. coli* maximizes its growth yield by coupling oxidation of low nutrient concentrations to respiration in the intestine. This may be a general strategy of facultative anaerobes, which generally grow well on simple sugars, but do not secrete polysaccharide-degrading enzymes. Thus, high-efficiency respiration may ensure the success of facultative anaerobes in the intestine, albeit always in lower numbers, by allowing them to maximize cell yield on scarce resources.

Since most mucosal pathogens are facultative anaerobes, our conclusions may extend to other mucosal pathogens. In support of this idea, *Mycobacterium tuberculosis* genes encoding cytochrome *bd* oxidase and the nitrate transporter were induced during mouse lung infection; a cytochrome *bd* oxidase mutant was attenuated during transition to chronic infection in mice (248). Likewise, *Shigella flexneri* cytochrome *bd* mutants showed decreased intracellular survival and attenuated virulence in mouse infections (301). These examples demonstrate the importance of respiration during infection by particular mucosal pathogens and support the idea that oxygen stimulates infectious disease by providing a competitive advantage for pathogens. Since there appears to be no distinction between enterohemorrhagic and commensal *E. coli* with respect to the respiratory pathways used *in vivo*, we suggest caution in targeting respiratory metabolism for combating EHEC infections because of potential collateral damage to commensal facultative anaerobes and the resulting instability of the intestinal microbiota (89).

In summary, we have shown that *E. coli* uses both aerobic and anaerobic respiratory pathways during colonization, which is an initial stage of intestinal infection. The results presented in this study support the conclusion that the intestine is microaerobic and that aerobic bacterial respiration in the intestine is essential for competition, and therefore successful colonization. Apparently, *E. coli* respires oxygen to optimize its reproduction in animals despite the low availability of its preferred carbon sources, which maximizes its colonization efficiency.

Chapter 3

Hierarchical Use of Terminal Electron Acceptors by *E. coli* in the Mouse Intestine

3.1 Introduction

Escherichia coli has a complex regulatory network that adjusts the respiratory system to compete in the mouse intestine to survive a wide variety of growth conditions. Metabolic efficiency under different environmental conditions depends on the flexibility of an organism to use electron acceptors that provide the highest energy yield. Some indigenous microbes of the GI tract, such as facultative anaerobes, have the ability to generate energy by oxidative phosphorylation. Therefore, these microorganisms may occupy habitats *in vivo* where oxygen offers an ecological advantage. These aerobic habitats may be in close proximity to epithelial cells where O_2 molecules are thought to pass from the blood through the epithelium to the adjacent bacteria. Facultative anaerobes, such as *E. coli*, may also play a role in creating and maintaining an anaerobic environment that favors the strict anaerobes present in the large bowel (232). The presence of alternative electron acceptors in the intestine, which also can be used, may provide stability to the *E. coli* population if oxygen is not consistently available, allowing it to successfully compete in the

intestine. In the last chapter, I showed that *E. coli* uses oxygen, nitrate and fumarate to efficiently colonize the intestine.

A natural inhabitant of the gastrointestinal tract, *E.coli* serves an important role in maintaining a stable and healthy intestinal microbiota. My focus in this study was to determine the preferred order of electron acceptors used by pathogenic *E. coli* O157:H7 and commensal *E. coli* K-12 strains during colonization of the murine intestine. *E. coli's* ability to respire both aerobically and anaerobically using multiple electron acceptors allows for successful colonization, thus ensuring its survival. Indeed the energy efficiency provided by respiration may contribute to the wide spread distribution (i.e., success) of *E. coli* as commensal inhabitants of the mammalian intestine. Invading pathogens must compete with the resident intestinal microflora in order to establish themselves in the densely populated gastrointestinal tract. Enterohemorrhagic *E. coli* (EHEC), an intestinal pathogen, has a low infectious dose and causes severe foodborne illnesses that can result in diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (135, 183). Thus, it is essential to understand how EHEC generates energy for growth, since little is known about the role of microbial respiration in pathogenesis during colonization. Furthermore, competition for the best available electron acceptors in the intestine ultimately confers a competitive advantage to support intestinal colonization.

The respiratory metabolism of *E. coli* in the GI tract had been ignored for years by many studying the physiology of this organism. However, more interest has developed recently regarding the ability of this organism to produce energy for growth and survival during colonization of the intestine. Indeed, prior studies have shown oxygen to be in the range of 2 to 7 % air saturation in the mouse intestine (113). Studies have also shown anaerobic bacteria that inhabit the intestinal tract to respire oxygen at nanomolar concentrations (113). Our previous findings that oxygen offers a competitive advantage for *E. coli in vivo* support the idea of microaerobic habitats in the intestine. We have previously shown that oxidative phosphorylation mediated by cytochrome oxidase, nitrate reductase, and fumarate reductases all contribute in varying degrees to colonization. Of the two cytochrome oxidases, only the high affinity, low oxygen tension cytochrome *bd* oxidase (CydAB and CydDC) is essential for competitive fitness in the intestine during growth from low to high numbers (initiation) and for persistence (maintenance stage), whereas cytochrome bo_3 (CyoAB) is not important. Mutations in nitrate reductase or fumarate reductase had no affect on initiation, but reduced fitness for competition with the wild-type during maintenance. Thus, success of *E. coli* in the gastrointestinal tract demands respiratory flexibility and use of the best available electron acceptor, i.e., oxygen.

Since *E. coli* must compete with the intestinal microflora for available resources, it will use the best available electron acceptor that yields the highest redox potential, thereby maximizing its energetic efficiency. *E. coli* has a preferred order for electron

acceptors that enables the organism to achieve maximum and efficient energy production. The established hierarchy of terminal electron acceptors used by *E. coli in vitro* is as follows: oxygen is more energetically favorable than nitrate, which is favored over fumarate (100, 249). This preferred order of electron acceptors follows the redox potential for the given acceptor where oxygen gives the maximal energy ($E^{\circ\prime}$ = + 0.82 V) followed by nitrate ($E^{\circ\prime}$ = + 0.42 V) and fumarate ($E^{\circ\prime}$ = + 0.031 V) (132). The use of these electron acceptors has been extensively studied *in vitro* (93). However, the hierarchy of these electron acceptors used *in vivo* has never been examined.

To compete in the GI tract, *E. coli* must be able to adapt to a variety of growth conditions. We have shown *E. coli* to respire aerobically using the high affinity cytochrome oxidase, and in the absence of oxygen switch to anaerobic respiration using alternative electron acceptors such as nitrate and fumarate. To address the hierarchical use of electron acceptors in the gut, we used a streptomycin-treated mouse colonization model. Our results demonstrate that oxygen is preferred over all other terminal electron acceptors in the intestine. The data agree with our previous finding that the gut is not strictly anaerobic and that oxygen scavenging is necessary for colonization. The ability of facultatively anaerobic bacteria, such as *E. coli*, to alter its respiratory metabolism suggests a type of respiratory flexibility that the organism demonstrates in the intestine. These data suggest that the intestine is not static, but instead is in a dynamic state with respect to fluctuations in electron

acceptor availability. Here we show the hierarchy of terminal electron acceptors used by *E. coli* in the microaerobic intestine.

3.2 Materials and Methods

Bacterial Strains and Growth Conditions. The bacterial strains used in this study were derived from *E. coli* MG1655 Str^r (streptomycin-resistant), a K-12 strain (176), and *E. coli* EDL933 Str^r, the prototypical O157:H7 strain (184). Cultures were grown at 37°C in Luria-Bertani (LB), with gyratory shaking at 250 rpm. Null alleles were constructed by using the allelic replacement method of Datsenko and Wanner (65), as described previously (39), such that target genes were deleted and replaced with kanamycin- or chloramphenicol-resistance cassettes (used as selectable markers in mouse colonization assays, as described below). The null allele strains are identified in the text by the genes that were deleted; single gene deletions began with the start codon and ended with the stop codon, and multiple gene deletions began with the start codon of the first gene deleted and ended with the stop codon of the last gene deleted. Strains containing multiple mutations were constructed by sequential allelic replacement; the first inserted cassette was removed with FLP recombinase (65), followed by subsequent allelic replacement(s) and removal of the insertion as necessary, leaving the selected marker in the last mutation made. Mutant strains were verified by phenotype analysis and DNA sequencing. The bacterial strains and plasmids used in this study are listed in Table A.2.1.

Phenotypic analysis. MOPS (3-(*N*-morpholino) propanesulfonic acid) defined medium was used to grow cultures for growth curves, as described previously (39). Anaerobic cultures were grown in culture tubes filled to the top with N_2-sparged media, sealed and incubated in Balch tubes. To test for nitrate or fumarate reductase activity, mutant strains were grown anaerobically overnight in MOPS medium with glycerol (1.6%) as the carbon source and either 50 mM nitrate or fumarate as the electron acceptor. Cell growth was monitored spectrophotometrically at 600 nm (OD_{600}).

HPLC Analysis. Anion analysis of mucus was made using a Dionex DX-500-Microbore system with an IonPac AS11 column and a NaOH gradient as eluent. Standards and blanks were analyzed and standard curves were developed for a 1 mg/ml sample of cecal mucus. Mouse cecal mucus was isolated from the cecum of CD-1 male mice and lyophilized as described previously (295). Regression coefficients for standard curves were calculated and used to demonstrate the linearity of the peak area with respect to concentration.

Mouse Colonization Experiments. The streptomycin-treated mouse model has been used extensively to study colonization of the mouse large intestine by *E. coli* and *Salmonella enterica* serovar Typhimurium (35, 45, 110, 279). Briefly, three CD-1 male mice, six weeks of age, were given drinking water containing streptomycin sulfate (5 g/L) for 24 h to remove the existing resident facultative microflora, and

then starved for food and water for 18 to 24 h. The mice were then fed approximately 10^5 colony-forming units (cfu) of both the wild-type and mutant strains in 1 ml of 20% sucrose. The wildtype strains were *E. coli* MG1655 Str^r Nal^r (nalidixic acid resistance) (176) and *E. coli* EDL933 Str^r Nal^r (184); Nal^r was used to distinguish the wildtype (reference strain) from the null allele mutants in fecal plate counts. After the bacterial suspension was ingested, food and streptomycin-water were restored and fecal plate counts were determined at 5 h, 24 h, and on every other day thereafter for 15 days. Fecal samples were homogenized and diluted in 1% tryptone broth and plated on MacConkey agar containing either streptomycin (100 μg/ml) and nalidixic acid (50 μg/ml) to count the wildtype or streptomycin and kanamycin (40 μg/ml) or chloramphenicol (30 μg/ml) to count the null allele mutants. Each colonization experiment was repeated, on separate occasions, and the plotted values (in figures) represent the average for 6 mice. The $\log_{10}$ mean number of CFU per gram of feces ± the standard error for each strain in the mice was calculated for each time point. The limit of detection in fecal plate counts was 10^2 cfu/g feces. To determine the role of strains during the maintenance stage of colonization, mice were colonized for 10 days with a mutant strain of *E. coli* EDL933, starved for food and streptomycin-water overnight, and fed 10^{10} CFU of the wild-type parent strain *E. coli* EDL933, after which food and streptomycin-water were replaced.

3.3 Results

Control of aerobic respiration is essential for colonization. ArcA (aerobic respiratory control) is a global regulator that activates the high-affinity cytochrome *bd* oxidase and represses the low-affinity cytochrome bo_3 oxidase under microaerobic conditions (2). Previously we showed that cytochrome *bd* oxidase and aerobic respiratory control were important for intestinal colonization. To determine whether control of aerobic respiration was important for colonization and if ArcA control of cytochrome *bd* was the reason for the observed colonization defect (Δ*arcA* mutant versus wild-type) reported in the last chapter, *E. coli* EDL933 and MG1655 Δ*arcA*::*kan* and Δ(*cydA*-*cydB*)::*cat* were fed low at a level of 10^5 CFU/mouse. The ArcA mutant was unable to initiate or maintain during competition with cytochrome *bd* oxidase mutants (Fig. 3.1A and Fig. A.3.1A). The observed 6 log difference between the two strains suggests that regulation under microaerobic conditions is important for competition of *E. coli* in the intestine. Since the Δ*arcA* mutant outcompeted the Δ*cydAB* mutant, we conclude that the observed colonization defect of the Δ*arcA* strain is not limited to the inability of the Δ*arcA* mutant to activate *cydAB* expression. This result is in keeping with similar results with transcriptional regulator mutants having a large defect in colonization (157). Thus, the observed defect is probably due to altered regulation of many ArcA-dependent genes.

Aerobic respiration is more important than anaerobic respiration for intestinal colonization. To determine if respiration of oxygen is more important than respiration of nitrate, we tested mutants that lack cytochrome *bd* oxidase for their

Figure 3.1. Aerobic respiratory global regulatory and cytochrome *bd* oxidase mutants exhibit colonization defects in competitive colonization assays. (A) *E. coli* EDL933 Δ*arcA* mutants are unable to compete during colonization with EDL933 Δ(*cydA-cydB*) mutant strains. (B) *E. coli* EDL933 Δ(*cydA-cydB*)::*cat* mutants are unable to compete during colonization with EDL933 Δ(*narG*)::*kan* mutant strains. (C) *E. coli* EDL933 Δ(*cydD-cydC*)::*cat* mutants are unable to compete during colonization with EDL933 Δ(*frdA*)::*kan* mutant strains.

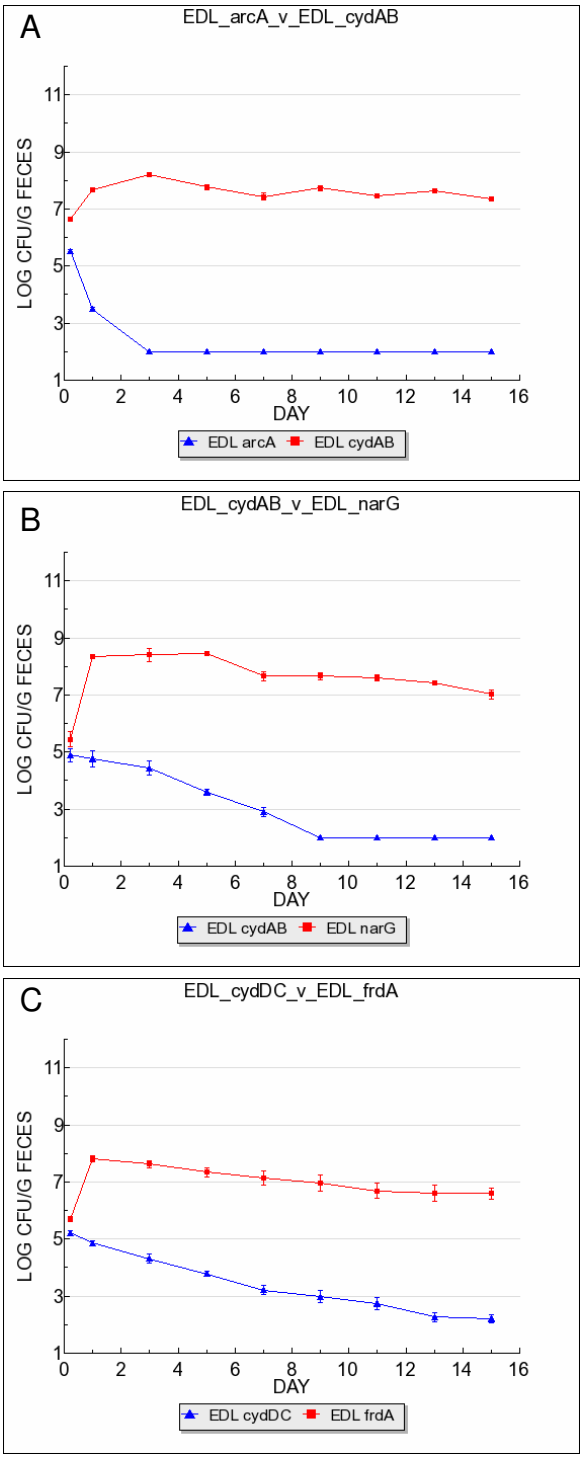


Figure 3.1. ArcA and cytochrome *bd* oxidase mutants of *E. coli* EDL933 exhibit colonization defects.

ability to compete with primary nitrate reductase mutants in the streptomycin-treated mouse colonization model. *E. coli* EDL933 and MG1655 Δ(*cydA*-*cydB*)::*cat* and Δ(*narG*)::*kan* were fed low at a level of 10^5 CFU/mouse. The cytochrome *bd* oxidase mutants were unable to initiate or maintain colonization when competed against the primary nitrate reductase mutants (Fig. 3.1B and Fig. A.3.1B). We previously showed that Δ*cyd* mutants can colonize alone indicating a fitness defect and not a growth defect (Chapter 2). Therefore, cytochrome *bd* oxidase is more important than nitrate reductase for *E. coli* colonization of the intestine. To test whether oxygen is more important than fumarate for colonization, we competed cytochrome *bd* oxidase mutants with fumarate reductase mutants. *E. coli* EDL933 and MG1655 Δ(*cydD*-*cydC*)::*cat* and Δ(*frdA*)::*kan* were fed low at a level of 10^5 CFU/mouse. The cytochrome *bd* oxidase mutants were unable to initiate or maintain colonization when competed against the fumarate reductase mutants, demonstrating that cytochrome *bd* oxidase is more important than fumarate reductase for *E. coli* to colonize the mouse intestine (Fig. 3.1C and Fig. A.3.1C). Since mutants lacking anaerobic respiratory pathways were found to outcompete mutants lacking aerobic respiratory pathways during colonization, we can conclude that oxygen respiration is more important than anaerobic respiration of nitrate and fumarate. Together, these findings imply that oxygen respiration is more advantageous for *E. coli* to colonize the mouse intestine compared to anaerobic respiration.

The primary nitrate reductase is most important of the three nitrate reductases for intestinal colonization. *E. coli* has three nitrate reductases (97, 247): a cytoplasmic primary nitrate reductase (encoded by *narG*), a cytoplasmic secondary nitrate reductase (encoded by *narZ*), and a periplasmic nitrate reductase (encoded by *napA*). We previously determined that the primary nitrate reductase mutant had a colonization defect during competition with the wild-type parent as shown in Chapter 2. However, no defects were observed for the other nitrate reductases during competiton unless they were in a Δ*narG* background. To determine if the primary nitrate reductase is the most important of the three, we competed a Δ*narG* mutant against mutants lacking the secondary and periplasmic nitrate reductases to determine the *in vivo* hierarchy of the three reductases. *E. coli* EDL933and MG1655 Δ(*narG*)::*kan* and Δ(*narZ*)::*cat* were fed low at a level of 10^5 CFU/mouse. Both nitrate reductase mutants were able to colonize initially (Fig. 3.2A and Fig. A.3.2A). However, by day 3 postfeeding, the *ΔnarG* strain was no longer able to compete against the Δ*narZ* strain. The primary nitrate reductase had a 3 log separation at day 3 postfeeding, and a 5 log separation by day 13 postfeeding relative to the secondary nitrate reductase. The observed fitness defect of *ΔnarG* in competition with *ΔnarZ* indicates that NarG is more important than NarZ.

Next, we competed the primary nitrate reductase mutant with a mutant lacking the periplasmic nitrate reductase. *E. coli* EDL933 and MG1655 Δ(*narG*)::*kan* and Δ(*napD-napA*)::*cat* were fed low at a level of 10^5 CFU/mouse. Both nitrate reductase

Figure 3.2. Primary nitrate reductase mutants exhibit colonization defects during competitive colonization assays with the secondary and periplasmic nitrate reductase mutants. (A) *E. coli* EDL933 Δ(*narG*)::*kan* mutants are unable to compete during colonization with EDL933 Δ(*narZ*)::*cat* mutant strains. (B) *E. coli* EDL933 Δ(*narG*)::*kan* mutants are unable to compete during colonization with EDL933 Δ(*napD-napA*)::*cat* mutant strains.

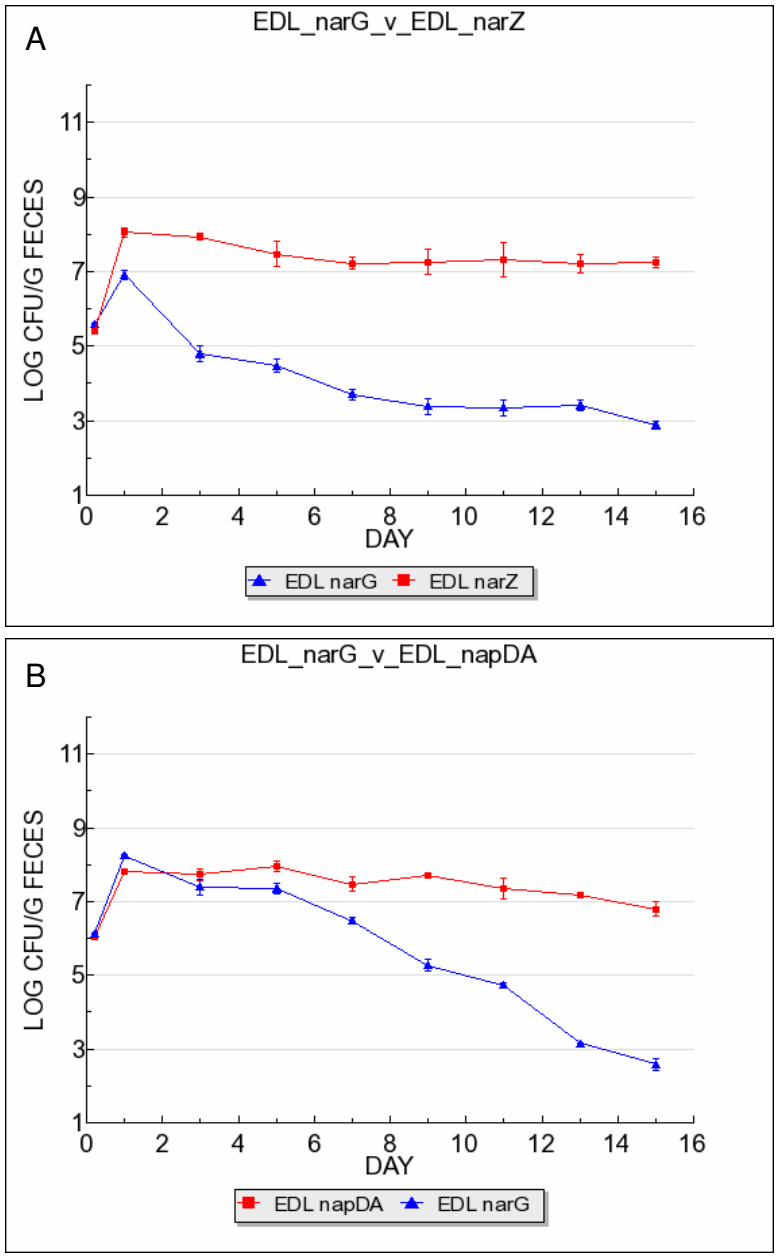


Figure 3.2. Primary nitrate reductase mutants outcompete secondary and periplasmic nitrate reductase mutants in *E. coli* EDL933.

mutants were able to colonize initially (Fig.3.2B and Fig. A.3.2B). The primary nitrate reductase mutant had a 3 log separation at day 9 postfeeding, and a 4 log separation by day 15 postfeeding relative to the secondary nitrate reductase. Again, the fitness defect observed for *ΔnarG* in competition with *ΔnapDA* suggests that NarG is more important than NapDA. Thus, the *in vivo* hierarchy for nitrate reductase is consistent with the previous finding that the primary nitrate reductase (NarG) is optimally expressed at nitrate levels greater than 2 mM *in vitro* and indicates that the largest flux of electrons to nitrate is through the primary nitrate reductase (300). We have previously measured nitrate in intestinal mucus and found nitrate to be present at the 2.62 mM range. Indeed, our finding nitrate at this concentration in cecal mucus agrees with the colonization data for the expression of NarG, where the nitrate level is high enough to make NarG the primary nitrate reductase used *in vivo*. In other words, the primary nitrate reductase is optimally expressed in cecal mucus since the nitrate concentration is >2mM. Thus, NarG is preferred over the other two nitrate reductase during intestinal colonization.

Respiration of nitrate is more important than respiration of fumarate for intestinal colonization. To determine the preferred order of alternative electron acceptors used for colonization of the mouse intestine, we competed a mutant in the primary and secondary nitrate reductases with a fumarate reductase mutant. *E. coli* EDL933 Δ*narG* Δ*narZ*::*cat* and Δ(*frdA*)::*kan* were fed together to mice at a level of 10^5 CFU/mouse. The nitrate reductase mutants were unable to colonize initially, but

Figure 3.3. Nitrate reductase mutant is outcompeted by a fumarate reductase mutant in maintenance not initiation during competitive colonization. *E. coli* EDL933 Δ*narG* Δ*narZ*::*cat* mutants are unable to compete initially during colonization with EDL933 Δ(*frdA*)::*kan* mutant strains, but able to maintain colonization in the mouse intestine.

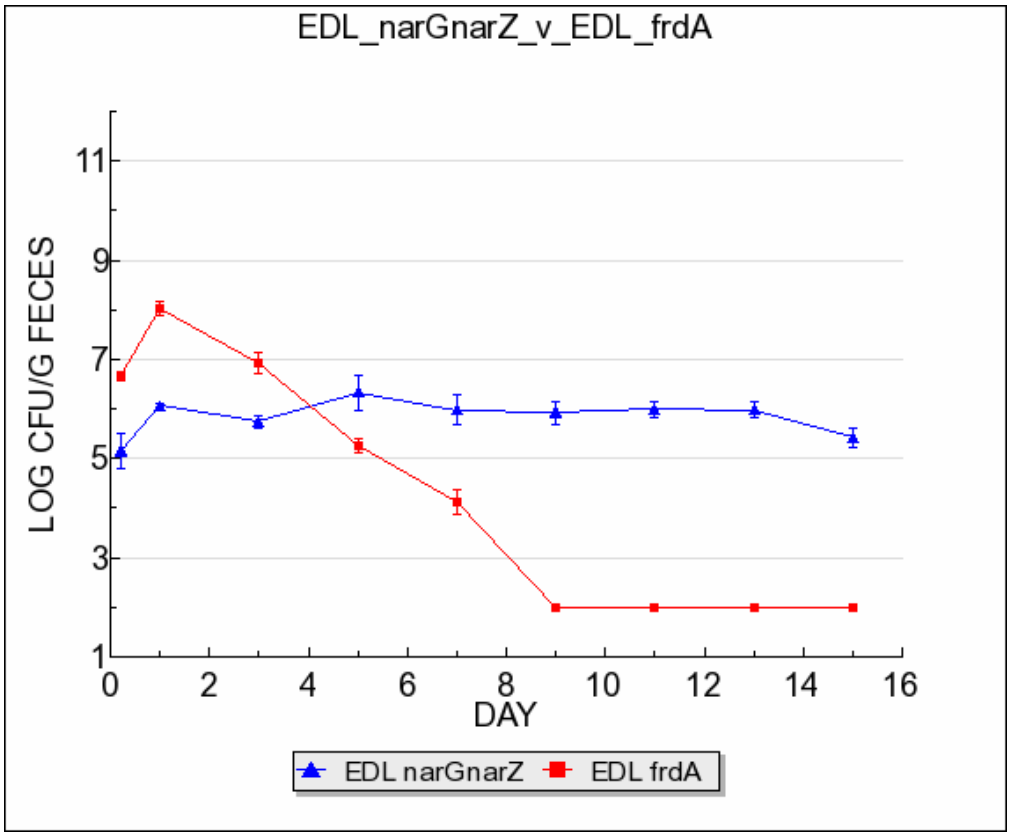


Figure 3.3. Fumarate reductase mutant outcompetes a nitrate reductase mutant of *E. coli* EDL933 in initiation but not maintenance.

by day 4 postfeeding the Δ*narGnarZ* mutants were able to grow up and subsequently outcompete the Δ*frdA* mutants, which declined by 3 logs relative to the Δ*narGnarZ* strains (Fig. 3.3 and Fig. A.3.3). This suggests that nitrate is being used as the primary anaerobic electron acceptor initially, and when nitrate is no longer readily available in the intestine, fumarate is used as an alternative electron acceptor. These results are consistent with the finding that nitrate represses the synthesis of fumarate reductase at concentrations greater than 2 mM (130, 133, 284). Again, this concentration of nitrate supports induction of *narG* and represses *frdA* (134, 159, 249).

Since nitrate and fumarate reductase mutants are able to initiate colonization and have colonization defects only during maintenance, we decided to focus our attention on the maintenance stage of colonization. We competed either the double nitrate reductase mutant or the fumarate reductase mutant with the wild-type EDL933 parent strain during maintenance to determine the *in vivo* hierarchy of nitrate and fumarate. *E. coli* EDL933 Δ*narG* Δ*narZ*::*cat* was fed low at a level of 10^5 CFU/mouse and then challenged with wild-type EDL933 after 10 days postfeeding. In a similar experiment, *E. coli* EDL933 Δ(*frdA*)::*kan* was fed low at a level of 10^5 CFU/mouse and then challenged with wild-type EDL933 after 10 days postfeeding. In both cases, the mutant strains were unable to compete effectively once challenged with high levels of the wild-type parent strain and declined in numbers to a 3 and 5 log difference by day 15 of the experiments (Fig. 3.4 A and B). These results suggest

Figure 3.4. Nitrate and fumarate reductase mutants exhibit colonization defects in maintenance during competition with wild-type *E. coli*. (A) *E. coli* EDL933 Δ*narG* Δ*narZ*::*cat* mutant is unable to compete during maintenance with wild-type EDL933 mutant strains. (B) *E. coli* EDL933 Δ(*frdA*)::*kan* mutant is unable to compete during maintenance with wild-type EDL933 mutant strains.

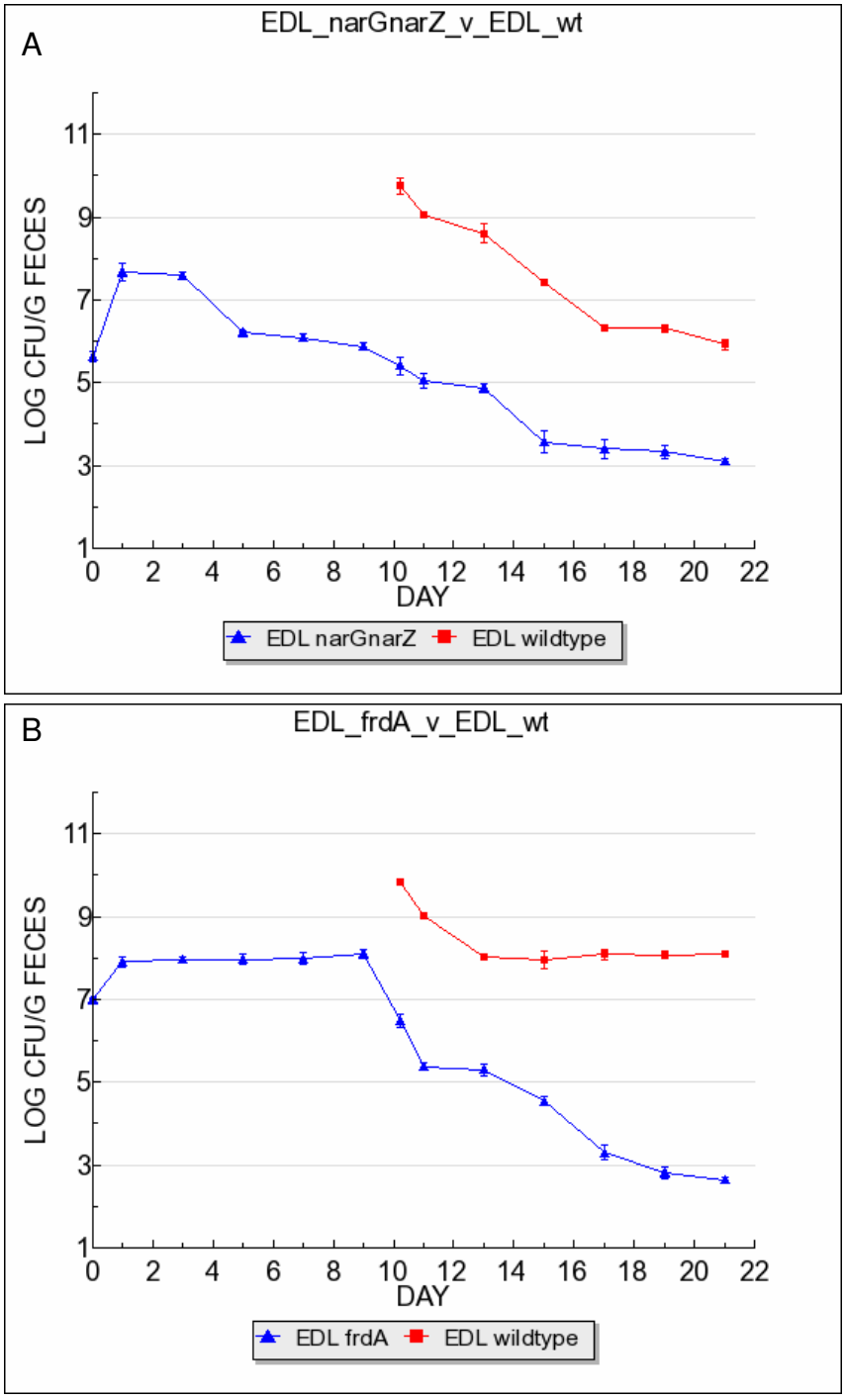


Figure 3.4. Nitrate and fumarate reductase mutants of *E. coli* EDL933 exhibit colonization defects in maintenance.

Figure 3.5. Nitrate reductase mutants are defective in competition with fumarate reductase mutants during maintenance in competitive colonization assays. (A) *E. coli* EDL933 Δ*narG* Δ*narZ*::*cat* pre-colonized mutant is unable to compete during maintenance with a *E. coli* EDL933 Δ(*frdA*)::*kan* mutant strain challenged at day 10 postfeeding. (B) Fig. 4D. *E. coli* EDL933 Δ(*frdA*)::*kan* pre-colonized mutant is able to compete during the maintenance stage of colonization with a *E. coli* EDL933 Δ*narG* Δ*narZ*::*cat* mutant strain challenged at day 10 postfeeding.

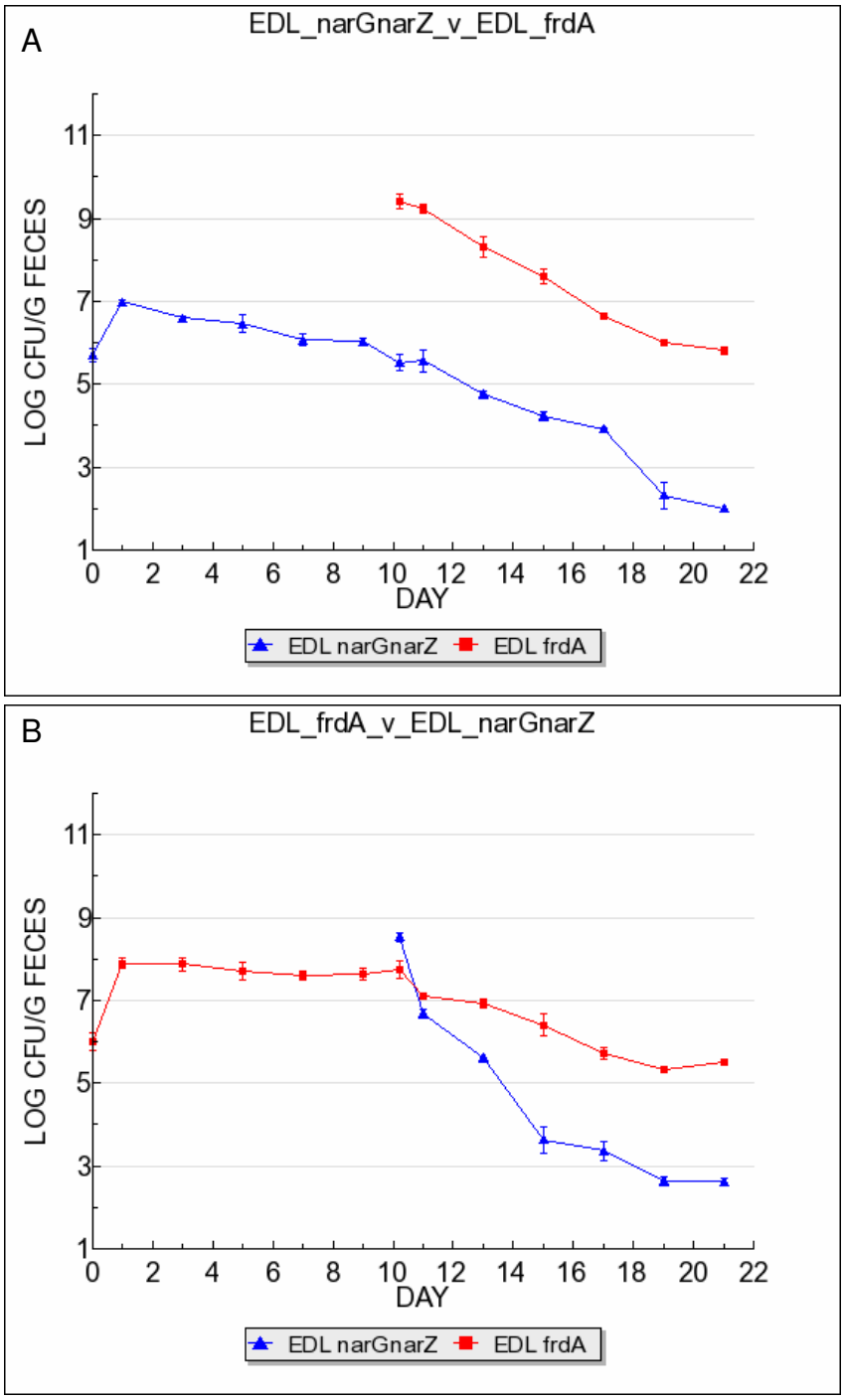


Figure 3.5. Nitrate reductase mutants outcompete fumarate reductase mutants of *E. coli* EDL933 when fed high in maintenance.

that both nitrate reductase and fumarate reductase are necessary for competition with the wild-type during maintenance of intestinal colonization.

To determine the *in vivo* hierarchy of nitrate and fumarate during maintenance, we competed a mutant in the primary and secondary nitrate reductases with a fumarate reductase mutant. *E. coli* EDL933 Δ*narG* Δ*narZ*::*cat* was fed low at a level of 10^5 CFU/mouse and then challenged with high levels of Δ(*frdA*)::*kan* after 10 days postfeeding. The nitrate reductase mutant was unable to maintain colonization after the fumarate reductase mutant was introduced (Fig 3.5A). In this competition, nitrate should have been readily available to the fumarate reductase mutant, which can respire nitrate. This result is best explained by the advantage afforded by the ability of the fumarate reductase mutant to consume the nitrate that should have been available in the animals that were precolonized with the nitrate reductase mutant. Therefore, the data suggest that nitrate provided a growth advantage *in vivo*. We next competed a fumarate reductase mutant against a double nitrate reductase mutant. *E. coli* EDL933 Δ(*frdA*)::*kan* was fed low at a level of 10^5 CFU/mouse and then challenged with high levels of Δ*narG* Δ*narZ*::*cat* after 10 days postfeeding. The fumarate reductase mutant was found to colonize at a steady level prior to introducing the nitrate reductase mutant (Fig. 3.5B). However, the nitrate reductase mutant was unable to colonize efficiently in competition with the pre-colonized fumarate reductase mutant, showing a 3 log difference and just above detectable levels (2 log

cfu/g feces). These results are consistent with our finding that nitrate is preferred over fumarate during the maintenance stage of colonization.

Nitrate analysis of cecal mucus. To address the availability of nitrate in the mouse intestine, we used anion exchange HPLC to determine the concentration of nitrate in cecal mucus at day 1, 5, and 11 of colonization. We previously found nitrate in intestinal mucus at a concentration of 2.62 ± 0. 21 mM (Table 2.2). Similarly, in mice colonized with a triple nitrate reductase mutant Δ*narG narZ* (*napD-napA*)::*cat*, nitrate was detected in intestinal mucus at levels of 2.76 ± 0.27 mM (day 1), 2.89 ± 0.33 mM (day 5), 2.65 ± 0.49 mM (day 11) (Table 3.1). These results agree with the nitrate concentrations detected in mucus prepared from uncolonized animals. By comparison, nitrate was undectable in mice colonized with the wild-type EDL933 strain at day 1, 5, and 11. The data suggest that *E. coli* does in fact respire nitrate in the intestine and lowers the nitrate to undetectable levels. Additionally, nitrate measurements were conducted for mice co-colonized with Δ*narG narZ* (*napD-napA*)::*cat* and Δ*frdA*::*kan* to determine how the *in vivo* concentration of nitrate might affect competition between the two strains. Initially, nitrate was found to be 0.77 ± 0.31 mM (day 1); at this level, nitrate would repress fumarate reductase, explaining how the fumarate reductase mutant could outcompete the nitrate reductase mutant during the first few days of the experiment. By day 5 of colonization, nitrate levels decreased to 0.31 ± 0.25 mM and were undetectable by day 11. Together, these findings imply that the fumarate reductase mutant uses nitrate as a terminal electron

Table 3.1. IC analysis of biologically relevant anions present in mouse cecal mucus.

Strain	NO_3^- Concentration in Mucus (mM)	Day of Colonization
EDL933 Str^R	ND	1
	ND	5
	ND	11
EDL933 Str^R	2.76 ± 0.27	1
Δ*narG narZ*(*napD-napA*)::*cat*	2.89 ± 0.33	5
	2.65 ± 0.49	11
EDL933 Str^R	0.77 ± 0.31	1
Δ*narG narZ*(*napD-napA*)::*cat*	0.31 ± 0.25	5
Δ*frdA*:: *kan*	ND	11

ND = not detected

acceptor until nitrate levels diminish and *frdA* is no longer repressed, at which time the nitrate reductase mutant increases in numbers in the intestine. Thus, during the latter days of the experiment the nitrate reductase mutant has an advantage over the Δ*frdA*::*kan* mutant, which is outcompeted because nitrate levels are too low to support its growth in the intestine. Thus, these results are in agreement with the established *in vitro* hierarchy where nitrate is the more energetically favorable electron acceptor than fumarate and is used first.

3.4 Discussion

We have previously defined the respiratory pathways used by *E. coli* during colonization of the mouse intestine (Chapter 2). We found the high affinity cytochrome oxidase to be essential for colonization in the microaerobic intestine. We also showed nitrate and fumarate reductase to be important *in vivo*. The experiments described in this study provide evidence that the known hierarchy of electron acceptors used by *E. coli in vitro* (93, 132) also applies during colonization of the mouse intestine. Presumably, the presence of multiple electron acceptors allows *E. coli* to compete in a densely colonized habitat such as the mammalian large intestine, where respiratory flexibility and diversity would be an advantage.

Since prior results demonstrated that aerobic respiration is important for colonization and suggested a microaerobic niche for *E. coli* in the intestine, we hypothesized that ArcA was necessary for competition with the wild-type because it

is needed to activate *cydAB* and *cydDC* (2, 269). To test this prediction, the *arcA* and *cydAB* mutants were competed in mice; the *cydAB* mutant was found to colonize at wild-type levels, while the *arcA* mutant was eliminated by day 3 of the experiment. Thus, our results indicate that the defect of strains lacking ArcA is not only due to failure to induce *cydAB*, but may also be caused by inappropriate expression of genes and pathways under negative control. Since ArcA is most active when oxygen is low, this result indicates that microaerobic conditions prevail in the intestine, or at least in the microhabitats occupied by *E. coli*. Furthermore, both Δ*narG* and Δ*frdA* mutants were found to outcompete the Δ*cydAB* and Δ*cydDC* mutants, suggesting that respiration of oxygen is more important for colonization than anaerobic respiration. Together, these results support previous findings that the intestine is microaerobic (138).

Given that *E. coli* has three nitrate reductase isoenzymes, we were interested to determine which of the nitrate reductases was most important. We found in a direct competition that Δ*narG* could initially colonize but not maintain, while Δ*narZ* was able to initiate and maintain colonization suggesting that NarG is the more important nitrate reductase. In a similar experiment, a Δ*napDA* mutant was found to outcompete the Δ*narG* mutant, suggesting that the primary nitrate reductase plays a significant role in colonization. These results are consistent with the report that the activation of *narG* requires at least >2 mM nitrate *in vitro* while activation of *napFDA* requires low levels of nitrate at about 0.5 to 1 mM for maximal expression

(251, 284). Indeed our previous detection of nitrate in intestinal mucus at the level of 2.62 mM agrees with our observations that NarG plays the primary role for nitrate reduction and is necessary for nitrate respiration *in vivo*. Therefore, it appears that an anaerobic niche exists in the intestine where nitrate is available to signal induction of the gene systems necessary for nitrate respiration, which contributes to the competitive advantage of *E. coli* or for the organism that can express NarG, which provides the major pathway for electron flow to nitrate. In contrast, the *narZ* mutation did not affect colonization, suggesting a backup role *in vivo* for this homologous nitrate reductase, which is induced by growth arrest instead of nitrate and is regulated by the stationary phase sigma factor, RpoS (40). Likewise, the periplasmic nitrate reductase, NapFDA, had no affect on colonization.

The competition of a nitrate reductase mutant against a fumarate reductase mutant revealed that nitrate is used initially in colonization while fumarate is used later during maintenance. Our results are consistent with prior studies that demonstrate nitrate repression of fumarate reductase synthesis while activating the synthesis of nitrate reductase (48, 92, 201, 202). Once nitrate levels have diminished, then synthesis of fumarate respiratory enzymes is no longer repressed, which would lead to the observed result of the competition between fumarate and nitrate reductase mutants where fumarate reductase mutants display a colonization defect. To address the role of nitrate in mediating this competition result, we measured nitrate levels in mouse cecal mucus colonized simultaneously with Δ*narG narZ* (*napD-napA*)::*cat* and

frdA::*kan* mutants. Our findings agree with the colonization data in that nitrate levels decrease during colonization until nitrate is no longer detectable in the animals. Once nitrate reaches undetectable levels, the fumarate reductase mutant gets outcompeted, suggesting that fumarate is the more important electron acceptor in the maintenance stage of colonization. The data also suggest that nitrate is used before fumarate and corroborate our previous finding that it is essential for intestinal colonization. Also, we found that wild-type *E. coli* uses the available nitrate in the intestine, while the triple nitrate reductase mutant is unable to. Since the nitrate level remains high if a wild-type *E. coli* strain is not present, this suggests that *E. coli* is the primary microbe that respires nitrate in the intestine.

Our results show that oxygen respiration is needed for both commensal and pathogenic *E. coli* to compete in the intestine. We have found cytochrome *bd* oxidase to provide a competitive advantage during competition with other oxidoreductase mutants. Furthermore, nitrate reductase was found to be more important during competiton with fumarate reductase up until *E. coli* respires the available nitrate, thereby removing nitrate from the mucus. Thus, we conclude that the hierarchy of terminal electron acceptors used by *E. coli in vivo* agrees with the *in vitro* hierarchy. Indeed, our previous observation that the entire population of *E. coli* in the intestine depends on both oxygen and nitrate support our finding that this hierarchy holds in the intestine. Therefore, success of *E. coli* in the lower gastrointestinal tract demands respiratory flexibility and use of the best available electron acceptor, in accordance

with the strict regulation imposed by oxygen and nitrate. Together, these experiments suggest a hierarchy of electron acceptor utilization in the intestine identical for both *E. coli* O157:H7 and K-12.

Chapter 4

Electron Flow through the Quinone Pool of *Escherichia coli* in the Mouse Intestine

4.1 Introduction

Quinones are lipid-soluble molecules that mediate the transfer of electrons between the protein components of the electron transport chain, ultimately transporting electrons to the appropriate terminal oxidoreductases. *E. coli* synthesizes three types of quinones: a benzoquinone, ubiquinone, and two napthoquinones, menaquinone and demethylmenaquinone (89, 170). Ubiquinone is presumed to be primarily involved in aerobic respiration while menaquinone is thought mainly to be involved in nitrate and anaerobic respiration (89, 146, 270, 295). A third quinone produced by *E. coli*, demethylmenaquinone, is considered to be involved in anaerobic respiration using terminal electron acceptors other than nitrate (89, 170, 270). However, previous studies have shown all three quinones to be present during both aerobic and anaerobic respiration, where only the relative concentrations of the quinones changes under varying growth conditions (89, 170). Given that the exact role of the individual quinones used during *E. coli* respiration remains unknown, our experiments aimed to identify the quinones needed for *E. coli* to colonize the gastrointestinal tract.

We have previously shown that respiration of oxygen is essential for intestinal colonization of the murine gastrointestinal tract. Additionally, respiration of nitrate and fumarate were found to be important for colonization during maintenance. We also found that the hierarchy of electron acceptors used *in vivo* agrees with the established *in vitro* hierarchy, where oxygen is most favored, followed by nitrate and fumarate. To examine the contribution of other electron transport chain components for colonization and to corroborate our previous finding that both aerobic and anaerobic respiration support colonization, we sought to identify the quinones used *in vivo*. Using the streptomycin-treated mouse model we performed fitness competitions between wild-type *E. coli* K-12 and O157:H7 strains and mutant strains deficient in biosynthesis of various components of the quinone pool. The experiments described in this study confirm the flow of electrons through the respiratory pathways used by commensal and pathogenic strains of *E. coli* to colonize the mouse intestine.

4.2 Materials and Methods

Bacterial Strains and Growth Conditions. The bacterial strains used in this study were derived from *E. coli* MG1655 Str^r (streptomycin-resistant), a K-12 strain (176), and *E. coli* EDL933 Str^r, the prototypical O157:H7 (184) strain. Cultures were grown at 37°C in Luria-Bertani (LB), with gyratory shaking at 250 rpm. The bacterial strains and plasmids used in this study are listed in Table A.4.1.

Mutant Constructions. Null alleles were constructed by using the allelic replacement method of Datsenko and Wanner (65), as described previously (39), such that target genes were deleted and replaced with kanamycin- or chloramphenicol-resistance cassettes (used as selectable markers in mouse colonization assays, as described below). The null allele strains are identified in the text by the genes that were deleted; single gene deletions began with the start codon and ended with the stop codon, and multiple gene deletions began with the start codon of the first gene deleted and ended with the stop codon of the last gene deleted. Mutant strains were verified by phenotype analysis and DNA sequencing.

Mouse Colonization Experiments. The streptomycin-treated mouse model has been used extensively to study colonization of the mouse large intestine by *E. coli* and *Salmonella enterica* serovar Typhimurium (35, 45, 110, 279). Briefly, three CD-1 male mice, six weeks of age, were given drinking water containing streptomycin sulfate (5 g/L) for 24 h to remove the existing resident facultative microflora, and then starved for food and water for 18 to 24 h. The mice were then fed approximately 10^5 colony-forming units (cfu) of both the wild-type and mutant strains in 1 ml of 20% sucrose. The wildtype strains were *E. coli* MG1655 Str^r Nal^r (nalidixic acid resistance) (176) and *E. coli* EDL933 Str^r Nal^r (184); Nal^r was used to distinguish the wildtype (reference strain) from the null allele mutants in fecal plate counts. After the bacterial suspension was ingested, food and streptomycin-water were restored and fecal plate counts were determined at 5 h, 24 h, and on every other day thereafter for

15 days. Fecal samples were homogenized and diluted in 1% tryptone broth and plated on MacConkey agar containing either streptomycin (100 μg/ml) and nalidixic acid (50 μg/ml) to count the wildtype or streptomycin and kanamycin (40 μg/ml) or chloramphenicol (30 μg/ml) to count the null allele mutants. Each colonization experiment was repeated, on separate occasions, and the plotted values (in figures) represent the average for 6 mice. The $\log_{10}$ mean number of CFU per gram of feces ± the standard error for each strain in the mice was calculated for each time point. The limit of detection in fecal plate counts was 10^2 cfu/g feces.

4.3 Results

Ubiquinone is not necessary for competitive colonization. The first step in the biosynthesis of ubiquinone is the formation of 4-hydroxybenzoate from chorismate (Fig. 4.1) (241). The conversion is mediated by chorismate pyruvate lyase and 4-hydroxybenzoate octaprenyltransferase encoded by the *ubiC* and the *ubiA* genes, respectively (154). Thus, *ubiCA* mutants make menaquinone and demethylmenaquinone, but not ubiquinone. To determine whether passage of electrons through ubiquinone (Q) is required for colonization, we competed Δ(*ubiC-ubiA*)::*cat* mutants with their respective wild-type parent strains in the streptomycin-treated mouse colonization model. *E. coli* EDL933 and MG1655 Δ(*ubiC-ubiA*)::*cat* were fed low at a level of 10^5 CFU/mouse. The mutants lacking ubiquinone were

Figure 4.1. Biosynthetic pathway for Q, DMK, and MK in *E. coli*. Number of arrows corresponds to number of steps not shown in biosynthetic pathway.

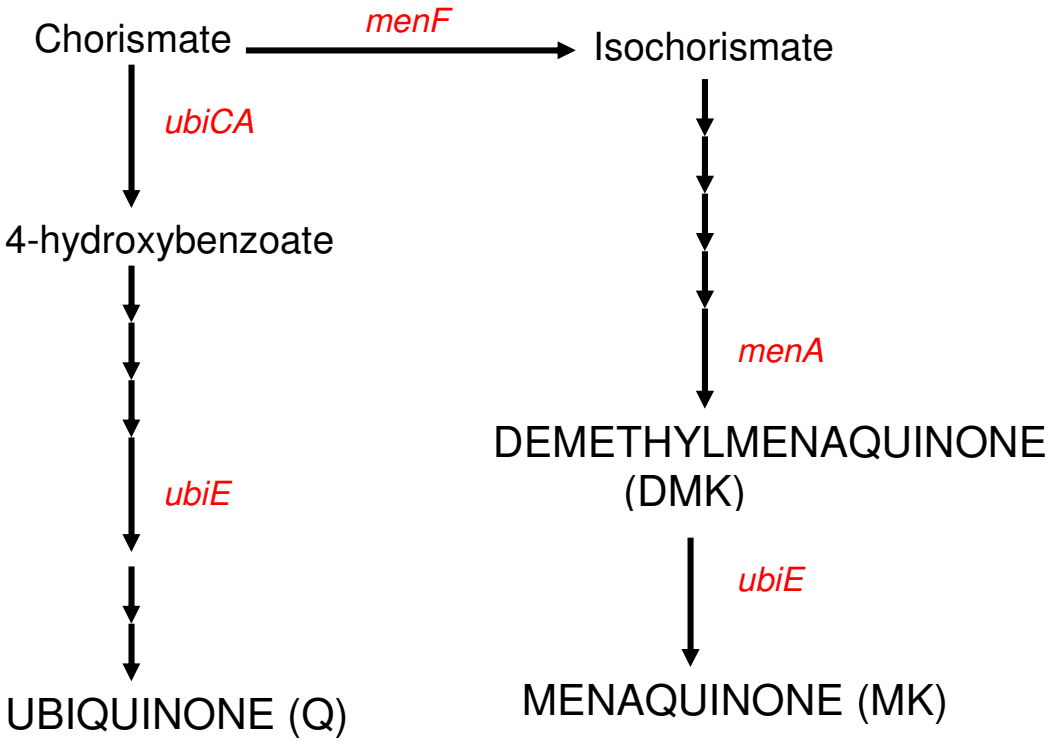


Figure 4.1. Simplified biosynthetic pathway showing mutational blocks in Q, DMK, and MK in *E. coli*.

found to co-colonize with wild-type strains (Table 4.1, Fig. 4.2, Fig. A.4.1). This implies that ubiquinone is not needed to support *E. coli* colonization of the intestine, because menaquinone and demethylmenaquinone are sufficient to carry electrons to the appropriate oxidoreductases.

Demethylmenaquinone alone is not sufficient for competitive colonization. *C*-methyltransferase, encoded by *ubiE*, is required for the biosynthesis of both ubiquinone and menaquinone (99, 153, 195). *ubiE* catalyzes the formation of ubiquinone from 4-hydroxybenzoate and menaquinone from demethylmenaquinone. Thus, *ubiE* mutants can make demethylmenaquinone, but not ubiquinone or menaquinone. Ubiquinone and menaquinone serve as the redox mediator in aerobic and nitrate respiration (280, 295). We tested Δ*ubiE*::*cat* mutants for their ability to compete with the wild-type strains. *E. coli* EDL933 and MG1655 Δ*ubiE*::*cat* were fed low at a level of 10^5 CFU/mouse. The mutants lacking *ubiE* were unable to initiate or maintain colonization when competed against wild-type strains (Table 4.1, Fig. 4.3A, Fig. A.4.2). The fitness defect observed for the Δ*ubiE*::*cat* strains was not merely a growth defect, since the mutants colonized when fed alone to mice (Fig. 4.3B). This result implies that demethylmenaquinone alone is not sufficient and that ubiquinone and menaquinone together are necessary for electron transport by *E. coli* during colonization of the intestine. Given that ubiquinone was found not to be necessary for efficient colonization, this implies that menaquinone is most important.

Table 4.1. Competitive colonization between quinone mutants and wild-type *E. coli* strains. [a]

		E. coli EDL933		*E. coli* MG1655	
Quinone Enzyme	**Mutant**	**Day 1**	**Day 9**	**Day 1**	**Day 9**
Ubiquinone biosynthesis	Δ(*ubiC-ubiA*)	0.2 ± 0.1	0.7 ± 0.1	0.5 ± 0.3	0.4 ± 0.2
Ubiquinone and menaquinone biosynthesis	Δ*ubiE*	**3.3 ± 0.1**	**5.8 ± 0.1**	**4.0 ± 0.1**	**5.7 ± 0.2**
Demethylmenaquinone biosynthesis	Δ*menA*	0.8 ± 0.2	**5.4 ± 0.2**	0.4 ± 0.2	**5.7 ± 0.1**
Demethylmenaquinone and RNase E inhibitor	Δ(*menA-menG)*	0.5 ± 0.1	**5.4 ± 0.2**	0.1 ± 0.1	**5.9 ± 0.1**
RNase E inhibitor (*rraA*)	Δ*menG*	0.7 ± 0.2	0.3 ± 0.1	0.6 ± 0.1	0.4 ± 0.2

[a] Mice were fed 10^5 CFU each of a mutant and its wild-type parent. Mice were transferred to fresh cages every day and feces no older than 24h were assayed every other day for 15 days. At each time point, for each mouse the Log_{10} CFU/gram of feces for the mutant was subtracted from the Log_{10} CFU/gram of feces for the wild-type. The average ± the standard error of the mean of Day 1 and Day 9 data from 6 mice are shown. Differences of at least one order of magnitude (10-fold) are in bold type.

Figure 4.2. Ubiquinone mutant co-colonizes with wild-type during competitive colonization assays. *E. coli* EDL933 Δ(*ubiC-ubiA*) mutants are able to compete with wild-type *E. coli* EDL933.

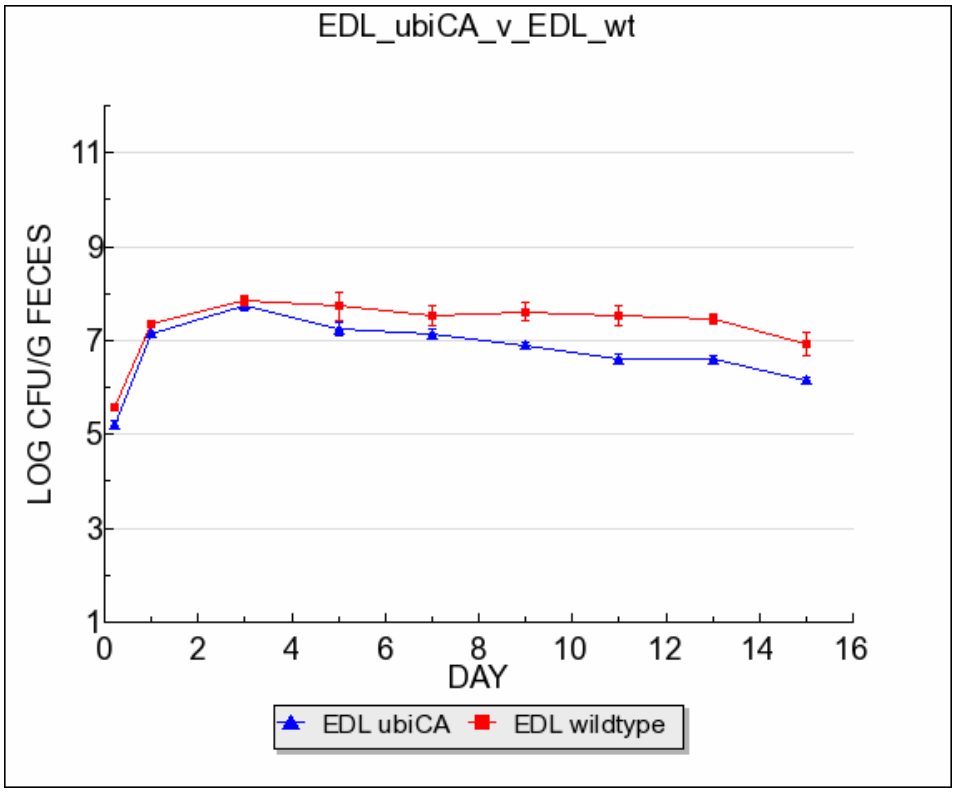


Figure 4.2. Mutant defective in ubiquinone synthesis of *E. coli* EDL933 co-colonize with wild-type *E. coli* EDL933.

Figure 4.3. Ubiquinone and menaquinone mutants exhibit colonization defects in competitive colonization assays. (A) *E. coli* EDL933 Δ*ubiE* mutants are unable to compete during initiation and maintenance with wild-type *E. coli* EDL933. (B) *E. coli* EDL933 Δ*ubiE* mutants are able to colonize when fed alone to mice.

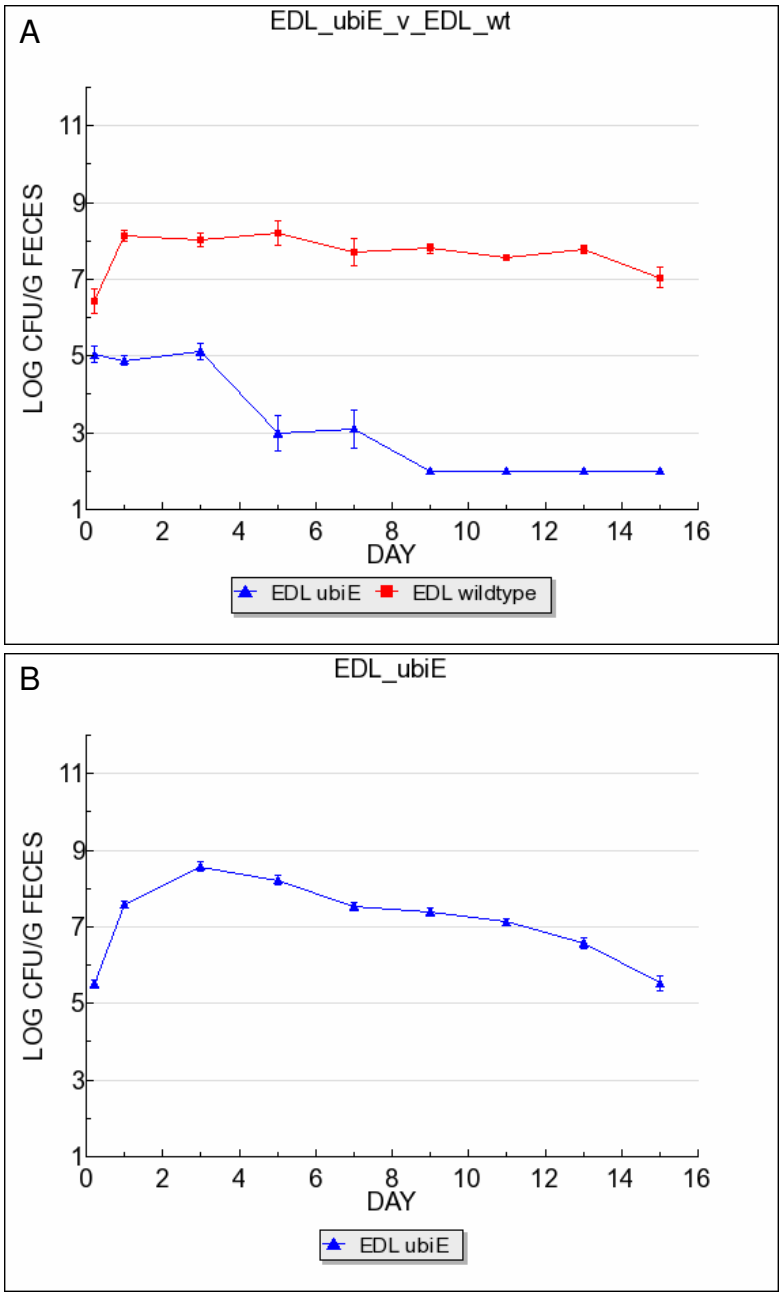


Figure 4.3. Mutants defective in ubiquinone and menaquinone synthesis of *E. coli* EDL933 exhibit colonization defects.

Menaquinone and demethylmenquinone are necessary for colonization. Biosynthesis of demethylmenaquinone from 2-succinylbenzoate involves the enzyme 4-dihydroxy-2-naphthoate octaprenyltransferase encoded by *menA*. Since demethylmenaquinone is a precursor of menquinone, deletion of the *menA* gene not only prevents DMK biosynthesis, the major electron carrier during anaerobic respiration using terminal electron acceptors other that oxygen and nitrate, but also prevents synthesis of MK, a major electron carrier during anaerobic respiration of nitrate (274). Thus, to determine the role of anaerobic electron transport pathways during colonization, we competed Δ*menA* mutants with wild-type strains in the mouse. *E. coli* EDL933 and MG1655 Δ*menA*::*cat* were fed low at a level of 10^5 CFU/mouse. The *menA* mutant was able to initiate, but unable to maintain colonization when competed against the wild-type parents (Table 4.1, Fig. 4.4, Fig. A.4.3). Since DMK primarily shuttles electrons to alternative anaerobic electron acceptors such as fumarate, DMSO, and TMAO, and since MK shuttles electrons primarily to nitrate, our results suggest that nitrate is necessary for competition in the mouse intestine during maintenance. These results confirm the importance of MK in intestinal colonization, where DMK plays a minor role to support competitiveness *in vivo* since the ability to initiate colonization is the major difference between the Δ*menA* mutant and the Δ*ubiE* mutant. Furthermore, Q may also be involved in initiation since Q is present in the Δ*menA* strain, but not the Δ*ubiE* strain.

Figure 4.4. Demethylmenaquinone mutants exhibit colonization defects in competitive colonization assays. (A) *E. coli* EDL933 Δ*menA* mutants are unable to compete with wild-type *E. coli* EDL933 during colonization of the mouse intestine. (B) *E. coli* EDL933 Δ*menA* mutants are able to colonize when fed alone to mice.

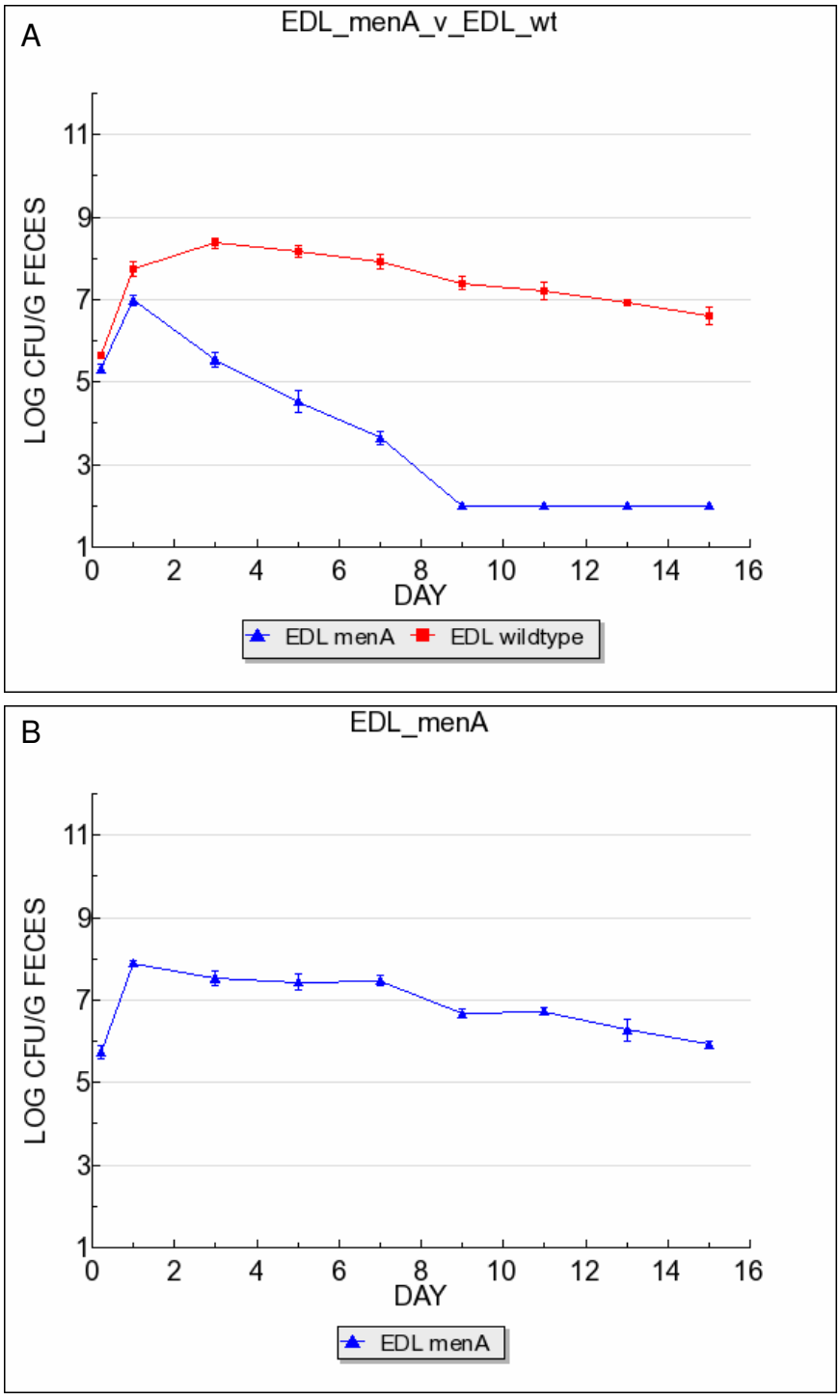


Figure 4.4. Mutant defective in demethylmenaquinone synthesis of *E. coli* EDL933 exhibits colonization defect.

***menAG* is necessary for competitive colonization.** We originally made a Δ(*menA*-*menG*) mutant, not knowing that *menG* is not involved in menaquinone biosynthesis. To verify the role of demethylmenaquinone and menaquinone *in vivo*, Δ(*menA*-*menG*) and Δ*menG* mutants were constructed and tested for their relative colonization fitness in the mouse intestine. *menG* was once thought to be involved in the biosynthesis of menaquinone, however, it has been shown more recently to encode for a ribonuclease E (RNase E) inhibitor protein (188). To test the possibility of involvement in quinone biosynthesis, *E. coli* EDL933 and MG1655 Δ(*menA*-*menG*)::*cat* were fed low at a level of 10^5 CFU/mouse. The mutant lacking DMK and MK was able to initiate, however unable to maintain colonization during competition with the wild-type strains (Table 4.1, Fig. 4.5, Fig. A.4.4). These results are consistent with our observation that DMK and MK are necessary for maintenance during *E. coli* colonization of the mouse intestine. In a similar experiment, *E. coli* EDL933 and MG1655 Δ*menG*::*cat* were fed low at a level of 10^5 CFU/mouse. The Δ*menG* mutants were found to co-colonize during competition with the wild-type strains. These results confirm prior findings that *menG* does not encode for menaquinone. Since Δ*menAG* mutants behave like Δ*menA*, and Δ*menG* has no colonization defect, these results confirm the Δ*menA* results.

4.4 Discussion

Figure 4.5. (A) *E. coli* EDL933 Δ(*menA*-*menG*)::*cat* mutant is unable to compete during colonization with EDL933 and wild-type strains. (B) *E. coli* EDL933 and Δ*menG*::*cat* mutants are able to compete during colonization with EDL933 and wild-type strains.

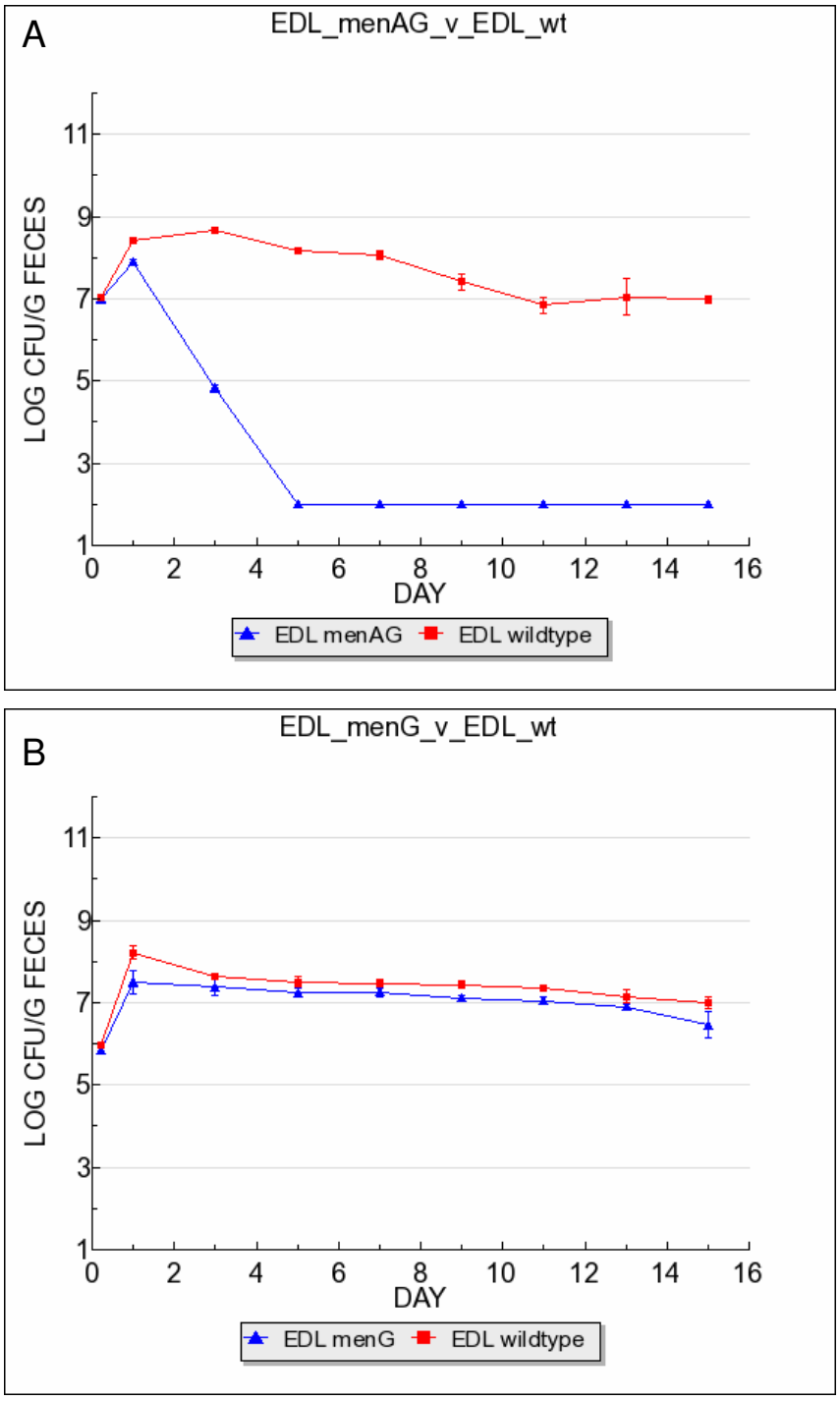


Figure 4.5. Mutant defective in demethylmenaquinone and menaquinone biosynthesis and not ribosome synthesis of *E. coli* EDL933 exhibits a colonization defect.

We previously found that respiration of low oxygen and nitrate is important for colonization based on mutational analysis of terminal oxidoreductases. We showed that colonization of the mouse intestine by *E. coli* depends upon its ability to use oxygen, nitrate, and fumarate as terminal electron acceptors (138). The mammalian intestine is presumed to be anaerobic due to the presence of a large number of anaerobes (4); however, there is a lack of evidence to support this theory. We have previously shown cytochrome *bd* to be important for colonization of the GI tract (138). This suggests that the intestinal environment is not strictly anaerobic, and prior studies have found oxygen to be present in the intestinal tract (113). Moreover, we have established that the known hierarchy of electron acceptor utilization, i.e. O_2 > nitrate > fumarate, which was established by investigation of *in vitro* grown cultures is also true *in vivo*. However, it was also clear that both aerobic and anaerobic processes contribute to successful colonization, most likely in response to intermittent availability of oxygen. To verify these results, we turned to a corroborative investigation of the quinone pool. The results presented in this study indicate that MK is the most important for intestinal colonization, followed by Q, since a Δ*ubiE* mutant, which eliminated both Q and MK, is unable to initiate and maintain colonization. DMK alone does not appear to contribute much to colonization, as the Δ*menA* mutant, which eliminated MK and DMK, is able to initiate but unable to maintain colonization.

The quinone pool in *E. coli* consists of three components, including ubiquinone,

menaquinone, and demethylmenaquinone. These quinones serve as the electron carriers between the appropriate dehydrogenases and oxidoreductases during both aerobic and anaerobic respiration. Q is primarily used under aerobic conditions, while MK is used mainly under microaerobic and anaerobic conditions where low oxygen and nitrate are available as electron acceptors (280, 289, 294). Alternatively, DMK serves as the major electron carrier during anaerobic respiration of alternative electron acceptors such as fumarate (294, 295). It has been observed that aerated cells contain about four or five times more Q than MK and DMK, whereas anaerobic cells contain about one-third as much Q as MK and DMK (170, 250). Biochemically, the quinones are used interchangeably to various extents to carry electrons to oxygen and alternative electron acceptors, as will be discussed in more detail below.

In order to verify our previous finding that aerobic respiration offers a competitive advantage during initiation of intestinal colonization, while anaerobic respiration is more important for maintenance, we conducted a systematic mutational analysis of quinone biosynthetic genes. Biosynthetic mutants lacking one or more quinones were tested for their ability to colonize the streptomycin-treated mouse model. Although it was not possible to construct strains to distinguish MK from DMK because the two mutations that prevented MK synthesis also blocked Q or DMK, we were able to separate electron flow through some of the quinones. Thus, the possible combinations eliminated Q and MK or DMK and MK. A Δ(*ubiC-ubiA*) mutation lacks Q, but contains MK and DMK, a Δ*ubiE* mutation lacks Q and MK, but contains

DMK, and a Δ*menA* mutation lacks MK and DMK, but contains Q (Table 4.2 and Fig. 4.1). These mutations ultimately allowed us to consider electron flow for the Δ(*ubiC-ubiA*) mutation, which is thought to be important under high oxygen tension or aerobic conditions, the Δ*ubiE* mutation important for microaerobic and anaerobic conditions using nitrate as a terminal electron acceptor, and the Δ*menA* mutation important for anaerobic respiration of alternative electron acceptors such as nitrate and fumarate. The selectivity of quinones for different terminal oxidoreductases can be explained by their binding site affinity. Binding affinities, demonstrated by K_m values, have been previously shown for the following: cytochrome *bo₃* for Q (0.048 mM), cytochrome *bd* for Q (0.23 mM), cytochrome *bd* for MK (1.67 mM), primary cytoplasmic nitrate reductase for Q (0.746 mM), primary cytoplasmic nitrate reductase for MK (0.916 mM), and fumarate reductase for MK/DMK (0.155 mM) (4, 33, 34, 96, 111, 173, 174, 225, 230, 255, 256, 304). The binding affinities, determined from steady state kinetic studies, indicate that cytochrome *bo₃* has the highest affinity for Q of all the oxidoreductases, cytochrome *bd* has a higher affinity for Q than MK, and nitrate reductase has similar affinities for both Q and MK. However, prior reports suggest that the cytochrome *bd* oxidase primarily uses MK for electron transfer (111). The enzymology of the anaerobic reductases has only been characterized using analogs of the reducing substrate for MK, since MK and DMK are very similar structurally and overlap in many instances (225). Thus, the reported affinities for MK can be extended to include DMK. Since all three quinones are able to carry electrons under different respiratory conditions, we can predict the preferred

Table 4.2. Presence or absence of quinones in quinone biosynthetic mutants of *E. coli*. N (No) indicates absence of quinones in mutant strains and Y (Yes) indicates presence of quinones in mutant strains.

	Quinones Present		
Mutant	**Q**	**MK**	**DMK**
Δ(*ubiC-ubiA*)	N	Y	Y
Δ*ubiE*	N	N	Y
Δ*menA*	Y	N	N
Δ(*menA-menG)*	Y	N	N
Δ*menG*	Y	Y	Y

use of specific quinones under aerobic and anaerobic conditions.

The pathway used for biosynthesis of all three quinones is shown in Figure 4.1. Although Q is used under conditions of high oxygen tension and has been found to be present at a higher concentration than both MK and DMK during aerobic respiration (89, 170), it has not been proven to be the sole quinone used when oxygen is available as a terminal electron acceptor. We found that a Δ(*ubiC-ubiA*) mutation, which blocks Q biosynthesis only, does not affect colonization of the streptomycin-treated mouse. Additionally, *ubiE*, involved in biosynthesis of both Q and MK (153), was found to have a major colonization defect during competition with the wild-type in both initiation and maintenance. This result suggests that Q is not the only quinone utilized by cytochrome *bd*, and that MK also serves as an electron carrier. In addition, the presence of MK and DMK allows the Δ(*ubiC-ubiA*) mutant to colonize and compete in the mouse intestine, suggesting that both MK and DMK can shuttle electrons during aerobic respiration *in vivo*. However, DMK alone is unable to serve as an electron carrier during intestinal colonization as seen in the Δ*ubiE* mutant. In a previous study, we found that the high affinity cytochrome bo_3 oxidase is not important for colonization. The finding that Q alone is not necessary for colonization supports our hypothesis that oxygen tension in the intestine is low because a Δ*ubiE* mutant, lacking both Q and MK, was unable to initiate or maintain colonization. This suggests that both Q and MK shuttle electrons to cytochrome *bd* oxidase and nitrate reductase during colonization and are together important for intestinal colonization.

Prior studies have led to the conclusion that Q is required when using oxygen as the electron acceptor, although MK has been demonstrated to be used when nitrate and other anaerobic electron acceptors are present (89, 170, 256). In addition, DMK has been shown to be present at a higher concentration during anaerobic respiration using fumarate, DMSO, or TMAO as an electron acceptor (89, 170). Our results show that a Δ*menA* mutant, lacking MK and DMK, has a major colonization defect during maintenance. This suggests that MK is necessary for intestinal colonization since a Δ*ubiE* mutant, which lacks Q and MK but contains DMK, was unable to initiate and maintain colonization. MK ultimately appears to be suited for carrying electrons to a mixture of electron acceptors, such as oxygen and nitrate. Thus, our findings are in accordance with our previous observations that aerobic respiration is essential for initiation and maintenance, while anaerobic respiration is essential for maintenance.

The specific quinones that couple NADH oxidation to aerobic, microaerobic, and anaerobic electron acceptors *in vivo* requires further investigation so that electron flow through the quinone pool can be better understood. In this study, we sought to further our understanding of the respiratory metabolism of *E. coli* in the intestine by using biosynthesis quinone mutants to eliminate electron transfer pathways to verify our previous terminal oxidoreductase mutant results. We have confirmed what we have previously learned about the relative importance of terminal oxidoreductases,

from what we have discovered here about the contribution of electron flow through the quinone pool. Here we show that the quinones used by both a pathogenic and commensal strain during colonization of the mouse intestine are nearly identical, and further suggest that the bioenergetics between pathogenic and commensal *E. coli* in the intestine are very similar. These studies confirm our previous finding regarding the respiratory pathways important for *E. coli in vivo*. Indeed, our results presented in this study support our conclusions that *E. coli* respires both aerobically and anaerobically during colonization of the mouse intestine, specifically that aerobic respiration is important during both initiation and maintenance, whereas anaerobic respiration is important only during maintenance.

Chapter 5

Maltose and Glycogen utilization of *Escherichia coli* O157:H7 in the Mouse Intestine

5.1 Introduction

Escherichia coli demonstrates remarkable flexibility in the metabolism of different sugars as carbon and energy sources (39). In general, bacterial existence in nature is thought to be one of feast and famine since the nutrients that support bacterial growth are rarely available continuously and almost always present in limiting amounts (146). It has been suggested that *E. coli* lives a feast and famine type of existence in the mammalian gastrointestinal tract (49). A feast and famine existence in the intestine is supported by the fact that *E. coli* grows and resides in the mucus layer, which despite being rich in carbohydrates and glycoproteins (157), *E. coli* leads a scavenging lifestyle using as many as seven different sugars to support its colonization (39). Apparently, nutrient availability in the intestine is not constant and more than likely varies where growth limiting nutrients are limited and variable. Thus, times between periods of abundant and limiting nutrients may require that intracellular energy stores be used by *E. coli* to successfully compete and persist in the mouse intestine. Glycogen is the primary carbon and energy storage molecule for many bacteria and since glycogen breakdown involves maltose and maltodextrins as intermediates, we thought to examine whether catabolism of exogenous maltose and

maltodextrins as well as glycogen support intestinal colonization. The importance of maltose utilization and glycogen as a carbon storage molecule, its synthesis and degradation, has not been tested *in vivo*.

Maltose consists of two glucose monosaccharides joined in an α-1-4 linkage, and maltodextrins are longer polysaccharides with the same structure. These sugars are most commonly derived from starch and glycogen. *E. coli* can grow on maltose and maltodextrins by taking up the sugars and cleaving them to release glucose and glucose 1-phosphate. At least nine genes encode proteins that are involved in the utilization of maltose and maltodextrins in *E. coli* (175). MalT is the maltose responsive transcription activator of the maltose system (175). The maltose inducible porin for maltodextrin transport is encoded by *lamB* and has a larger pore size than those of the porins normally present in the outer membrane (297). The periplasmic maltose-binding protein that facilitates maltose transport is encoded by *malE* (103-105). The transmembrane pore encoded by *malF*, *malG*, and *malK* constitute the active transport system for maltose and are located in the inner membrane (103-105). *malP* (maltodextrin phosphorylase) and *malQ* (amylmaltase) encode enzymes for maltodextrin and maltose metabolism, respectively.

Amylomaltase (MalQ) cleaves glucosyl and dextrinyl residues from the nonreducing end of incoming or exogenous maltodextrins and transfers them again onto glucose and maltodextrins. In this way, starting with any maltodextrin including

maltose, a mixture of maltodextrins and glucose is obtained. Amylomaltase is a strict transferase where the total number of glycosidic linkages remains constant during the cleavage and transfer reaction. Glucose released by amylomaltase can then enter glycolysis after being phosphorylated by glucokinase. The accumulation of maltodextrins, however, is prevented by the action of maltodextrin phosphorylase encoded by *malP*. This enzyme phosphorylytically cleaves glucosyl residues from the nonreducing end of maltodextrins forming α-glucose 1-phosphate, with maltotetraose being the smallest substrate. α-Glucose 1-phosphate then enters glycolysis after having been transformed to glucose 6-phosphate by phosphoglucomutase. Thus, the combined action of amylomaltase and maltodextrin phosphorylase yields glucose and glucose 1-phosphate from maltodextrin.

In addition to metabolizing carbohydrates for energy production, *E. coli* can also store carbon in the form of glycogen. Glycogen is a polysaccharide consisting of branched polymers that is primarily produced under growth conditions when nutrients become limiting (7, 197). Glycogen is the fundamental energy storage compound for most organisms, including bacteria. However, the importance of glycogen in the form of energy storage for *E. coli in vivo* has never been explored. Glycogen metabolism is encoded by *glgA* encoding for glycogen synthase, *glgS* a predicted glycogen synthesis protein, and *glgP* encoding for glycogen degradation (62, 211, 212). Glycogen is synthesized during times when carbon is abundant but low availability of other nutrients limits growth. GlgA, glycogen synthase, is responsible

for the transfer of a glucosyl unit of ADP-glucose to maltodextrin or glycogen primer to form a new α-1,4-glucosidic linkage (153). GlgS, on the other hand, has no defined role in glycogen synthesis except that it is RpoS dependent and its overproduction stimulates glycogen synthesis (114). Glycogen degradation occurs when carbon sources become limiting, however, the role of glycogen phosphorylase is not well understood (3). The degradation of glycogen is stimulated during periods of substrate deprivation. GlgP, glycogen phosphorylase, catalyzes glycogen breakdown and affects glycogen structure by removing glucose units from the polysaccharide outer chain to yield glycogen containing short chains that consist of four glucosyl units. In support of the hypothesis that glycogen metabolism is important for colonization, we previously found that one of the genes involved in glycogen metabolism, *glgS*, is highly induced in *E. coli* K-12, strain MG1655, when growing on mucus (39).

We have previously identified the carbon sources utilized by enterohemorrhagic *E. coli* (EHEC) and commensal *E. coli* K-12 during colonization of the mouse intestine and found *E. coli* to use several monosaccharides during intestinal colonization (39). Sugar usage by *E. coli* in the mouse intestine has been studied extensively in our laboratory. Genes involved in maltose metabolism were previously shown to be induced in *E. coli* K-12 when growing on intestinal mucus, conditions that are thought to mimic nutrient availability in the intestine (39). Since glycogen breakdown involves maltose and maltodextrins as intermediates, we thought to

examine whether catabolism of exogenous maltose and maltodextrins as carbon sources supports colonization.

In this study, we sought to investigate the role of maltose utilization, glycogen storage, and glycogen degradation *in vivo*. Here we explore the possible role of a feast and famine lifestyle for *E. coli* in the mouse intestine where maltose utilization and the contribution and role of endogenous energy sources for survival inside the host was examined. Our results demonstrate that both maltose and glycogen are needed for intestinal colonization. Furthermore, glycogen synthesis and glycogen degradation appear to both be important during colonization. This suggests that endogenous carbon stores might ensure survival during brief periods of carbon limitation and success inside the host. *E. coli* may experience intermittent nutrient starvation when switching carbon sources during infection of animal hosts. Thus, *E. coli* has more than likely adapted its nutritional preferences *in vivo* to nutrient availability in the host, where carbon storage facilitates its ability to switch to available nutrients found in the intestine. Investigating the use of complex sugars and energy storage for colonization will ultimately increase our understanding of nutrient utilization *in vivo* and provide new insight into host-pathogen interactions.

5.2 Materials and Methods

Bacterial Strains and Growth Conditions. The bacterial strains used in this study were derived from *E. coli* MG1655 Str^r (streptomycin-resistant), a K-12 strain (176), and *E. coli* EDL933 Str^r, a streptomycin resistant derivative of the prototypical O157:H7 strain (184). Cultures were grown at 37°C in Luria-Bertani (LB), with gyratory shaking at 250 rpm. The bacterial strains and plasmids used in this study are listed in Table A.5.1.

Mutant Constructions. Null alleles were constructed by using the allelic replacement method of Datsenko and Wanner (65), as described previously (39), such that target genes were deleted and replaced with kanamycin- or chloramphenicol-resistance cassettes (used as selectable markers in mouse colonization assays, as described below). The null allele strains are identified in the text by the genes that were deleted; single gene deletions began with the start codon and ended with the stop codon, and multiple gene deletions began with the start codon of the first gene deleted and ended with the stop codon of the last gene deleted. Strains containing multiple mutations were constructed by sequential allelic replacement; the first inserted cassette was removed with FLP recombinase (65), followed by subsequent allelic replacement(s) and removal of the insertion as necessary, leaving the selected marker in the last mutation made. Mutant strains were verified by phenotype analysis and DNA sequencing.

Phenotypic analysis. MOPS (3-(*N*-morpholino) propanesulfonic acid) defined medium was used to grow cultures for growth curves, as described previously (39). To test for the ability to use maltose, mutant strains were grown overnight in MOPS medium with maltose (0.2%) supplied as the carbon source. Cell growth was monitored spectrophotometrically at 600 nm (OD_{600}). Iodine stains were conducted to test for the ability of mutant strains to synthesize glycogen. Strains were grown overnight on MOPS medium with 50 mM glucose and flooded with 4 % iodine. A yellow color reaction was the negative determinant for glycogen production, where as a brown color reaction was the positive determinant for glycogen production.

Microarray experiments and statistics. Total RNA from MG1655 Str^r Nal^r (nalidixic acid-resistant) or EDL933 Str^r Nal^r was extracted where glucose (0.2% wt/vol) was the sole carbon source. Culture samples were diluted into DNA-RNA Protect (Sierra Diagnostics) to inhibit RNA degradation and the RNA was purified using RNeasy minikits with the optional DNase treatment (Qiagen). The RNA was converted to cDNA using Superscript II (Invitrogen) and random hexamers, according to the manufacturer's specifications. The resulting cDNA was fragmented and biotinylated (Enzo Kit, Roche Diagnostics), according to the Affymetrix prokaryotic labeling protocol. Biotinylated samples were hybridized to Affymetrix GeneChip custom microarrays for 16 hours at 60°C. The custom GeneChips contained probe sets for all genes on the *E. coli* MG1655 and *E. coli* EDL933 genomes. Hybridized arrays were stained using the Affymetrix protocol

(ProkGE_WS2v2_450). Biological replicates of cultures were used for all microarrays. After hybridization, microarrays were scanned and .cel files were further analyzed. All biological replicates were averaged after RMA processing (quartile normalization). Microarrays of EDL933 Str^r Nal^r grown on glucose were used as the experimental condition and MG1655 Str^r Nal^r grown on glucose were used as the control condition. Significantly induced genes were binned and identified as being greater than 2 standard deviations above the rest of the transcriptome.

Mouse Colonization Experiments. The streptomycin-treated mouse model has been used extensively to study colonization of the mouse large intestine by *E. coli* and *Salmonella enterica* serovar Typhimurium (35, 45, 110, 279). Briefly, three CD-1 male mice, six weeks of age, were given drinking water containing streptomycin sulfate (5 g/L) for 24 h to remove the existing resident facultative microflora, and then starved for food and water for 18 to 24 h. The mice were then fed approximately 10^5 colony-forming units (cfu) of both the wild-type and mutant strains in 1 ml of 20% sucrose. The wildtype strains were *E. coli* MG1655 Str^r Nal^r (nalidixic acid resistance) (176) and *E. coli* EDL933 Str^r Nal^r (184); Nal^r was used to distinguish the wildtype (reference strain) from the null allele mutants in fecal plate counts. After the bacterial suspension was ingested, food and streptomycin-water were restored and fecal plate counts were determined at 5 h, 24 h, and on every other day thereafter for 15 days. Fecal samples were homogenized and diluted in 1% tryptone broth and plated on MacConkey agar containing either streptomycin (100 μg/ml) and nalidixic

acid (50 μg/ml) to count the wildtype or streptomycin and kanamycin (40 μg/ml) or chloramphenicol (30 μg/ml) to count the null allele mutants. Each colonization experiment was repeated, on separate occasions, and the plotted values (in figures) represent the average for 6 mice. The $\log_{10}$ mean number of CFU per gram of feces ± the standard error for each strain in the mice was calculated for each time point. The limit of detection in fecal plate counts was 10^2 cfu/g feces.

5.3 Results

Comparative transcriptomics of *E. coli* EDL933 to *E. coli* MG1655 grown in glucose. To colonize the intestine, *E. coli* EDL933 grows from low to high numbers in the presence of *E. coli* MG1655 (184). The basis of this competition is thought to be nutritional. Our previous results demonstrated that there are significant nutritional differences between *E. coli* EDL933 and *E. coli* MG1655 during colonization of the mouse intestine (despite having nearly identical nutrient preferences *in vitro*) (Fabich *Submitted* 2007). While initial transcriptomics comparisons between *E. coli* EDL933 and *E. coli* MG1655 when grown on mucus compared to glucose revealed minimal differences reflecting nutrient availability in mucus, the differences that exist between these two strains must have some basis on a gene expression level. To address any differences that might exist between *E. coli* EDL933 and *E. coli* MG1655, we determined the gene expression profile for these strains during steady-state growth on glucose and made a direct comparison in gene expression. Custom Affymetrix

GeneChips show the genes involved in carbohydrate catabolism that were induced in glucose-grown *E. coli* EDL933 (Fig. 5.1). Genes involved in the transport and metabolism of maltose were found to be more upregulated in *E. coli* EDL933 than *E. coli* MG1655. Specifically, *malE* (1.119), *malK* (1.051), *malP* (0.781), and *malQ* (0.614) were all induced. Previously, maltose catabolism genes (*malPQ*) were also shown to be induced in *E. coli* MG1655 when grown on mucus (39). Together, these findings led us to consider the importance and involvement of maltose uptake and utilization during colonization of the mouse intestine.

Maltose transport is necessary for intestinal colonization. The main focus of this study was to investigate the role of an exogenous supply of a complex sugar, such as maltose, and its use and metabolism *in vivo*. We tested mutants unable to transport maltose for their ability to compete with the wild-type in the streptomycin-treated mouse colonization model. Mutants were constructed that deleted the periplasmic maltose ABC transport system *malE*, *malF*, and *malG* genes, the phenotype of which was described previously as able to grow on maltose (26, 27). *E. coli* EDL933 and MG1655 Δ(*malE-malG*)::*cat* were fed low at a level of 10^5 CFU/mouse. During competition with the respective wild-types, *E. coli* EDL933 Δ(*malE-malG*)::*cat* and *E. coli* MG1655 Δ(*malE-malG*)::*cat* were able to initiate at day 1 post-feeding but unable to maintain colonization (Table 5.1, Fig.5.2A, Fig. A.5.1A). A 4 log difference was observed during maintenance, suggesting that maltose transport is important for colonization. This observation led us to further explore the use of

Figure 5.1. Maltose genes expressed during steady state growth. Genes involved in carbohydrate catabolism that were induced in glucose-grown *E. coli* EDL933. Gene expression is shown as the log2 ratio of EDL933 compared to MG1655.

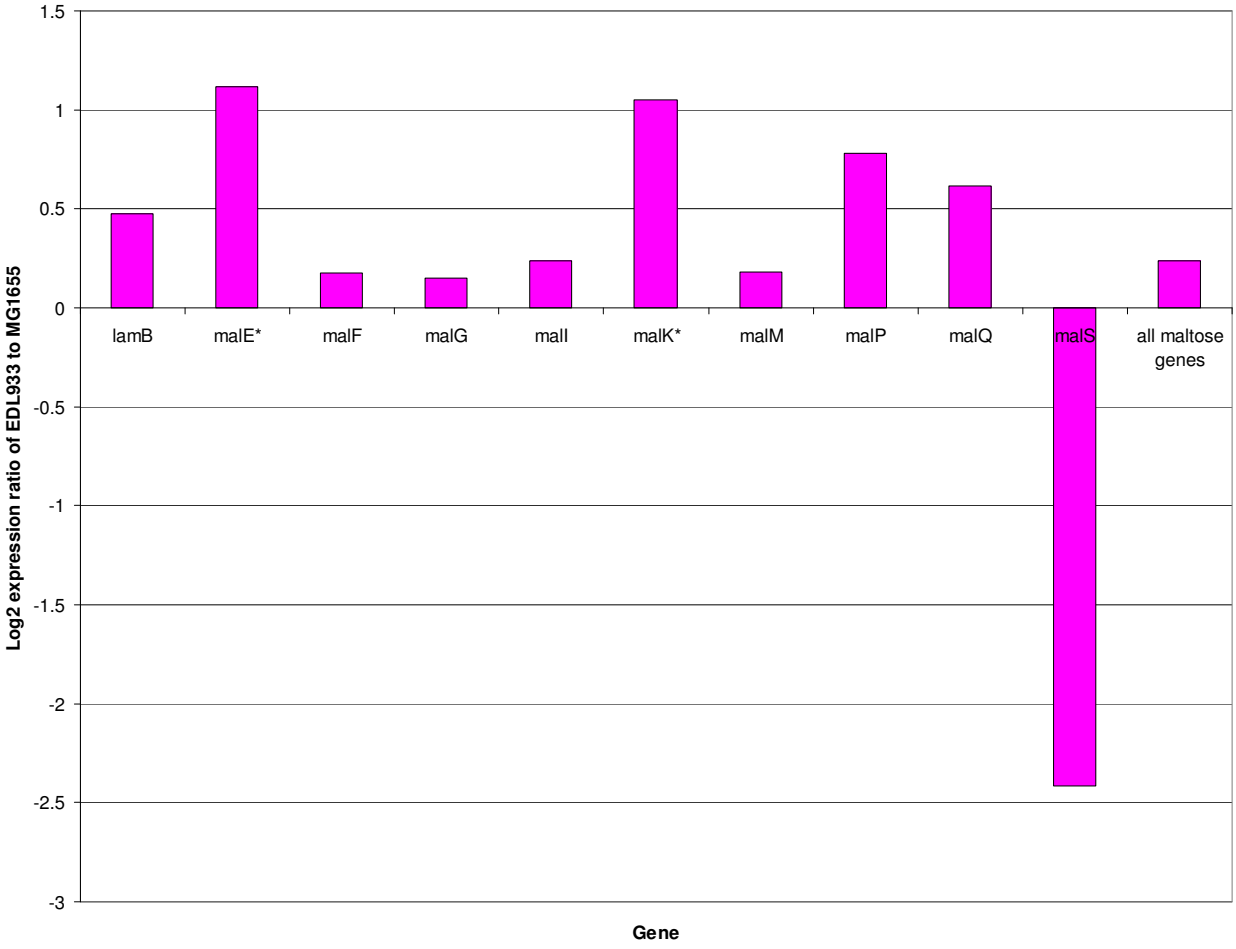


Figure 5.1. The log2 expression ratio of maltose genes in EDL933 compared to MG1655.

Table 5.1. Competitive colonization between maltose and glycogen mutants and wild-type *E. coli* strains. [a]

		E. coli EDL933		*E. coli* MG1655	
Enzyme	**Mutant**	**Day 1**	**Day 15**	**Day 1**	**Day 15**
Maltose specific transport	Δ(*malE-malG*)	0.4 ± 0.1	**3.3 ± 0.2**	0.0 ± 0.1	1.3 ± 0.3
Maltose transport PTS system	Δ(*malX*)	0.4 ± 0.1	0.2 ± 0.2	0.6 ± 0.2	0.6 ± 0.1
Maltose specific and PTS transport	Δ(*malE-malG*) Δ*malX*	0.2 ± 0.1	**2.3 ± 0.2**	0.6 ± 0.2	**2.5 ± 0.3**
Maltodextrin phosphorylase and amylomaltase	Δ(*malP-malQ*)	0.8 ± 0.1	**3.7 ± 0.1**	0.8 ± 0.3	**4.5 ± 0.3**
Maltodextrin phosphorylase	Δ*malP*	0.3 ± 0.1	1.4 ± 0.5	ND	ND
Amylomaltase	Δ*malQ*	0.3 ± 0.1	**3.3 ± 0.4**	ND	ND
Maltodextrin transport	Δ*lamB*	0.0 ± 0.2	0.0 ± 0.2	0.0 ± 0.2	0.1 ± 0.2
Glycogen phosphorylase	Δ*glgP*	0.3 ± 0.1	**2.4 ± 0.2**	0.5 ± 0.2	**3.2 ± 0.1**
Glycogen synthase	Δ*glgA*	0.2 ± 0.1	**2.3 ± 0.2**	0.2 ± 0.1	**2.9 ± 0.2**
Glycogen synthesis	Δ*glgS*	0.2 ± 0.1	**1.9 ± 0.1**	0.4 ± 0.2	**1.6 ± 0.2**

[a] Mice were fed 10^5 CFU each of a mutant and its wild-type parent. Mice were transferred to fresh cages every day and feces no older than 24h were assayed every other day for 15 days. At each time point, for each mouse the Log_{10} CFU/gram of feces for the mutant was subtracted from the Log_{10} CFU/gram of feces for the wild-type. The average ± the standard error of the mean of Day 1 and Day 9 data from 6 mice are shown. Differences of at least one order of magnitude (10-fold) are in bold type.

ND = Not Determined

Figure 5.2. Mutants in the ATP-dependent ABC transporter of the maltose system display colonization defects. (A) *E. coli* EDL933 Δ(*malE-malG)* mutants are unable to compete during colonization with EDL933 wild-type strains**.** However, the PTS system of maltose transport exhibits no phenotype in the intestine. (B) *E. coli* EDL933 Δ*malX* Δ(*malE-malG)* mutants are unable to compete during colonization with EDL933 wild-type strains as was seen in the single Δ(*malE-malG*) mutation.

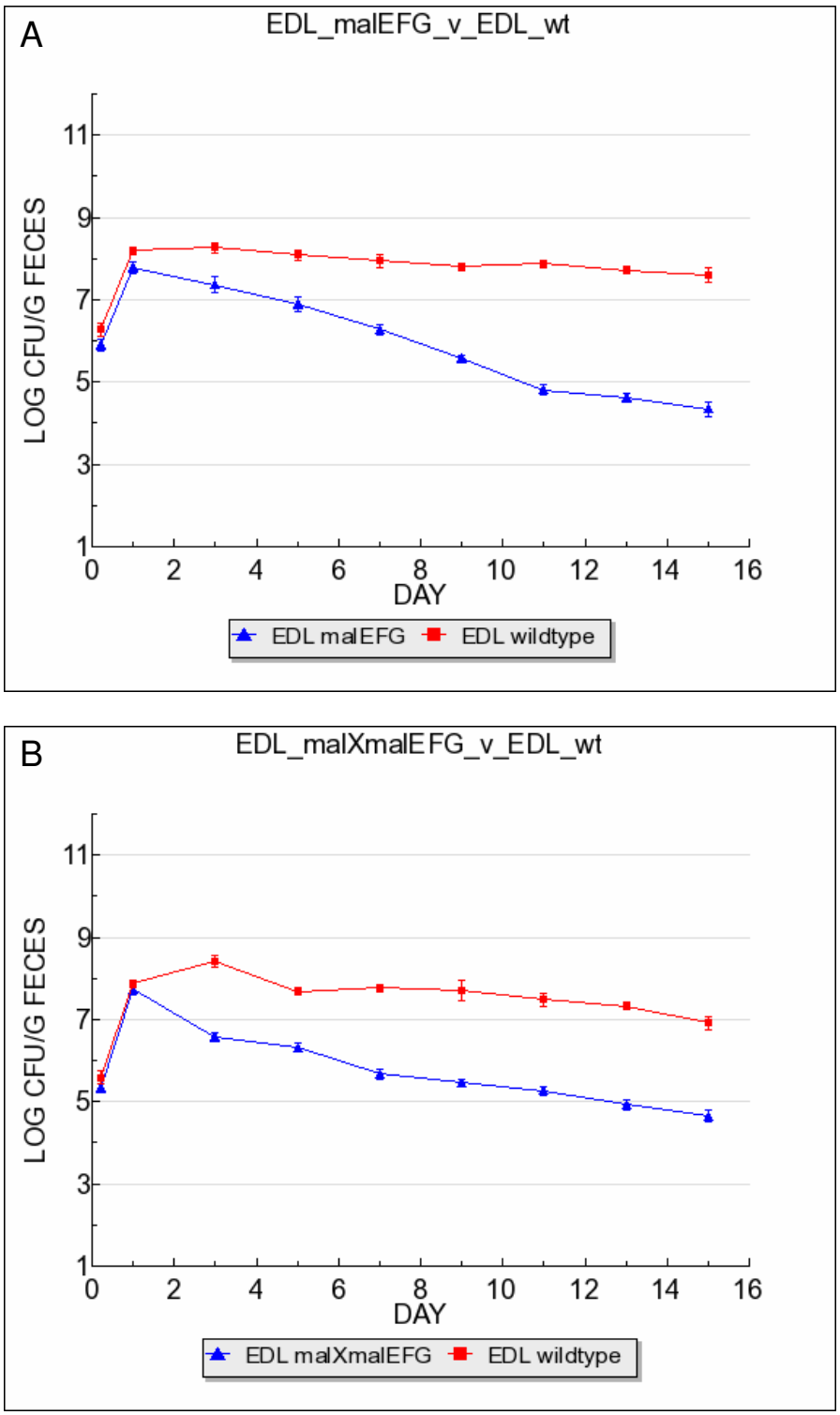


Figure 5.2. Maltose transport mutants of *E. coli* EDL933 exhibit colonization defects.

maltose *in vivo*.

Given that *E. coli* has multiple transporters for maltose, mutants were also constructed in the PTS system for maltose transport (*malX*). These mutant strains are able to grow on maltose, as previously shown (217). *E. coli* EDL933 and MG1655 Δ*malX*::*cat* mutants were fed low at a level of 10^5 CFU/mouse with the respective wild-type strains. The mutant strains were found to co-colonize during competition with the wild-type parents, suggesting that the maltose PTS transport system is not necessary for colonization (Fig. A.5.1B and Fig. A.5.1C). However, in order to test the importance of maltose transport, a double mutant was constructed in both maltose uptake systems (*malEFG* and *malX*) which eliminated the ability of the strain to transport maltose. *E. coli* EDL933 and MG1655 Δ(*malE-malG*) *malX*::*cat* mutants were fed low at a level of 10^5 CFU/mouse with wild-types. The Δ*malE-malG malX*::*cat* mutants were initially able to colonize but declined in numbers after day 1 post-feeding (2.5 to 3 log difference) (Table 5.1, Fig. 5.2B, Fig. A.5.1D). Thus, these findings are nearly identical to the Δ(*malE-malG*)::*cat* colonizations. We, therefore, conclude from these results that MalX plays a minor role *in vivo*, whereas MalEFG has a more significant role for maltose transport during *E. coli* colonization.

Maltose catabolism is important for intestinal colonization. Next we examined the importance of maltose utilization during colonization by testing mutants unable to metabolize maltose for their ability to compete with the wild-type. Mutants were

constructed that deleted the *malP* (encoding maltodextrin phosphorylase) and *malQ* (encoding amylmaltase) genes as described previously, where *malP* mutants are unable to grow on maltodextrins and *malQ* mutants are unable to grow on maltose (29). *E. coli* EDL933 Δ(*malP-malQ*)::*cat* and *E. coli* MG1655 Δ(*malP-malQ*)::*cat* were unable to initiate or maintain colonization during competition with the respective wild-types (Table 5.1, Fig. 5.3A, Fig. A.5.2). Initially, a 3 log difference was observed during competition that ultimately resulted in a 4 log difference in maintenance. In order to test whether the observed colonization defect was a result of the inability of the Δ(*malP-malQ*)::*cat* mutants to grow in the intestine or compete with the wild-type, mutant strains were fed alone to mice. Mutants were found to colonize the streptomycin-treated mouse at wild-type levels suggesting that the ability to utilize maltose, and not the inability of the mutant strain to grow, contributes to successful competition in the mouse intestine (Fig. 5.3B). Thus, the inability of the Δ(*malP-malQ*)::*cat* mutants to initiate colonization is a result of their inability to compete with the wild-type. Our results suggest that maltose catabolism is indeed important for *E. coli* colonization of the intestine.

To determine if the utilization of maltose or maltodextrin caused the *malPQ* strain to have a colonization defect, we further characterized the strain *in vivo* by deleting the gene encoding for either maltodextrin phosphorylase (*malP*) or amylmaltase (*malQ*) as previously described (27, 226, 228) and testing their ability to compete with the wild- type in the mouse intestine. However, when Δ*malQ*::*cat* mutants were

Figure 5.3. Utilization of maltose and not maltodextrins is essential for competitive colonization in the mouse intestine. (A) *E. coli* EDL933 Δ(*malP-malQ*) mutants are unable to compete during colonization with EDL933 wild-type strains, (B) but is able to colonize when fed alone. (C) *E. coli* EDL933 Δ*malQ* mutants are unable to compete during colonization and display colonization defects during competition with EDL933 wild-type strains. (D) *E. coli* EDL933 Δ*malP* mutants are able to compete and co-colonize with EDL933 wild-type.

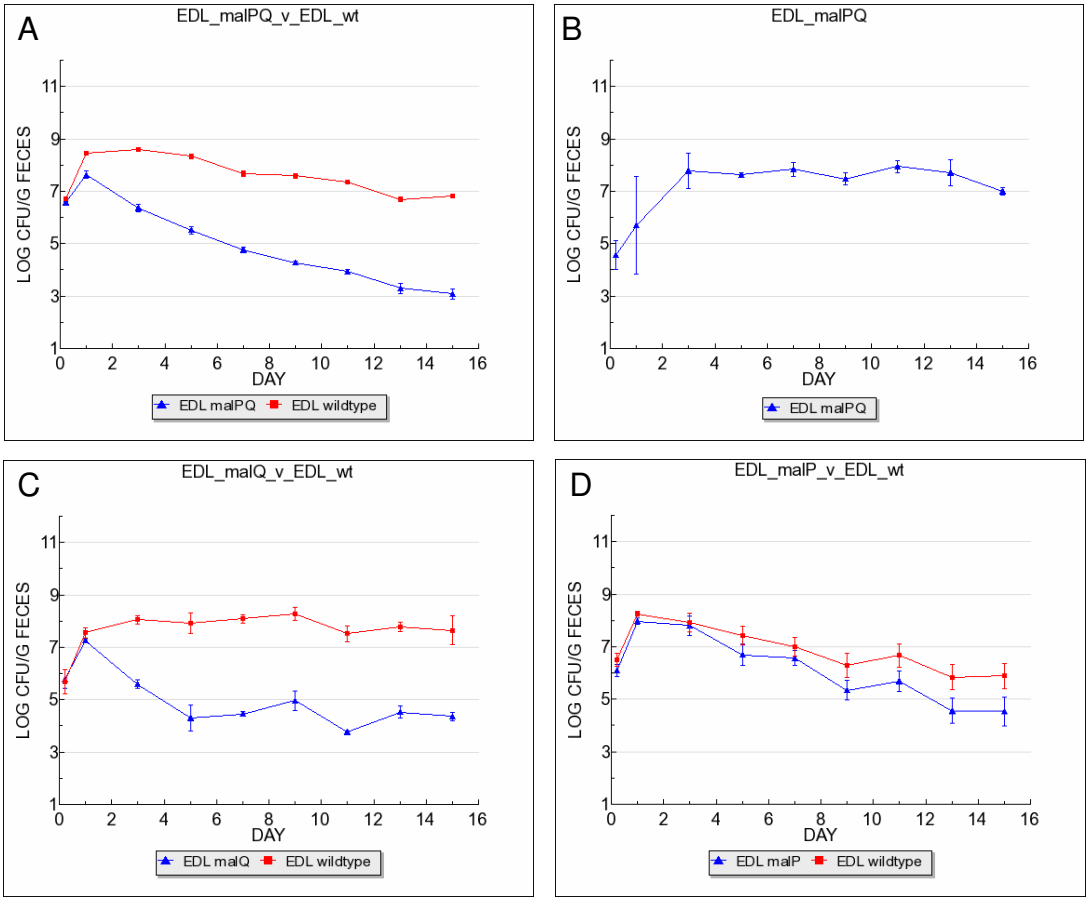


Figure 5.3. Maltose utilization mutants of *E. coli* EDL933 exhibit colonization defects.

fed with their respective wild-types, they were found to initiate poorly and could not maintain colonization as seen by a 4 log colonization defect (Table 5.1 and Fig. 5.3C). Δ*malP*::*cat* mutants co-colonized with their wild-type parents, indicating that maltodextrins are not important for colonization of the intestine (Table 5.1 and Fig. 5.3D). These results suggest that the ability to use maltose, but not maltodextrins, is important for intestinal colonization. Thus, from these findings we conclude that maltose transport and catabolism are needed to support colonization of the intestine.

Transport of and use of maltodextrin is not necessary for intestinal colonization. The *malP* result suggested that maltodextrin catabolism does not contribute to colonization. To confirm this finding, mutants unable to transport maltodextrins from the medium to the cytoplasm were tested for their relative colonization fitness. Mutants were constructed to knock out LamB (maltoporin, glycoporin), encoded by *lamB*, a maltodextrin-specific homotrimeric porin found in the outer membrane. *lamB* mutants are unable to grow on maltodextrins as described previously (276). *E. coli* EDL933 Δ*lamB*::*cat* and *E. coli* MG1655 Δ*lamB*::*cat* mutants were fed together with the respective parent strains and found to co-colonize (Table 5.1 and Fig. A.5.3). From these results, we conclude that transport and catabolism of maltodextrins is not important for *E. coli* to colonize the murine intestinal tract.

Maltose is necessary for EHEC to initiate colonization during competition with commensal *E. coli*. Since *E. coli* EDL933 was found to upregulate genes involved in

the catabolism and transport of maltose, we wanted to test the importance of maltose for the pathogenic strain *E. coli* EDL933 (EHEC) during competition with the commensal *E. coli* MG1655 strain in the mouse intestine. If both the pathogen EDL933 and commensal strain MG1655 are fed low at 10^5 CFU/mouse they initiate equally well, but the pathogen eventually declines in numbers relative to the commensal strain (Fig 5.4A). Additionally, if the pathogenic strain is fed in low numbers and the commensal strain is fed high, then the pathogen is able to initiate and outcompete the commensal strain for the first 3 days post-feeding, but then declines in numbers relative to the commensal strain as previously shown (Fig. 5.4B) (184). To test the role of maltose catabolism during initiation of *E. coli* EDL933 in the presence of high numbers of wild-type *E. coli* MG1655, a *malQ* mutant was constructed in *E. coli* EDL933 and tested for its ability to compete with wild-type *E. coli* MG1655 in the mouse colonization model. The *E. coli* EDL933 Δ*malQ*::*cat* mutant was fed low at 10^5 CFU/mouse and wild-type MG1655 was fed high at 10^{10} CFU/mouse. The results show that the *E. coli* EDL933 Δ*malQ*::*cat* mutant was unable to initiate in competition with wild-type *E. coli* MG1655 during colonization (Compare Fig. 5.4B and Fig. 5.4C). This implies that maltose is important for pathogenic *E. coli* to grow from low to high numbers while attempting to gain residence in the mouse intestine when higher numbers of commensal *E. coli* (i.e., MG1655) are present.

Figure 5.4. Utilization of maltose is necessary for *E. coli* EDL933 to compete with *E. coli* MG1655 in the mouse intestine. (A) *E. coli* EDL933 wild-type colonizes initially in competition with *E. coli* MG1655 wild-type when fed low at the same level and eventually is eliminated. (B) *E. coli* EDL933 wild-type is able to colonize and outcompete *E. coli* MG1655 wild-type initially, when *E. coli* EDL933 is fed low and *E. coli* MG1655 is fed high. (C) A Δ*malQ* mutant in *E. coli* EDL933 wild-type is unable to initiate colonization during competition with *E. coli* MG1655 wild-type.

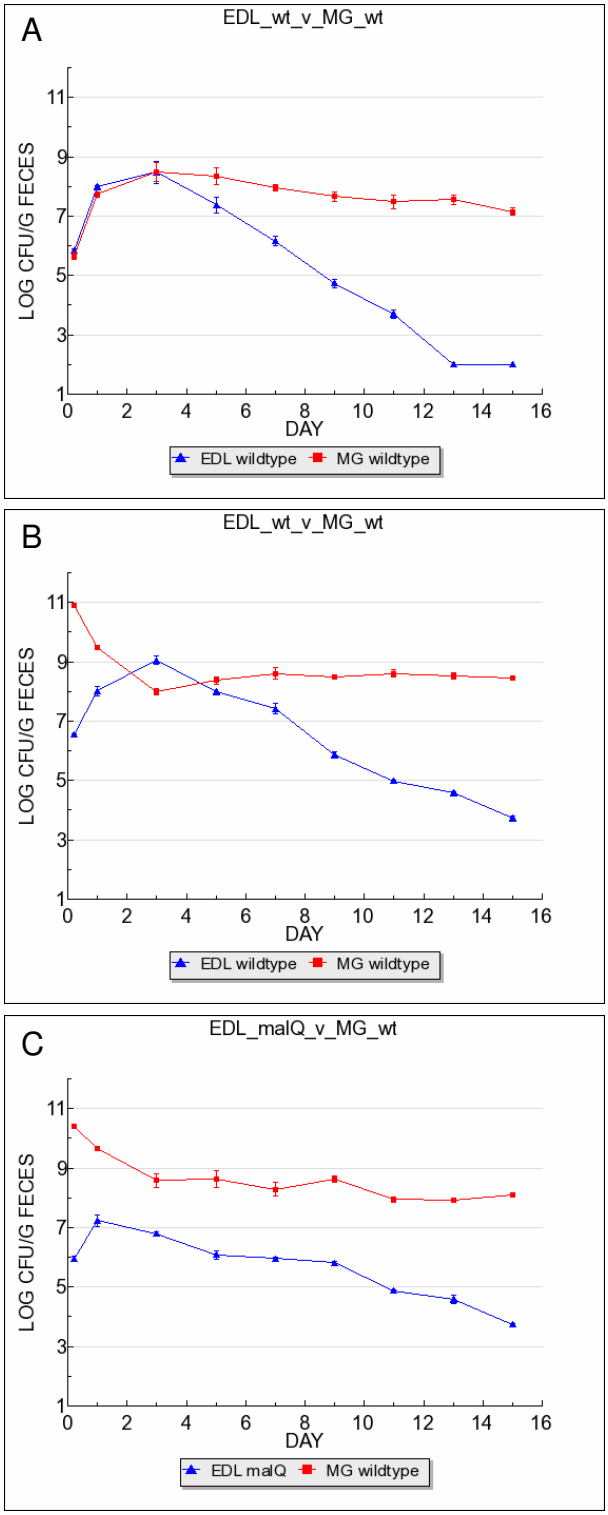


Figure 5.4. Maltose utilization mutant of *E. coli* EDL933 exhibits a colonization defect in competition with wild-type *E. coli* MG1655.

Glycogen synthesis and degradation are important for colonization and competition in the intestine. To test the involvement of carbon storage during colonization in the form of glycogen, mutants were constructed in enzymes encoding for glycogen synthesis (*glgA* and *glgS*), the phenotypes of which were previously described where *glgA* mutants are unable to synthesize glycogen (108, 185). *E. coli* EDL933 Δ*glgA*::*cat* and *E. coli* MG1655 Δ*glgA*::*cat* were fed low at a level of 10^5 CFU/mouse with the wild-type parents. The mutant strains were able to initiate but unable to maintain colonization, ultimately resulting in a 2 log defect (Table 5.1, Fig. 5.5A, Fig. A.5.4A). Likewise, *E. coli* EDL933 Δ*glgS*::*cat* and *E. coli* MG1655 Δ*glgS*::*cat* were fed low at a level of 10^5 CFU/mouse together with the respective parent strains. These mutants also had a 2 log defect in the maintenance stage during competition with the wild-type (Table 5.1, Fig. 5.5B, Fig. A.5.4B). Next we tested the importance of glycogen degradation (*glgP*) in the form of stored energy in the mouse intestine. Mutants were constructed in *glgP*, which are unable to breakdown glycogen, the phenotype of which was described previously (300). *E. coli* EDL933 Δ*glgP*::*cat* and *E. coli* MG1655 Δ*glgP*::*cat* were fed low at a level of 10^5 CFU/mouse. We found that the *glgP* mutant could initiate but not maintain colonization, marked by a 2 log colonization defect by day 9 post-feeding (Table 5.1, Fig. 5.6, Fig. A.5.5). These results indicate that glycogen is an important carbon store used for intestinal colonization and suggest that *E. coli* encounters times of carbon limitation that can be offset by catabolism of glycogen. To confirm this possibility, we added gluconate to the drinking water of mice throughout the duration of the

Figure 5.5. Glycogen synthesis mutants are unable to effectively compete in the mouse intestine. (A) *E. coli* EDL933 Δ*glgA* mutants exhibit colonization defects in maintenance during competitive colonization with *E. coli* EDL933 wild-type strains. (B) *E. coli* EDL933 Δ*glgS* mutants also exhibit colonization defects in maintenance during competitive colonization with *E. coli* EDL933 wild-type strains.

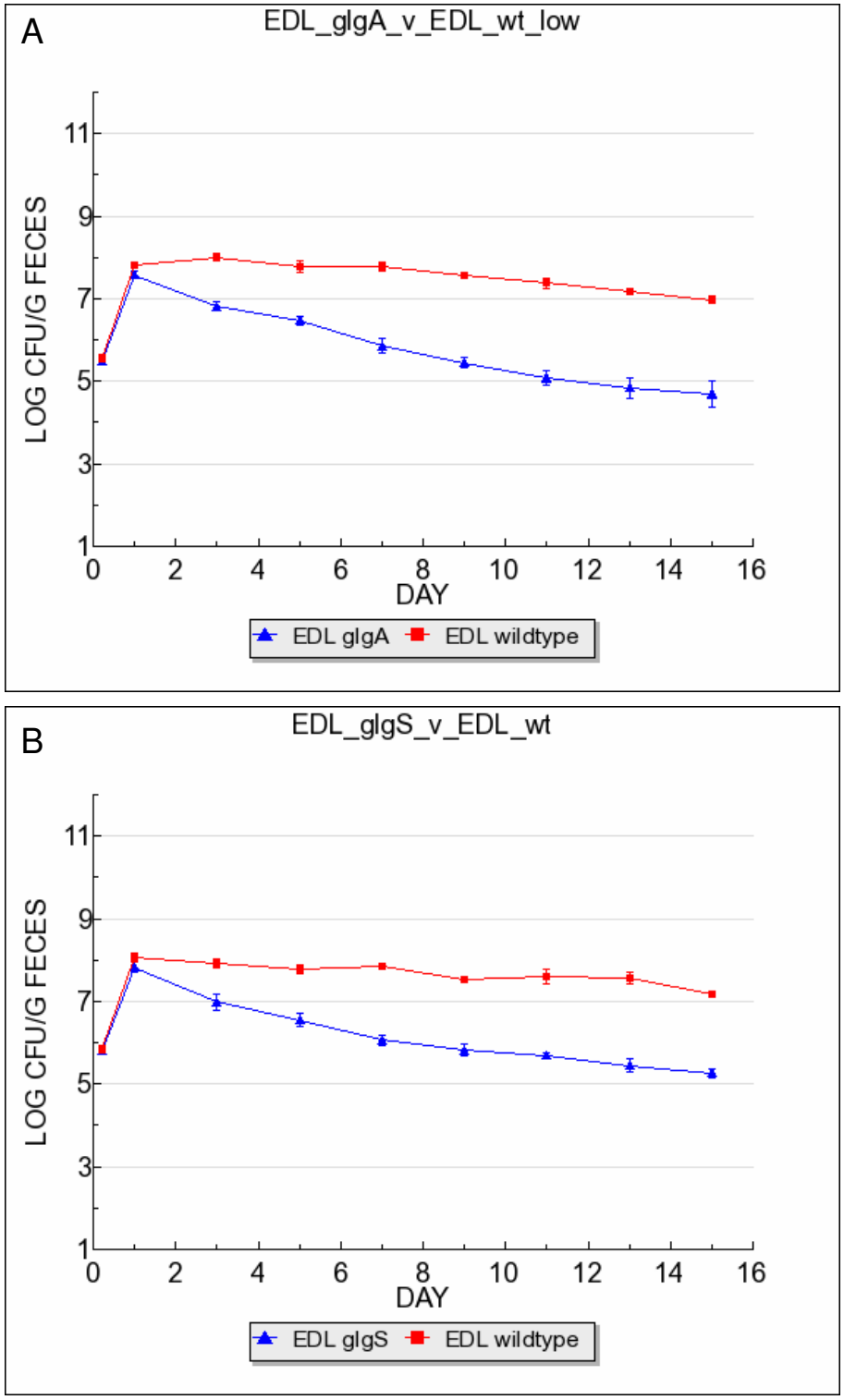


Figure 5.5. Mutants in glycogen synthesis of *E. coli* EDL933 exhibit colonization defects.

Figure 5.6. Glycogen degradation mutants are unable to compete in the mouse intestine. *E. coli* EDL933 Δ*glgP* mutants exhibit colonization defects during competitive colonization with *E. coli* EDL933 wild-type.

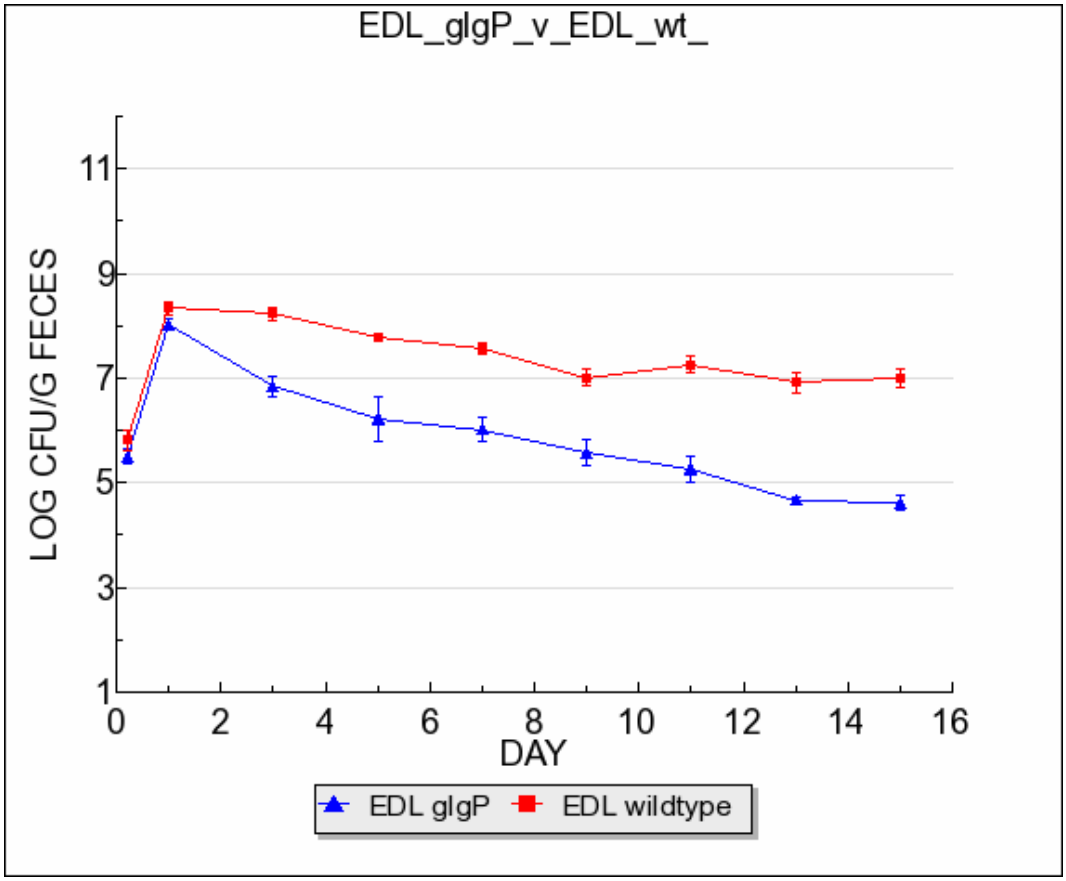


Figure 5.6. Mutants in glycogen degradation of *E. coli* EDL933 exhibit colonization defects.

experiment (Fig. A.5.6). We previously showed that gluconate, which supports growth of *E. coli* but is not absorbed by the host, is able to affect the outcome of experiments that depend on competition for carbon sources (160) and reasoned that the constant availability of an excess supply of gluconate would obviate the dependence on stored glycogen. Indeed, the *glgP* mutant was equally fit for colonization in competition with the wild-type parent strain when gluconate was supplied in the drinking water. Thus, this result supports the conclusion that glycogen is important for maintaining the highest possible energy levels during times of famine in the intestine.

5.4 Discussion

In this study, we examined the nutritional role of maltose and glycogen for pathogenic and commensal *E. coli* to colonize the mouse intestine. Our results show that maltose utilization is essential for competitive intestinal colonization, whereas glycogen degradation and synthesis is important for competition in the intestine. Furthermore, we found that the utilization of maltose *in vivo* gives EHEC a competitive advantage during colonization with the commensal K-12 strain, since the inability of EHEC to use maltose as a carbon and energy source eliminated the pathogen's ability to compete effectively in the intestine. Together, these results suggest a role for maltose competition *in vivo* between pathogenic and commensal bacteria.

The maltose and maltodextrin systems in *E. coli* are a model for understanding bacterial sugar utilizing systems (27, 67, 68). Maltose and maltodextrin utilization in *E. coli* is complex. Extensive work has been done involving the regulatory network of the *mal* operon (67, 68); however, the importance of maltose as a sugar source for *E. coli* has remained an open question (175). *E. coli* has several genes that are involved in maltose and maltodextrin utilization (175). We found that genes involved in maltose transport and utilization (*malQ* and *malEFG*) are necessary for colonization. Conversely, the catabolism and transport of maltodextrins (*malP* and *lamB*) was found not to be important for colonization of the mouse intestine.

The use of complex sugars for energy storage in the form of glycogen synthesis and degradation was used to determine the lifestyle of *E. coli* in the intestine with respect to a feast or famine existence. The observed 2 log fitness defect of mutants unable to degrade or synthesize glycogen during competition in the intestine suggests that *E. coli* experiences intermittent states of nutritional starvation, during which stored carbon ensures success and survival *in vivo*. Previously we have found *E. coli* to be flexible in its energy yielding capabilities in the form of respiration. We have also found that *E. coli* is equally flexible in its ability to utilize different sugars as carbon sources for energy production and, therefore, successful colonization of its host. *E. coli* has previously been shown to display a nutritional preference for available sugars *in vivo*. Indeed, to ensure survival and maximal growth in the intestine *E. coli* has to display flexibility energetically.

Successful colonization of a host relies on a bacterium's ability to utilize available nutrients to generate energy and grow. Many microorganisms oxidize carbohydrates for their main source of cellular energy. Recently, an increased support and appreciation of the role of fundamental metabolic processes during bacterial pathogenesis has been gained. Previous studies have shown maltodextrin acquisition to be the main factor in the ability of group A *Streptococcus* (GAS) to successfully colonize and infect the oropharynx, which is the major site of GAS infection in humans (230-233). Specifically, the maltodextrin binding protein MalE was found to be essential for GAS to colonize the oropharynx. These findings suggest that maltose transport via MalE is a key component for initial colonization of mucosal surfaces. Other reports have shown that deletions in carbon metabolism genes, such as in the maltose operon, led to a decreased production of virulence factors including capsular polysaccharide and cholera toxin in both *S. pneumoniae* and *Vibrio cholerae* (91, 148). Additionally, the ability of *Mycobacterium tuberculosis* to transition to latent infection was shown to be affected by the inactivation of isocitrate lyase, an enzyme involved in the glyoxylate shunt pathway, further suggesting the importance of metabolic genes involved in pathogenesis (167, 178, 179). Thus, it is reasonable to speculate that the utilization of carbohydrates, specifically maltose, may contribute to the ability of pathogenic bacteria to cause disease in humans.

Previous reports have also show glycogen reserves to be important for virulence and biofilm formation of *Salmonella enteritidis* (24). However, until recently the

relative importance of energy reserves for pathogenic microorganisms has remained unknown (178). In *E. coli*, glycogen has been found to be involved in extending survival during starvation (271). Bacterial growth rate may certainly vary in the intestine, and glycogen stores may supply a temporary source of carbon for cellular energy when other sources may be scarce. Indeed, glycogen was found to be important for intestinal colonization of *E. coli*. Thus, this suggests that nutrient availability is intermittent and the use of carbon stores is important during times of transition from one carbon source to the next in the intestine.

In conclusion, we used results of the transcriptome analysis to focus our attention on maltose transport and utilization *in vivo*. Here we report that the use of a complex sugar, as well as stored energy in the form of glycogen, is critical for mucosal colonization and competition in the intestine. Our data further demonstrate that *malEFG* and *malQ* are essential for *E. coli* to successfully colonize the intestinal tract. Additionally, glycogen synthesis and degradation are needed to support intestinal colonization during times of nutritional transitions for *E. coli* to successfully compete when nutrients are limiting in the intestine. Our findings, therefore, suggest that carbohydrate utilization of maltose (exogenous carbon supply) and carbon storage (endogenous carbon supply) both contribute to the ability of pathogens to successfully colonize and infect their host.

Taken together, these findings open new areas for investigations into bacterial colonization and pathogenesis of host mucosal surfaces. The examination of understudied areas, such as carbohydrate physiology and their contribution to pathogenesis, may yield broadly applicable and novel insights into microbial host-pathogen interactions and bacterial pathogenesis. Since the mucosal surface of the gastrointestinal tract is a major site of bacterial colonization and infection, further investigation into carbohydrate transport, utilization, and the metabolic pathways used *in vivo* is essential for understanding the underlying mechanisms that enable pathogens to successfully colonize their hosts. Furthermore, many of the nutrient acquisition mechanisms used by *E. coli* are also used by other human mucosal pathogens. Therefore, such studies are crucial for understanding pathogenesis and will aid in finding new and effective treatments.

Chapter 6

Conclusions

Mammals are aerobes that harbor an intestinal ecosystem dominated by large numbers of anaerobic microorganisms. The role of oxygen in the intestinal ecosystem is largely unexplored. My research provided evidence that aerobic respiration is required for commensal and pathogenic *E. coli* to colonize mice. A systematic mutational analysis was used to determine the role of respiratory metabolism in the streptomycin-treated mouse model of intestinal colonization. Our results showed that mutants lacking ATP synthase, which is required for all respiratory energy-conserving metabolism, were eliminated by competition with respiratory-competent wild-type strains (Table 6.1). Additionally, mutants lacking the high affinity cytochrome *bd* oxidase, which is used when oxygen tensions are low, also failed to colonize (Table 6.2). However, the low affinity cytochrome bo_3 oxidase, which is used when oxygen tension is high, was found not to be necessary for colonization (Table 6.2). Mutants lacking either nitrate reductase or fumarate reductase also had major colonization defects (Table 6.2). The results showed that the entire *E. coli* population was dependent on both microaerobic and anaerobic respiration, consistent with the hypothesis that the *E. coli* niche is alternately microaerobic and anaerobic, rather than static. Taken together, the results indicate

Table 6.1. Summary of colonization data for respiratory mutants. Major colonization defects are shown in red.

Mutation (Gene Name)	**Electron Chain Component**	**Colonization Defect**	**Phase of Colonization**
arcA	Regulates aerobic respiration	Major	Initiation, Maintenance
Fnr	Regulates anaerobic respiration	Major	Maintenance
atpAG	ATP synthase	Major	Initiation, Maintenance

Table 6.2. Summary of colonization data for respiratory oxidoreductase mutants. Major colonization defects are shown in red.

Mutation (Gene Name)	**Electron Chain Component**	**Colonization Defect**	**Phase of Colonization**
cyoAB	Cytochrome *bo3* oxidase	None	
cydAB, cydDC	Cytochrome *bd* oxidase	Major	Initiation, Maintenance
narG	Nitrate reductase	Minor	Maintenance
narZ	Nitrate reductase	None	
napDA	Nitrate reductase	None	
narG/narZ	Nitrate reductase	Significant/Major	Maintenance
narG/narZ/napDA	Nitrate reductase	Major	Maintenance
nirBD	Nitrite reductase	None	
frdA	Fumarate reductase	Major	Maintenance
dmsAB	DMSO reductase	None	
torCA	TMAO reductase	None	

that success of the facultative anaerobes in the intestine depends on their respiratory flexibility. Despite competition for relatively scarce carbon sources, the energy efficiency provided by respiration may contribute to the wide spread distribution (i.e., success) of *E. coli* as commensal inhabitants of the mammalian intestine.

The availability of terminal electron acceptors in the gastrointestinal tract allows *Escherichia coli* to colonize a densely populated microhabitat. After establishing which respiratory pathways are used by *E. coli* strains O157:H7 and K-12 to colonize the mammalian intestine, i.e., which specific respiratory oxidoreductases are important, my next task was to determine the hierarchy of preferred electron acceptors used by *E. coli* in the intestine. Respiratory pathways were systematically eliminated by gene knockouts and the mutants were tested for their relative fitness for colonization in the streptomycin-treated mouse. We determined the relative fitness of cytochrome *bd* mutants in competition with Δ*narG*::*kan* and Δ*frdA*::*kan* mutants and found that oxygen is more important than the alternative electron acceptors nitrate and fumarate. Since *E. coli* has three nitrate reductases, we tested their relative contribution to colonization and found that Δ*narG* mutants lacking the primary nitrate reductase are outcompeted by mutants lacking either NarZ or NapDA, which are the secondary cytoplasmic and periplasmic nitrate reductases, respectively. Next we tested the preference for nitrate or fumarate respiration by co-colonizing Δ*narG narZ*::*cat* and Δ*frdA*::*kan* mutants and observed a switch in relative fitness where the fumarate reductase mutant outcompeted the nitrate reductase double mutant during

initiation, but the fumarate reductase mutant was more competitive during maintenance. The observed switch can best be explained by nitrate availability in the intestine, where nitrate represses synthesis of fumarate reductase until nitrate levels diminish and fumarate reductase is no longer repressed. Taken together, the results indicate that the *in vivo* hierarchy of terminal electron acceptors (O_2>NO_3>fumarate) reflects the known regulation of the respective respiratory pathways *in vitro*. This display of respiratory flexibility during colonization presumably allows *E. coli* to adapt to the availability of electron acceptors present in the intestine, and for the first time demonstrates the hierarchy of electron acceptors used *in vivo*.

The mammalian intestine is presumed to be anaerobic, since the microflora is dominated by strict anaerobes. However, we have showed that cytochrome *bd* oxidase, in addition to nitrate reductase and fumarate reductase, is important for *E. coli* colonization of the gastrointestinal tract. This suggests that the intestinal environment is not strictly anaerobic, but instead contains aerobic microhabitats. In order to complete our understanding of the respiratory pathways used for colonization of the mouse intestine, we turned our attention to the intra-membrane electron carriers, the quinones. The *E. coli* quinone pool consists of ubiquinone (Q), the biosynthesis of which is encoded by *ubiCA* and *ubiE*, menaquinone (MK), the biosynthesis of which is encoded by *ubiE*, and demethylmenaquinone (DMK) the biosynthesis of which is encoded by *menA*. These quinones serve as electron carriers between the appropriate dehydrogenases and oxidoreductases during both aerobic and

anaerobic respiration. A systematic mutational analysis of quinone pool biosynthetic pathways was used to determine the colonization fitness of strains competing in the streptomycin-treated mouse model. We performed fitness competitions with mutant strains lacking each of the three quinones in both *E. coli* K-12 and *E. coli* O157:H7 backgrounds. We found that Δ(*ubiC-ubiA*)::*cat* mutants co-colonize with the wild-type parent strain, indicating that Q, which is used primarily during growth when oxygen tension is high, is not important for colonization (Table 6.3). Our results show that electron flow under low oxygen tension, which is the primary role of MK, is essential for colonization since the Δ*ubiE*::*cat* mutant failed to initiate colonization in competition with the wild-type and was eliminated from mice (Table 6.3). In addition, electron flow via DMK to alternative anaerobic electron acceptors is required for maintenance of colonization, as the Δ*menA*::*cat* mutant initiated colonization but could not persist in the intestine (Table 6.3). Together these results confirm our previous findings that aerobic respiration of low oxygen is important for *E. coli* to grow from low to high numbers during the initiation stage of colonization and that anaerobic respiration is important during the maintenance stage.

It is important to understand how gastrointestinal pathogens acquire the nutrients necessary to infect their hosts and initiate the disease process. Carbohydrate utilization has recently been demonstrated to be important for microbial pathogenesis. However, the role of complex sugars, such as maltose, and energy storage compounds, such as glycogen, during colonization of the mammalian large intestine

Table 6.3. Summary of colonization data for quinone biosynthetic mutants. Major colonization defects are shown in red.

Mutation (Gene Name)	**Electron Chain Component**	**Colonization Defect**	**Phase of Colonization**
ubiCA	Q	None	
ubiE	Q/MK	Major	Initiation, Maintenace
menA	DMK/MK	Major	Maintenance

is poorly understood. In this study, we sought to investigate the role of maltose utilization, glycogen storage, and glycogen degradation *in vivo*. Chapter 5 of this dissertation explored the possible role of a feast and famine lifestyle for *E. coli* in the mouse intestine, where the role of endogenous energy sources for survival inside the host was examined. Our results demonstrate that both maltose and glycogen are needed for intestinal colonization. Furthermore, we conclude that bacterial growth rate may vary in the intestine, and glycogen stores may supply a steady source of carbon for cellular energy when other sources are limiting. Thus, this further suggests that the need for glycogen indicates periods of famine or carbon limitation in the intestine. *E. coli* almost certainly lives a feast and famine existence during infection of animal hosts. Understanding the use of complex sugars and energy storage *in vivo* should build on our knowledge of model systems such as the one discussed here.

In summary, the capacity to respire various terminal electron acceptors provides the respiratory flexibility that bacteria need to adapt their energy yielding metabolism to changing environmental conditions. Respiratory flexibility appears to be key to maximizing growth yield *in vivo*. Respiratory flexibility has been found to be important for *Mycobacterium tuberculosis*, where adaptation of the organism involves three respiratory states (248). Here we provide evidence that respiratory metabolism of *E. coli* O157:H7 and K-12 is critical for successful colonization of the mouse intestine. Furthermore, we showed that the respiratory metabolism of *E. coli*

O157:H7 and *E. coli* K-12 during colonization of the mouse intestine depends upon both aerobic respiration of oxygen and anaerobic respiration of nitrate and fumarate.

While the GI tract is presumed to be an anaerobic environment, our results suggest the presence of aerobic microhabitats. It has been assumed that strictly anaerobic GI bacteria would not require respiratory cytochrome oxidases (11). However, studies have shown that strict anaerobes, such as *Bacteroides fragilis Desulfovibrio gigas* and *Moorella thermoacetica*, contain a cytochrome *bd* oxidase that is advantageous under conditions of nanomolar oxygen concentrations (11, 64). Together, these findings suggest that the intestine is microaerobic, and indeed He et al. (113) found oxygen in low concentrations in the intestine. Our results further indicate that oxygen scavenging may be vital for initiation and maintenance of facultative anaerobes and suggests an important role for *E. coli* in making the GI tract anaerobic. Additionally, it appears that oxygen is important for establishing a high population and alternate electron acceptors are important for maintaining colonization. It remains to be determined if the apparent use of these three electron acceptors is a property of the entire population, in which each bacterium is using all three simultaneously, or if individual cells each use different electron acceptors, perhaps in distinct microhabitats.

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APPENDIX

Table A.2.1. Strains and plasmids used in this study.

Strain or plasmid	Relevant Characteristic(s)	Source or reference
MG1655	Wild-type (CGSC no. 7740)	*E. coli* Genetic Stock Culture Collection, Yale University
MG1655 Str^r	Spontaneous streptomycin-resistant mutant of MG1655	176
MG1655 Str^r Nal^r	Spontaneous nalidixic acid-resistant mutant of MG1655 Str^r	176
MG1655 Str^r Δ*arcA*::*kan*	*arcA* deletion mutant of MG1655 Str^r, carrying km-resistance cassette	This study
MG1655 Str^r Δ*fnr*::*kan*	*fnr* deletion mutant of MG1655 Str^R, carrying km-resistance cassette	This study
MG1655 Str^r Δ(*atpA-atpG*)::*cat*	*atpAG* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ(*cyoA-cyoB*)::*cat*	*cyoAB* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ(*cydA-cydB*)::*cat*	*cydAB* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ(*cydD-cydC*)::*cat*	*cydDC* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ*narG*::*kan*	*narG* deletion mutant of MG1655 Str^r, carrying km-	This study

	resistance cassette	
MG1655 Str^r Δ*narZ*::*cam*	*narZ* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ(*napD-napA*)::*cat*	*napDA* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ*narG narZ*::*cat*	*narGnarZ* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ*narG naZ*(*napD-napA*)::*cat*	*narGnarZnapDA* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ*frdA*:: *kan*	*frdA* deletion mutant of MG1655 Str^r, carrying km-resistance cassette	This study
MG1655 Str^r Δ(*dmsA-dmsB*)::*cat*	*dmsAB* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ(*torC-torA*)::*cat*	*torCA* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ(*nirB-nirD*)::*cat*	*nirBD* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
EDL933	Wild-type O157:H7	Alison O' Brien
EDL933 Str^r	Spontaneous streptomycin-resistant mutant of EDL933	174
EDL933 Str^r Nal^r	Spontaneous nalidixic acid-resistant mutant of EDL933 Str^r	174
EDL933 Str^r Rif^r	Spontaneous rifampicin-	This study

	resistant mutant of EDL933 Str^r	
EDL933 Str^r Δ*arcA*::*cam*	*arcA* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ*fnr*::*kan*	*fnr* deletion mutant of EDL933 Str^r, carrying km-resistance cassette	This study
EDL933 Str^r Δ(*atpA-atpG*)::*cat*	*atpAG* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ(*cyoA-cyoB*)::*cat*	*cyoAB* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ(*cydA-cydB*)::*cat*	*cydAB* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ(*cydD-cydC*)::*cat*	*cydDC* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ*narG*::*kan*	*narG* deletion mutant of EDL933 Str^r, carrying km-resistance cassette	This study
EDL933 Str^r Δ*narZ*::*cam*	*narZ* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ(*napD-napA*)::*cat*	*napDA* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ*narG narZ*::*cat*	*narGnarZ* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ*narG naZ*(*napD-napA*)::*cat*	*narGnarZnapDA* deletion mutant of EDL933 Str^r, carrying cm-	This study

	resistance cassette	
EDL933 Str^r Δ*frdA*:: *kan*	*frdA* deletion mutant of EDL933 Str^r, carrying km-resistance cassette	This study
EDL933 Str^r Δ(*dmsA-dmsB*)::*cat*	*dmsAB* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ(*torC-torA*)::*cat*	*torCA* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ(*nirB-nirD*)::*cat*	*nirBD* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
pKD3	Template plasmid, contains chloramphenicol resistance cassette flanked by FLP recombinase target sites; *bla cat*	61
pKD4	Template plasmid, contains kanamycin resistance cassette flanked by FLP recombinase target sites; *bla kan*	61
pKD46	Temperature-sensitive plasmid, contains arabinose-inducible λ phage red recombinase gene for linear DNA exchange; *bla*	61

Table A.2.2. Competitive colonization between respiratory mutants and wild-type *E. coli* strains. [a]

		E. coli EDL933		*E. coli MG1655*	
Respiratory Enzyme	**Mutant**	**Day 1**	**Day 9**	**Day 1**	**Day 9**
Dimethyl sulfoxide reductase	Δ*dmsAB*	0.6 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Trimethylamine N-oxide reductase	Δ*torCA*	0.6 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
Fumarate reductase	Δ*frdA*	0.8 ± 0.1	**3.1 ± 0.2**	0.1 ± 0.1	**2.8 ± 0.2**

[a] Mice were fed 10^5 CFU each of a mutant and its wild-type parent. Mice were transferred to fresh cages every day and feces no older than 24h were assayed every other day for 15 days. At each time point, for each mouse the Log_{10} CFU/gram of feces for the mutant was subtracted from the Log_{10} CFU/gram of feces for the wild-type. The average ± the standard error of the mean of Day 1 and Day 9 data from 6 mice are shown. Differences of at least one order of magnitude (10-fold) are in bold type; all values shown in bold are statistically significant: $P < 0.005$ (student's t-test).

Figure A.2.1. Respiratory mutants exhibited colonization defects in competitive colonization assays. (A) *E. coli* MG1655 ATPase mutants showed colonization defects during competition with wild-types, (B) but were able to colonize when fed alone. (C) *E. coli* MG1655 cytochrome *bd* oxidase mutants showed colonization defects during competition with wild-types, (D) but were able to colonize when fed alone.

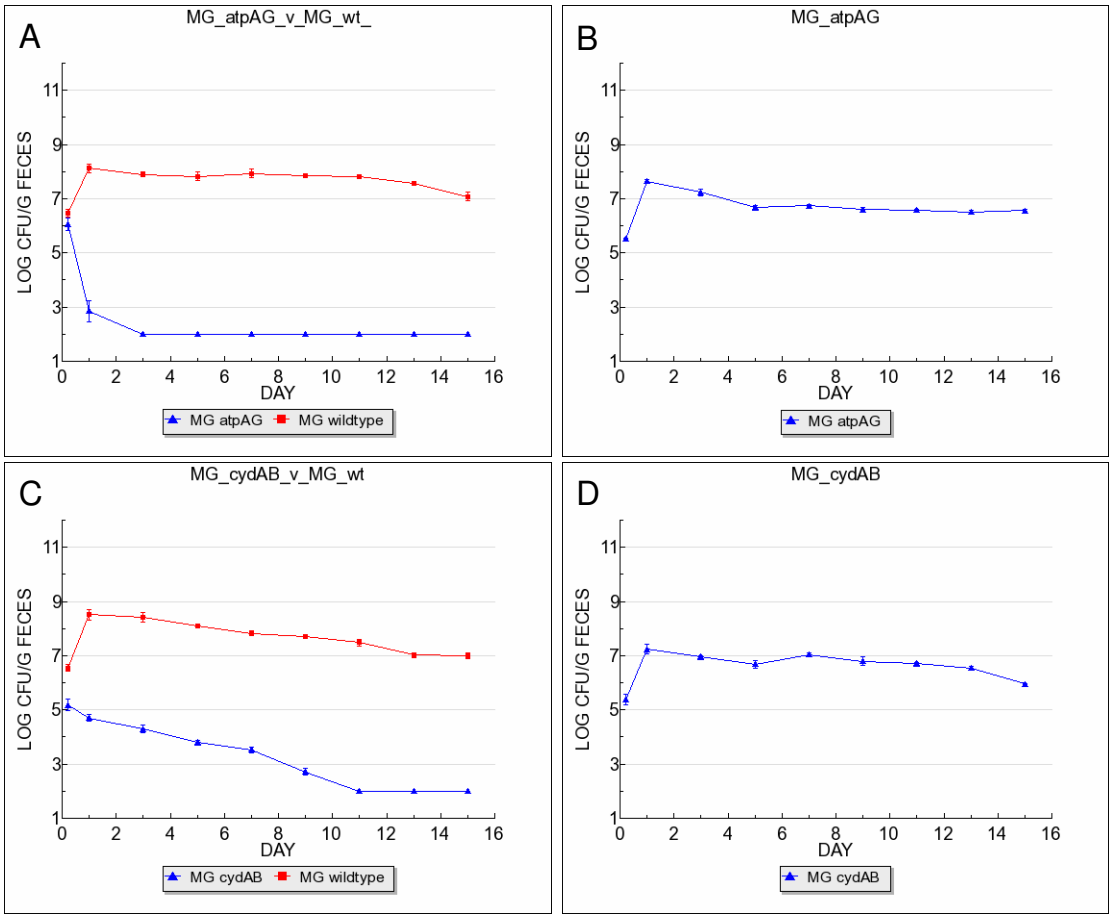

Figure A.2.1. ATPase and cytochrome *bd* oxidase mutants of *E. coli* MG1655 exhibit colonization defects.

Figure A.2.2. Cytochrome *bd* oxidase assembly mutants of *E. coli* EDL933 exhibited colonization defects in competitive colonization assays. (A) *E. coli* MG1655 Δ(*cydD-cydC*) mutants showed colonization defects during competition with wild-type *E. coli* MG1655, (B) but was able to colonize when fed alone to mice.

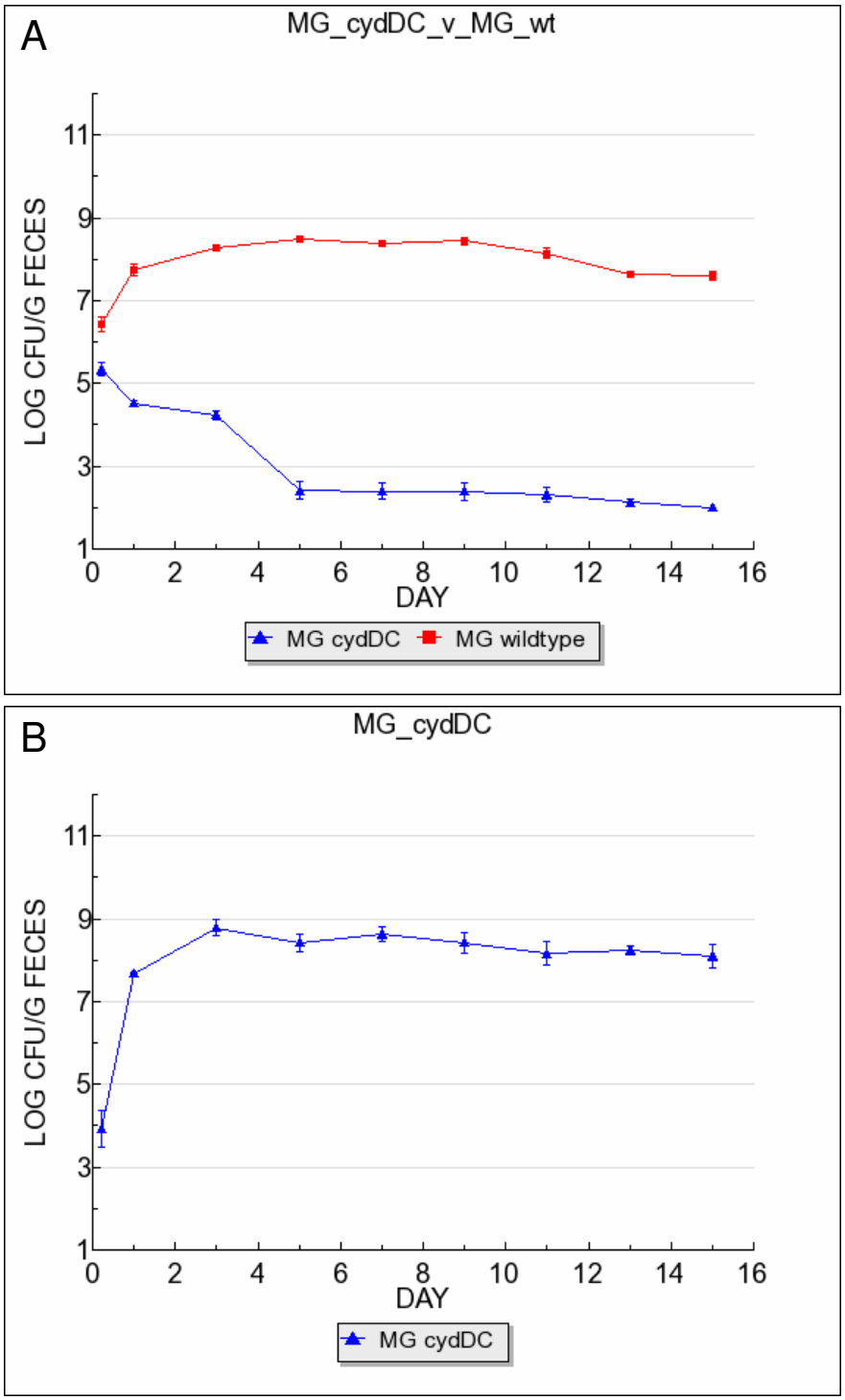

Figure A.2.2. Cytochrome *bd* oxidase assembly mutants of *E. coli* MG1655 exhibit colonization defects.

Figure A.2.3. Cytochrome bo_3 oxidase mutants compete with wild-type strains in competitive colonization assays. (A) *E. coli* EDL933 Δ(*cyoA-cyoB*) mutants co-colonized and displayed no colonization defect during competition with wild-type *E. coli* EDL933. (B) *E. coli* MG1655 Δ(*cyoA-cyoB*) mutants co-colonized and displayed no colonization defect during competition with wild-type *E. coli* MG1655.

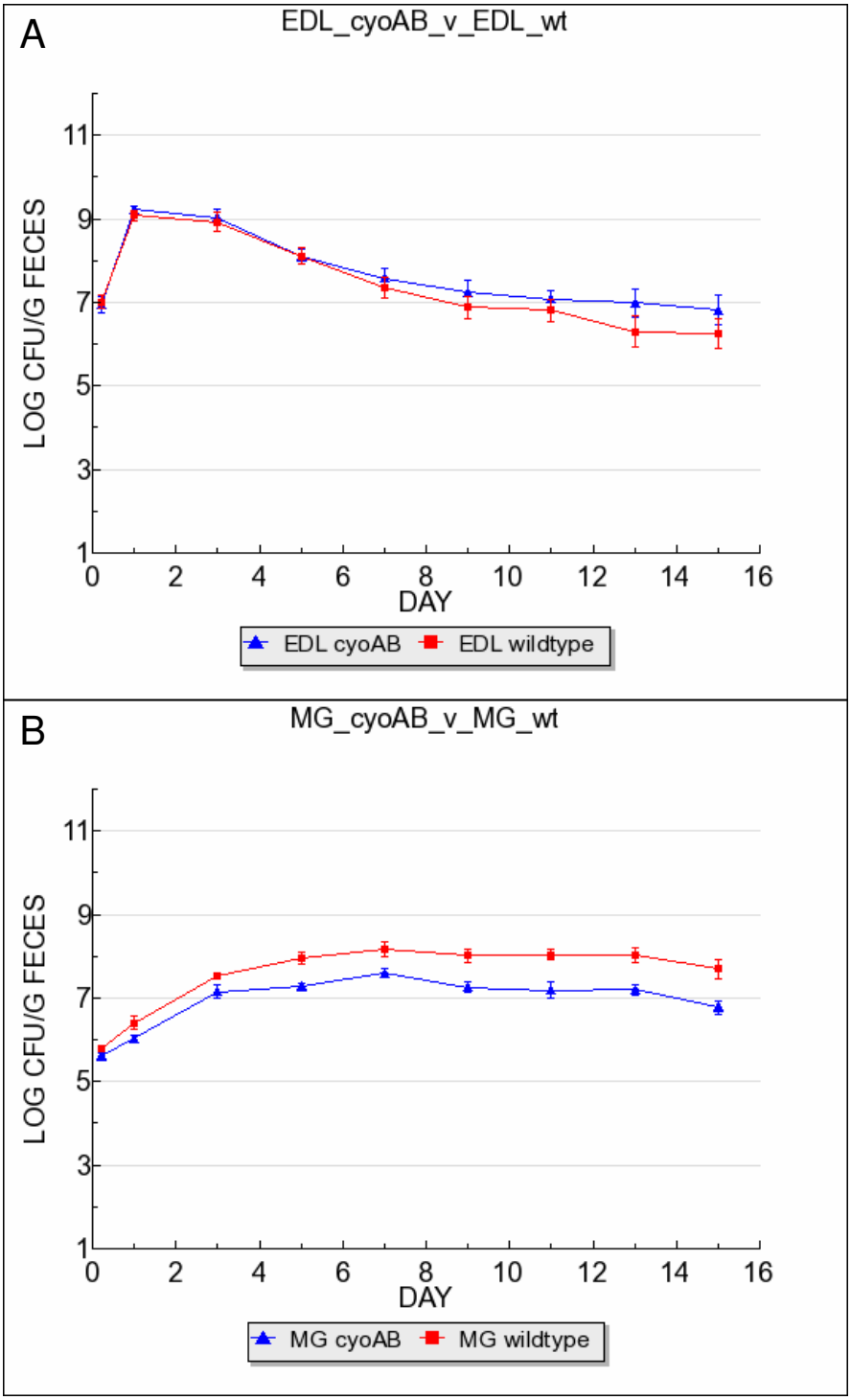

Figure A.2.3. *E. coli cyoAB* mutants co-colonized with wild-types.

Figure A.2.4. Aerobic respiratory and anaerobic global regulatory mutants exhibit colonization defects in competitive colonization assays. (A) *E. coli* MG1655 Δ*arcA* was eliminated during competition with wild-type *E. coli* MG1655, (B) but was able to colonize when fed alone. (C) *E. coli* MG1655 Δ*fnr* was eliminated during competition with wild-type *E. coli* MG1655, (D) but was able to colonize at wild-type levels when fed alone to mice.

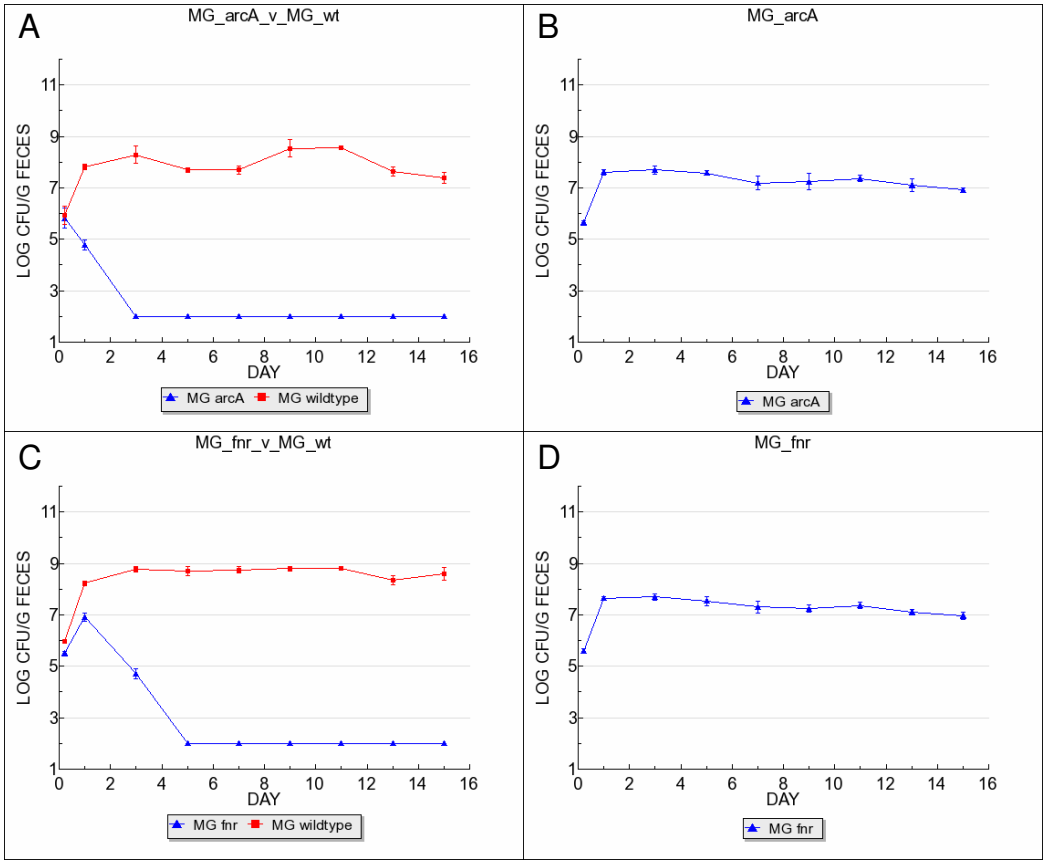

Figure A.2.4. ArcA and Fnr mutants of *E. coli* MG1655 exhibit colonization defects.

Figure A.2.5. Primary nitrate reductase mutants display colonization defects with wild-type strains in competitive colonization assays. *E. coli* MG1655 Δ*narG* mutants are defective in maintenance during competition with wild-type *E. coli* MG1655.

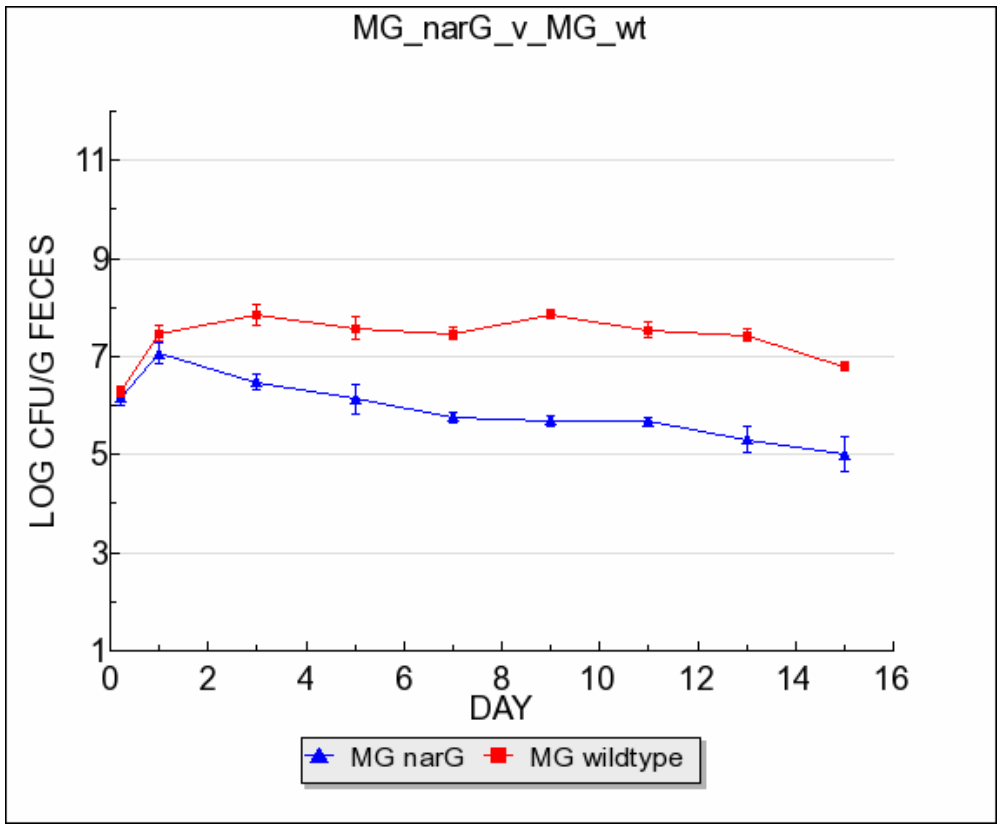

Figure A.2.5. *E. coli* primary nitrate reductase mutant exhibits a colonization defect.

Figure A.2.6. Nitrate reductase mutants in the primary and secondary reductase display colonization defects in competitive colonization assays. (A) *E. coli* EDL933 Δ*narG narZ* was defective in competition during maintenance with wild-type *E. coli* EDL933, (B) *E. coli* MG1655 Δ*narG narZ* was defective in competition during maintenance with wild-type *E. coli* MG1655.

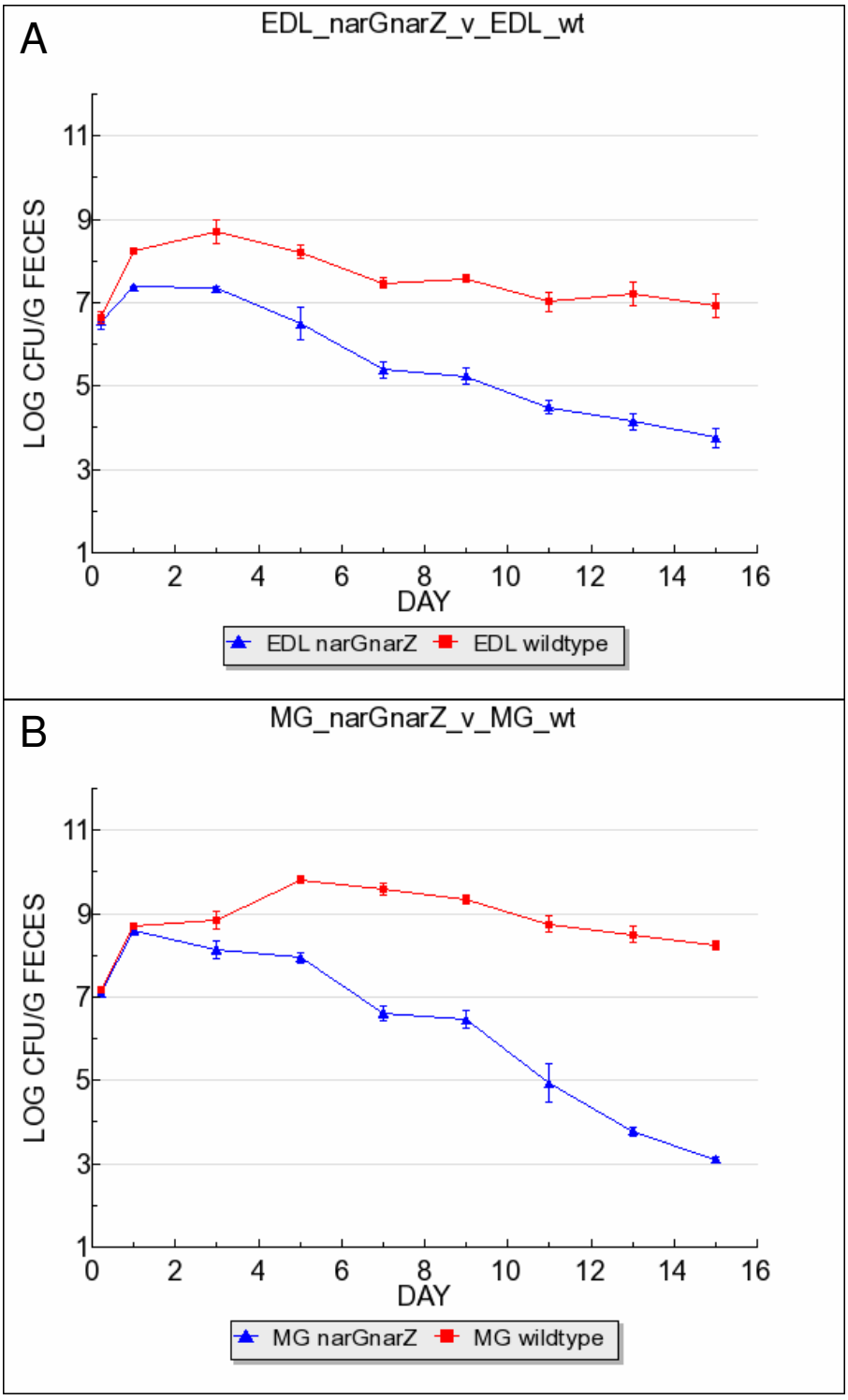

Figure A.2.6. *E. coli* double nitrate reductase mutants exhibit colonization. defects.

Figure A.2.7. Nitrate reductase mutants in the primary and secondary and periplasmic reductase display colonization defects in maintenance during competitive colonization assays. (A) *E. coli* EDL933 Δ*narG narZ* (*napD-napA*) was defective in competition with wild-type *E. coli* EDL933, (B) *E. coli* MG1655 Δ*narG narZ* (*napD-napA*) was defective in competition with wild-type *E. coli* MG1655.

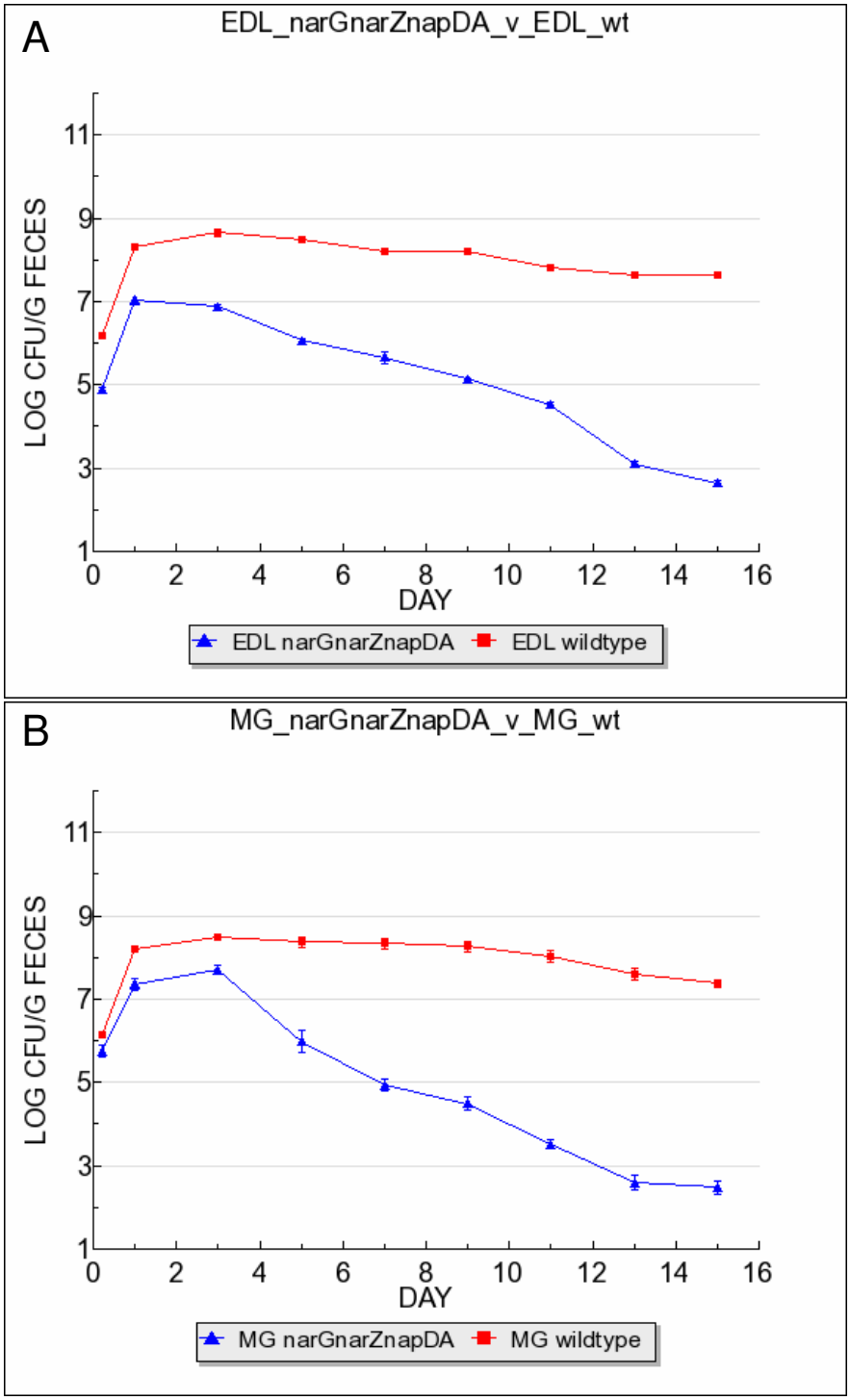

Figure A.2.7. *E. coli* triple nitrate reductase mutants show colonization defects.

Figure A.2.8. Nitrate reductase mutants in the secondary reductase are able to compete in competitive colonization assays. (A) *E. coli* EDL933 Δ*narZ* was able to co-colonize during competition with wild-type *E. coli* EDL933, (B) *E. coli* MG1655 Δ*narZ* was able to co-colonize during competition with wild-type MG1655.

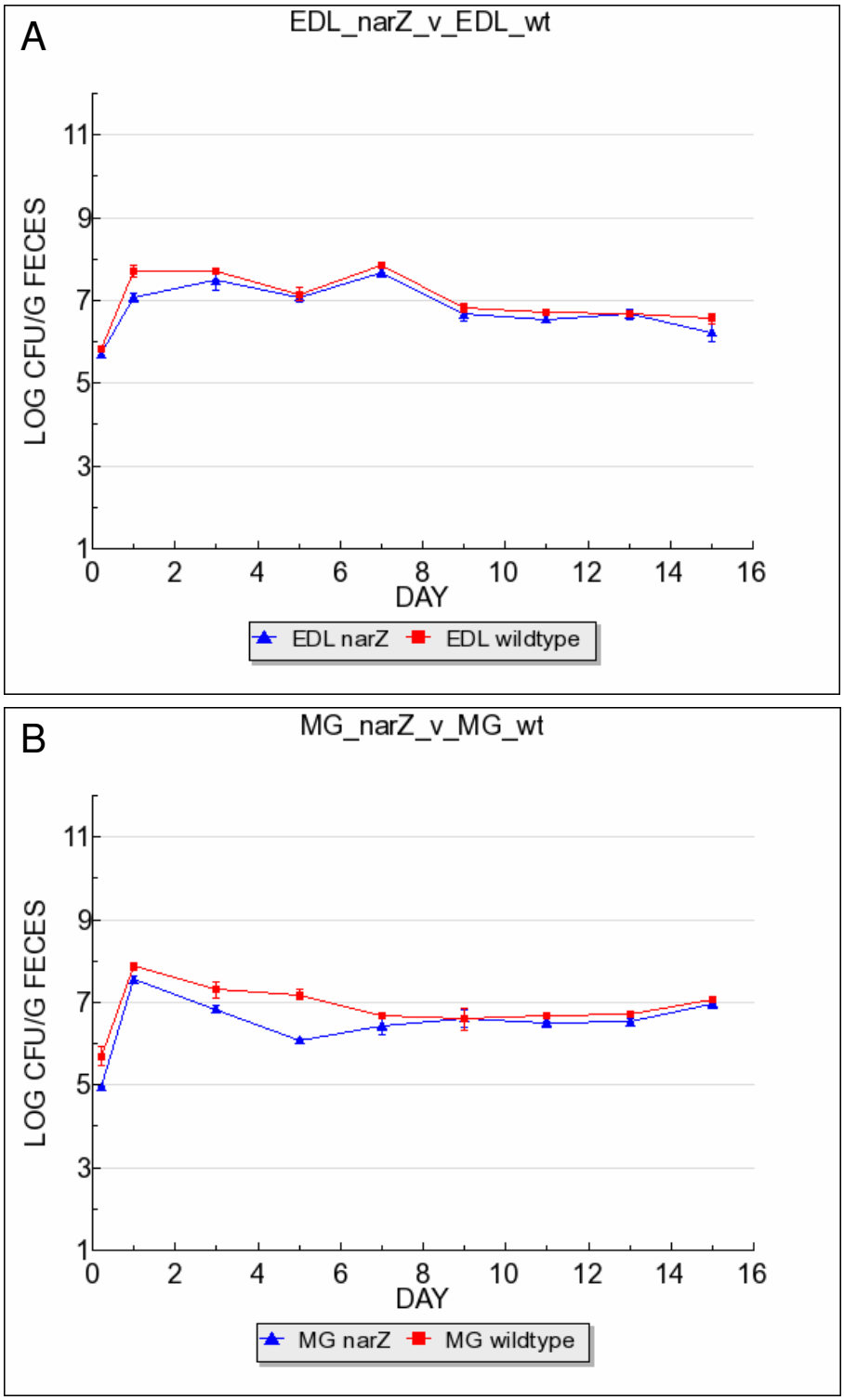

Figure A.2.8. *E. coli* secondary nitrate reductase mutants do not exhibit colonization defects.

Figure A.2.9. Nitrate reductase mutants in the periplasmic nitrate reductase are able to compete in competitive colonization assays. (A) *E. coli* EDL933 Δ(*napD-napA*) was able to co-colonize during competition with wild-type *E. coli* EDL933, (B) *E. coli* MG1655 Δ(*napD-napA*) was able to co-colonize during competition with wild-type *E. coli* MG1655.

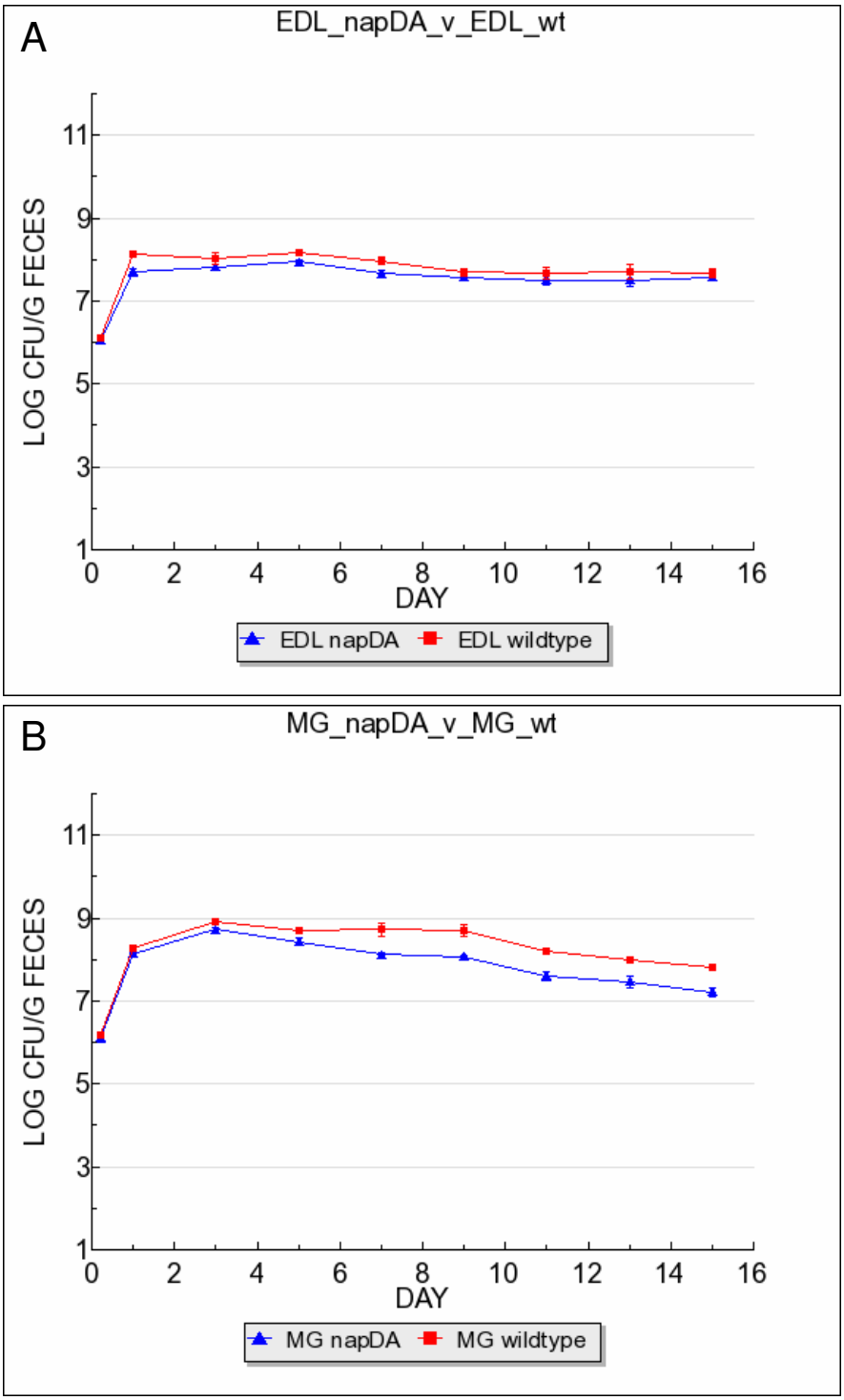

Figure A.2.9. *E. coli* periplasmic nitrate reductase mutants do not exhibit colonization defects.

Figure A.2.10. Fumarate reductase mutants display colonization defects in competitive colonization assays. *E. coli* MG1655 Δ*frdA* was defective in maintenance during competition with wild-type *E. coli* MG1655.

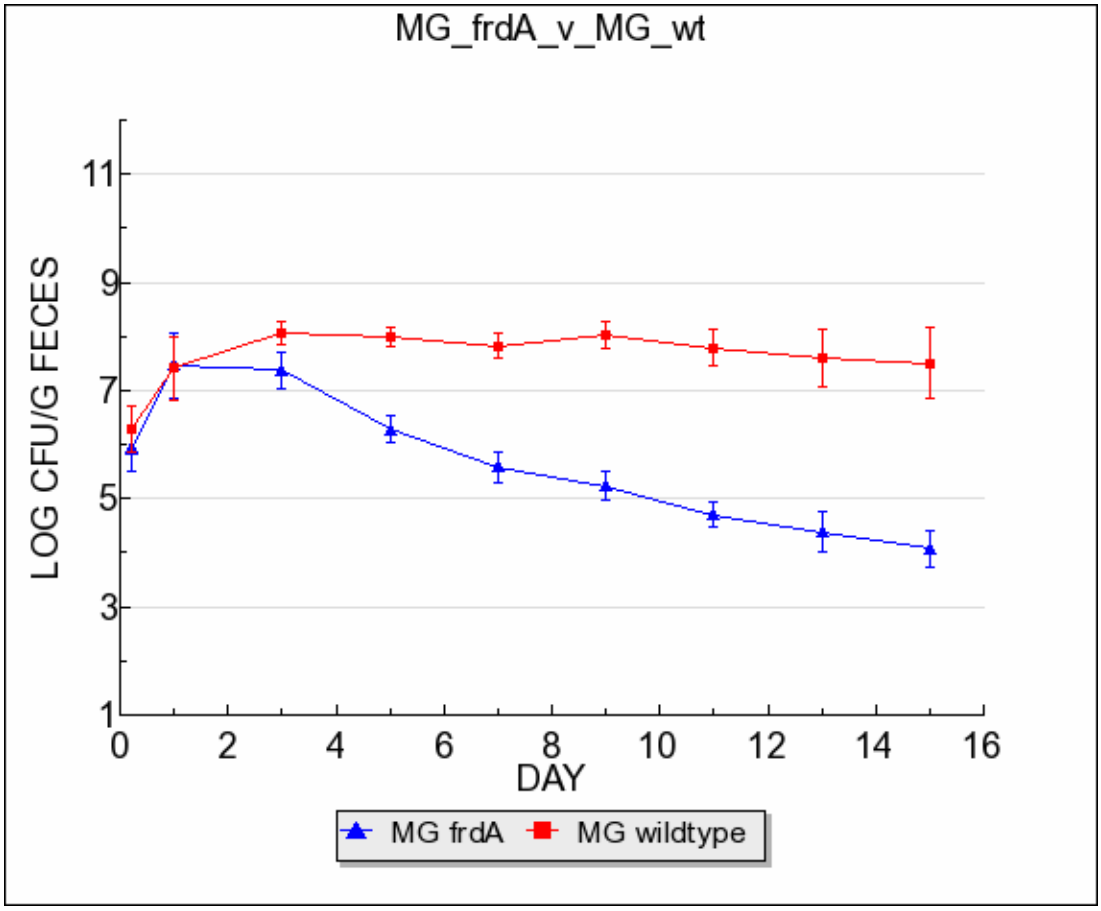

Figure A 2.10. *E. coli* fumarate reductase mutants exhibit colonization defects.

Figure A.2.11. DMSO reductase mutants are able to compete in competitive colonization assays. (A) *E. coli* EDL933 Δ(*dmsA-dmsB*) was able to co-colonize during competition with wild-type *E. coli* EDL933, (B) *E. coli* MG1655 Δ(*dmsA-dmsB*) was able to co-colonize during competition with wild-type MG1655.

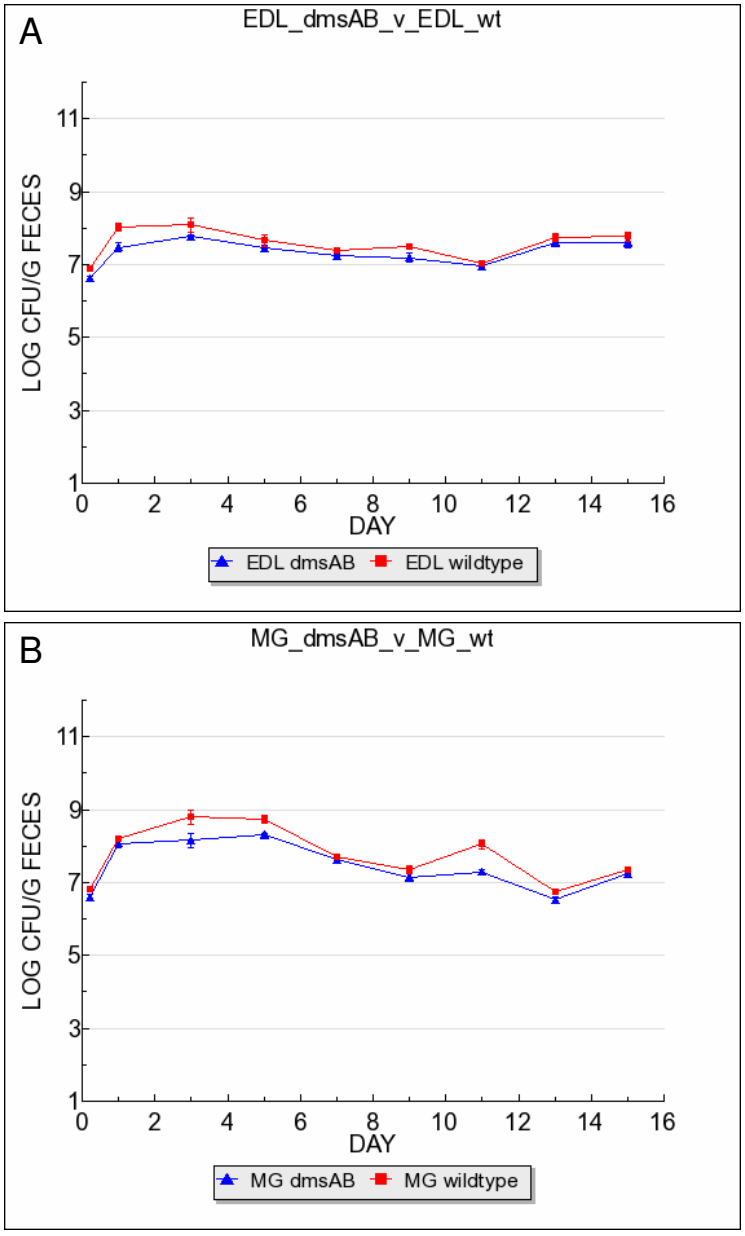

Figure A.2.11. *E. coli* alternative terminal reductase mutants co-colonized with wild-types.

Figure A.2.12. TMAO reductase mutants are able to compete in competitive colonization assays. (A) *E. coli* EDL933 Δ(*torC-torA*) was able to co-colonize during competition with wild-type *E. coli* EDL933, (B) *E. coli* MG1655 Δ(*torC-torA*) was able to co-colonize during competition with wild-type MG1655.

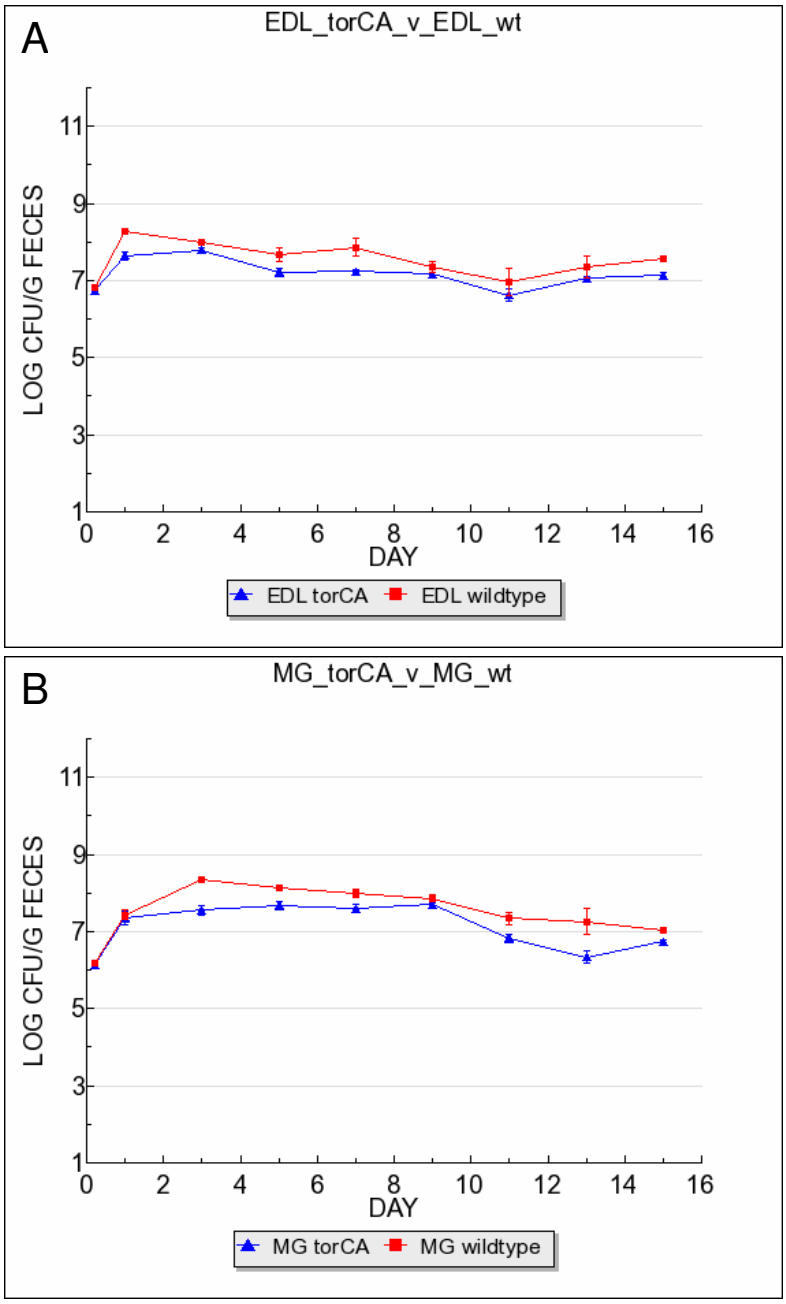

Figure A.2.12. *E. coli* alternative terminal reductase mutants co-colonized with wild-types.

Figure A.2.13. Nitrite reductase mutants are able to compete in competitive colonization assays. (A) *E. coli* EDL933 Δ(*nirB-nirD*) was able to co-colonize during competition with wild-type *E. coli* EDL933, (B) *E. coli* MG1655 Δ(*nirB-nirD*) was able to co-colonize during competition with wild-type MG1655.

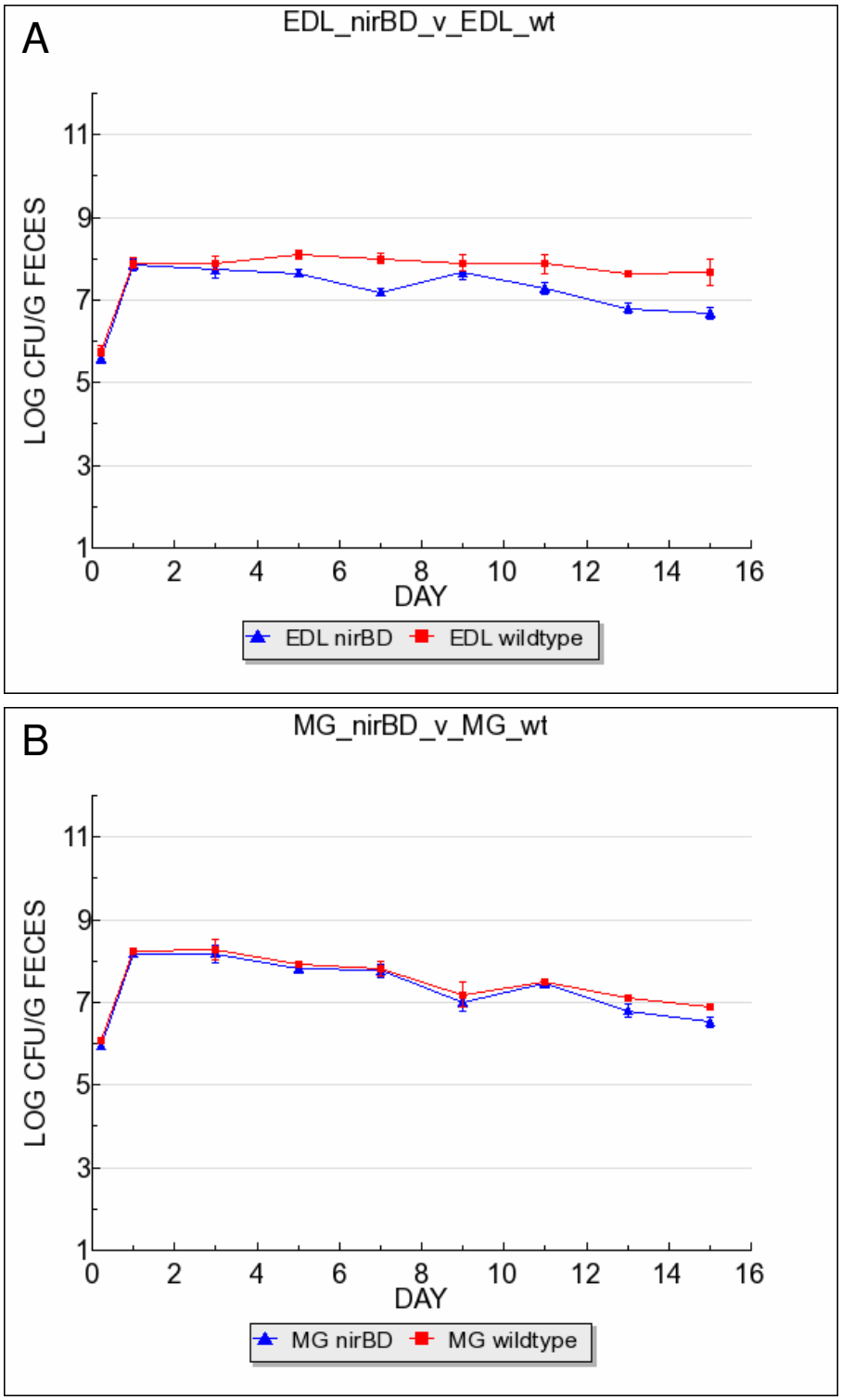

Figure A.2.13. *E. coli* alternative terminal reductase mutants co-colonized with wild-types.

Figure A.3.1. Aerobic respiratory global regulatory and cytochrome *bd* oxidase mutants exhibit colonization defects in competitive colonization assays. (A) *E. coli* MG1655 Δ*arcA* mutants are unable to compete during colonization with MG1655 Δ(*cydA*-*cydB*) mutant strains. (B) *E. coli* MG1655 Δ(*cydA*-*cydB*)::*cat* mutants are unable to compete during colonization with MG1655 Δ(*narG*)::*kan* mutant strains. (C) *E. coli* MG1655 Δ(*cydD*-*cydC*)::*cat* mutants are unable to compete during colonization with MG1655 Δ(*frdA*)::*kan* mutant strains.

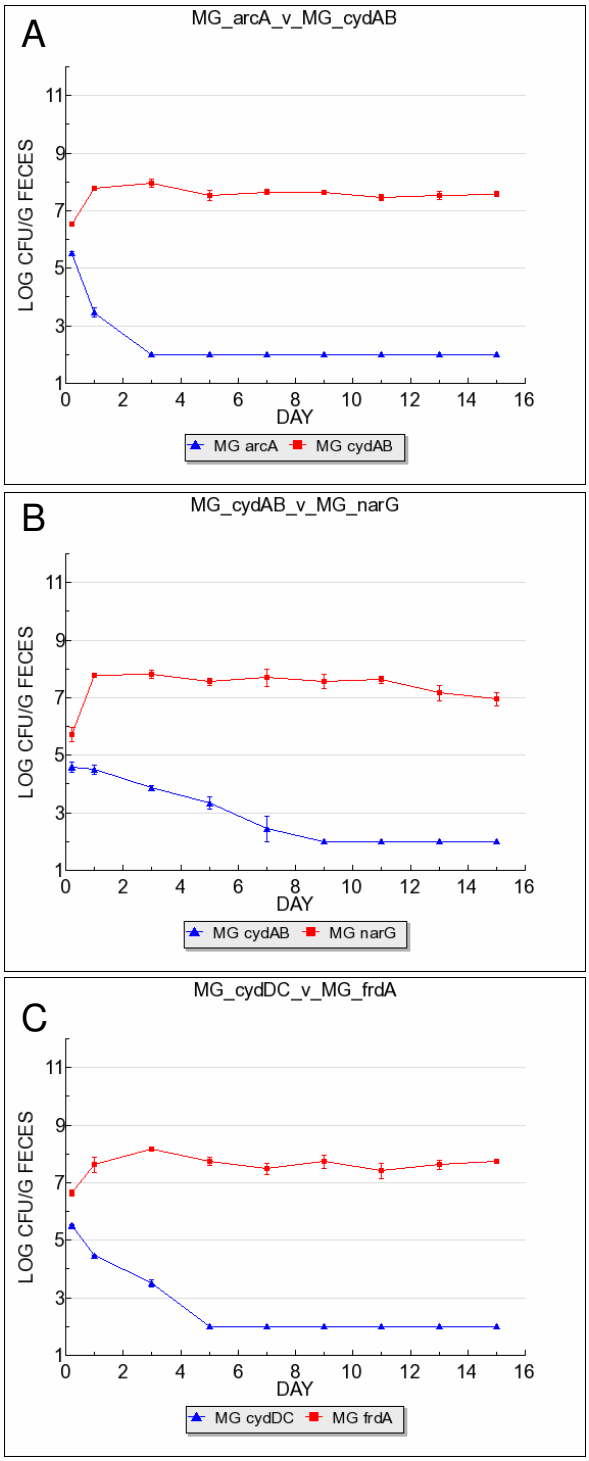

Figure A.3.1. ArcA and cytochrome *bd* oxidase mutants of *E. coli* MG1655 exhibit colonization defects during competitive competition.

Figure A.3.2. Primary nitrate reductase mutants exhibit colonization defects during competitive colonization assays with the secondary and periplasmic nitrate reductase. (A) *E. coli* MG1655 Δ(*narG*)::*kan* mutants are unable to compete during colonization with MG1655 Δ(*narZ*)::*cat* mutant strains. (B) *E. coli* MG1655 Δ(*narG*)::*kan* mutants are unable to compete during colonization with MG1655 Δ(*napD-napA*)::*cat* mutant strains.

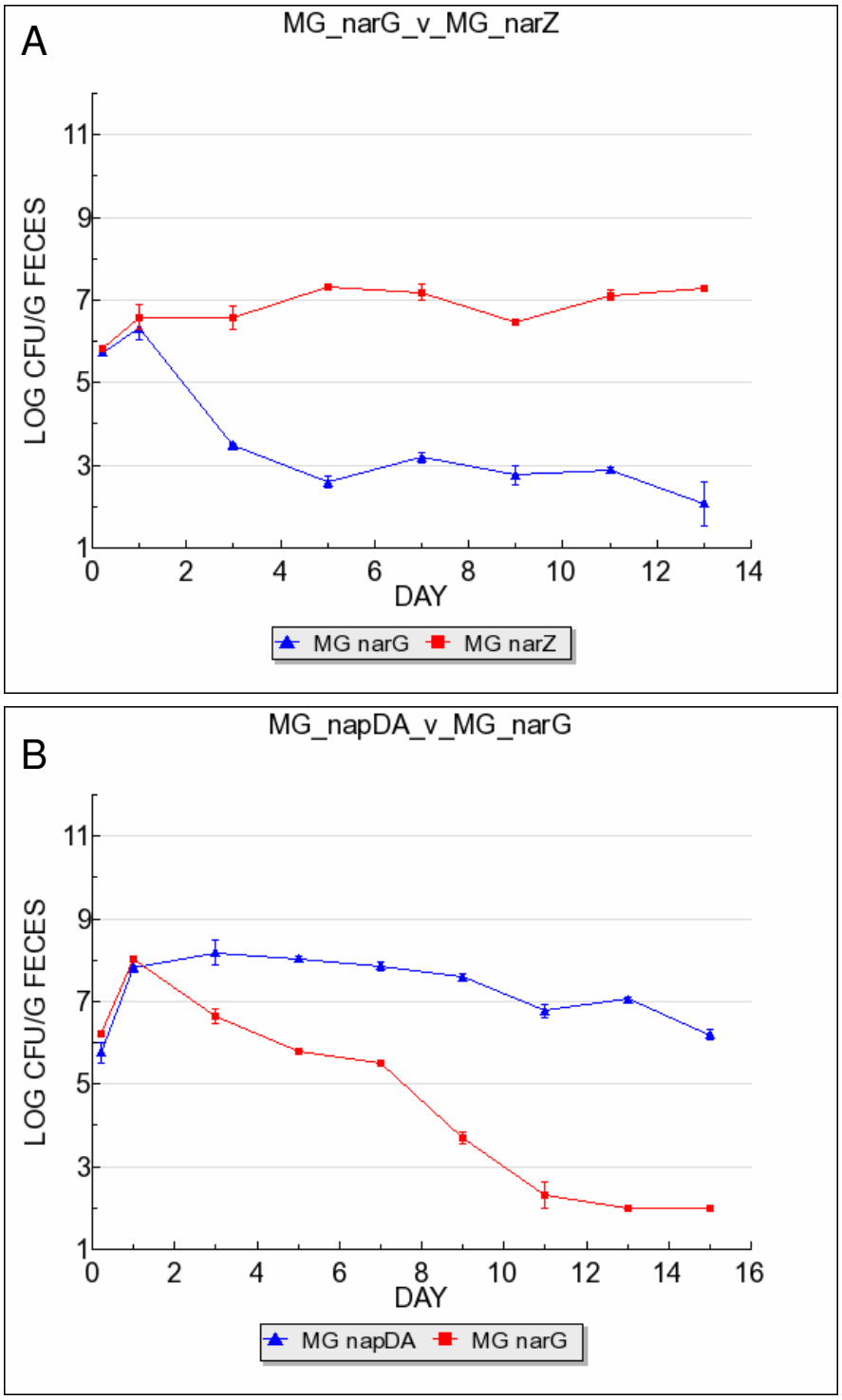

Figure A.3.2. Primary nitrate reductase mutants outcompete secondary and periplasmic nitrate reductase mutants in *E. coli* MG1655.

Figure A.3.3. Nitrate reductase mutant is outcompeted by fumarate reductase in maintenance not initiation during competitive colonization. *E. coli* MG1655 Δ*narG* Δ*narZ*::*cat* mutants are unable to compete initially during colonization with MG1655 Δ(*frdA*)::*kan* mutant strains, but able to maintain colonization in the mouse intestine.

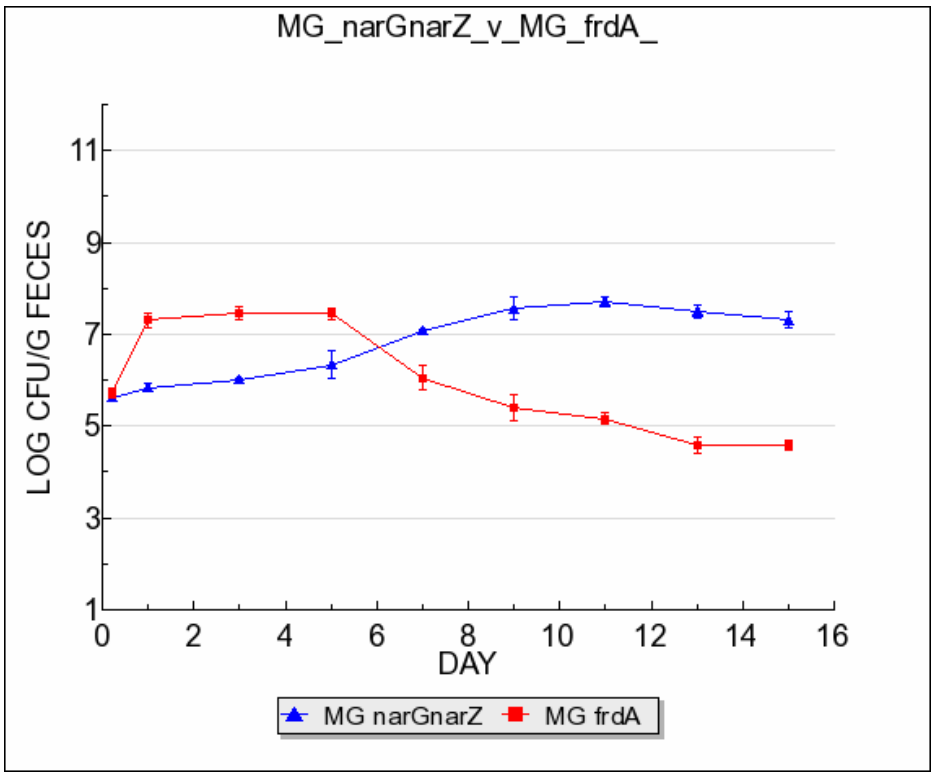

Figure A.3.3. Fumarate reductase mutant outcompetes a nitrate reductase mutant of *E. coli* MG1655 in initiation but not maintenance.

Table 4.1. Strains and plasmids used in this study.

Strain or plasmid reference	Relevant Characteristic(s)	Source or
MG1655	Wild-type (CGSC no. 7740)	*E. coli* Genetic Stock Culture Collection, Yale University
MG1655 Str^r	Spontaneous streptomycin-resistant mutant of MG1655	176
MG1655 Str^r Nal^r	Spontaneous nalidixic acid-resistant mutant of MG1655 Str^r	176
MG1655 Str^r Δ(*ubiC-ubiA*)::*cat*	*ubiCA* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ*ubiE*::*cat*	*ubiE* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ*menA*::*cam*	*menA* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ(*menA-menG*)::*cat*	*menAG* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ*menG*::*cat*	*menG* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
EDL933	Wild-type O157:H7	Alison O' Brien
EDL933 Str^r	Spontaneous streptomycin-resistant mutant of EDL933	174
EDL933 Str^r Nal^r	Spontaneous nalidixic acid-resistant mutant of EDL933 Str^r	174

EDL933 Str^r Δ(*ubiC-ubiA*)::*cat*	*ubiCA* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ*ubiE*::*cam*	*ubiE* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ*menA*::*cat*	*menA* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ(*menA-menG*)::*cat*	*menAG* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ*menG*::*cat*	*menG* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
pKD3	Template plasmid, contains chloramphenicol resistance cassette flanked by FLP recombinase target sites; *bla cat*	61
pKD46	Temperature-sensitive plasmid, contains arabinose-inducible λ phage red recombinase gene for linear DNA exchange; *bla*	61

Figure A.4.1. Ubiquinone mutants co-colonize during competitive colonization assays. *E. coli* MG1655 Δ(*ubiC-ubiA*) mutants are able to compete and display no colonization defects during competition with wild-type *E. coli* MG1655.

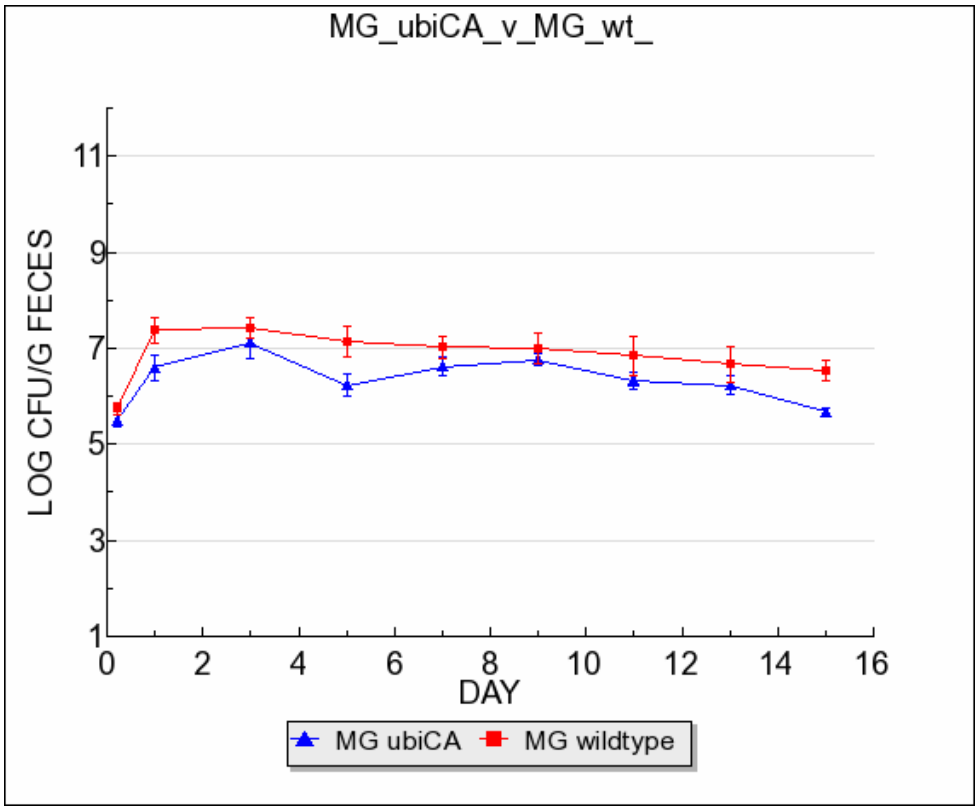

Figure A.4.1. Mutant defective in ubiquinone synthesis of *E. coli* MG1655 co-colonize.

Figure A.4.2. Ubiquinone and menaquinone mutants exhibit colonization defects in competitive colonization assays. (A) *E. coli* MG1655 Δ*ubiE* mutants are unable to compete with wild-type *E. coli* MG1655. (B) *E. coli* MG1655 Δ*ubiE* mutants are able to colonize when fed alone to mice.

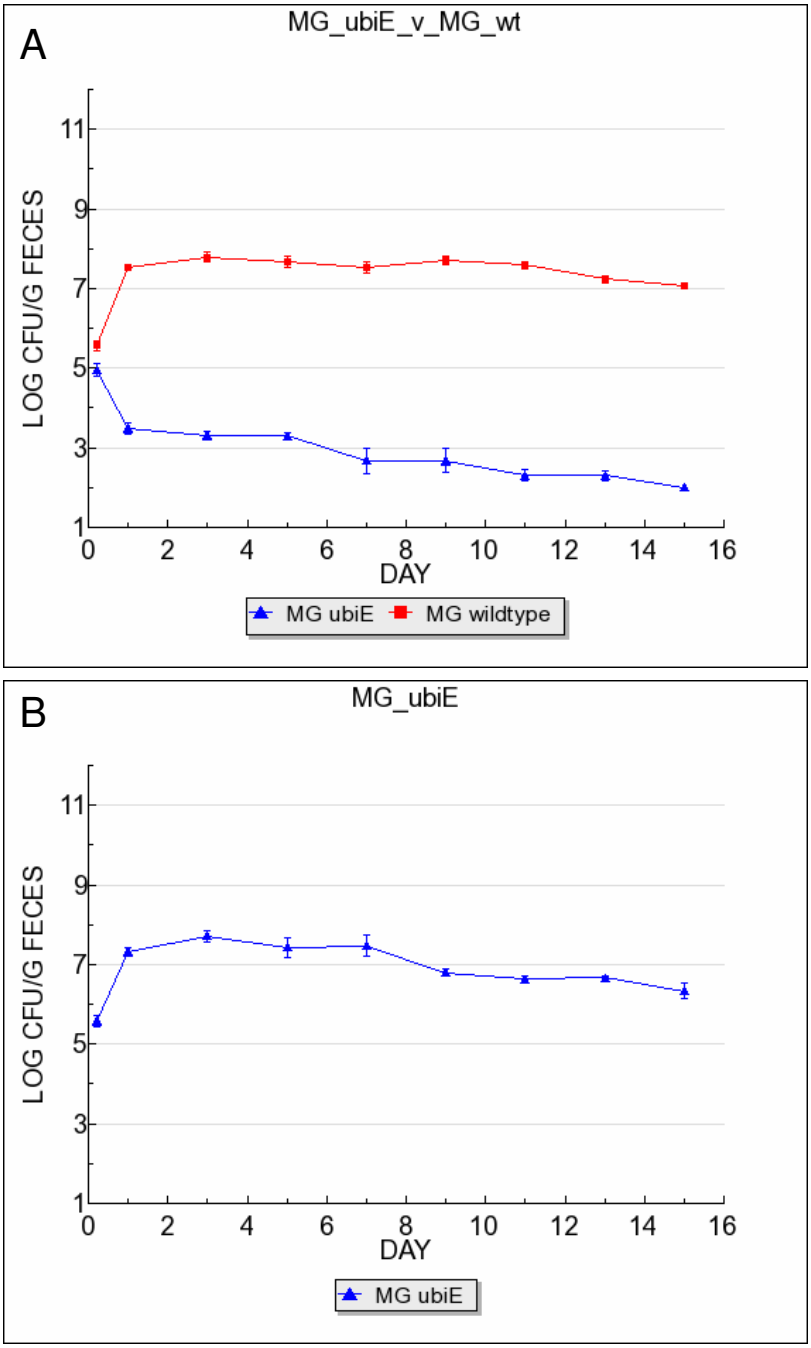

Figure A.4.2. Mutant defective in ubiquinone and menaquinone synthesis of *E. coli* MG1655 exhibit colonization defects.

Figure A.4.3. Demethylmenaquinone mutants exhibit colonization defects in competitive colonization assays. (A) *E. coli* MG1655 Δ*menA* mutants are unable to compete with wild-type *E. coli* MG1655. (B) *E. coli* MG1655 Δ*menA* mutants are able to colonize at wild-type levels when fed alone to mice.

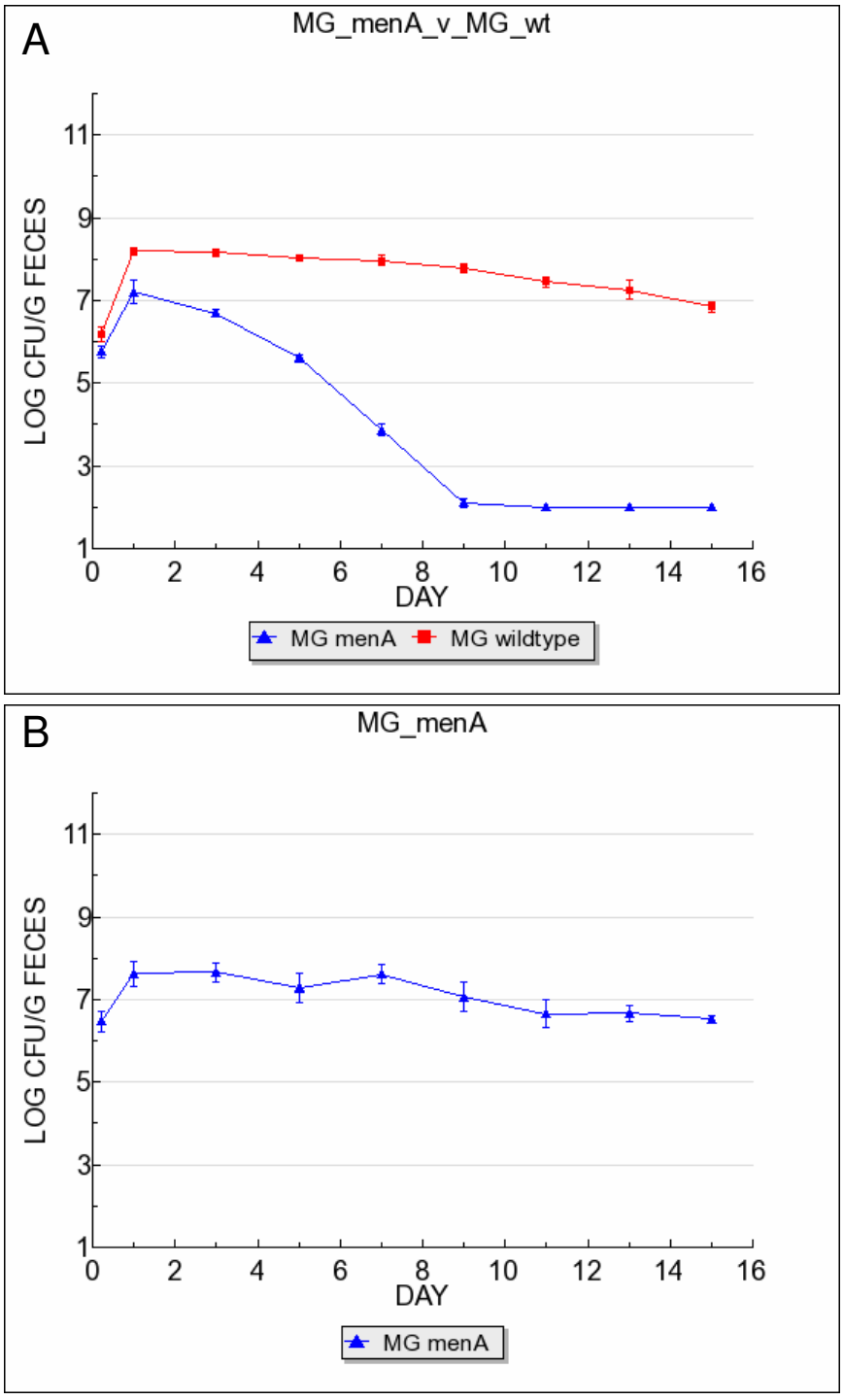

Figure A.4.3. Mutant defective in demethylmenaquinone synthesis of *E. coli* MG1655 exhibits colonization defect.

Figure A.4.4. (A) *E. coli* MG1655 Δ(*menA-menG*)::*cat* mutant is unable to compete during colonization with MG1655 and wild-type strains. (B) *E. coli* MG1655 and Δ*menG*::*cat* mutants are able to compete during colonization with MG1655 and wild-type strains.

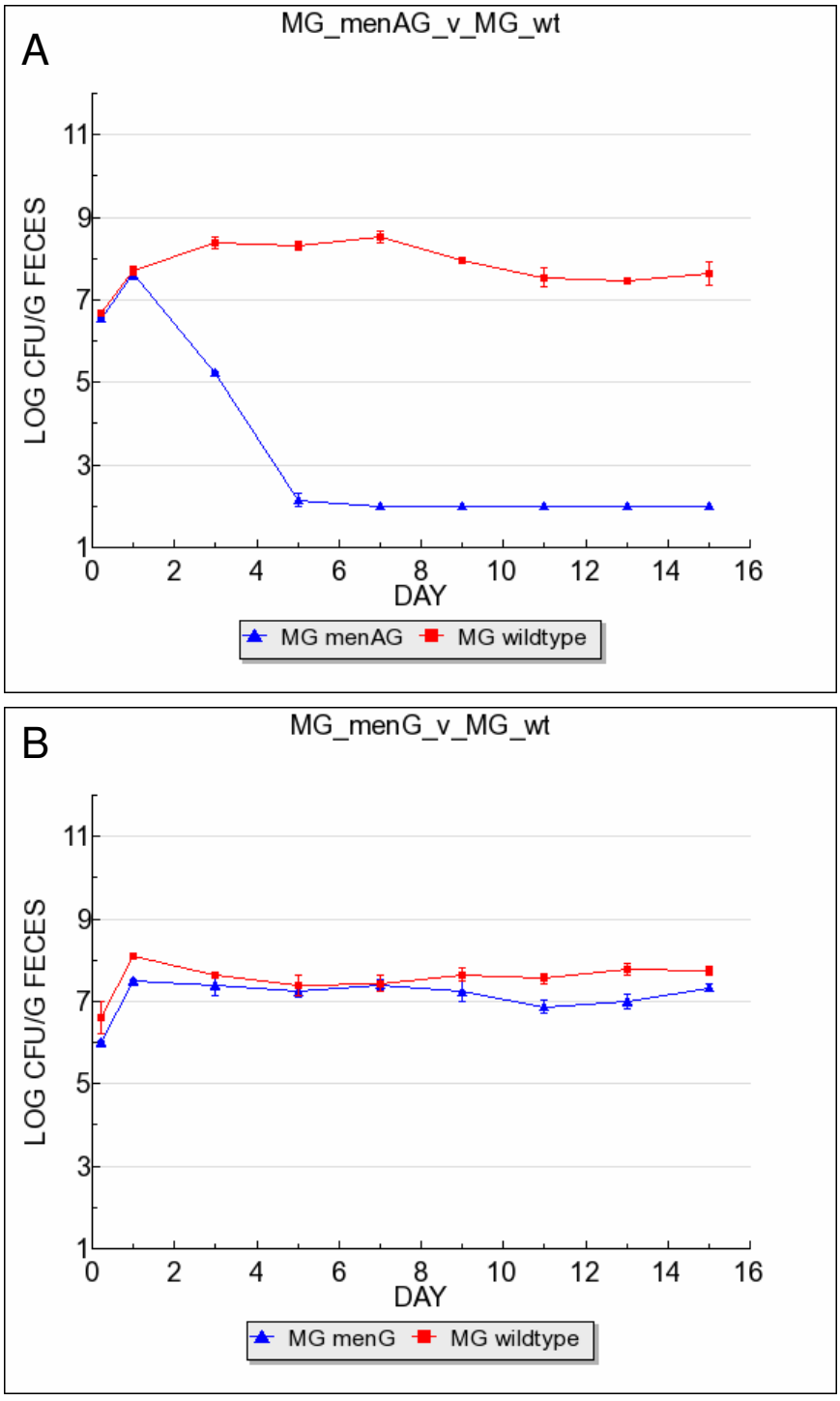

Figure A.4.4. Mutant defective in demethylmenaquinone synthesis not ribosome synthesis of *E. coli* MG1655 exhibits colonization defect.

Table A.5.1. Strains and plasmids used in this study.

Strain or plasmid reference	Relevant Characteristic(s)	Source or
MG1655	Wild-type (CGSC no. 7740)	*E. coli* Genetic Stock Culture Collection, Yale University
MG1655 Str^r	Spontaneous streptomycin-resistant mutant of MG1655	176
MG1655 Str^r Nal^r	Spontaneous nalidixic acid-resistant mutant of MG1655 Str^r	176
MG1655 Str^r Δ(*malE-malG*)::*cat*	*malEFG* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ*malX*::*cat*	*malX* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ(*menE-menG*) *malX*::*cat*	*menEFG malX* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ(*malP-malQ*)::*cat*	*malPQ* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ*lamB*::*cam*	*lamB* deletion mutant of MG1655 Str^r, carrying km-resistance cassette	This study
MG1655 Str^r Δ*glgP*::*cam*	*glgP* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ*glgA*::*cat*	*glgA* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study

MG1655 Str^r Δ*glgS*::*cat*	*glgS* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
MG1655 Str^r Δ*glgP malP*::*cat*	*glgP malP* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study
EDL933	Wild-type O157:H7	Alison O' Brien
EDL933 Str^r	Spontaneous streptomycin-resistant mutant of EDL933	174
EDL933 Str^r Nal^r	Spontaneous nalidixic acid-resistant mutant of EDL933 Str^r	174
EDL933 Str^r Δ(*malE-malG*)::*cat*	*malEFG* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ*malX*::*cat*	*malX* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ(*menE-menG*) *malX*::*cat*	*menEFG malX* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ(*malP-malQ*)::*cat*	*malPQ* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ*malP*::*cat*	*malP* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ*malQ*::*cam*	*malQ* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ*lamB*::*cat*	*lamB* deletion mutant of MG1655 Str^r, carrying cm-resistance cassette	This study

EDL933 Str^r Δ*glgP*::*cam*	*glgP* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ*glgA*::*cat*	*glgA* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ*glgS*::*cat*	*glgS* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
EDL933 Str^r Δ*glgP malP*::*cat*	*glgP malP* deletion mutant of EDL933 Str^r, carrying cm-resistance cassette	This study
pKD3	Template plasmid, contains chloramphenicol resistance cassette flanked by FLP recombinase target sites; *bla cat*	61
pKD4	Template plasmid, contains kanamycin resistance cassette flanked by FLP recombinase target sites; *bla kan*	61
pKD46	Temperature-sensitive plasmid, contains arabinose-inducible λ phage red recombinase gene for linear DNA exchange; *bla*	

Figure A.5.1. Mutants in the ATP-dependent ABC transporter of the maltose system display colonization defects. (A) *E. coli* MG1655 Δ(*malE-malG)* mutants are unable to compete during colonization with MG1655 wild-type strains. However, the PTS system of maltose transport exhibits no phenotype in the intestine. (B) *E. coli* MG1655 Δ*malX* mutants are able to co-colonize in competition with MG1655 wild-type strains. (C) *E. coli* EDL933 Δ*malX* mutants are able to co-colonize in competition with EDL933 wild-type strains (D) *E. coli* MG1655 Δ*malX* Δ(*malE-malG)* mutants are unable to compete during colonization with MG1655 wild-type strains as was seen in the single Δ(*malE-malG*) mutation.

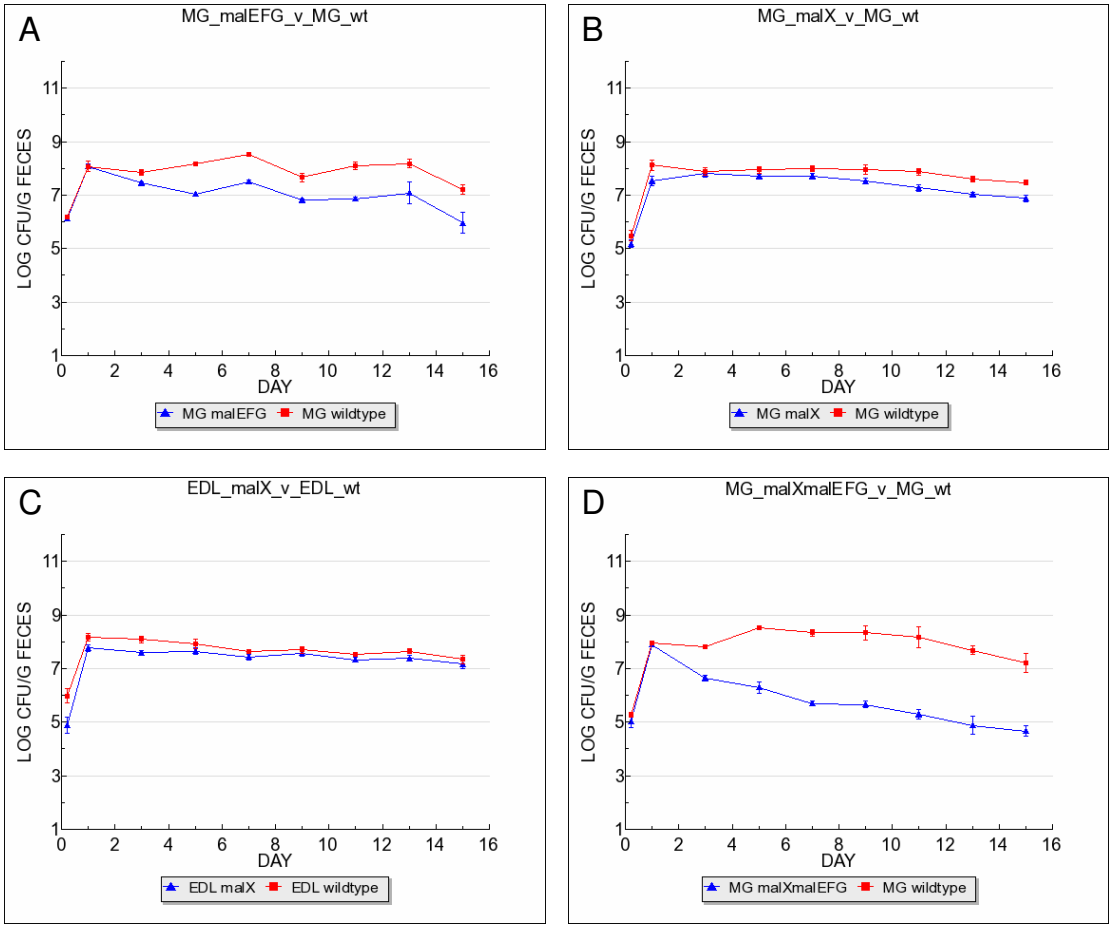

Figure A.5.1. Mutants in PTS maltose transport system in *E. coli* EDL933 and MG1655 co-colonize, while ATP dependent maltose transport mutants display colonization defects in *E. coli* MG1655.

Figure A.5.2. Utilization of maltose is essential for competitive colonization in the mouse intestine. (A) *E. coli* M G1655 Δ(*malP-malQ*) mutants are unable to compete during colonization with MG1655 wild-type strains.

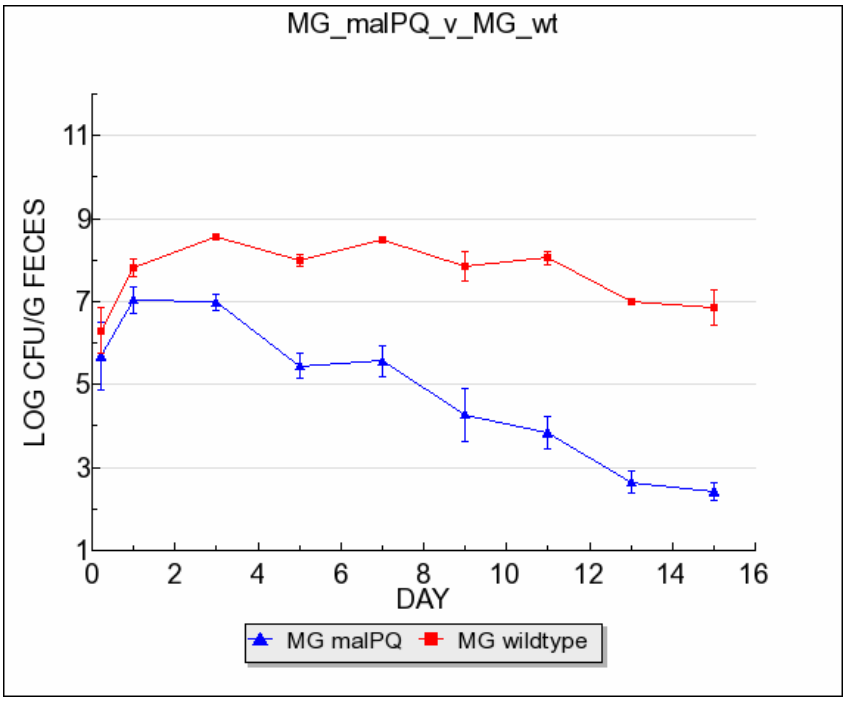

Figure A.5.2. Maltose utilization mutant in *E. coli* MG1655 exhibits a colonization defect.

Figure A.5.3. Mutants in the lambda receptor or maltoporin for maltodextrin transport exhibit no colonization defects and colonize the mouse intestine during competition. (A) *E. coli* EDL933 Δ*lamB* mutants are able to co-colonize with *E. coli* EDL933 wild-type strains. (B) *E. coli* MG1655 Δ*lamB* mutants are able to co-colonize in competition with *E. coli* MG1655 wild-type strains.

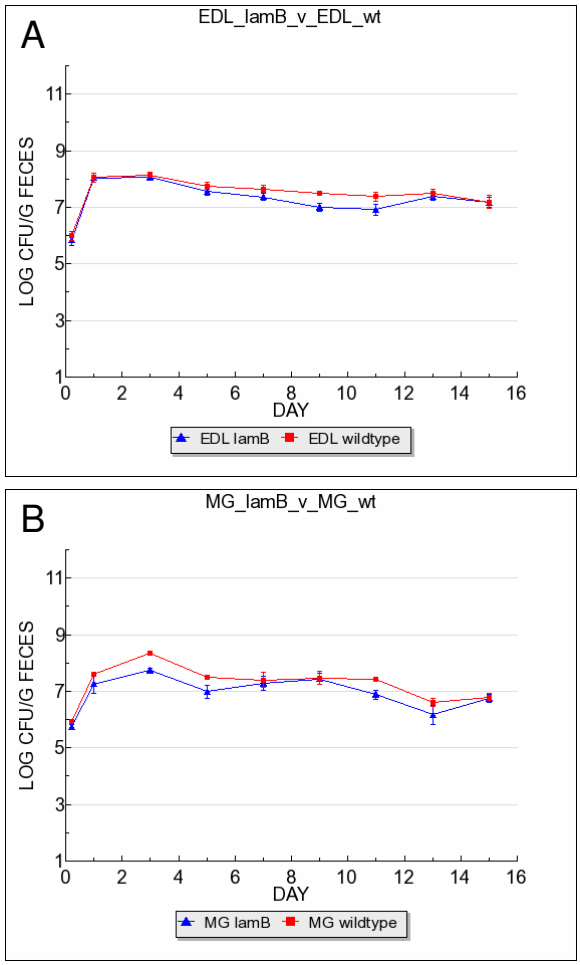

Figure A.5.3. Mutants in maltodextrin transport system in *E. coli* EDL933 and MG1655 co-colonize.

Figure A.5.4. Glycogen synthesis mutants are unable to effectively compete in the mouse intestine. (A) *E. coli* MG1655 Δ*glgA* mutants exhibit colonization defects in maintenance during competitive colonization with *E. coli* MG1655 wild-type strains. (B) *E. coli* MG1655 Δ*glgS* mutants also exhibit colonization defects in maintenance during competitive colonization with *E. coli* MG1655 wild-type.

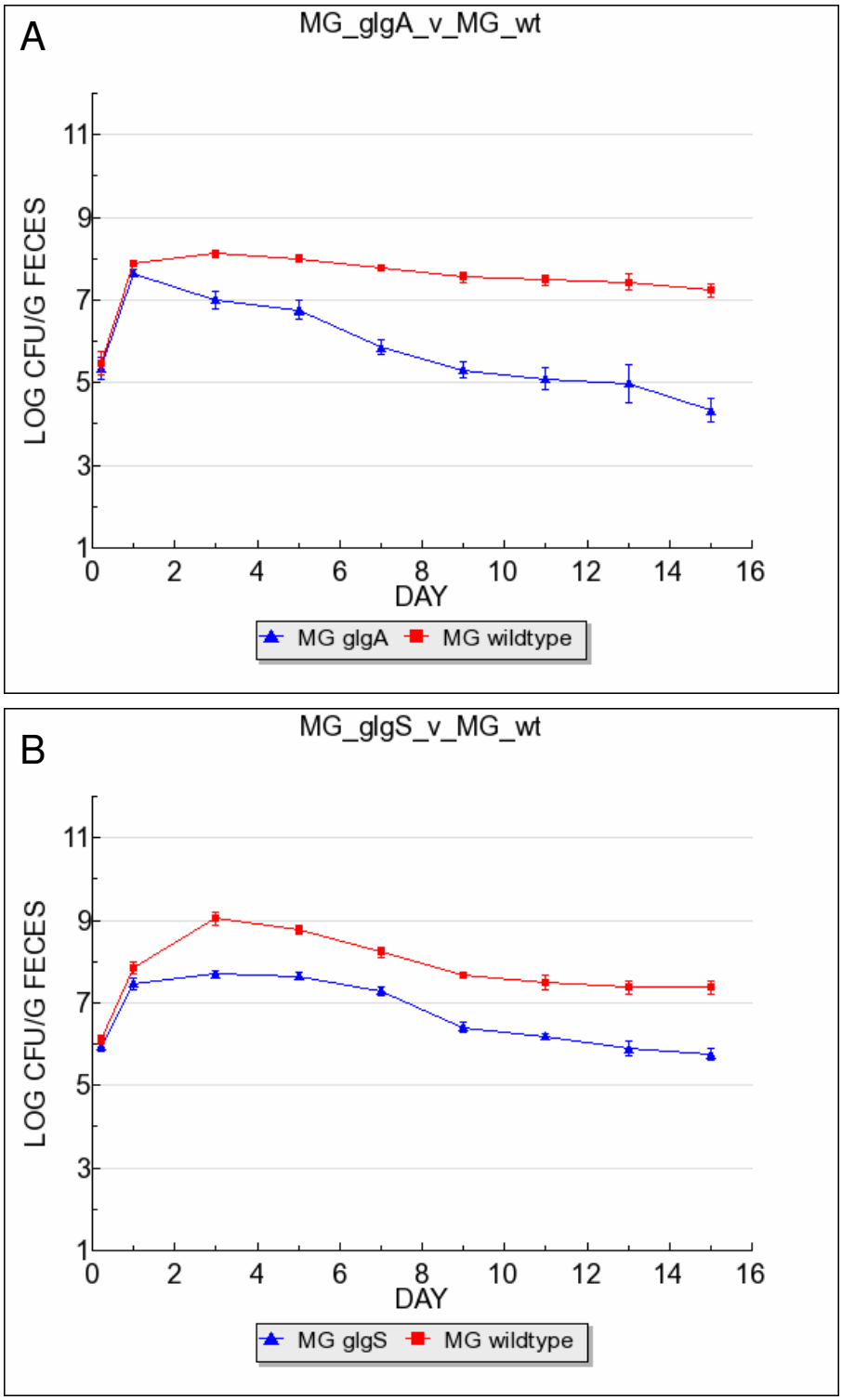

Figure A.5.4. Mutants in glycogen synthesis of *E. coli* MG1655 exhibit colonization defects.

Figure A.5.5. Glycogen degradation mutants are unable to compete in the mouse intestine. *E. coli* MG1655 Δ*glgP* mutants exhibit colonization defects during competitive colonization with *E. coli* MG1655 wild-type.

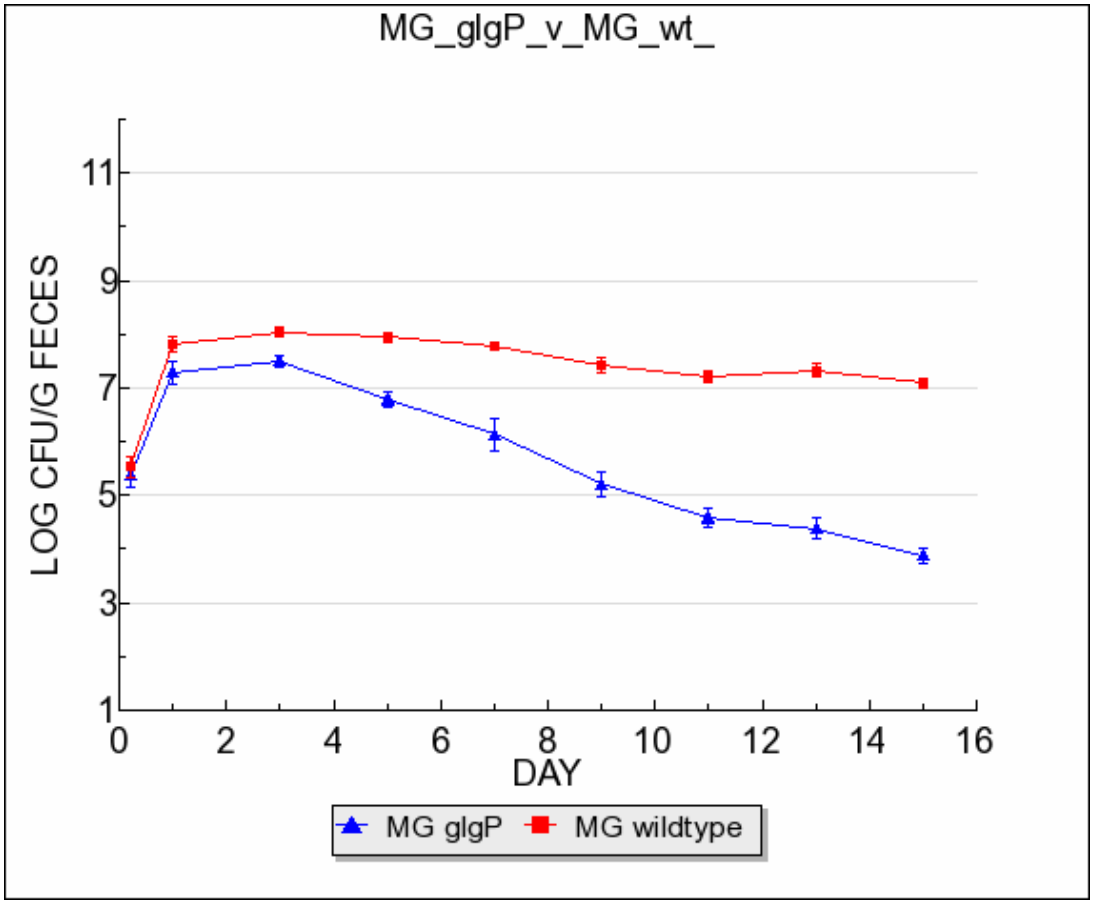

Figure A.5.5. Mutant in glycogen degradation of *E. coli* MG1655 exhibits a colonization defect.

Figure A.5.6. Gluconate rescues the colonization defect observed for glycogen degradation mutants during competition in the mouse intestine. *E. coli* MG1655 Δ*glgP* mutants co-colonized in competitive colonization with *E. coli* MG1655 wild-type.

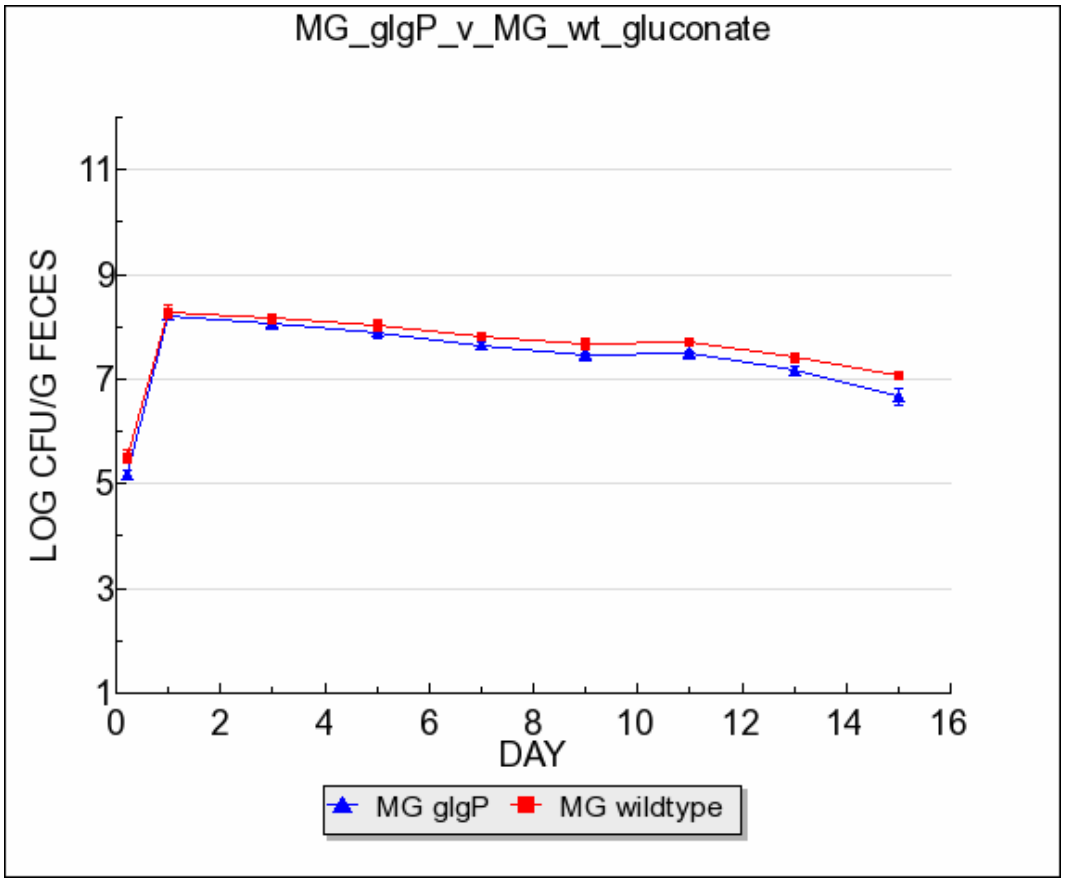

Figure A.5.6. Mutant in glycogen degradation of *E. coli* MG1655 colonizes with wild-type *E. coli* MG1655 when excess gluconate is supplied.