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AEROBIC RESPIRATION AND ECOLOGY OF *Escherichia coli*
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Abstract

The gastrointestinal tract contains a complex microbial ecosystem that exists in a symbiotic relationship with its host and plays an important role in maintaining human health. *Escherichia coli*, a facultative anaerobe, is a commensal bacterial resident of the gastrointestinal tract and has demonstrated the importance of aerobic respiration for its survival in this competition rich ecosystem. The gastrointestinal tract is also host to a diverse and numerically dominant population of obligately anaerobic bacteria that coexist with *E. coli* in the mucus layer adjacent to the intestinal wall. This dissertation explores the relationship between *E. coli* and its oxygen sensitive microbial cohorts in mouse cecum mucus. We produced and *in silico* model of oxygen diffusion from the intestinal wall into the mucus layer in the absence and presence of aerobically respiring *E. coli*. The model predicts a decrease in the oxygen concentration of the mucus layer in the presence of aerobically respiring *E. coli*. In order to confirm the prediction of our model, we performed T_2^* weighted Magnetic Resonance Imaging (MRI) on the ceca of conventional mice, mice that had their native facultative anaerobes removed by treatment with streptomycin sulfate, and mice that were treated with streptomycin sulfate and subsequently colonized with *E. coli* MG1655 wild-type Nal^r Str^r or *E. coli* MG1655Δ*cydAB* Δ*cyoAB::cat* Str^r, and isogenic cytochrome oxidase mutant that is unable to respire oxygen but is otherwise identical to the wild-type. T_2^* weighted MRI confirmed the prediction of our model in that conventional and wild-type colonized mice indicate lower cecal oxygen concentration than streptomycin sulfate only mice or those colonized with

the cytochrome oxidase mutant. In order to examine the hypothesis that aerobic respiration in *E. coli* has an impact on the ecology of cecum mucus, we conducted 16S rRNA pyrosequencing surveys of the same experimental conditions for the MRI experiment. The results indicate a difference in β-diversity between our streptomycin treated only mice and each of those that had been treated with streptomycin and subsequently colonized with the wild-type or the cytochrome oxidase mutant. The results of this work lay the foundation for examinations of the affect of probiotic *E. coli* strains on the total intestinal microbiota.

Chapter 1: Literature review and dissertation overview

The work presented in this dissertation originates from a renewed effort to understand intestinal microbial ecology resulting from the recent boom in sequencing and information technology and more specifically, how the activities of a signal species might influence the entire ecosystem to the benefit of human health.

The gastrointestinal tract and its commensal microbiota.

The gastrointestinal system is home to a dense, species rich microbial population (45, 61, 64, 67). The relationship between the host and its commensal microbiota is symbiotic and governed by a variety of factors including nutrient availability, resistance to pathogens, and immune system development (32, 69). Newborn infants are rapidly colonized with simple microbial communities that chaotically mature into populations that remain relatively stable in adulthood (32, 34, 54, 56). The process of bacterial succession following birth supports ecological theories regarding community development and succession, in which opportunistic species facilitate the arrival of and are gradually replaced by those more suited for long-term survival in that environment (8, 50). The final composition of the mature microbiota can be effected, positively or negatively, by host genetics, method of birth, diet, antibiotics, immune responses, invading pathogens, prebiotics, and probiotics (14, 32, 33, 54). Homeostasis between host and commensal microbiota and within the various sub-populations of the intestinal microbiota contributes to its ability to resist pathogenic invasion.

Most of the communication between host and the intestinal microbiota occur in the mucus layer lining the epithelium of the intestinal wall (13). The gastrointestinal mucus layer is continuously secreted by intestinal epithelial cells and varies in thickness (200 – 800 μm) from stomach to colon (3). It provides a protective barrier to the epithelial lining and governs oxygen diffusion as well as spatial distribution of the microbiota (63, 72). The outer layer is continuously sloughing as food passes through the lumen, and it is a readily available nutrient source for many of the bacterial residents of the gastrointestinal tract (40). There is a relatively thin, firmly adherent layer (116 ± 51 μm), which provides an important physical and chemical barrier to luminal contents including pathogenic microorganisms (1, 3, 38). The bacterial community residing in the mucus layer is distinct from those found in the lumen (49).

The gastrointestinal tract operates like an *in vivo* chemostat. However, it is a surprisingly heterogeneous ecosystem in which competition for resources or an ability to overcome the physical effects of peristalsis and mucus secretion from the intestinal wall are key for microbial species to maintain stable populations (18). The cecum comprises the anterior portion of the ascending colon, receives digesta from the small intestine, and has a higher concentration of fatty acids and lower pH than the descending colon (42). Facultative anaerobes and obligate or aerotolerant anaerobes are two distinct sub-populations of bacteria found in the cecum that have functionally distinct ecosystem roles. Obligate and aerotolerant anaerobes are primarily located in the large intestine and degrade complex carbohydrates by way of exo-enzymes.

Their metabolic end-products provide nutrients to the host or neighboring microorganisms in addition to chemically altering the environment to benefit the host (69). Facultative anaerobes can be found throughout the gastrointestinal tract and have been implicated as necessary for establishment of commensal obligate anaerobes in infants and as useful probiotic treatments for a variety of intestinal disorders (12, 35, 54). Together, these sub-populations work in concert to regulate succession of the intestinal microbiota in newborns, promote stability of the microbial population, and provide colonization resistance to invading pathogenic bacteria (22, 83). The Firmicutes are the largest clade of anaerobic bacteria in the intestine and, along with the Bacteroidetes phylum, constitute the largest population of anaerobic bacteria in large intestine. The Bacillus and the Gammaproteobacteria both include several genera of facultative anaerobes that are known to inhabit various regions of the gastrointestinal tract (16).

Influence of Escherichia coli and oxygen in the intestine.

Escherichia coli, a well-known model bacterium, is a facultative anaerobic member of the Gammaproteobacteria and is a commensal resident of both human and mouse intestines (5). It is a relatively minor constituent of the intestinal microbial population, just 0.1% (1×10^8 cells/ml intestinal mucus) of the total bacterial load in mice, and resides in the mucus layer coating the epithelial cells lining the intestinal wall (59, 65). It is ubiquitous in the intestines of mammals, and has demonstrated biochemical and probiotic benefits to its host (4, 36). The pan-genome of *E. coli* represents ~13,000 genes, and some serotypes are emerging pathogens that will cause disease if they escape the

intestine or supplant commensal strains in the intestine (31, 60). *Shigella* sp., a close relative of *E. coli*, is also notorious for causing disease, though it is not considered an intestine commensal (25). A pathogenic *E. coli* strain must overcome colonization resistance imparted by commensal strains through nutrient niche-defined occupation in an otherwise healthy host (36, 78).

E. coli, is highly adaptable to changes in its environment, and can respire aerobically or anaerobically as well as use a variety of fermentative pathways, but its regulatory pathways are precisely regulated to allow it to take full advantage of even nanomolar concentrations of oxygen (68, 70, 71). *E. coli* has two cytochrome oxidases, cytochrome *bd* oxidase and cytochrome bo_3 oxidase, that it selectively uses under low or high concentrations of oxygen respectively (20). Both of these oxidases are heme proteins, which catalyze the reduction of oxygen to water as the terminal step in aerobic respiration (19, 46). Under anaerobic conditions, they will switch to fermentation or can use several alternative electron acceptors including: nitrate, fumarate, DMSO, and TMAO in order of preference (20). *E. coli* resides amongst obligate anaerobic bacteria in the mucosal layer 20 - 60 μm from the intestinal wall (9, 47, 48, 59, 72, 79). Oxygen enters the gastrointestinal tract by way of swallowed air and by diffusion from the dense capillary beds underlying the intestinal epithelial layer (7, 10, 11, 26-28, 37, 80, 81). Oxygen tension near the luminal wall of the intestine (~15 mmHg) is high enough to support aerobic respiration in *E. coli* (7, 10, 37, 57, 77). The ability to respire oxygen provides a competitive advantage *in vivo*,

suggesting that this microorganism may reduce the oxygen content of its environment (30).

Outstanding questions

The gastrointestinal tract is home to a complex microbial ecosystem that has an enormous impact on the health of its host (17, 51, 79). To date only a fraction of the microorganisms from this ecosystem have been cultivated and classified, and our knowledge of their *in vivo* associations and activities is limited (15). Obligate anaerobes, microorganisms that are highly sensitive to oxygen, dominate the gastrointestinal ecosystem. However, the concentration of oxygen in the mucus layer, where many of these bacteria thrive, should be inhibitory to these oxygen sensitive bacteria (28, 37). The native microbiota reduce oxygen tension in the intestine, but it is unclear which bacteria are responsible for this activity (6, 7). Facultative anaerobes, such as *E. coli*, reside in the gastrointestinal tract, and are well equipped to both benefit energetically from oxygen and prevent damage from toxic radicals. Other, anaerobic residents of the intestine are able to tolerate the toxic effects of oxygen, through production of superoxide dismutase or catalase, and a few have recently been found to respire oxygen in nanomolar concentrations, most likely as a means to protect anaerobic pathways (55, 62, 71, 76). Concurrently, these preventative measures may also reduce the molecular oxygen content of the environment. At least one member of the other large group of facultative anaerobic bacteria found in the intestine, the Lactic Acid Bacteria, do not express genes for aerobic respiration *in vivo* (41). Therefore, *E. coli* may be one of the few inhabitants of the gastrointestinal tract

using aerobic respiration as a means of survival in this competition rich and nutrient replete ecosystem.

Aerobes and aerobically respiring facultative anaerobes, including *E. coli*, have demonstrated the ability to create conditions *in vitro* in which obligate anaerobes can grow under oxygen tensions they could not survive alone (21, 24, 39). Previous *in vitro* studies have indicates that microniches are most likely an important aspect of microbial ecology, and it is probable that they are vital to the survival of the numerically dominant obligate anaerobes, particularly in the gastrointestinal mucus where the oxygen tension is highest (39, 58, 84). Even though *E. coli* is a relatively minor constituent of the intestinal microbiota, oxygen scavenging by this microorganism may be a vital ecosystem function. The ability of the commensal microbiota to protect its host from invading pathogens depends on the stability of the microbial population as a whole (2, 22, 66, 73, 83). Macro-ecologists have demonstrated that a loss of one member of a food web often leads to loss of diversity in the entire ecosystem (53). Additionally, loss of diversity has been linked to disease in the gastrointestinal tract, despite its apparent functional redundancy (52, 75, 85). It would be interesting to know if *E. coli* is promoting diversity, stability and health by reducing oxygen tension in the gastrointestinal ecosystem.

Preamble to Chapter two: aerobic respiration in *Escherichia coli* influences oxygen tension in the mucus layer of the intestine.

When I started my graduate student career with Dr. Conway in the fall of 2005, Dr. Shari Jones, another of Dr. Conway's graduate students at that time,

was in the middle of a series of mouse colonization experiments investigating the respiration of *E. coli* in the mouse intestine. She had recently discovered that a *cydAB* (cytochrome *bd* oxidase) mutant was unable to compete with its wild type parent, indicating that oxygen scavenging in the intestine might be important for *E. coli* to survive in this competition rich and nutrient depleted environment (29). *E. coli* is finely tuned to take full energetic advantage of any oxygen available in its environment. To maximize its aerobic potential, *E. coli* possess two enzymes, which it uses to reduce oxygen to water in the terminal respiration step (20). Despite previous conception of the gastrointestinal tract as an anaerobic digester, there is enough oxygen available to support aerobic respiration in *E. coli* (7). A computer model of oxygen diffusion from the capillary beds underlying the intestinal epithelial layer provided a conceptual starting point for modeling oxygen diffusion into the lumen of the intestine (23). The work described in the second chapter of this dissertation is an effort to calculate oxygen availability and the ability of *E. coli* to respire oxygen in the intestine. This work produced a computer model of oxygen diffusion from the epithelial layer of the colonic intestinal wall and the influence *E. coli* has on the distance oxygen is able to diffuse into the lumen of the intestine. The biological implications of the resulting prediction are discussed.

Preamble to Chapter three: T_2^* weighted magnetic resonance imagining can detect *Escherichia coli* cytochrome oxidase activity in the mouse cecum.

Having developed a computer model of the impact *E. coli* aerobic respiration has on oxygen diffusion from the intestinal wall, we wanted to confirm the predictions experimentally using the least invasive methods available, especially since the theoretical impact *E. coli* oxygen respiration has on the distance oxygen diffuses from the epithelial layer may be pathogenically significant (43, 44). The presence of oxygen in the gastrointestinal tract has been determined many times using point measurements with an oximetric probe, but this technique is invasive and limited in its scope. One group used electromagnetic pulse resonance imagine to measure oxygen tension throughout the gastrointestinal tract in a live animal, which is more informative and noninvasive, though it still requires the use of an ingested probe (27). After some discussion, Dr. Conway and I decided to bring our idea to the attention of our colleagues in Dr. Rheal Towner's lab at the Oklahoma Medical Research Foundation. They presented a novel application of T_2^* weighted magnetic resonance imaging (MRI) as a potential completely noninvasive method for measuring oxygen availability in the mouse intestine. T_2^* weighted MRI is sensitive to the arrangement of protons surrounding iron atoms, which are the principle component of the heme molecules in cytochrome oxidases (19, 74, 82). *E. coli* employs two cytochrome oxidases: cytochrome bo_3 oxidase has a low affinity for oxygen and has optimal activity in fully aerobic conditions; cytochrome *bd* oxidase has a relatively high affinity for oxygen and has optimal activity in microaerophilic conditions. The work described in the third chapter of this dissertation follows an effort to measure oxygen tension under various

experimental conditions designed to validate the prediction of the computer model from chapter two. We used T_2^* weighted MRI to examine oxygen availability in the ceca of: conventional mice, streptomycin treated only mice, and mice treated with streptomycin and subsequently colonized with *E. coli* MG1655 wild-type Nal^r Str^r or *E. coli* MG1655Δ*cydAB* Δ*cyoAB::cat* Str^r. We found that conventional mice and mice colonized with the wild-type had significantly more oxygen bound to cytochrome oxidase than streptomycin treated mice and mice colonized with the isogenic cytochrome oxidase mutant. These results confirmed the prediction of the computer model and suggested that oxygen scavenging by *E. coli* in the intestine could have an impact on gastrointestinal ecology.

Preamble to chapter four: *Escherichia coli* influences gastrointestinal ecology.

Having determined that *E. coli* reduces oxygen tension in the intestine, we wanted to examine the influence this process has on the ecology of the intestine. This is the focus of Chapter 4. The gastrointestinal tract is home to a diverse and numerically dominant anaerobic bacterial community (16). Many of these organisms are negatively affected by oxygen in their environment, thus it is interesting to examine the impact *E. coli* oxygen respiration has on the composition of the anaerobic bacterial community. In order to do this we performed 16S rRNA pyrosequencing of DNA isolated from cecum mucus under the same experimental conditions as for the T_2^* weighted MRI experiment: conventional, streptomycin treated only, and mice colonized with *E. coli* MG1655

wild-type or *E. coli* MG1655 Δ*cydAB* Δ*cyoAB::cat*. The results suggest that *E. coli* does influence the obligately anaerobic bacterial community, but aerobic respiration alone is insufficient to explain all of the changes. Implications of this result are discussed.

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Chapter Two: Modeling oxygen diffusion and aerobic respiration in *E. coli* in the mouse cecum.

INTRODUCTION

The mouse intestine is host to hundreds, possibly thousands, of different species of bacteria, which, together, have an enormous impact on the health of their host (15, 43). To date only a fraction have been cultivated and classified, and our knowledge of their roles in their natural environment is limited (14). Obligate anaerobes, microorganisms that are highly sensitive to oxygen, are numerically dominant in the gastrointestinal ecosystem (20). However, the stomach and lumen of the intestines contains oxygen, with a decreasing amount as ingested material travels from the stomach to rectum (27). Facultative anaerobes also reside in the gastrointestinal tract, and are better able to survive oxygen, as well as use it to their advantage in a highly competitive, resource limited environment. *Escherichia coli* could be considered the most well known facultative anaerobic resident of both human and mouse intestines (5). In fact, it has been found in the intestines of almost every mammal investigated (52). Very little is known about the effect of oxygen-respiring facultative anaerobes on the anaerobic population in this ecosystem. Removal of one function from a food web can result in loss of diversity and destabilization of an ecosystem (45, 54). Although *E. coli* is not the only member of the intestinal ecosystem capable of respiring oxygen, this is an ecosystem function with limited redundancy in the large intestine due to the relative scarcity of strict aerobes and the demonstration of a preference for fermentation in other facultative anaerobes known to inhabit the intestine, such as the Lactic Acid Bacteria (35). A clear understanding of the relationship between facultative anaerobes, such as *Escherichia coli*, and the

oxygen sensitive obligate anaerobe population is important, because host resistance to infectious disease depends on the functionality of the microbial population as a whole (19, 54, 59, 69).

Facultative anaerobes will preferentially respire oxygen for growth if it is available, because it is energetically favorable to do so. *E. coli* has two cytochrome oxidases: cytochrome *bd* oxidase and cytochrome bo_3 oxidase, which it selectively uses under low or high concentrations of oxygen respectively (17). Commensal *E. coli* lives amongst the obligate anaerobic bacteria in the mucosal layer 20 - 60 μm from the intestinal wall (9, 38, 48, 58). The gastrointestinal mucus layer is continuously secreted by intestinal epithelial cells and varies in thickness from stomach to colon (200 – 800 μm). It provides a protective barrier to the epithelial lining and governs oxygen diffusion as well as spatial distribution of the microbiota. The outer layer is continuously sloughing as food passes through the lumen, but there is a relatively thin, firmly adherent layer, which provides a more stable physical and chemical barrier to luminal contents including pathogenic microorganisms. In the colon the firmly adherent layer is 116 ± 51 μm thick (1, 3, 33, 51, 58). Oxygen readily diffuses from the capillary beds across the epithelial layer of the intestinal wall into the lumen (11, 24, 67, 68). Several studies have demonstrated that oxygen tensions near the luminal wall of the intestine (~15 mmHg) are above the minimum required for the cytochrome oxidases of *E. coli* (7, 10, 32, 63). Incidentally, this relatively high concentration of oxygen should be inhibitory to many if not all of the obligate anaerobic residents of the gastrointestinal tract (29, 32).

Recent studies investigating intestinal colonization of respiratory mutants revealed the importance of aerobic respiration in this nutrient replete, highly competitive ecosystem (30). *E. coli* is a minor constituent of the intestinal microbiota, but oxygen scavenging may be a pivotal ecosystem function, i.e., *E. coli* will preferentially use oxygen as an electron acceptor and create an environment more suitable for the numerically dominant obligate anaerobes. Germ-free animals have higher oxygen tensions than conventional animals (6, 7), which supports the concept that the native microbiota, *E. coli* included, are able to reduce the oxygen tension of the colon. A theoretical examination of whether or not the resident *E. coli* population is capable of altering the oxygen content of the intestinal lumen could begin to address this hypothesis. Modeling is a valuable technique for investigating biological systems, which cannot be easily studied *in vivo* or for predicting outcomes in well defined systems under theoretical conditions (22). Mathematical models have been employed to understand ecosystem dynamics, substrate limitation, and microbial population dynamics (8, 13, 22, 31, 49, 50, 55, 56, 60, 61). Several *in silico* investigations of the intestinal microbiota have provided insights into infection, host-microbiota interactions, and oxygen diffusion (12, 21, 71). Models of aerobic respiration by *E. coli* under a variety of *in vitro* conditions have also been reported (40, 73). We designed an *in silico* model of oxygen diffusion from the capillary beds just below the epithelial cell layer lining into the lumen of the colon with and without aerobically respiring *E. coli*. Since enough oxygen is available at the intestinal wall to support aerobic respiration by *E. coli*, we hypothesize that *E. coli* cells

residing in the adjacent mucus layer will reduce the oxygen tension as it diffuses from the intestinal wall toward the lumen, thus providing a more anaerobic environment for the numerically dominant obligate anaerobe bacteria.

MODEL DESIGN AND IMPLEMENTATION

Mathematical description. We constructed a deterministic, two-part model to examine first, how much oxygen diffuses from the epithelial layer of the intestinal wall into the lumen, and second, to what degree aerobic respiration in *E. coli* influences the oxygen tension in the mucus layer. The diffusion process involves a change in the concentration of a solute over time inside the spatial interval Δx and is treated mathematically as an ordinary differential equation known as Fick's Second Law of diffusion (Eq. 1) (16).

$$\frac{\partial[O_2]}{\partial t} = -D\frac{\partial^2[O_2]}{\partial x^2} \qquad (1)$$

The rate of diffusion is determined by calculating the diffusion of oxygen from its starting point, the intestinal wall, and is governed by the diffusivity of oxygen in intestinal mucus, *D*. Diffusivity is governed by the viscosity of the medium through which a given substance is diffusing and the temperature of the system. The respiration of oxygen by actively respiring *E. coli*, is determined by Monod kinetics (39, 64, 72).

$$-\frac{\partial[O_2]}{\partial t} = kN\frac{[O_2]}{K_S + [O_2]} \qquad (2)$$

Where k = the maximum uptake rate for oxygen, N = the number of cells in intestinal mucus, and K_S = the half-saturation constant of oxygen.

Combined in Eq. 3, the two processes determine how much oxygen is able to diffuse from the surface of the intestinal wall into the lumen, where a dense, oxygen sensitive anaerobic bacterial population resides.

$$\frac{\partial[O_2]}{\partial t} = -D\frac{\partial^2[O_2]}{\partial x^2} - kN\frac{[O_2]}{K_S + [O_2]} \quad (3)$$

The values for the model parameters are reported in Table 1. Given a known *[O_2]* at *t=0* and *x=0*, we can compute Eq. 1 to examine how much oxygen will diffuse into the lumen in the absence of *E. coli* and Eq. 3 in the presence of *E. coli.* We restricted aerobic respiration in *E. coli* to oxygen concentrations above 0.0121 (or 1/10th the K_S). Thus, we can measure the theoretical impact *E. coli* oxygen scavenging has on the oxygen tension in the lumen of the intestine.

Conceptualization and implementation. A conceptual representation of the model can be seen in Fig. 1, where the influx of oxygen from the capillary beds across the epithelial layer is influenced by the diffusivity of oxygen in the mucus layer and the rate of oxygen consumption by *E. coli*. The intestinal environment was divided into layers that are 5 μm thick, with a maximum distance from the epithelial wall set at 2.5 mm (an estimate of the radius of an average mouse cecum). An algorithm was constructed to aid in development of a program to calculate the both equations (Fig. 2). Each layer is allowed to reach equilibrium before the calculation for the next layer begins. A theoretical demonstration of the resulting oxygen diffusion for each layer is illustrated in Fig. 3. Input parameters were taken from the relevant literature. In order to simplify the computations several assumptions were made regarding various modules of

the system and are reported in Table 2. Assumptions 1, 4, and 8 were required, because available literature does not report values completely appropriate for this model. Assumptions 2, 3, 6, and 7 were made in order to simplify the programming, but there is enough data in available literature to add these factors to a more complicated algorithm. Assumption 5 was necessary simply because there is not enough data available regarding oxygen consumption by other intestinal microorganisms to make calculations feasible. We implemented this equation in the C# programming language using Visual Studio® 2010 (Microsoft Corporation, Redmond, WA). Each simulation ran for the same amount of time. The code is reported in Appendix 1.

RESULTS

A graphical representation of the model output is shown in Fig. 4. The solid line depicts oxygen diffusion from the intestinal epithelium into the lumen as governed by Fick's Second Law of Diffusion (Eq. 1). The dashed line subtracts oxygen respired by *E. coli* from the amount of available oxygen in each layer as diffusion is taking place (Eq. 3). When *E. coli* is respiring oxygen, anaerobic conditions occur much closer to the epithelial layer than when *E. coli* is absent. In the time allowed, the *E. coli* simulation was able to reach equilibrium. In the absence of any output, if "Diffusion" had been given enough time to reach equilibrium we would have found $[O_2]$ = 659.64 μM for all distances. Instead, we chose to limit both to an equally short amount of time in order to illustrate a more meaningful measure of the impact of *E. coli* on the diffusion process. In the presence of *E. coli* within 20 μm of the epithelium $[O_2]$ is essentially 0.

DISCUSSION

Despite the theoretical limitations of this model, a difference in diffusion of oxygen into the lumen of the intestine is observed when *E. coli* is respiring oxygen. This result supports the hypothesis that *E. coli*, a representative facultative anaerobe, is removing oxygen from the lumen of the intestine and therefore, may provide anaerobic growth conditions for the obligate anaerobes. Obligate anaerobic bacteria grow in the intestine under what should be inhibitory levels of oxygen. If *E. coli* is removing oxygen from the system to the extent predicted by this model, then it is quite possible that oxygen scavenging, facultative anaerobes are providing the anaerobic conditions necessary for growth of the obligate anaerobic residents of the gastrointestinal tract.

At first glance the degree to which *E. coli* reduces oxygen tension in intestinal mucus would seem to be extreme. However, germ-free rats have been found to have 13 – 53% lower intra-colonic oxygen tensions than conventional rats (6, 7). Additionally, it has been demonstrated that oxygen uptake rates in *E. coli* are higher under limited oxygen (< 5 mmHg) than when oxygen is in excess (5 – 150 mmHg) (26), which was not taken into account in this model. It is conceivable that under conditions of limiting oxygen both cytochrome oxidases are active, which would explain the increase in uptake rates (62). It is not possible to determine from our results whether the reduction in oxygen is local or systemic. Microniches created by the metabolic activities of resident bacteria have noted import in many ecosystems and therefore, may also be part of the intestinal ecosystem (70). Even if *E. coli* is not the only oxygen scavenger in the

gastrointestinal tract, this model predicts that *E. coli* is capable of creating anaerobic microniches sufficient to support its obligate anaerobe neighbors and perhaps reducing oxygen tension in the lumen of the gastrointestinal tract (18, 23, 28, 34, 47, 53)

It would improve the accuracy of these results if the diffusivity of oxygen in mucus could be determined experimentally or extrapolated for normal mouse body temperature (4, 25). Including diffusion in Δy and Δz and influx/efflux due to peristalsis would all directly influence oxygen tension in the lumen, and most likely reduce the degree of the impact *E. coli* has on oxygen tension in the lumen. Calculating the velocity of oxygen in intestinal mucus would enable more accurate determination of the size of the anoxic zone surrounding an individual *E. coli* cell or the distance a single burst of oxygen travels from the epithelial layer through mucus containing dispersed cells. Such results may increase our understanding of intestinal states that encourage pathogenesis in microorganisms that rely on oxygen availability to cue virulence factors (36, 37). Within the constraints of this model's ability to make such a prediction, *E. coli* is capable of providing an anaerobic environment for the numerically dominant anaerobic bacterial residents of the gastrointestinal tract. However, it is important to verify the predictions of any model (42), which is the intent of the experiments outlined in Chapter 3.

DISCLAIMER

"Modeling oxygen diffusion and aerobic respiration in *E. coli* in the mouse cecum." is an independent publication and is not affiliated with, nor has it been authorized, sponsored, or otherwise approved by Microsoft Corporation.

Table 1. Notation, description, units and references for parameters used in model.

Parameter	Value	Reference
Maximum oxygen utilization rate[a]	9.33×10^{-13} µmol O_2 cells^{-1} s^{-1}	(65)
Diffusion coefficient of oxygen in intestinal mucus[b]	2.38×10^{-2} µm^2 s^{-1}	(51)
Number of *E. coli* cells in intestinal mucus[c]	1.1×10^{11} cells L^{-1}	(48)
Oxygen concentration at epithelial surface[d]	6.5964×10^{2} µM	(7)
Monod half-saturation constant for *E. coli* on oxygen[e]	1.21×10^{-1} µM	(57)

[a]Reported as 12 mmol O_2 g (dry weight)$^{-1}$ h^{-1}. Converted to µmol O_2 cells^{-1} s^{-1} using the following relationships: 1.0×10^{3} mmol O_2 = 1.0 µmol O_2, 2.8×10^{-13} g (dry weight) = 1 *E. coli* cell (41), and 1 h = 3600 s. Oxygen uptake rates have been experimentally determined for anaerobic growth on a variety of substrates and found to be 14 - 20 mmol O_2 per g (dry weight) per h (2, 44, 46, 65, 66). One study determined that at a specific growth rate of 0.701 h^{-1} (very close to that reported as the actual growth rate of *E. coli* in intestinal mucus by Poulsen et al. 1995 as 0.69 h^{-1}) the oxygen uptake rate is 12 mmol per g (dry weight) per h (65). We chose to use this value in our diffusion model even though it is lower than other reported *in vitro* values, because of its apparent biological relevance.

[b]Reported as 2.38×10^{-6} cm^2 s^{-1} at 25ºC. Converted to µm^2 s^{-1} using the following relationship 1.0 cm = 1.0×10^{4} µm.

[c]Reported as 1.1×10^{8} cells µL^{-1}. Converted to cells L^{-1} using the following relationship 1.0 µL = 1.0×10^{-6} L.

[d]Reported as 12.8 mmHg. Calculated µM by assuming O_2 behaves like an Ideal Gas at 38ºC (normal mouse body temperature [reference]) and use the Ideal Gas Law PV=nRT -> P/RT=n/V to calculate µM of O_2 [reference]. R is the Ideal Gas Constant = 62.363 L mmHg K^{-1} mol^{-1} [reference]. 38ºC = 311.15 K [reference]. 1 µmol = 1×10^{-6} mol.

[e]Reported as 121 nM. Converted to µM using the following relationship 1.0 nM = 1×10^{-3} µM.

Table 2. Main assumptions and simplifying considerations in the simulation.

1. Concentration of oxygen found in germ free rats is equal to that in mice and is a valid representation of the oxygen available to *E. coli* in a conventional mouse at the luminal boundary of the intestinal epithelial cells.
2. Advection, i.e. oxygen influx from swallowed air and efflux due to peristalsis, has been excluded.
3. Ignores diffusion of oxygen from neighboring dimensions.
4. Diffusivity of oxygen in mucus at 25ºC is not significantly different from diffusivity of oxygen in mucus at 38ºC, normal mouse body temperature.
5. Oxygen respiration by all other bacterial and human cells present in the system is not taken into account.
6. The maximum uptake rate of oxygen by *E. coli* is not self-limiting as oxygen availability decreases.
7. If oxygen encounters *E. coli* it will automatically be used by the cell.
8. There is an even distribution of mucus and *E. coli* throughout the system.

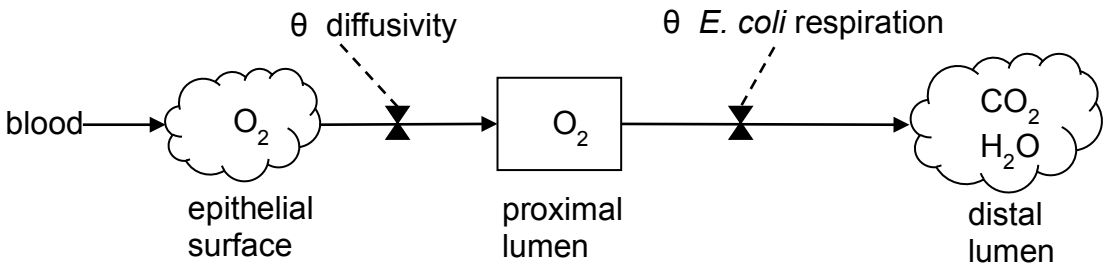

Figure 1. Conceptual model of oxygen diffusion and *E. coli* respiration in the intestine. θ indicates a process included in the mathematics of the model.

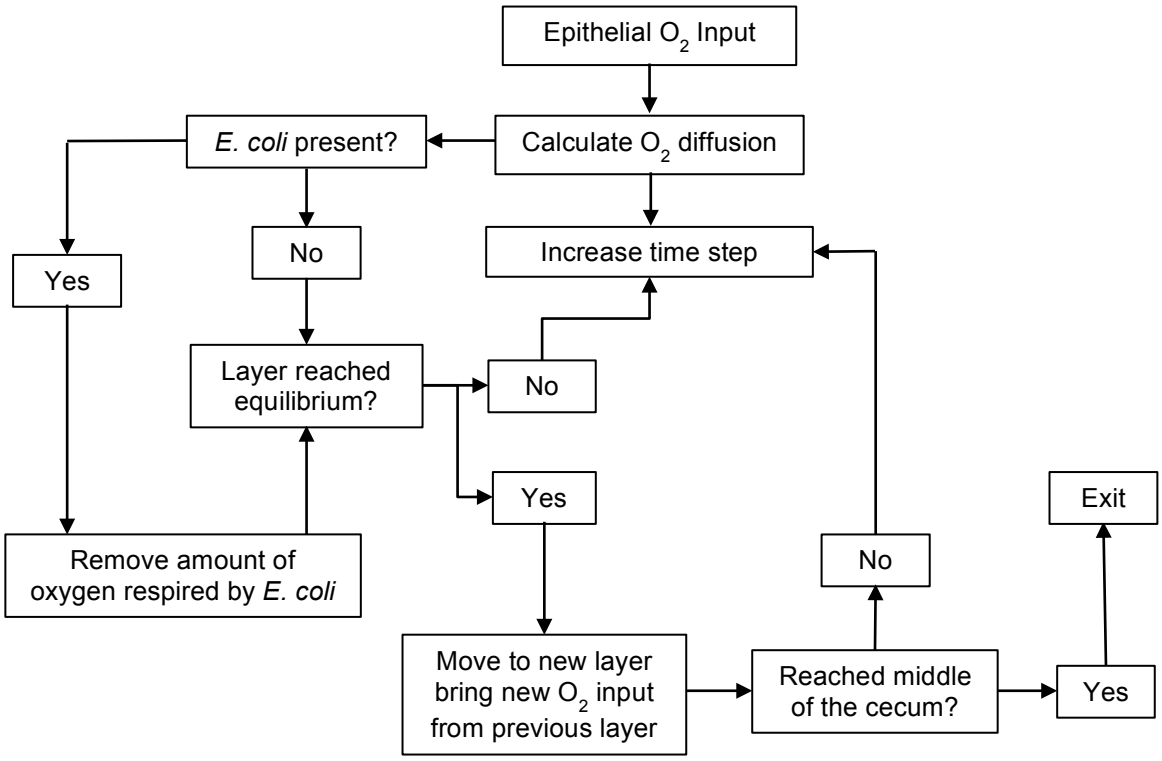

Figure 2. Computer algorithm used to calculate the influence of oxygen scavenging by *E. coli* on oxygen diffusion from the epithelial layer of the intestinal wall according to Fick's Second Law of diffusion and Monod kinetics.

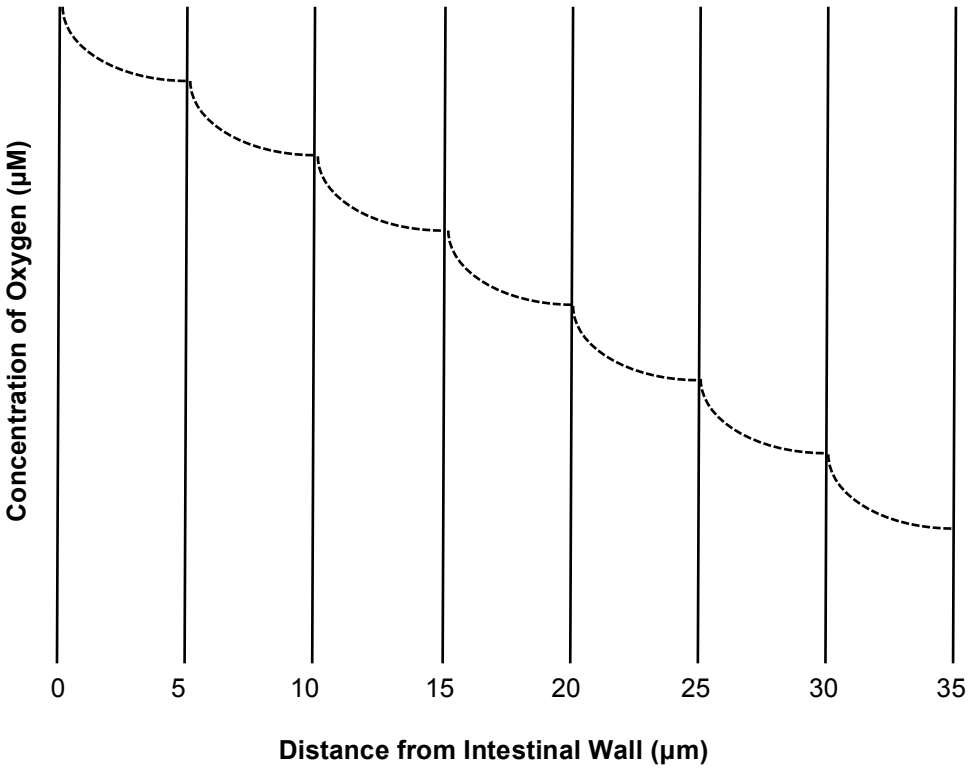

Figure 3. Illustration of output for each layer. Only the values at each boundary are reported.

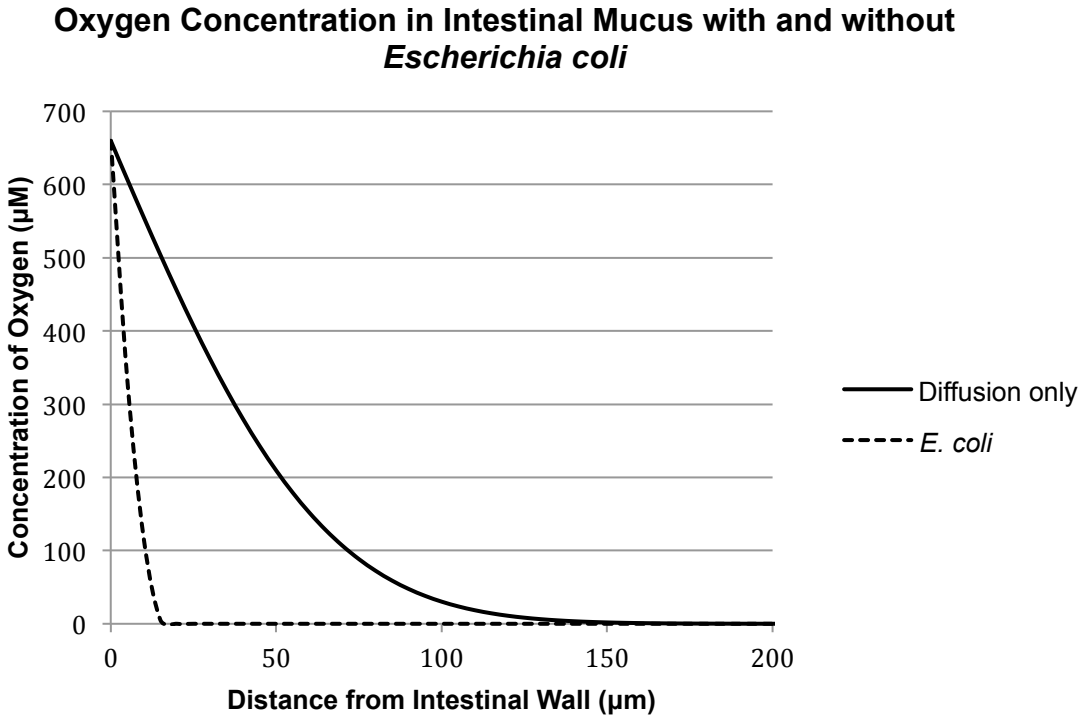

Figure 4. Distance from intestinal epithelial wall (µm) vs. oxygen concentration (µM). The solid line represents oxygen diffusion in the absence of *E. coli*. The dashed line represents oxygen diffusion in the presence of aerobically respiring *E. coli*.

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Chapter Three: Oxygen level dependence and T_2^* weighted MRI of aerobic respiration in *Escherichia coli* in the mouse cecum

INTRODUCTION

While it is well documented that the intestinal microbiota plays an important role in health of the host, the intestinal ecosystem remains poorly understood (17, 44, 56). 16S rRNA and metagenomic studies have begun the process of identifying intestinal microbiota and evaluating their physiological potential, but the ecological role of commensal microorganisms in this complex ecosystem is largely understudied (13, 15, 16, 19, 34, 35, 48). Even though anaerobic bacteria are numerically dominate, the lumen of the mouse intestine contains oxygen (9, 14, 24, 25, 33, 61), thus it is important to understand the function of oxygen-respiring facultative anaerobes in this ecosystem. Facultative anaerobes are found in the mucus layer adjacent to the intestinal epithelium, where oxygen tension is highest, in intimate contact with their anaerobic neighbors (41, 42, 56). A clear understanding of the relationship between facultative anaerobes, such as *Escherichia coli*, and the numerically dominant anaerobic population is important, because host resistance to infectious disease depends on the stability of the microbial population as a whole (3, 24, 50, 53, 59).

E. coli has a complex regulatory network that adjusts its respiratory metabolism to compete in challenging environments and increase survival by adapting to a wide variety of growth conditions (51, 52). *E. coli* prefers aerobic respiration in the presence of sufficient oxygen, and employs two different cytochrome oxidases: cytochrome bo_3 oxidase and cytochrome *bd* oxidase, for respiration under high and low oxygen tension, respectively, in order to maximize its aerobic respiratory potential (23). Both of these oxidases are heme proteins,

which catalyze the reduction of oxygen to water as the terminal step in aerobic respiration (22, 40). Previous studies indicated that oxygen tension in the colon is sufficient to support aerobic respiration in *E. coli* (47). Additionally, we have demonstrated that the ability to respire oxygen provides a competitive advantage *in vivo* (29). Since *E. coli* appears capable of consuming oxygen in the intestine, we hypothesize that *E. coli* positively impacts the anaerobic community by scavenging oxygen to provide an anaerobic environment for the obligate anaerobic residents of the mouse intestine.

Oxygen tension in the intestine has been used to assess tissue function and health, but most of the historical data comes from invasive techniques, which limit their application for ecological investigations (25). There has been a recent surge in the use of imaging techniques to measure tissue hypoxia. Of these, T_2^* weighted MRI provides information about oxygen without the use of a contrasting agent or oximetry probe, which is an important consideration when investigating bacterial activity *in vivo* (57). Originally developed to examine blood oxygenation, this technique has since been used to examine tissue oxygenation in many biological systems (37, 43, 49, 60). T_2^* weighted MRI does not provide quantitative measurements of $[O_2]$ (6), but does provide high resolution, user defined, qualitative information regarding the interaction of oxygen molecules with the iron containing heme molecules found in many bacterial aerobic respiratory enzymes (57). In oxyheme, iron is diamagnetic, and in deoxyheme, iron is paramagnetic. This paramagnetic state causes increased magnetic susceptibility in the sample, which in turn causes local inhomogeneities of the

magnetic field. T_2^* is a measure of the time (relaxation time) it takes for spins to diphase in a region of interest. If the region has significant magnetic inhomogeneity, the spins will quickly diphase, i.e. have a short relaxation time, indicating a predominance of deoxyheme (54). Thus, we can collect evidence for bacterial cytochrome oxidase interactions with oxygen by determining relaxation times under various experimental conditions, and deduce the availability of molecular oxygen in the cecum in the presence or absence of aerobically respiring *E. coli*.

In order to test our hypothesis, we designed an *in silico* model of oxygen diffusion from the capillary beds underlying the epithelial cell layer into the lumen of the colon, with or without aerobic respiration in *E. coli*. The model predicts a decrease in oxygen availability in the lumen of the intestine in the presence of actively respiring *E. coli* (as previously discussed in Chapter 2). In order to confirm the model's prediction, T_2^* weighted MRI experiments were conducted using: conventional animals (CN), streptomycin treated animals (ST), and animals that were streptomycin treated and then individually colonized with *E. coli* MG1655 wild-type (WT) or *E. coli* MG1655 Δ*cydAB* Δ*cyoAB::cat* (DM, for double mutant).

MATERIALS AND METHODS

Magnetic resonance imaging. A gradient echo experiment was used to visualize the intestinal tract of the mouse. Acquisition parameters were as follows: image matrix size of 256 x 128; repetition time (TR) = 2 s; initial echo time (TE) = 2.88 ms; number of averages = 8; field of view (FOV) = 40 x 30 mm;

and slice thickness = 1.0 mm. Accordingly, the in-plane resolution was 156 x 234 μm. While this is significantly larger than the micro-architecture of the intestinal wall, the breadth of signal intensity change, ΔS, due to Δa in this environment is expected to occupy all voxels contributing to the observable signal. Consequently, a change in *S/N* and therefore, *a*, is expected to signal the hypothesized anaerobiosis of the micro-environment caused by introducing *E. coli*. Initially two sets of images were collected to produce a dual echo image set. The echo times were, *a* = (4.0, 10.0) ms. Final images were collected with 12 echoes, with the first echo collected after 2.88 ms and 4.71 ms for each subsequent echo, i.e. the echo spacing after the first was 4.71 ms. While respiratory gating can be used during the acquisition, we have not done so because unnecessary delays can be introduced into the acquisition time caused by respiratory rate variability. Instead, motion artifacts were reduced by signal averaging over 8 scans. Post-processing motion correction was not used in analysis, but could be used if images are heavily tainted with motion artifacts.

Signal Intensity Change During Low Oxygen Delivery. Under normal acquisition conditions, mice are anesthetized under a flow of 100% medical grade oxygen. Thus the blood oxygen concentration presumably is saturated. Under these conditions, isoflurane can be administered at ~0.5 - 1.0% of total gas flow. This is sufficient to maintain a respiratory rate in the range of 35 - 60 breaths per minute. When nitrogen gas is introduced to dilute total oxygen delivery, and thus whole body blood oxygen concentration, isoflurane delivery has to be adjusted to maintain a respiratory rate in the range of 35 - 60 bpm. We

have found that this requires an increase of about 3% compared to delivery under 100% oxygen. Whilst these parameters are kept constant, S/N changes can be directly attributed to the blood oxygen level, which is directly proportional to tissue oxygen concentration. However, it is somewhat difficult to obtain a direct measure of such concentrations. Accordingly we report here concentrations relative to baseline S/N from animals anaesthetized with 1% isofluorane delivered in 100% oxygen at a flow rate of 1.0 L/min.

Animal Studies. We chose the streptomycin-treated mouse as a model intestinal ecosystem, because it has been used to study the physiology of facultative anaerobes *in vivo* (4, 11, 18, 21, 28, 30-32, 38, 39, 41, 42). Streptomycin sulfate is an aminoglycoside that interferes with carbohydrate metabolism, terminal respiratory pathways, and cellular division in susceptible bacteria (5, 26, 45). Additionally, streptomycin uptake only occurs in facultative anaerobes that are actively respiring and has previously been noted to selectively remove facultative anaerobes from the intestine (55, 59). Therefore, streptomycin treatment removes resident facultative anaerobes, which opens a niche for our strains of *E. coli* to colonize, and leaves behind a dense anaerobic community (7, 27).

Preliminary studies were conducted on four groups of 5 - 6 wk old CD-1 male mice (Charles River Laboratories, Kingston, NY). A baseline group (n = 4) of untreated mice, which are naturally colonized with *E. coli* and other facultative anaerobes (28), were imaged using the gradient echo imaging protocol as described above. Images were also collected for groups of four mice, colonized

individually with wild-type *E. coli* MG1655 wild-type Str^r Nal^r (42) or *E. coli* MG1655Δ*cydAB* Δ*cyoAB::cat* Str^r (this study: an isogenic cytochrome *bd* oxidase and cytochrome bo_3 oxidase double mutant), or streptomycin-treated mice (5 g/L), not colonized with *E. coli*. Strains used in this study are reported in Table 1. Briefly, mice were treated with 5 g/L streptomycin sulfate in their drinking water for 24 hours and then had all food (Harlan Teklad Mouse and Rat Diet, Madison, WI) and water withheld for 16 hours. Mice then voluntarily consumed *E. coli* MG1655 wild-type and mutant strains at a level of 1 x 10^4 cells/mL in 20% sucrose. All strains colonized at 1 x 10^6 – 1 x 10^8 cells/g feces by twenty-four hours post-feeding. At this time mice were analyzed using T_2^* weighted MRI and the intestinal tract was examined. Animals were imaged using a 7 Tesla-30 cm USR horizontal-bore magnet (Bruker BioSpin MRI Gmbh, Ettlingen, Germany).

Image Analysis. A total of 20 slices were taken from each animal between the kidneys and the bladder. Three consecutive images with visible cross sections of the cecum were selected. For each of these images the cecum was outlined by hand to select the region of interest (ROI). Attention was paid to the location of the cecal wall. An example is displayed in Fig. 1. Each ROI was then analyzed using the ISA tool in Paravision (Brucker BioSpin Corp., Billerica, MA) to determine the relaxation time according to the following equation: $SI = S_0 e^{-t/T2}$, where S_0 (integer machine units) is the initial signal intensity measured at t = 0 (1, 2, 20, 46, 49, 58). Data points more than two standard deviations outside of the mean, were excluded from further analysis. An

independent two-tailed Student's t-test was used to determine the significance of differences between experimental conditions.

RESULTS

In order to investigate oxygen scavenging by *E. coli* in the mouse intestine we employed the streptomycin treated mouse model along with T_2^* weighted MRI to qualitatively determine [O_2] the ceca of mice. In this model mice are treated prior to colonization with 5 g/L streptomycin sulfate, which removes all of the native facultative anaerobes, opening a niche for colonization of strains of interest. Four experimental conditions were examined: conventional mice, mice treated with streptomycin but not colonized, and streptomycin treated mice that were subsequently colonized with *E. coli* MG1655 wild-type or *E. coli* MG1655Δ*cydAB* Δ*cyoAB::cat*. T_2^* weighted MRI is sensitive to oxy- and deoxy-species of heme proteins, which include the cytochrome oxidases employed by *E. coli* during aerobic respiration. It follows that if oxygen is bound to cytochrome oxidase, then *E. coli* is actively respiring oxygen and the cecum will be more anaerobic than it would be in the absence of aerobic respiration. We expect the ceca of conventional mice, which have their native facultative anaerobes intact, and *E. coli* MG1655 wild-type Str^r colonized mice to be more anaerobic than streptomycin treated only or *E. coli* MG1655Δ*cydAB* Δ*cyoAB::cat* Str^r colonized mice.

The results of this experiment are shown in Table 2, which reports the relaxation time (T_2^*) for each experimental condition. WT mice (T_2^* = 13.12 ms) did not have relaxation times significantly different from CN mice (T_2^* = 13.65

ms). ST mice (T_2^* = 11.15 ms) as well as DM mice (T_2^* = 12.01 ms) had significantly shorter ($p \leq 0.05$) relaxation times than either CN or WT mice as determined by an independent two-tailed Student's t-test.

In T_2^* weighted MRI, longer relaxation times indicate a predominance of oxyheme in the system. The results indicate that the ceca of conventional mice and *E. coli* MG1655 wild-type colonized mice are more anoxic than the ceca of streptomycin treated or *E. coli* MG1655Δ*cydAB* Δ*cyoAB::cat* colonized mice. The data support our hypothesis that *E. coli* positively impacts the anaerobic community by scavenging oxygen to provide an anaerobic environment for the obligate anaerobic residents of the mouse intestine.

DISCUSSION

We chose T_2^* weighted MRI to observe the effects of *E. coli* colonization on the oxygen in the mouse cecum, because it is non-invasive and does not involve the use of an ingested probe as in EPR, which may disturb ecosystem function. Interpretation of the results requires careful consideration of all potential sources of heme molecules inside the cecum, which include but are not limited to: sloughed epithelial cells and hemocytes from the host, resident and transient microorganisms, and ingested material. The similarity between Con and WT animals indicate that cytochrome *bd* oxidase and cytochrome bo_3 oxidase from *E. coli* account for the majority of the oxygen-bound bacterial heme in the cecum. However, from this study, we cannot rule out the possibility that cytochrome oxidase activity from other facultative anaerobes found in conventional mice contributes to oxygen scavenging.

It stands to reason that if there is indication of significant aerobic respiratory activity, then the [O_2] of the cecum would be less than in the absence of this activity. Previous comparisons of intracolonic oxygen tension between conventional and gnotobiotc rats indicate a 13 – 53% lower [O_2] in conventional animals with their native *E. coli* than in gnotobiotic animals (8, 9). The results of this study suggest that there is a significant reduction in oxygen tension in the cecum in the presence of wild-type *E. coli* (Con and WT) as compared to Str and DM. The implications of these results are two-fold. First, they suggest that *E. coli* is actively respiring oxygen in the cecum and likely throughout the gastrointestinal tract. Secondly, *E. coli* is capable of restoring conventional levels of anoxia in the mouse cecum in the absence of all other facultative anaerobes, although, from this study. However, from our results, we cannot conclude that *E. coli* is the only aerobically respiring microorganism in the cecum of a conventional animal.

The evidence gathered from this study directly support our hypothesis that *E. coli* positively impacts the anaerobic intestinal community by scavenging oxygen to provide an anaerobic environment for the obligate anaerobic residents of the mouse intestine. The other dominant group of facultative anaerobes known to inhabit the gastrointestinal tract, the lactic acid bacteria, can benefit energetically from reducing oxygen in aerobic conditions, but have demonstrated a preference for fermentation *in vivo* (10, 12, 36). Additionally, it is tempting to consider oxygen scavenging by *E. coli* as a keystone process driving development and maintenance of a healthy anaerobic microbiota in this highly

competitive, resource limited ecosystem (28). Further investigation of the anaerobic community's responses to oxygen scavenging by *E. coli* is needed to confirm this prediction and will be examined further in Chapter 4.

One limitation of this study is that $[O_2]$ cannot be directly measured using T_2^* weighted MRI. It would be informative to correlate the results of this study with more direct measurements of the $[O_2]$ under similar experimental conditions (6). From this we would learn how much oxygen is removed from the ecosystem directly by *E. coli*.

In conclusion, this work demonstrates a novel application of T_2^* weighted MRI to investigate the availability of oxygen in the gastrointestinal tract and the respiratory physiology of *E. coli* in its natural habitat. This study underlines the potential of non-invasive investigations of microbial ecosystem ecology with applications in gastrointestinal health.

Table 1. Strains and plasmids used in this study.

Strain	Genotype or Phenotype	Reference
MG1655	Wild type (CGSC no. 7740)[a]	CGSC
MG1655 Str^r	Spontaneous Str^r	(42)
MG1655 Str^r Nal^r	Spontaneous Nal^r	(42)
Nissle 1917 Str^r Nal^r	Spontaneous Str^r and Nal^r	(4)
Derivatives of MG1655 Str^r		
cydAB cyoAB[b]	Δ*cydAB* Δ*cyoAB::cat*	This study

[a] CGSC, *E. coli* Genetic Stock Culture Collection, Yale University.
[b] For simplicity, strains are named in the text by strain and mutation(s).

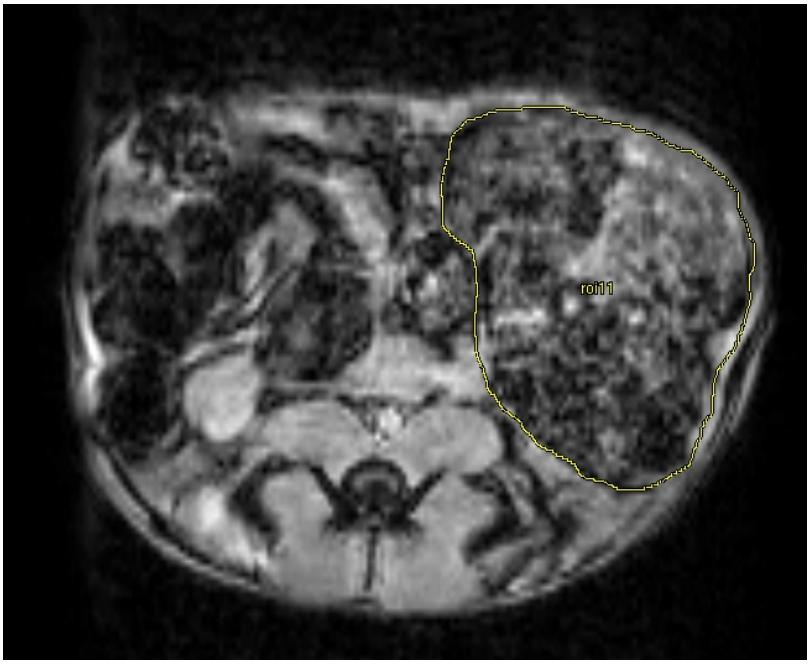

Figure 1. Example of “free-hand” ROI selection of the mouse cecum in one slice.

Table 2. Relaxation time in milliseconds for each experimental condition. CN = conventional, WT = *E. coli* MG1655 Str^r Nal^r, ST = streptomycin sulfate treatment only, DM = *E. coli* MG1655Δ*cydAB* Δ*cyoAB::cat* Str^r.

Experimental Condition	Mean ± SD
CN	13.65321 ± 0.91448
ST	11.15912** ± 1.32964
WT	13.12529 ± 0.51065
DM	12.00781* ± 0.90328

*$p \leq 0.05$ and **$p \leq 0.001$, statistical significance compared to conventional mice, using in an independent two-tailed Student's t-test.

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Chapter Four: The influence of aerobic respiration in *Escherichia coli* on the bacterial community structure of mouse cecum mucus.

INTRODUCTION

The human body is host to a myriad of microorganisms. The lumen of the large intestine is home to bacterial population composed of 10^{11} - 10^{12} cells/ml contents and hundreds of different species. Fungi and a few archaea have also been identified as members of the intestinal microbiota (51, 70, 75, 78). The commensal microbiota provide several important functions to the host: increased nutrient availability, colonization resistance to invading pathogens, proper immune system development, and healthy systemic biochemisty (34, 80). Early succession in newborns begins with initially simple communities, that develop into a more complex, mature population in response to a variety of environmental inputs in addition to host and resident bacterial regulatory pressures (34, 36, 60, 62). This process is remarkably similar to macro-ecological theories regarding community development and succession, in which opportunistic species facilitate the arrival of and are gradually replaced by those more suited long-term survival in that ecosystem (11, 57). The composition of the mature microbiota can be effected, positively or negatively, by host genetics, method of birth, diet, antibiotics, immune responses, invading pathogens, prebiotics, and probiotics (17, 34, 35, 60). Homeostasis between host and commensal microbiota and within the various sub-populations of the intestinal microbiota contribute to its ability to resist pathogenic invasion, but this balance can be incredibly delicate and alterations may lead to disease.

Despite the fact that the gastrointestinal tract functions similarly to an *in vivo* chemostat, it is a surprisingly heterogeneous, nutrient replete ecosystem in

which competition for resources or an ability to overcome the physical effects of peristalsis and mucus secretion from the intestinal wall are key for microbial species to maintain stable populations. The intestine is not a homogeneous system, and environmental conditions change from stomach to colon and from intestinal wall to lumen (54, 56, 76). From the perspective of the microbiota a variety of niches exist throughout the gastrointestinal tract, governed by pH, oxygen tension carbon source and electron acceptor availability, microbial antimicrobial production, and host immune system activity.

There are two distinct sub-populations of bacteria in the gastrointestinal ecosystem, which have been identified as functionally unique: facultative and obligate anaerobes. Together these sub-populations dictate succession of the intestinal microbiota in newborns, lend to overall stability of the microbial population, and provide colonization resistance to invading pathogenic bacteria (23, 91). The numerically dominant, obligate anaerobes include members of phyla the Firmicutes and Bacteroidetes. Facultative anaerobes include several genera from the Lactobacilliaceae and the Enterobacteriaceae (18). Obligate anaerobes are primarily located in the large intestine and are the metabolic work-horses of the intestinal ecosystem. They degrade complex carbohydrates by way of exo-enzymes, and their metabolic end-products may be absorbed by the host or neighboring microorganisms in addition to chemically altering the environment to benefit the host (80). Facultative anaerobes may be necessary for establishment of commensal obligate anaerobes in infants and have

demonstrated potential as beneficial prophylaxes to a variety of internal stressors (37).

Escherichia coli, a commensal facultative anaerobic gamma-proteobacteria, is a relatively minor constituent of the intestinal microbial population: 1 x 10^8 cells/ml mucus reside in the mucus layer coating the epithelial cells lining the intestinal wall (66). *E. coli* is a commensal inhabitant of the gastrointestinal tract and provides several benefits to its host. However, certain strains of *E. coli* are emerging pathogens and will cause disease if they escape the intestine or supplant commensal strains in the intestine (33). *Shigella*, a close relative of *E. coli*, is also notorious for causing disease (25). Establishment of a pathogenic *E. coli* strain in an otherwise healthy host requires over-coming colonization resistance imparted by commensal strains through nutrient niche-defined occupation (39). Therefore, the appropriate combination of commensal *E. coli* strains is capable of preventing colonization by a pathogenic strain if they occupy the same nutrient niche as the pathogen (39). Additionally, commensal *E. coli* strains have been strongly implicated in proper succession of the microbiota in infants, presumably, due to its ability to alter the environment sufficiently to allow the establishment of obligate anaerobes (14, 60).

Oxygen is present in the intestine due to air swallowed during normal ingestion and by diffusion from hemoglobin in the dense capillary beds underlying the intestinal wall (8, 12, 13, 27, 28, 31, 40, 87, 88). Some facultative anaerobes are capable of both aerobic and anaerobic respiration as well as

fermentation of various carbon sources, but because aerobic respiration is more energetically favorable, *E. coli* will take advantage of this ability if sufficient oxygen is available in the environment. Mutants lacking cytochrome *bd* oxidase, important for aerobic respiration under low oxygen tension, are unable to compete with their isogenic parent in colonization models indicating that *E. coli* scavenges oxygen in the intestinal ecosystem (32). Very little is known about the potential systemic effect *E. coli* has on the oxygen content of the intestinal environment or its oxygen sensitive anaerobic residents.

Studies using gnotobiotic mice indicate that introduction of native microbiota reduce oxygen tension, but implications regarding specific species cannot be made from currently available data (7, 8). There are two possible explanations for these results that are not mutually exclusive. First, obligate anaerobic inhabitants of the intestine possess a wide array of mechanisms to resist the toxic effects of oxygen, which may also reduce the molecular oxygen content of the surrounding environment. These mechanisms include: production of superoxide dismutase, productions of catalase, and a few have recently been found to respire oxygen in nanomolar concentrations, most likely as a means to protect anaerobic pathways (61, 73, 81, 83). Secondly, microniches created by the metabolic activities of different members of an ecosystem have been established as an important aspect of microbial ecology (41, 65, 93). Facultative anaerobes, including *E. coli*, have demonstrated the ability to create conditions *in vitro* in which obligate anaerobes can grow under oxygen tensions they could not survive alone (24). It is entirely possible that a similar effect is occurring in the

intestine, which would help explain why *E. coli* remains a small but ubiquitous member of the ecosystem. Gene expression profiling of *Lactobacillus plantarum* in gnotobiotic mice revealed that they only express genes for fermentation *in vivo* (47). These results indicate that *E. coli* may be one of the few inhabitants of the gastrointestinal tract using aerobic respiration as a means of survival in this competition rich and nutrient replete ecosystem.

Interest in the composition of the intestinal microbiota has produced many surveys conducted using laser capture micro-dissection, 16S rRNA pyrosequencing and Sanger sequencing from clones libraries of PCR products from feces and intestinal contents, fluorescent in situ hybridization (FISH), terminal restriction length polymorphism (t-RFLP), and traditional culturing techniques involving a variety of selective techniques. The gastrointestinal tracts of a variety of animals, including humans, have been examined using such techniques. Recent metagenomic surveys have begun to reveal the metabolic potential of the gastrointestinal microbiota (20, 55, 96). Each of these techniques has inherent advantages and disadvantages for experiments designed to examine the nuances of an ecosystem contained entirely within another living being. Pyrosequencing of 16S rRNA amplified from total environmental DNA has proven invaluable for its ability to elucidate bacterial populations of medium to high diversity, which often are dominated by microorganisms that are difficult to characterize using available cultivation techniques. The intestine has proven to be such an environment, because it is of medium diversity. Additionally, it is difficult to examine *in situ* without disturbing the native ecology.

The streptomycin treated mouse model has been used to examine respiration of *E. coli in vivo* (32). Streptomycin sulfate is an aminoglycoside that interferes with carbohydrate metabolism, terminal respiratory pathways, and cellular division in susceptible bacteria (4, 29, 59). Anaerobic bacteria are intrinsically resistant to aminoglycoside antibiotics, such as streptomycin (21). Additionally, streptomycin uptake only occurs in facultative anaerobes that are actively respiring and has previously been noted to selectively remove facultative anaerobes from the intestine (84, 91). We took advantage of this knowledge to create a model system in which we could examine the impact of oxygen scavenging by *E. coli* on the composition of bacterial population of mouse cecum mucus. It is clear from studies involving streptomycin sulfate treatment of animals, that the composition of the intestinal microbiota is altered beyond removal of facultative anaerobes, but it is unclear if this shift is driven by a metabolic, environmental, or undefined secondary effects on the population (30). Previous investigations have revealed that *E. coli*, operating in the absence of other facultative anaerobes, is capable of significantly reducing the oxygen content of the intestine (see Chapters 2 and 3). In order to investigate the hypothesis that *E. coli* oxygen scavenging influences the composition of the bacterial population of the large intestine, I used 16S rRNA pyrosequencing of DNA isolated from mouse cecum mucus to examine the bacterial populations of conventional animals (CN), animals that were streptomycin treated and then individually colonized with *E. coli* Nissle 1917 wild-type Nal^r Str^r (NS), a commensal isolate used as a control for strain effects, *E. coli* MG1655 wild-type

Nal^r Str^r (MG), a laboratory adapted commensal strain, or *E. coli* MG1655 Δ*cydAB* Δ*cyoAB::cat* Str^r (DM), an isogenic cytochrome oxidase mutant that is unable to respire oxygen but has the same carbon-use profile and the same fermentative and anaerobic respiration capabilities as its wild-type parent *in vitro*.

MATERIALS AND METHODS

Mouse Colonization and Sample Collection. All mice were CD-1 male mice, 6 wks of age, obtained from Charles River Laboratories, Kingston, New York. Animals were allowed a minimum of 48 hrs to acclimate following shipment prior to start of experiment. Mice colonized with strains of *E. coli* were housed in groups of three until inoculation, during and after which they were housed individually. All animals received autoclaved 7012 Teklad LM-485 Mouse/Rat Sterilizable Diet (Harlan Laboratories, Inc., Indianapolis, IN) and had Premium KLEENEX® sheets for bedding. Feces were removed from each cage and a fresh kleenex sheet was provided daily. Cages were changed every other day.

Strains (Table 1) for colonization were prepared by 24 hrs incubation at 37°C on Luria broth agar (Becton, Dickson and Co., Sparks, MD) containing antibiotics appropriate to the strain. A 250 ml Erlenmeyer flask containing 10 ml Luria broth (Becton, Dickson and Co., Sparks, MD) and antibiotics appropriate to the strain was inoculated and incubated at 250 rpm and 37°C for 16 – 18 hrs.

Mice were handled as follows:

Conventional (CN) mice were given sterile food and water *ad libidum* for 10 d. No other treatment was given.

Day 0 (D0) mice were given sterile drinking water containing streptomycin sulfate (5 g/L) and sterile mouse chow *ad libidum* for 24 hrs. Food and water were withheld for 16 hrs, prior to inoculation.

Nissle 1917, MG1655, and MG1655 Δ*cydAB* Δ*cyoAB::cat* (MG, NS, and DM): mice were given sterile drinking water containing streptomycin sulfate (5 g/L) and sterile mouse chow *ad libidum* for 24 hrs. Food and water were withheld for 16 hrs, prior to inoculation.

Inoculation preparation and procedure: A dilution of 1 x 10^5 cells/ml of the appropriate strain in 20% sucrose was provided to each animal for voluntary inoculation (the mice typically consumed the entire inoculum within 10 min). Sterile food and water containing streptomycin sulfate (5 g/L) were provided *ad libidum* for 10 d.

Plate counts were conducted using serial dilutions in 1% tryptone (Becton, Dickson and Co., Sparks, MD) onto MacConkey agar (Becton, Dickson and Co., Sparks, MD) with appropriate antibiotics for the 5 hr, Day 1, every other day thereafter until colonization levels reached a steady state, and on Day 10 for each animal. Each strain colonized at a minimum of 1 x 10^6 cells/g feces.

On Day 10, the mice were euthanized by CO_2 asphyxiation, dissected within 30 min post mortem, and the cecum removed. Cecal contents were removed from the tissue by manual manipulation, taking care to leave the adherent mucus layer intact. The cecum tissue with intact mucus layer was divided into two pieces and each piece placed in an individual PowerBead Tube

(MO BIO Laboratories, Inc., Carlsbad, CA). DNA extraction proceeded immediately.

DNA isolation, PCR amplification, and sequencing. Genomic DNA was isolated from the cecum mucus using a PowerSoil DNA Isolation kit according to the manufacturer's instructions (MO BIO Laboratories, Inc., Carlsbad, CA). The final elution was quantified using a DU 800 Spectrophotometer (Beckman Coulter, Inc., Brea, CA). The V3 region of the 16S rRNA gene (16S rDNA) was amplified from the bulk community DNA in a 50-µl reaction mixture containing (final concentration) 20 ng of extracted DNA, 1x PCR buffer (Invitrogen Corp., Carlsbad, CA), 2.0 mM $MgSO_4$, a mixture of 0.2 mM concentrations of each deoxynucleoside triphosphate, 0.3125 U of High Fidelity Platinum Taq polymerase (Invitrogen Corp., Carlsbad, CA), and 0.17 µM concentrations of each of the forward and reverse primers. A separate mixture of 0.25 µM concentrations of each of the 454 FLX-Titanium pyrosequencing and barcoded 16S rRNA primers for each sample was made. The primers (Invitrogen Corp., Carlsbad, CA) used for amplifying the V3 region of 16S rRNA genes for the first twenty cycles were the forward primer 338F (5′ ACTCCTACGGGAGGCAGCAG 3′) and the reverse primer 518R (5′ ATTACCGCGGCTGCTGG 3′), followed by another 8 cycles with the addition of the forward primer 338F (5' *CCTATCCCCTGTGTGCCTTGGCAGTCTCAG*tc ACTCCTACGGGAGGCAGCAG 3' 338HF.B), which has a two base linker sequence ('tc') between the FLX-Titanium 454 pyrosequencing B primer (Roche Diagnostics Corp., Indianapolis, IN) and the 16S rRNA primer to improve 16S

rRNA priming (26) and the reverse primer 518R (5' *CCATCTCATCCCTGCGTGTCTCCGACTCAG*NNNNNNNNATTACCGCGGCTG CTGG 3'), which had FLX-Titanium 454 pyrosequencing primers (Roche Diagnostics Corp., Indianapolis, IN) followed by a unique 8-nt error-correcting Hamming barcode (26) for each sample (Table 2). PCR amplification was carried out on a GeneAmp PCR System 9700 thermocycler. The 16S rRNA amplification used a protocol involving initial denaturation for 5 min at 94°C and 28 cycles of 94°C for 0.75 min, 55°C for 0.75 min, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. After cycle 20 was complete, the thermocycler was paused and 1 μL of the tagged and barcoded primer mixture corresponding to each sample was added. The thermocycler was started again and allowed to complete the 28 cycle program. Replicate amplicons were either pooled or not (depending on the protocol) and visualized on 2% agarose gels using SYBR Safe DNA gel stain (Invitrogen Corp., Carlsbad, CA). The ~250 bp band for each sample was excised and purified using a gel extraction kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions with the following exception: the final elution was performed in 20 μL of 50°C sterile, distilled, deionized water for 30 mins. The cleaned amplicons were quantified using a DU 800 Spectrophotometer (Beckman Coulter, Inc., Brea, CA) to make sure each had a minimum of 200 ng total product. Size and concentration of the PCR product for each sample was analyzed using a 2100 Expert High Sensitivity DNA Assay (Agilent Technologies, Inc., Santa Clara, CA). Sample preparation and pyrosequencing was carried out according to the protocol outlined by the

manufacturer, Roche (48). In brief, samples were amplified using emulsion PCR and pyrosequencing was performed using primer A on a 454 Life Sciences Genome Sequencer FLX-Titanium Instrument (Roche Diagnostics Corp., Indianapolis, IN).

Phylogenetic analysis. Sequence analysis was performed using QIIME (10). In brief, sequences were removed from analysis if they were < 100 nt, > 250 nt, had a quality score < 25, contained ambiguous characters, an uncorrectable barcode, or did not contain the forward primer sequence. Remaining sequences were split into libraries according to their 8-nt barcodes. Similar sequences were clustered into OTUs with a minimum identity of 97%. Representative sequences for each OTU were aligned using PyNAST (9) and the Greengenes database (15). This alignment was later used to generate phylogenetic trees using FastTree (68). Taxonomy was assigned using the Ribosomal Database Project (RDP) Naive Bayesian rRNA Classifier at an 80% confidence threshold (90). OTUs containing < 2 sequences, chloroplast, or mitochondrial DNA were removed from downstream analyses. Representative sequences for OTUs which could not be assigned to the genus level by QIIME, were uploaded into the Seqmatch tool on the RDP website (46) and classified against database isolates using the RDP Naive Bayesian rRNA Classifier at an 80% confidence threshold.

Community diversity. To control for sampling effort in diversity measures, the number of sequences from each sample was rarified according to the size of the smallest library. α-diversity was examined using rarefaction

curves made by using the observed_species metric available in QIIME, and calculated using Chao1 (species richness) and Shannon (richness and evenness) indices. Diversity between samples (β-diversity) was examined using both the weighted (quantitative, taking into account community composition and abundance) and the unweighted (qualitative, taking into account only community composition) UniFrac metrics (19, 42-44). Distance matrices generated by UniFrac were used for hierarchal clustering of libraries using Unweighted Pair Group Method with Arithmetic mean (UPGMA) along with jackknifing support from 75% of the sequences from each reduced library.

Phylogenetic comparison. For all samples, the top 10 OTUs by percent of total population or all of those representing ≥ 1% of the total population whichever was greater for that sample (the dominant OTUs) were examined and compared to the remaining phylotypes (the rare biosphere). In order to identify possible strain effects on community architecture a list of dominant OTUs from each pooled sample (CN, D0, DM, MG, and NS) was compared and examined for unique, dominant, and rare phylotypes. Dominant OTUs for each condition were used to construct a phylogenetic tree along with reference sequences obtained using RDPs Seqmatch, a PyNast alignment to the Greengenes database, and FastTree (uses a heuristic variant of neighbor-joining and infers approximately maximum-likelihood phylogenetic trees from Jukes-Cantor alignments with local support values for each node) (67, 68). *Nitrospira moscoviensis* was used as the root.

RESULTS

We examined the 16S rRNA data from conventional mice, mice which had been treated with streptomycin, and mice that had been treated with streptomycin and individually colonized with *E. coli* Nissle 1917 wild-type Nal^r Str^r (NS), a commensal isolate used as a control for strain effects, *E. coli* MG1655 wild-type Nal^r Str^r (MG), a laboratory adapted commensal strain, or *E. coli* MG1655 Δ*cydAB* Δ*cyoAB::cat* Str^r (DM), an isogenic cytochrome oxidase mutant, which is unable to respire oxygen but has the same carbon-use profile and the same fermentative and anaerobic respiration capabilities as its wild-type parent.

Across all conditions at a 97% similarity threshold we detected members of 8 bacterial phyla, 99.06% of which were included in only 4 phyla: Bacteroidetes (8.2%), Deferribacteres (7.7%), Firmicutes (84.0%), and Proteobacteria (0.1%). The remaining four 4 phlya included: Actinobacteria, TM7, Tenericutes, and Verrucomicrobia. These results are consistent with previous investigations of intestinal microbiota (69, 82). The distribution of the phyla across pooled samples is illustrated in Fig. 1. Fimicutes represents ~83.2% in D0 and DM, ~92.0% in CN and NS, and 66.3% in MG. Bacteroidetes represents ~3.5% in CN, D0, and NS, 10.3% in DM, and 32.2% in MG. These results indicate that *E.* coli MG1655 is either directly or indirectly affecting the Firmicutes to Bacteroidetes ratio. Three OTUs are present in the Bacteroidetes found in mice colonized with *E.* coli MG1655: one clusters with *Barnsiella intestinohominis*, a Gram negative, catalase and oxidase negative, obligate anaerobe (53) and the other two cluster with *Parabacteroides goldsteinii*

(formerly *Bacteroides goldsteinii*) a Gram negative, obligate anaerobe (74, 79). Deferribacteres represents ~4.4% in CN, DM, and NS, 14.2% in D0, and 1.2% in MG. Proteobacteria represents ~0.2% in D0, DM, MG, and NS and a tiny fraction of the phylotypes in CN. None of the phylotypes were unique to only one animal, which was interpreted as indicating that the core bacteria in all of the mice was sufficiently sampled.

The differences between CN and D0 indicate that streptomycin treatment alters the bacterial population (increase in proportion of Deferribacteres and Bacteroides, and reduction of Firmicutes) and that each of the *E. coli* strains we introduced influence the succession of intestinal microbiota differently following introduction of and during maintenance of streptomycin pressure on the ecosystem microbiology. Of the 1499 phylotypes detected across both pooled and replicate samples, 1254 (84%) represented phylotypes that could not be assigned beyond the family taxonomic level by the RDP naïve Bayesian rRNA Classifier at a 80% confidence threshold - the majority of these in the order Clostridiales. This is consistent with previous studies using 16S rRNA 454 pyrosequencing of samples derived from the large intestine (18). 10 phylotypes appeared in all samples: one *Parabacteroides*, one *Mucispirillum*, five novel Clostridia, one *Clostridium* and two novel Lachnospiraceae. There are several explanations for the presence of this core group which may be prevalent as a result of either direct or indirect streptomycin sulfate or *E. coli* strain effects: first they are not negatively influenced by the introduction of streptomycin sulfate; second, they are constantly reintroduced into the ecosystem in numbers high

enough to overcome selective pressure against their establishment (mice are known coprophages (1); third, they are not negatively impacted by the loss or reintroduction of facultative anaerobes.

We generated rarefaction curves at 97% sequence similarity for each of the pooled samples using the observed_species metric available in QIIME. CN has the highest diversity and MG has the lowest, NS and DM are similar with D0 being slightly higher than either of these (Fig. 2). These results indicate that at the level of 97% sequence similarity, we were least successful in fully sampling conventional mice. α-diversity and richness indices within pooled samples are reported in Table 3. The Chao1 index indicates that CN mice had the highest community richness and D0 mice had the lowest, with DM, MG and NS being in various states of diversity recovery. These results indicate that streptomycin treatment reduced the species richness of intestinal microbiota and different *E. coli* strains were successful in restoring the diversity to varying degrees. The Shannon indices indicate that CN mice have the highest species richness and evenness, followed closely by D0, DM, and NS mice, while MG mice have the lowest. Since the Shannon index provides information regarding diversity and evenness of the community, these results indicate that that streptomycin treatment had a less severe impact on the diversity of the rare biosphere than on the dominant phylotypes. Additionally, *E. coli* MG1655 wild-type was least successful in stimulating the diversity of the rare biosphere, indicating that it may have an ecological and prey species in macro-ecological investigations of food-

web complexity and species diversity (Fig. 3). Removal of one level of the food web results in a decrease in species diversity of the entire system (58).

β-diversity was examined, first by rarifying all samples to the size of the smallest library. Unweighted (community composition) and weighted (community structure) distance matrices were constructed using UniFrac. Both were individually used for hierarchal clustering using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) with jackknifing support of node placement from 75% of the rarified sample. In the unweighted tree (Fig. 4) we find that all samples are a subset of CN, which indicates that there are no external microbiota taking advantage of streptomycin pressure to invade the intestinal ecosystem of our mice. All of the MG samples cluster independently from NS, DM, and D0, and replicates cluster with their respective pools with ≥ 50% jackknifing support. This indicates that each of our replicates have similar community composition, which is properly represented by their respective pools. In the weighted tree (Fig. 5), we find CN and NS clustering with DM, which reflects the similarity of their α-diversity indices, particularly the Shannon index (richness and evenness). All replicates cluster with their pools with ≥ 75% jackknifing. This indicates that each of our replicates have similar community architecture, which is equivalent to their respective pools.

In order to undertake a detailed analysis of community architecture in each of our pooled samples, we limited each library to 10 OTUs or those representing ≥ 1% of the total population in a given sample, whichever was greater. The remaining sequences in each sample we call the rare biosphere,

which represent 31% of the phylotypes in CN, 12% in D0, 7% in DM, 6% in MG, and 14% in NS. These results are consistent with our Shannon α-diversity measures, which indicated that CN had the largest rare biosphere and MG the smallest (Fig. 3). A visual representation of all pooled samples can be seen in Figures 6a, b, and c. Three dominant phylotypes were common across all conditions: *Mucispirillum* (14% of D0 libraries, significantly lower in CN, DM, MG, and NS), a novel Clostridiales_4066 (18% of D0 and 21% of DM libraries, significantly lower in CN, MG, and NS), and a novel Lachnospiraceae_3983 (34% of CN, 24% of DM, and 6% of NS libraries, significantly lower D0 and MG). These phylotypes must not be strongly influenced by streptomycin treatment or the presence/absence of facultative anaerobes. Ten additional phylotypes were present in all libraries, though only found in the rare biosphere of at least one sample in each instance: a *Parabacteroides*, 3 novel Clostridiales, a *Clostridium*, three novel Lachnospiraceae, and one *Oscillobacter*. No dominant or rare phylotypes were unique only to streptomycin treated animals, indicating that streptomycin sulfate treatment does not overly influence any one phylotype, beyond elimination of facultative anaerobes. One phylotype was dominant in all animals we colonized with *E. coli*, a novel Clostridiales (OTU 1484), though it can be found in the rare biosphere of CN and D0. It is possible that streptomycin treatment either directly or indirectly eliminated the niche competition for this Clostridiales and introduction of *E. coli* was unable to restore its competition; it is most closely related to *Clostridium difficile*, a Gram positive, spore-forming, obligate anaerobe. Although *C. difficile* is normally found in low numbers in the

large intestines and feces of healthy humans, it is an opportunistic pathogen, which is notorious for causing disease in the intestine following antibiotic treatment (38, 63). Another phylotype, a novel Lachnospiraceae (OTU 391), was found only in mice containing wild-type *E. coli*, the native facultative anaerobes in CN included. It is dominant in CN and found in the rare biosphere of animals we colonized with *E. coli*. This Lachnospiraceae must be positively influenced, either directly or indirectly, by the presence of facultative anaerobes; it is most closely related to *Clostridium polysaccharolyticum*. One phylotype, a *Barnsiella* (OTU 3225), was not found in D0 and was present in the rare biosphere of all animals containing *E. coli*, the native facultative anaerobes in CN included. This *Barnsiella* sp. must be positively influenced, either directly or indirectly, by the presence of facultative anaerobes, independent of their ability to respire oxygen; it is most closely related to *B. intestinihominis*. The dominant OTUs of CN contained 7 unique phylotypes, all of them novel Lachnospiraceae. The dominant OTUs of D0 contained 5 unique phylotypes, 1 novel Lachnospiraceae, and 4 novel Clostridiales. The dominant OTUs of DM contained 7 unique phylotypes, *Barnsiella*, a novel Clostridium, 2 novel Lachnospiraceae, a novel Porphyromondaceae, a novel Ruminococcaeae, and a novel Bacteroidiales. The dominant OTUs of MG contained 2 unique phylotypes, *Parabacteroides*, and a novel Clostridiales. The dominant OTUs of NS contained 3 unique phylotypes, 2 novel Clostridiales and a novel Lachnospiraceae. Table 4 shows a detailed examination of the dominant OTUs in each pooled sample. Each condition has at least two unique, dominant phylotypes, and no two conditions stimulate the

dominant microbiota in the same manner. The differences between DM, MG, and NS indicate strain specific stimulation of the obligate anaerobes. Fig. 7a and b show a phylogenetic tree of the dominant OTUs in the pooled samples using FastTree and a Greengenes alignment with cultured isolates obtained from RDPs SeqMatch tool that are most similar to each OTU identified in Table 4. Importantly, the differences between DM, MG, and NS indicate the possibility of strain specific stimulation of the obligate anaerobes.

DISCUSSION

The gastrointestinal tract is an incredibly complex ecosystem. The results of this experiment support our hypothesis that oxygen scavenging in *E. coli*, a commensal facultative anaerobic resident of the gastrointestinal tract, impacts the cecum mucus bacterial community structure. It was not possible to quantitatively determine if clades of obligate anaerobes were responding differently than clades of aerotolerant anaerobes, but qualitative examination provided three possible explanations for responses of different phylotypes, regardless of their known sensitivity to oxygen. First, the phylotype is not adversely affected by streptomycin treatment either because it is intrinsically resistant or is not negatively influenced by the primary or secondary loss of those populations, which are unable to survive the post-treatment conditions. In another study, no alteration in the anaerobic composition of the fecal microbiota was found immediately following perfusion of the colon with oxygen, but anaerobic metabolism was effected (6). This alteration in biochemistry of the intestinal environment may be compounded as time progresses, irreversibly

altering the composition of its microbial constituents (16). Second, competition for the nutrient niche the phylotype naturally occupies has changed, either directly or indirectly, as a result of environmental changes or alterations in the other microbiota following streptomycin treatment. Competition for resources in the gastrointestinal tract is one of the primary driving forces dictating species fitness in this environment (22). Third, the affected phylotype is capable of surviving the alteration in its ecosystem by forming novel associations with members of the microbiota that provide a microniche and/or micro-environment sufficient for its maintenance in the absence of the native microbiota which streptomycin treatment either directly or indirectly removed from the ecosystem. Functional redundancy has been implicated in the stability of the intestinal microbiota (82, 95). We noted the presence of a small number of Alphaproteobacteria following streptomycin treatment, and all of the bacteria they cluster most closely to make their living respiring aerobically. Additionally, as previously discussed, other anaerobic bacteria can mitigate the toxic effects of oxygen. *E. coli* may not be the only bacteria reducing oxygen tension in the in the streptomycin-treated mouse intestinal ecosystem.

Colonization resistance in the intestine is a well-known phenomenon, and we have learned that different strains of *E. coli* must occupy different nutrient niches in the intestine in order to coexist (39). It is obvious from our results that all three strains of *E. coli* we used to investigate responses in the obligate anaerobes are stimulating different subpopulations within the microbiota. *E. coli* Nissle 1917 was much better at restoring the native microbiota than either of our

E. coli MG1655 strains in the time allowed. This may help explain why it has proven to be such a strong colonizer of animals despite native competitors for its niche (77). This strain may have evolved to stimulate the same microbiota as its host – a perfect "predator" in its ecosystem whose function ultimately promotes diversity and stability. It may be informative then to repeat our aerobic respiration replicates experiment with this strain to monitor the influence of aerobic respiration on the obligate anaerobes in the intestine, as the inconclusiveness of our results from *E. coli* MG1655 may simply be due to its relative inadequacy to stimulate the native microbiota. *E. coli* MG1655 also promoted a bloom in the Bacteroidetes with a concurrent decrease in the Firmicutes. Similar microbial community structure has been strongly correlated with Inflammatory Bowel Disease (IBD) (89). We also noted the presence of a core group of 10 phylotypes detectable in all of our mice, three of which represented ≥ 1% of the total bacterial population [a *Mucisprillum* sp. (OTU 1086 clusters with *M. schaedleri*, a novel member of the Lachnospiraceae (OTU 3983 clusters with *Hepsiella porcina*, catalase and oxidase negative Gram positive obligate anaerobes (92)) family, and a novel member of the order Clostridiales (OTU 4066 clusters with *C. polysaccharolyticum*)]. Culturing and identifying these organisms may provide insight into their adaptability and fitness in the mucus layer of the large intestine. We also identified two phylotypes that were found only in mice harboring *E. coli* (the native *E. coli* of conventional animals included), one clustered with *B. intestinihominis* and the other with *C. polysaccharolyticum.* It would be interesting to learn if these three

microorganisms form a microcosm with one another *in vivo*, and what the nature of their association is, particularly in the light of the importance of butyrate production to host intestinal health. Many of the phylotypes identified in this study fall in in the *Clostridium* Cluster XIVa that contains many bacteria known to produce butyrate in the gastrointestinal tract of healthy humans (3). Butyrate is beneficial to host health, because it provides an energy source to colonic epithelial cells, enhances intestine mucosal barrier function, and helps prevent proliferation of cancerous colonic epithelial cells (49, 64, 86). Additionally, it is most likely produced by bacteria that do not adhere to food particles and degrade mucin or starch as *C. polysaccharolyticum* is known to do (45, 72, 85). Understanding the responses of these bacteria to a disturbance in the intestinal ecosystem may have important applications in human health.

The scope of our investigation did not cover host-mediated responses to ecosystem disturbance, which have demonstrated the ability to alter the intestinal environment in favor of pathogens (94). We noted the prominence of one phylotype (1484) that clustered with *C. difficile* in all of the animals we treated with streptomycin and subsequently colonized with *E. coli*. Interestingly it was only a member of the rare biosphere in conventional animals and those that had only been treated with streptomycin for 24 hrs. It is possible the prevalence of this clade in streptomycin treated animals may be aided by an inflammatory response of host cells to the alteration in microbiota seen in our D0 mice, perhaps as a result of the dramatic increase in the *Mucispirillum* phylotype. *M. schaedleri* is specifically adapted to colonize intestinal mucus, because of its

spiral shape and motility, giving it a selective advantage over competitors for this space. It is not currently identified with disease, but two other bacteria, that share its physiology and propensity for mucosal association are: *Helicobacter* and *Campylobacter* (71). Previous studies have shown that presence of a pathogen increases oxygen uptake in host cells, resulting in marked anoxia (50). Unfortunately for the host, in this case, anoxia would only serve to promote over-growth of either *Mucispirillum*, which is unable to grow in aerobic or microaerophilic conditions, or the phylotype clustering with *C. difficile*, an obligate anaerobic pathogen notorious for causing disease following antibiotic treatment. It is difficult to place casual relationships on host-mediated responses *in situ*. However, these results provide an interesting basis for ecological investigations of indirect promotion of obligate anaerobic pathogens by host-mediated response following antibiotic treatment.

In conclusion, although *E. coli* is a relatively minor constituent of the gastrointestinal ecosystem, it has a significant impact on the architecture of the bacterial community in the mucus layer adjacent to the intestinal wall. The influence of this facultative anaerobe cannot be entirely explained by oxygen scavenging, possibly because the effect may be indirect or secondary and difficult to determine using 16S rRNA surveys of the microbiota. Additionally, different strains of *E. coli* influence succession of the microbiota following a disturbance, in our case antibiotic treatment, in very different ways. We limited this experiment to 10 days. It is entirely possible that given more or less time we would get a better picture of how succession proceeds towards a climax

community in the intestine. Physiological conditions may be restored to their native state within 3 - 5 days, but it may take the native microbiota much longer to completely recover, if complete recovery is possible in the absence of a conventional donor (1, 5). The results of this research provide a launching point for further investigation of probiotic applications of *E. coli* in order to prevent or ameliorate a variety of gastrointestinal disease that result from the disturbance of the native microbiota.

Table 1: Strains and plasmids used in this study.

Strain	Genotype or Phenotype	Reference
MG1655	Wild type (CGSC no. 7740)[a]	CGSC
MG1655 Str^r	Spontaneous Str^r	(52)
MG1655 Str^r Nal^r	Spontaneous Nal^r	(52)
Nissle 1917 Str^r Nal^r	Spontaneous Str^r and Nal^r	(2)
Derivatives of MG1655 Str^r		
cydAB cyoAB[b]	Δ*cydAB* Δ*cyoAB::cat*	This study

[a] CGSC, *E. coli* Genetic Stock Culture Collection, Yale University.
[b] For simplicity, strains are named in the text by strain and mutation(s).

Table 2: Samples and associated primers used for sequencing.

Sample	Sample Name	Primer Name	Hamming Barcode
Conventional (J15-16 and J28-30 pooled)	CN	518R.A.TS1	AACCAACC
Day 0 (J55-58 pooled)	D0	518R.A.TS6	ATAACCGC
MG1655 double mutant (J49-54 pooled)	DM	518R.A.TS4	AGGAACCA
MG1655 WT (J19-20 and J39-43 pooled)	MG	518R.A.TS3	AGACAGTG
Nissle WT (J59-63 pooled)	NS	518R.A.TS5	ACCAACCA
Day 0 55 (J55)	D055	518R.A.TS3	AGACAGTG
Day 0 56 (J56)	D056	518R.A.TS4	AGGAACCA
Day 0 57 (J57)	D057	518R.A.TS5	ACCAACCA
Day 0 58 (J58)	D058	518R.A.TS6	ATAACCGC
MG1655 double mutant 49 (J49)	DM49	518R.A.TS5	ACCAACCA
MG1655 double mutant 50 (J50)	DM50	518R.A.TS6	ATAACCGC
MG1655 double mutant 51 (J51)	DM51	518R.A.TS1	AACCAACC
MG1655 double mutant 52 (J52)	DM52	518R.A.TS2	ACACACAC
MG1655 WT 39 (J39)	MG39	518R.A.TS1	AACCAACC
MG1655 WT 41 (J41)	MG41	518R.A.TS2	ACACACAC
MG1655 WT 42 (J42)	MG42	518R.A.TS3	AGACAGTG
MG1655 WT 43 (J43)	MG43	518R.A.TS4	AGGAACCA

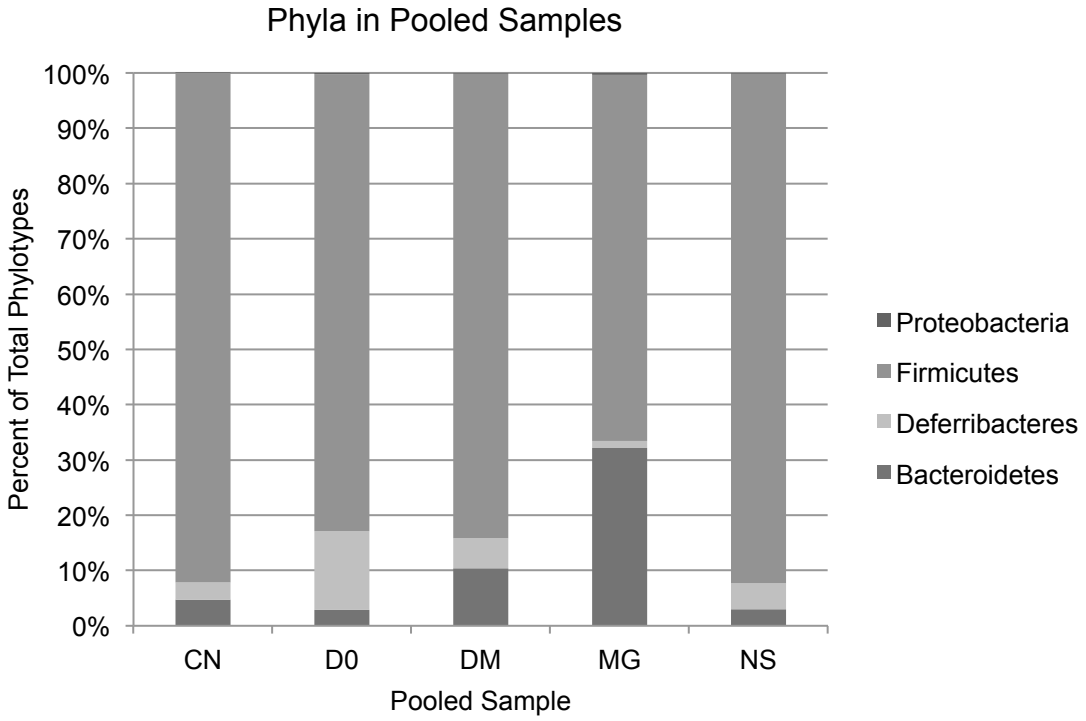

Figure 1. Dominant phyla found in pooled samples. The Proteobacteria are such a small percentage of each library that they are barely detectable above the Firmicutes bars.

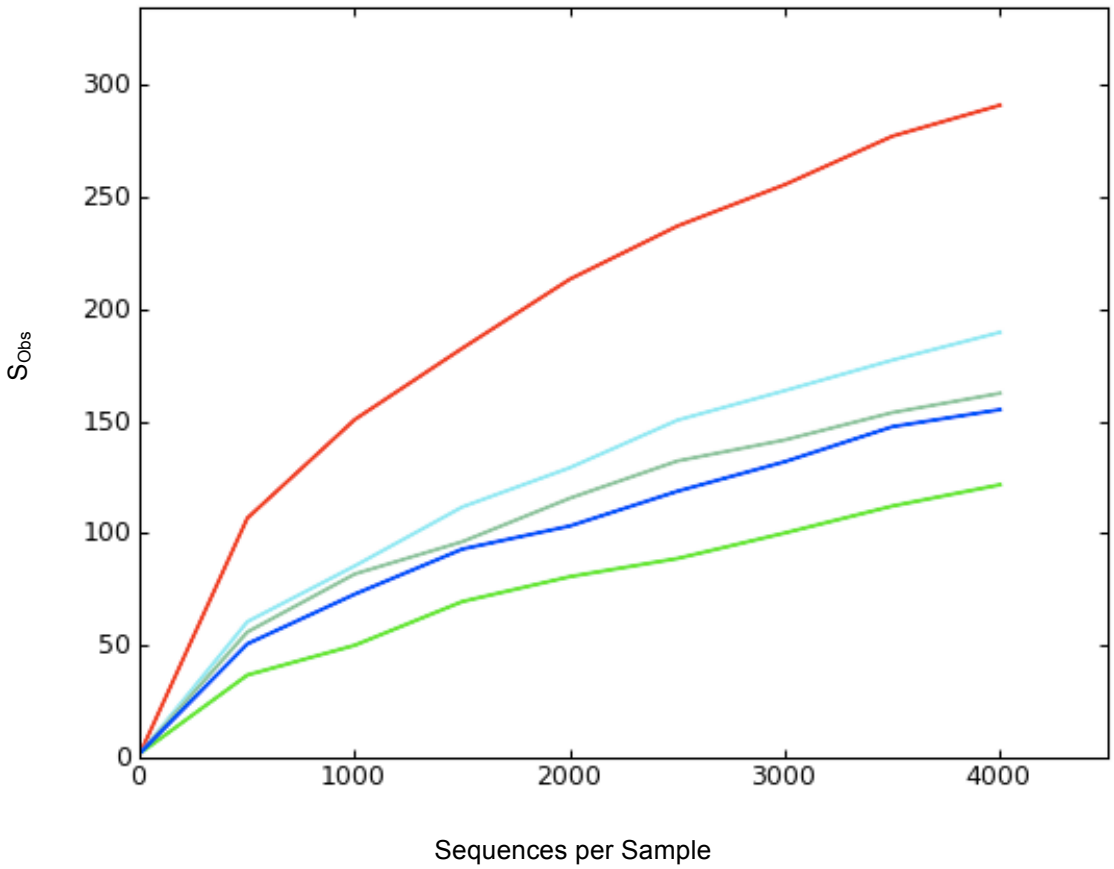

Figure 2. Rarefaction curves for pooled samples. CN = red, DM = light blue, NS = blue green, D0 = blue, and MG = green.

Table 3. α-diversity indices for pooled samples.

Sample	Chao1*	Shannon ± SD
CN	490.03 (484.24,495.81)	5.127 ± 0.052
D0	276.09 (274.44,277.73)	4.151 ± 0.008
DM	372.49 (369.19,375.79)	4.184 ± 0.019
MG	254.44 (248.85,260.04)	2.856 ± 0.025
NS	316.41 (310.50,322.32)	3.933 ± 0.024

*95% confidence interval shown in parentheses.

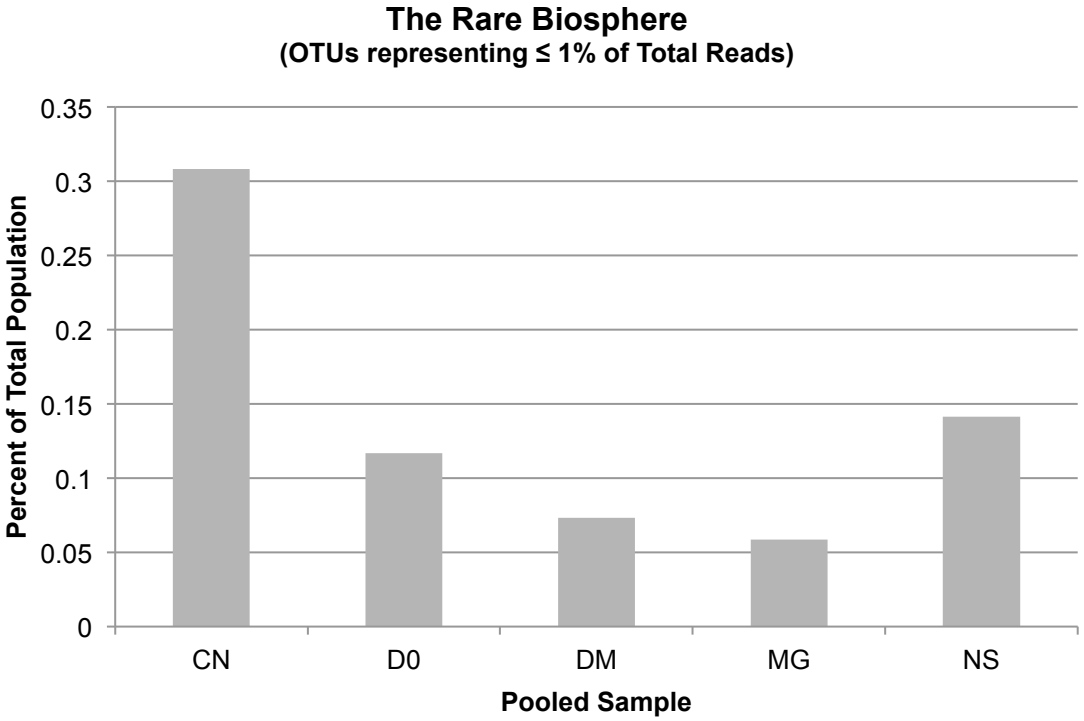

Figure 3. Table showing the size of the rare biosphere in each pooled sample.

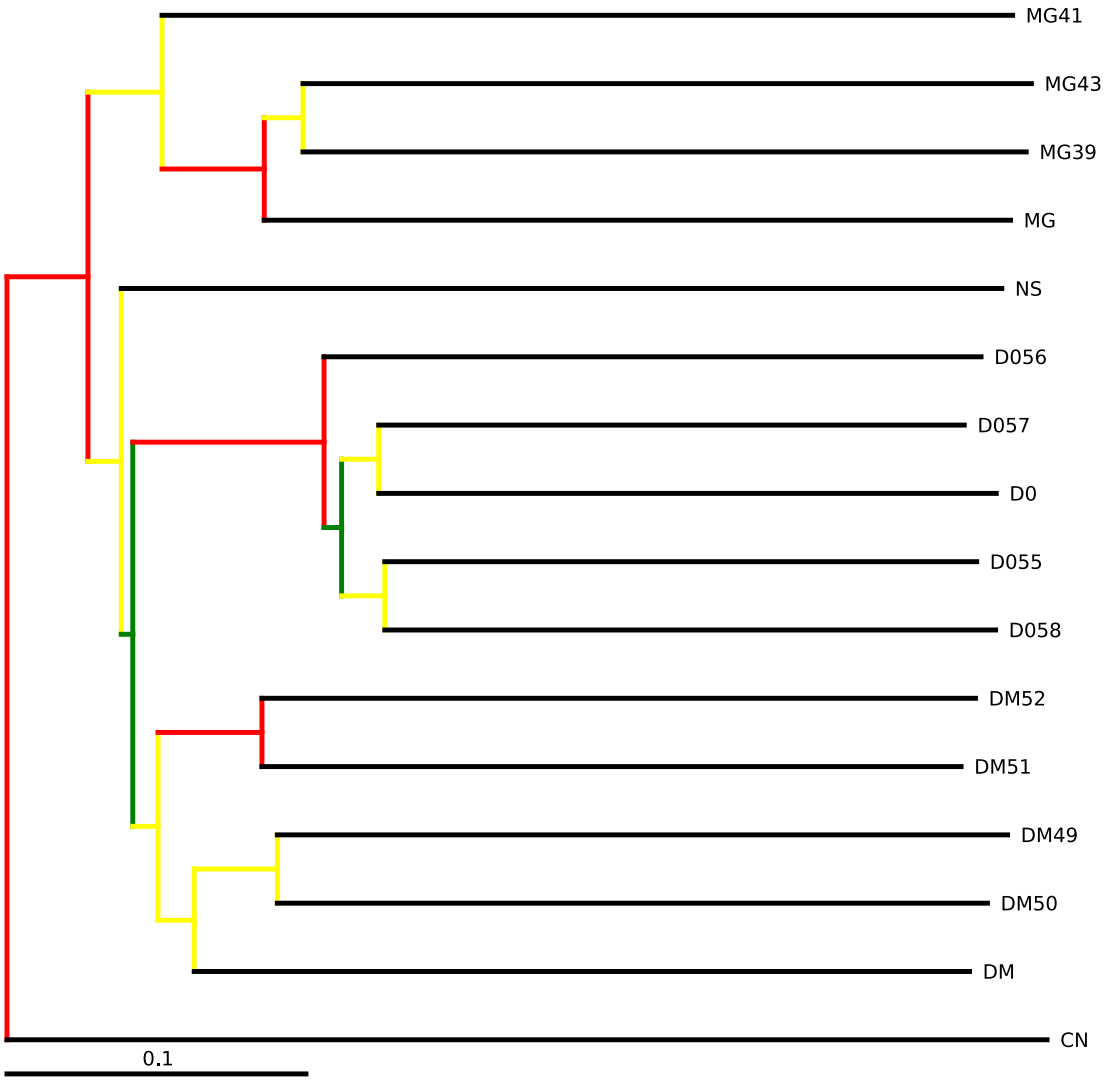

Figure 4. β-diversity of all samples as measured by UPGMA hierarchical clustering of an unweighted Unifrac distance matrix (community membership). 10 jackknife replicates from a subset of a rarified OTU table, were used to estimate the uncertainty in node placement. Red indicates 75 - 100% confidence, yellow for 50 – 75%, green for 25 – 50%, and blue indicates < 25% confidence in node placement.

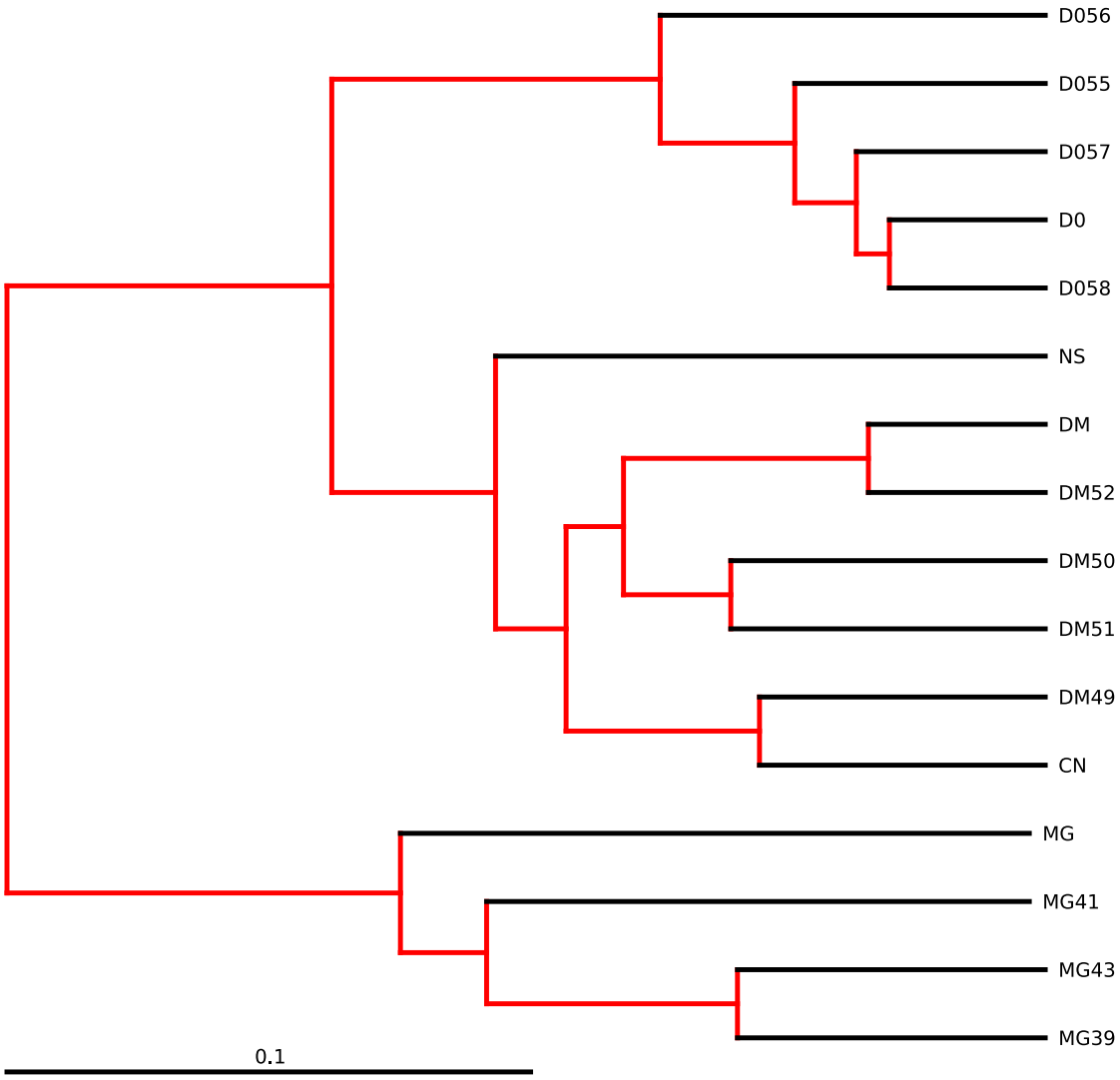

Figure 5. β-diversity of all samples as measured by UPGMA hierarchical clustering of a weighted Unifrac distance matrix (community structure). 10 jackknife replicates from a random subset of a rarified OTU table, were used to estimate the uncertainty in node placement. Red indicates 75 - 100% confidence in node placement.

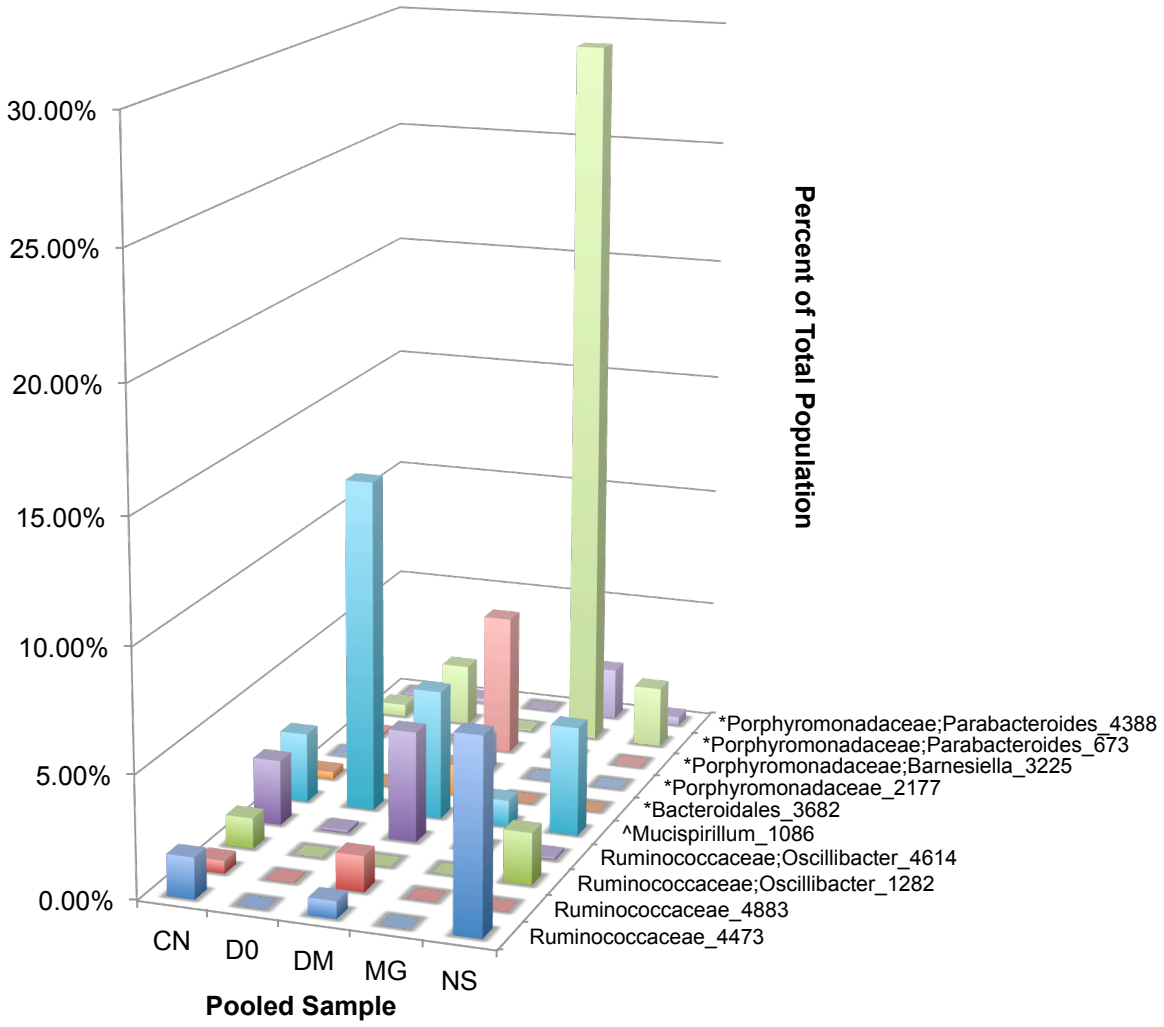

Figure 6a. Phylotypes representing ≥ 1% from at least one pooled sample. *Bacteroidetes;Bacteroidia;Bacteroidales;x, ^Deferribacteres;Deferribacteres;Deferribacterales;Deferribacteraceae;x, and Firmicutes;Clostridia;Clostridiales;x

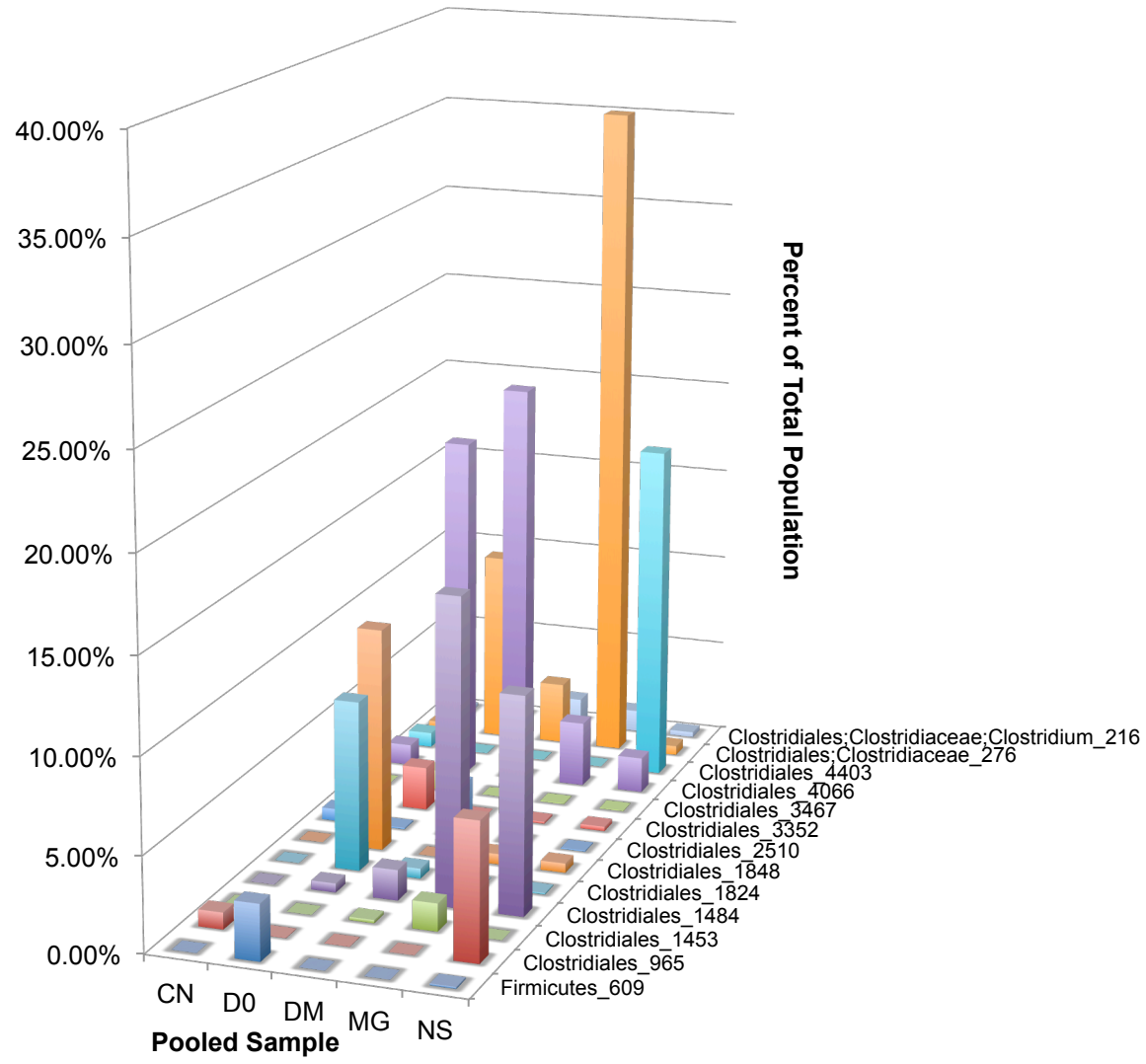

Figure 6b. Phylotypes representing ≥ 1% from at least one pooled sample. A novel Firmicutes (OUT ID 609) and Firmicutes;Clostridia;x

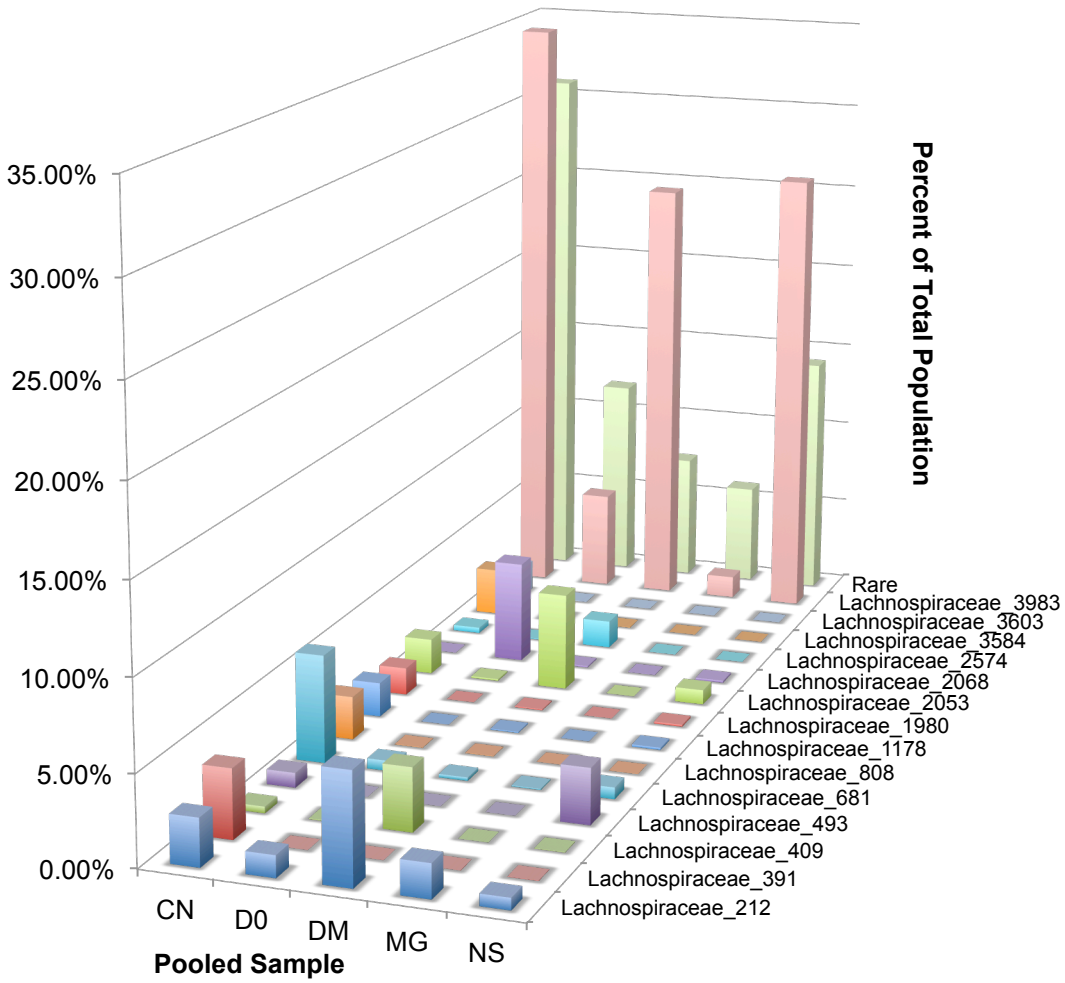

Figure 6c. Phylotypes representing ≥ 1% from at least one pooled sample. Rare biosphere and Firmicutes;Clostridia;Clostridiales;x.

Table 4. Dominant OTUs present in each condition listed in order from highest percent of total population to lowest. Corresponding taxonomy is reported in Table 5.

CN	D0	DM	MG	NS
3983	4066	3983	276	3983
681*	1086	4066	673	4403
391*	1848	212	1484	1484
1086	276	3225°	4066	4473
3584*	1824^	2053	4388``	965‡
4614	2068^	1086	212	1086
212	3983	4614	1453``	493‡
808*	216	409°	3983	673
2053	609^	276	216	1282
1178*	673	2510°	1086	4066
3603*	3352^	1484	1848	2053
4473	212	2574		
1980*	3467^	2177°		
1282		216		
4066		4883°		
		3682°		

*unique to CN, ^unique to D0, °unique to DM, ``unique to MG, ‡unique to NS

Table 5. RDP assigned taxonomy (80% confidence threshold) for OTU IDs in Table 5. A phylogenetic tree constructed from cultured isolates most similar to each OTU is shown in Fig. 7a and b.

OTU ID	Consensus Lineage
212	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae_212
216	Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Clostridium
276	Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiaceae
391*	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae_391
409	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae_409
493‡	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae_493
609^	Bacteria;Firmicutes
673	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Parabacteroides_673
681*	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae_681
808	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae_808
965‡	Bacteria;Firmicutes;Clostridia;Clostridiales_965
1086	Bacteria;Deferribacteres;Deferribacteres;Deferribacterales;Deferribacteraceae;Mucispirillum
1178*	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae_1178
1282	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Oscillibacter_1282
1453	Bacteria;Firmicutes;Clostridia;Clostridiales_1453
1484	Bacteria;Firmicutes;Clostridia;Clostridiales_1484
1824^	Bacteria;Firmicutes;Clostridia;Clostridiales_1824
1848	Bacteria;Firmicutes;Clostridia;Clostridiales_1848
1980*	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae_1980
2053	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae_2053
2068^	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae_2574
2177°	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae
2510°	Bacteria;Firmicutes;Clostridia;Clostridiales_2510
2574	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae_2574
3225°	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Barnesiella
3352^	Bacteria;Firmicutes;Clostridia;Clostridiales_3352
3467^	Bacteria;Firmicutes;Clostridia;Clostridiales_3467
3584*	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae_3584
3603*	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae_3603
3682°	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales
3983	Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae_3983
4066	Bacteria;Firmicutes;Clostridia;Clostridiales_4066
4388	Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Parabacteroides_4388
4403	Bacteria;Firmicutes;Clostridia;Clostridiales_4403
4473	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae_4473
4614	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Oscillibacter_4614
4883°	Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae_4883

*unique to CN, ^unique to D0, °unique to DM, ``unique to MG, ‡unique to NS

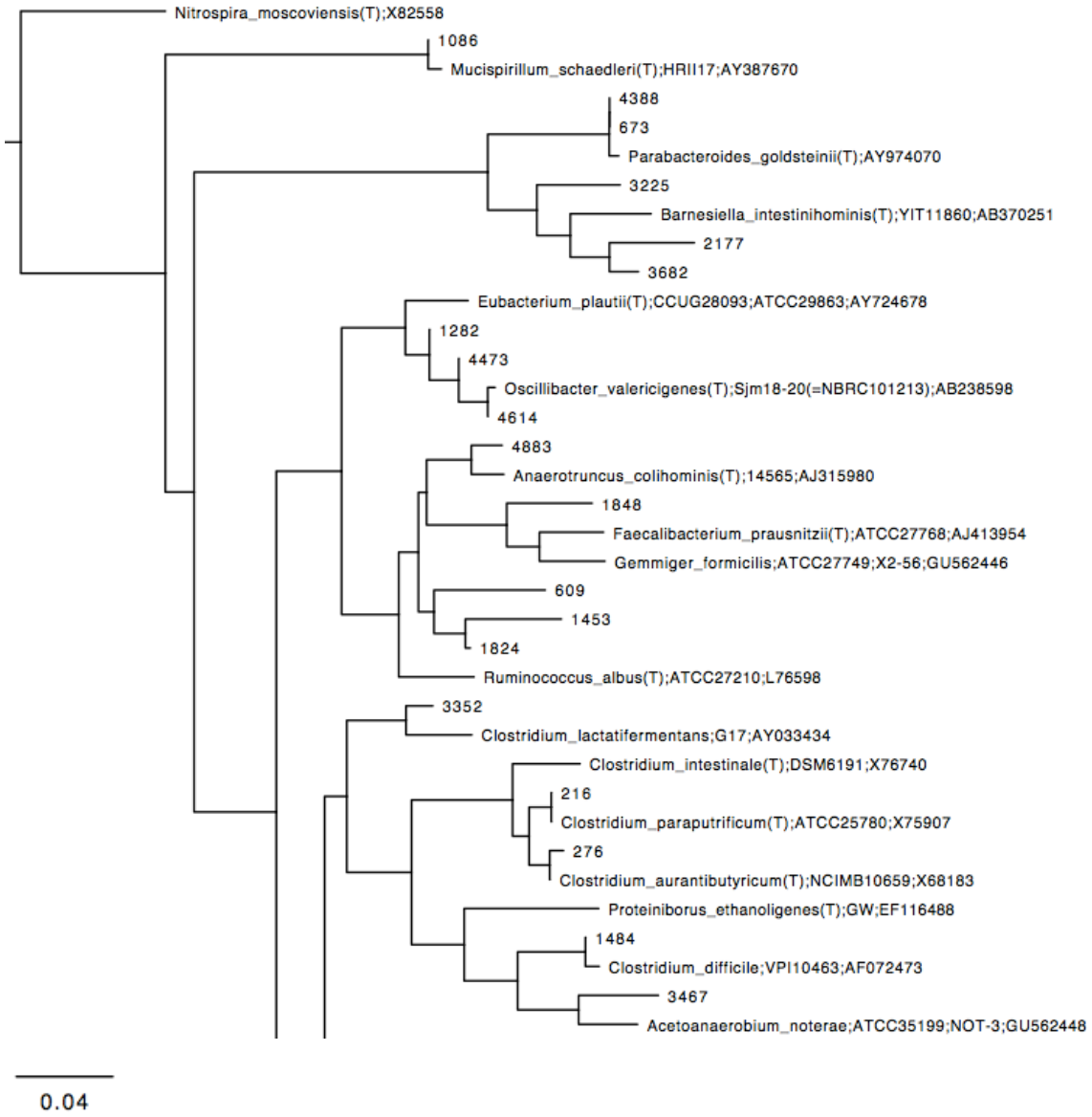

Figure 7a. A phylogenetic tree constructed using FastTree with cultured isolates obtained from RDPs SeqMatch tool that are most similar to each OTU identified in Table 6. *Nitrospira moscoviensis* was used as the root.

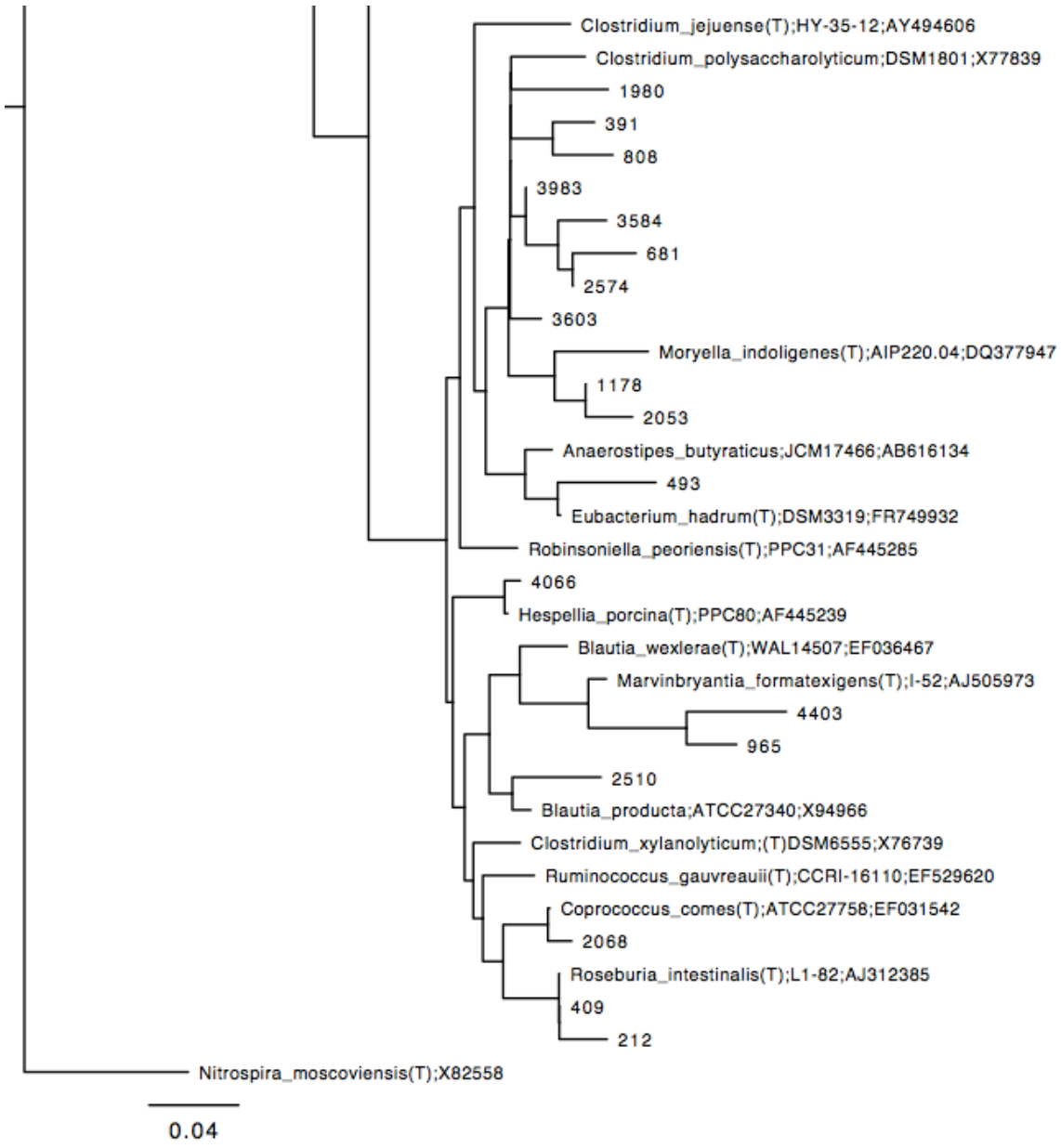

Figure 7b. A phylogenetic tree constructed using FastTree with cultured isolates obtained from RDPs SeqMatch tool that are most similar to each OTU identified in Table 6. *Nitrospira moscoviensis* was used as the root.

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Chapter 5: Conclusions and outstanding questions

The experiments described in this dissertation have presented *in silico* and *in vivo* evidence for the ability of *Escherichia coli* to aerobically respire in the large intestine and have demonstrated that different strains of *E. coli* place unique fingerprints on the bacterial residents of this complex, host-associated microbial ecosystem. In this final chapter, the highlights of the previous chapters are reviewed and general conclusions are drawn. Outstanding questions and potential directions for future work are also presented.

Chapter two summary. The model presented in Chapter two investigates the potential for *E. coli* cells residing in the mucus layer near the intestinal wall to reduce the concentration of oxygen in their environment. We combined Fick's Second Law of Diffusion with Monod growth kinetics to simulate oxygen diffusion from the epithelial cells of the intestinal wall into the luminal contents in the absence and presence of aerobically respiring *E. coli*. The model predicts a decrease in the oxygen tension of the mucus to 0 μM within 20 μm of the intestinal wall when *E. coli* are present. The model was purposefully restricted in order to simplify the involved calculations, but the results are congruent with data available in the literature. Additionally, we achieved an *in silico* result that warranted *in vivo* verification

Chapter three summary. The experiment outlined in Chapter three validates the prediction of the model presented in Chapter two. *E. coli* has two cytochrome oxidases, which it uses to respire oxygen under atmospheric or microaerophilic concentrations. Cytochrome oxidases are heme proteins with iron molecules at their center, which interact with oxygen during aerobic

respiration. T_2^* weighted MRI is sensitive to the arrangement of protons around iron molecules. This arrangement is altered when oxygen is present. We used a novel application of T_2^* weighted MRI to investigate oxygen availability in the ceca of conventional mice, streptomycin treated mice (selectively removes aerobically respiring Gram negative facultative anaerobes), and mice that were treated with streptomycin and then colonized with *E. coli* MG1655 wild-type Nal^r Str^r or its isogenic cytochrome oxidase mutant *E. coli* MG1655 Δ*cydAB* Δ*cyoAB::cat* Str^r. We found that mice with their native microbiota and those we colonized with wild-type *E. coli* had significantly more oxygen bound to heme than mice without aerobically respiring facultative anaerobes. By deduction, we may then conclude that oxygen tension is significantly lower in the ceca of mice harboring their native microbiota or wild-type *E. coli* than in mice without their native microbiota or those colonized with the cytochrome oxidase mutant. Additionally, the significant difference we found between conventional and streptomycin treated animals is consistent with data previously reported in the literature for intracolonic oxygen tension in gnotobiotic and conventional mice, indicating that microorganisms removed by streptomycin treatment are most likely responsible for this difference. Though it is not possible to conclude that *E. coli* is the only aerobically respiring facultative anaerobe in conventional mice, our results indicate that this organism is capable of producing oxygen tensions in the cecum equal to that found in mice with their native microbiota intact.

Chapter four summary. The experiments presented in Chapters two and three revealed the ability of aerobic respiration in *E. coli* to reduce oxygen

tension in the lumen of the gastrointestinal tract. The experiment presented in Chapter four investigates the influence of *E. coli* oxygen scavenging on the numerically dominant obligate anaerobic bacteria of the mouse cecum. The gastrointestinal tract is an environmentally stratified, competition rich microbial ecosystem with small nutrient pool size. The symbiotic relationship of the gastrointestinal microbiota with its host is dependent, in large part, upon the stability of the relationship between the numerically dominant obligate anaerobes and the facultative anaerobes. *E. coli* constitutes only 0.1% of the total bacterial population and is ubiquitous in mammalian intestines. The fact that it is one of the few aerobically respiring microbial residents of the gastrointestinal tract presents the intriguing possibility that it may be providing anaerobic conditions necessary for the survival of its obligate anaerobic neighbors. We investigated this hypothesis by conducting 16S rRNA surveys of conventional mice (CN), mice that had been treated with streptomycin to remove their native facultative anaerobes (D0), and mice that had been treated with streptomycin and then colonized for ten days with *E. coli* MG1655 wild-type Nal^r Str^r (MG), its isogenic cytochrome oxidase mutant *E. coli* MG1655 Δ*cydAB* Δ*cyoAB::cat* Str^r (DM), or *E. coli* Nissle 1917 wild-type Nal^r Str^r (NS), a control for effects not related to aerobic respiration. Disturbance of the gastrointestinal ecosystem results in a complex response from the remaining microbiota that may diverge from the conventional homeostasis. These responses may be either direct or indirect and have primary or secondary influences on succession. Streptomycin treatment reduces bacterial diversity in the intestine, and the recovery of this diversity,

particularly that of the rare biosphere, is affected more by the niche occupied by the experimentally introduced *E. coli* than by aerobic respiration. The native microbiota has sufficient functional redundancy to allow some obligate anaerobes to cope with the increased oxygen tension following the loss of aerobically respiring facultative anaerobes. Additionally, we detected phylotypes that seem to be stimulated by the presence of *E. coli*, and a core group of phylotypes that are constitutively present in the mucus of the mouse cecum. To our knowledge, this is the first 16s RNA investigation of the influence of *E. coli* on the microbial inhabitants of intestinal mucus.

Synthesis and outstanding questions. It was my initial goal to determine if *E. coli* influences oxygen tension in the intestine. Aerobic respiration under microaerophilic conditions had previously been identified in this microorganism as important for niche competition *in vivo* (6). Oxygen tension in the gastrointestinal tract is sufficient to support aerobic respiration in this microorganism (5, 14). Additionally, the Lactic Acid Bacteria, the other major group of facultative anaerobes in the intestine, do not express genes for aerobic respiration *in vivo* (8). It follows then, that if *E. coli*, a commensal facultative anaerobic resident of the gastrointestinal tract, did in fact respire oxygen in this environment, it may be one of the few, if not the only intestinal microorganism doing so. Considering that the large intestine harbors 5×10^{11} bacteria per ml of contents, the vast majority of which have been designated obligate anaerobes, aerobic respiration in *E. coli* may constitute a keystone function in this ecosystem. With our model we were able to demonstrate the potential for *E. coli*

to reduce the oxygen tension in intestinal mucus (Chapter 2, Fig. 3). We validated this prediction by measuring *E. coli* cytochrome oxidase interactions with oxygen *in vivo* using T_2^* weighted MRI (Chapter 3, Fig. 2) and deduced that *E. coli* lowers the oxygen tension of the mouse cecum. Interpretation of these results provides two theoretical presentations of the influence *E. coli* oxygen scavenging may have on the intestinal ecosystem that are not necessarily exclusive. First, if the cells are evenly distributed throughout the mucus layer, the *E. coli* population probably decreases the oxygen tension of the entire environment. Second, individual *E. coli* cells may be creating anaerobic microniches within approximately 20 μm of themselves. The implications of either of these theories are intriguing in that they would influence the obligately anaerobic residents of the gastrointestinal tract in different ways. One would provide an anaerobic blanket and promote dispersal of oxygen sensitive microorganisms throughout the mucus layer while the other would encourage more direct association between *E. coli* individual obligate anaerobes. It may also be that both are occurring simultaneously to varying degrees, depending on the distribution of *E. coli*. Examination of gastrointestinal biofilms has provided evidence for both distributions (9). Neither of these experiments directly measured the alteration in the oxygen tension in the intestine by aerobic respiration in *E. coli*. Doing so may provide a glimpse into how commensal bacteria promote resistance invasion of pathogens that rely on environmental cues to signal up-regulation of virulence factors (12). Fine-tuning the mathematics in our model to include multi-dimensional diffusion of oxygen along

with influx due to swallowed air and efflux due to peristalsis and mucus shedding would improve the biological relevance of its prediction.

The gastrointestinal tract is an incredibly complex ecosystem. A stable symbiotic relationship, between the host and its commensal microbiota has a major impact on the livelihood of both (10). Part of this stability depends upon interaction between facultative anaerobes and obligate anaerobes during succession following birth and as a mechanism of resistance to invading pathogens throughout the life of the host (2, 11). *E. coli* has a significant impact on the composition of the bacterial community in the cecum mucus (Fig. 6a, b, and c.), despite being a minor constituent of the intestinal microbiota. This result is highly reproducible (Fig. 4, 5), and further supports the use of mice as models for the human intestinal microbiota (3, 13).

We were able to prove our hypothesis that aerobic respiration in *E. coli* influences mouse cecum mucus bacterial community structure. We offer three possible explanations for the responses of commensal bacterial species to an ecosystem disturbance. First, the population of that species is not adversely affected by a disturbance either because it is intrinsically resistant to the disturbance or is not negatively influenced by the loss of those that are unable to survive conditions created post disturbance. Second, the niche this microorganism naturally occupies is positively or negatively altered as a result of this disturbance. Third, it is capable of surviving the alteration in its ecosystem by forming novel associations with members of the microbiota that provide a microniche and/or micro-environment sufficient for its maintenance in the

absence of the native microbiota. We were able to identify a core group of bacteria that were present in all conditions, including three phylotypes that constituted ≥ 1% of the total bacterial population in all of our mice. Almost all of these phylotypes are novel bacteria. What characteristics do these bacteria have that allow them to maintain populations despite the introduction of ecosystem-wide disturbance? We also identified phylotypes found only in the presence of *E. coli*. Is *E. coli* forming species-specific associations in the gastrointestinal tract?

We were not able to prove that aerobic respiration in *E. coli* residing in intestinal mucus directly promotes recovery of the native microbiota. This may be an unexpected result of the discovery that *E. coli* MG1655 wild-type was unable to promote recovery of the native microbiota (Fig. 1 - 3). The fact that *E. coli* Nissle 1917 came much closer to restoring the bacterial community in the time allowed suggests that niche influences of different strains outweigh that of aerobic respiration. Additionally, this result provides ecological evidence for the ability of certain strains to provide better colonization resistance or broader probiotic applications than others (4, 7). Investigating the effect of aerobic respiration on the obligate anaerobic residents of the gastrointestinal tract using *E. coli* Nissle 1917 may prove to be more insightful than the results we obtained using *E. coli* MG1655. It is also possible that the duration of our experiment was insufficient for complete restoration of the native microbiota to occur and this time might be strain dependent. In concert with providing additional proof or disproof of this concept, conducting a series of experiments designed to follow succession over time may yield important information related to efficacy of probiotc treatment

of a variety of intestinal diseases. There is a new interest, if not vital need, in replacing antibiotic treatments with natural manipulations of the intestinal microbiota in order to prevent or recover from disease. Probiotic bacteria working alone or in concert are capable of restoring the native microbiota from a disturbed or diseased state. Conversely, there is a high degree of functional redundancy in the native intestinal microbiota, and if properly stimulated the microbiota may be able to return to a healthy state on its own (1). We have just begun to scratch the surface of its therapeutic potential.

I hope the information contained in this work advances the understanding of intestinal microbial ecosystem ecology, particularly as it relates to human health.

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APPENDIX 1: Supplementary information to accompany Chapter 2.

```
#region Copyright (c) 2011, Philip J. Ludington
/*  All Right Reserved.
   http://www.PhilipLudington.com
 */
#endregion
using System;
using System.Collections.Generic;
using System.IO;
using MathNet.Numerics.LinearAlgebra;

namespace RespirationModelLib
{
   public class OxygenRespirationModel
   {
      // Effect of E. coli oxygen resipration in the mouse intestine
      // Assuming maximal growth rates of E. coli, diffusivity of O2 in intestinal
      // contents is equal to that of water, assuming cytochorme bo3 and bd
      // opporate independently of each other

      // This is a micrometer model
      // 1 micrometers (um,micron) = 10^-6 meters

      public const int NumberOfLayers = 500; // number of layers
      public const double totalDistance = 2500.0; // (um)
      public const double DistanceBetweenLayers = totalDistance / NumberOfLayers; // distance between layers
            (distance/layers)
      public const double D = 0.0238; // um^2/s  (Saldena, 2000)
      public const double N = 110000000000; // cells/L (1.1x10^11)  (Poulsen, 1995)
      public const double Ks = 0.121; // uM oxygen (Stolper, 2010)
      public const double o2con = 659.64;  // uM oxygen (starting oxygen concentration) (Bornside, 1976)
      public const double k = 0.000000000000933; //umol oxygen per cells per s (Varma, 1993)

      public void Calculate(int reportingInterval, bool use_bd, bool use_bo3, bool use_Ks, string filenamePrefix)
      {
         Console.Out.WriteLine("Begin Calculation");

         Matrix A = new Matrix(NumberOfLayers, NumberOfLayers);

         // calculate matrix A
         double dxToThePowerOf2 = DistanceBetweenLayers * DistanceBetweenLayers; // distance between layers^2
            (distance/layers)
         for (int i = 0; i < NumberOfLayers; i++)
         {
            A[i, i] = -2.0 * D / dxToThePowerOf2;

            if (i < NumberOfLayers - 1)
            {
               A[i, i + 1] = D / dxToThePowerOf2;
            }

            if (i > 0)
            {
               A[i, i - 1] = D / dxToThePowerOf2;
            }

         }

         A[0, 0] = -D / dxToThePowerOf2;
         A[NumberOfLayers - 1, NumberOfLayers - 1] = -D / dxToThePowerOf2;

         Console.Out.WriteLine("Finished Matrix");

         // (uM, mole aka molar) = 11.1 mmHg; [O2] on surface of epithelium (Bornside, 1976)(pressure conversion)
         double o2con = 659.64;

         // Calculate vector O2
         Matrix data = new Matrix(NumberOfLayers, 1); // Both enzymes
```

```
data[0, 0] = o2con;

// (this is time, we need to give the model a “Start up” time before it can reach equilibrium)
DateTime lastPast = DateTime.Now;
ReportingLayer[] report = new ReportingLayer[NumberOfLayers];
int NumberOfTimeSlices = Int32.MaxValue;
double last_val = 0;
for (int j = 0; j < NumberOfTimeSlices; j++) // steps for diffusion
{
   // update O2 concentration
   data = A * data + data;

   // recover the surface layer's [O2]
   data[0, 0] = o2con;

   data[NumberOfLayers - 1, 0] = 0.0;

   // microbial oxygen consumption (Haefner, 2005; Monod, 1942; van Unden, 1967; Williamson, 1975)
   for (int i = 0; i < NumberOfLayers; i++)  // increasing distance from epithelium
   {
      // Initialize Report if it's new
      if (report[i] == null)
      {
         report[i] = new ReportingLayer();
         report[i].Distance = i * DistanceBetweenLayers;
      }
      report[i].StartingO2Concentration = data[i, 0];

      // Ks
      double ksPart = 0;
      if (use_Ks)
      {
         // d[oxygen]/d(t) = D[oxygen]/x ((second order derivative)) - C([current oxygen conc.] /(Ks + [current oxygen
               conc.]) [email, Barksdale, Thu, Oct 6, 2011 at 4:53 PM]
         ksPart = k * (N * data[i, 0]) / (Ks + data[i, 0]);
         if (data[i, 0] < 0.0121)
         {
            ksPart = 0;
         }
      }

      data[i, 0] = data[i, 0] - ksPart;

      // Save for Reporting
      report[i].Ks = ksPart;

      // Save for Reporting
      report[i].EndO2Concentration = data[i, 0];
   }

   // How long did the pass take?
   double milliseconds = (DateTime.Now - lastPast).TotalMilliseconds;
   //Console.Out.WriteLine(string.Format("Pass No. {0} took {1:00.0000} milliseconds", j, milliseconds));
   lastPast = DateTime.Now;

   // Every 2500 pass report the results
   /*
   bool doreport = false;
   if (use_Ks == false &&  report[NumberOfLayers - 2].StartingO2Concentration > 0)
   {
       doreport = true;
   }
   else if (use_Ks == true && j == 52546)
   {
      doreport = true;
   }
    */

   if (j % reportingInterval == 0 || j + 1 == NumberOfTimeSlices)
   {
```

```
                string date = DateTime.Now.ToShortDateString().Replace('/', '-');

                string directory = Path.GetFullPath(string.Format("C:\\Users\\axin\\Desktop\\joy
                        barksdale\\RespirationModelData\\{0} Data", date));

                if (Directory.Exists(directory))
                {
                    // Do nothing
                }
                else
                {
                    // Create it
                    Directory.CreateDirectory(directory);
                }

                string filename = string.Format("{0}_RespirationModel_{1}_{2}.CSV", filenamePrefix, date, j);
                string path = Path.Combine(directory, filename);

                using (StreamWriter streamWriter = new StreamWriter(File.Create(path)))
                {
                    streamWriter.WriteLine("Distance (x), Starting O2 Concentration, bd Part, bo3 Part, Ks Part, End O2
                            Concentration (y)");

                    for (int i = 0; i < NumberOfLayers; i++)
                    {
                        streamWriter.Write(report[i].Distance);
                        streamWriter.Write(", ");
                        streamWriter.Write(report[i].StartingO2Concentration);
                        streamWriter.Write(", ");
                        streamWriter.Write(report[i].Ks);
                        streamWriter.Write(", ");
                        streamWriter.WriteLine(report[i].EndO2Concentration);
                    }
                }
                //if (doreport == true) break;
            }

        }

        Console.Out.WriteLine("Finished Calculation");
    }
  }
}
```