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Anaerobic Respiration of *Escherichia coli* in the Mouse Intestine[▽]

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The intestine is inhabited by a large microbial community consisting primarily of anaerobes and, to a lesser extent, facultative anaerobes, such as *Escherichia coli*, which we have shown requires aerobic respiration to compete successfully in the mouse intestine (S. A. Jones et al., Infect. Immun. 75:4891-4899, 2007). If facultative anaerobes efficiently lower oxygen availability in the intestine, then their sustained growth must also depend on anaerobic metabolism. In support of this idea, mutants lacking nitrate reductase or fumarate reductase have extreme colonization defects. Here, we further explore the role of anaerobic respiration in colonization using the streptomycin-treated mouse model. We found that respiratory electron flow is primarily via the naphthoquinones, which pass electrons to cytochrome *bd* oxidase and the anaerobic terminal reductases. We found that *E. coli* uses nitrate and fumarate in the intestine, but not nitrite, dimethyl sulfoxide, or trimethylamine *N*-oxide. Competitive colonizations revealed that cytochrome *bd* oxidase is more advantageous than nitrate reductase or fumarate reductase. Strains lacking nitrate reductase outcompeted fumarate reductase mutants once the nitrate concentration in cecal mucus reached submillimolar levels, indicating that fumarate is the more important anaerobic electron acceptor in the intestine because nitrate is limiting. Since nitrate is highest in the absence of *E. coli*, we conclude that *E. coli* is the only bacterium in the streptomycin-treated mouse large intestine that respires nitrate. Lastly, we demonstrated that a mutant lacking the NarXL regulator (activator of the NarG system), but not a mutant lacking the NarP-NarQ regulator, has a colonization defect, consistent with the advantage provided by NarG. The emerging picture is one in which gene regulation is tuned to balance expression of the terminal reductases that *E. coli* uses to maximize its competitiveness and achieve the highest possible population in the intestine.

The mouse colon is home to at least several hundred bacterial species and more than 100 billion bacteria per g of contents, a microbial community dominated by anaerobes with essentially no aerobes (2, 38, 57, 68). It is becoming increasingly clear that facultative anaerobes consume oxygen and thereby modify their host environment. For example, kidney infection caused by uropathogenic *Escherichia coli* results in local ischemia (47). The facultatively anaerobic enteric pathogen *Shigella flexneri* responds to oxygen in the gut by modulating virulence gene expression (45). It has been postulated that the importance of *Salmonella* motility for chemotaxis through the mucus layer (61) may be due to aerotaxis (44). That *E. coli* requires cytochrome *bd* oxidase to gain a competitive advantage implies that the colon is not the anaerobic environment that many consider it to be (30). Here, we explore the possibility that efficient oxygen scavenging by *E. coli* in the intestine causes it to depend also on anaerobic respiration.

To maximize their energy efficiency, enteric bacteria such as *E. coli* possess elaborate genetic regulatory networks for sensing oxygen (32), the redox state of the electron transport system (19, 43, 58), and the availability of alternative anaerobic electron acceptors (70). We previously showed that the anaerobic transcription factor Fnr, as well as the aerobic respiratory controller ArcA, is required for commensal and pathogenic *E. coli* strains to colonize the intestine in competition with wild-type *E. coli* parent strains (30). We further showed that mutants lacking ATPase (completely respiration deficient) and mutants lacking terminal reductases for low oxygen, nitrate, or fumarate each are at an extreme disadvantage in competition with their wild-type parents for colonization of the streptomycin-treated mouse intestine (30). On the basis of these results, we hypothesize that *E. coli* maximizes its cell yield from limited carbon sources by using the most energy-efficient respiratory electron acceptors that are available to it in the intestine.

There is clear evidence to indicate that oxygen is present in the gut (24, 37). We hypothesize that oxygen-scavenging facultative anaerobes such as *E. coli* create an anaerobic environment for the numerically dominant intestinal anaerobes, in analogy to the situation in anaerobic digesters (28). Our research supports this idea. Nevertheless, our experiments also indicate that reduction of the anaerobic electron acceptors nitrate and fumarate is essential for *E. coli* to maintain its competitive advantage in the intestine (30). We take this as evidence that the ability to efficiently respire oxygen and maintain an anaerobic environment (into which oxygen continuously diffuses) likewise requires maximum efficiency under anaerobic conditions. Here, we explore the possibility that additional alternative electron acceptors, i.e., nitrite, dimethyl sulfoxide (DMSO), and trimethylamine *N*-oxide (TMAO), support *E. coli* intestinal colonization. The results indicate that nitrate and fumarate are the only anaerobic electron acceptors used by commensal and pathogenic strains of *E. coli* to colo-

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TABLE 1. Strains used in this study

Strain	Relevant genotype, phenotype, or description[a]	Source or reference
EDL933	Wild-type O157:H7	Alison O'Brien
EDL933 Str^r	Spontaneous Str^r	51
EDL933 Str^r Nal^r	Spontaneous Nal^r	51
MG1655	Wild type (CGSC 7740)	CGSC[b]
MG1655 Str^r	Spontaneous Str^r	52
MG1655 Str^r Nal^r	Spontaneous Nal^r	52
VJS4322	Δ(*narP)253*::Tn*10d*(*cat*)	Valley Stewart
VJS2458	Δ(*narXL)240*::Kn^r	Valley Stewart
Derivatives of EDL933 Str^r		
cydAB mutant	Δ(*cydAB*)::*cat*	This study
cydDC mutant	Δ(*cydDC*)::*cat*	This study
dmsAB mutant	Δ(*dmsAB*)::*cat*	This study
frdA mutant	Δ*frdA*::Kn^r	This study
napDA mutant	Δ(*napDA*)::*cat*	This study
narG mutant	Δ*narG*::Kn^r	This study
narZ mutant	Δ*narZ::cat*	This study
narG narZ mutant	Δ*narG* Δ*narZ::cat*	This study
narG narZ napDA mutant	Δ*narG* Δ*narZ* Δ(*napDA*)::*cat*	This study
ubiCA mutant	Δ(*ubiCA*)::*cat*	This study
ubiE mutant	Δ*ubiE::cat*	This study
Derivatives of MG1655 Str^r		
cydAB mutant	Δ(*cydAB*)::*cat*	This study
cydDC mutant	Δ(*cydDC*)::*cat*	This study
dmsAB mutant	Δ(*dmsAB*)::*cat*	This study
dmsAB ynfEFGH mutant	Δ(*dmsAB*) Δ(*ynfEH*)::*cat*	This study
frdA mutant	Δ*frdA*::Kn^r	This study
napDA mutant	Δ(*napDA*)::*cat*	This study
narG mutant	Δ*narG*::Kn^r	This study
narZ mutant	Δ*narZ::cat*	This study
narG narZ mutant	Δ*narG* Δ*narZ::cat*	This study
narG narZ napDA mutant	Δ*narG* Δ*narZ* Δ(*napDA*)::*cat*	This study
narP mutant	Δ(*narP)253*::Tn*10d*(*cat*)	This study
narXL mutant	Δ(*narXL)240*::Kn^r	This study
nirBD mutant	Δ(*nirB-nirD*)::*cat*	This study
nrfA mutant	Δ*nirfA::cat*	This study
nrfA nirBD mutant	Δ*nirfA* Δ(*nirBD*)::*cat*	This study
torCA mutant	Δ(*torCA*)::*cat*	This study
torYZ mutant	Δ(*torYZ*)::*cat*	This study
torCA torYZ mutant	Δ(*torCA*) Δ(*torYZ*)::*cat*	This study
torCA torYZ dmsAB mutant	Δ(*torCA*) Δ(*torYZ*) Δ(*dmsAB*)::*cat*	This study
ubiCA mutant	Δ(*ubiCA*)::*cat*	This study
ubiE mutant	Δ*ubiE::cat*	This study

[a] Nal^r, nalidixic acid resistance; Str^r, streptomycin resistance; Kn^r, kanamycin resistance.
[b] *E. coli* Genetic Stock Culture Collection, Yale University.

nize the intestine. In addition, we present further evidence that anaerobic electron transport chain components, as well as the NarX-NarL two-component system that governs gene expression for anaerobic respiratory systems, are necessary for competitive fitness in the streptomycin-treated mouse intestine.

MATERIALS AND METHODS

Bacterial strains and growth conditions. In Table 1 we list the *E. coli* mutants used in this study; these were derived from *E. coli* MG1655 Str^r (streptomycin resistant), a K-12 strain isolated from a human (4), and *E. coli* EDL933 Str^r, the prototypical O157:H7 strain (53). For colonization experiments, the wild-type strains were *E. coli* MG1655 Str^r Nal^r (nalidixic acid resistant) (52) and *E. coli* EDL933 Str^r Nal^r (51); Nal^r is used to distinguish the wild type from null allele mutants. Null alleles were constructed by using the allelic replacement method of Datsenko and Wanner (14) as described previously (8), such that target genes were deleted and replaced with kanamycin or chloramphenicol resistance cassettes (used as selectable markers in mouse colonization assays described below). The null allele strains are identified in the text by the genes that were deleted; single gene deletions began with the start codon and ended with the stop codon, and multiple gene deletions began with the start codon of the first gene deleted and ended with the stop codon of the last gene deleted. Strains containing multiple mutations were constructed by sequential allelic replacement; removal of the first inserted cassette with FLP recombinase (14) was followed by subsequent allelic replacement(s) and removal of the insertion as necessary, leaving the selectable marker in the last mutation made. It is possible that mutations were inadvertently introduced elsewhere on the genome during strain construction. However, we would argue that this was not the case because the allelic replacements described herein were obtained with a frequency that varied less than 1 order of magnitude. In addition, the results obtained in two different genetic backgrounds (EDL933 and MG1655) were essentially identical. The *narXL* and *narP* mutations were moved from strains VJS2458 and VJS4322, respectively, into the *E. coli* MG1655 Str^r background by allelic replacement. Mutations were verified by phenotype and DNA sequence analyses.

Phenotype analysis. MOPS [3-(*N*-morpholino)propanesulfonic acid] defined medium was used for phenotype analysis, as described previously (8). Anaerobic cultures were grown in sealed Balch culture tubes filled to the top with N_2-sparged medium. To test for nitrate or fumarate respiration, mutant strains were grown anaerobically overnight in MOPS medium with glycerol (1.6%) as the carbon source and either 50 mM nitrate or 50 mM fumarate as the electron acceptor. Cell growth was monitored spectrophotometrically at 600 nm (as the optical density at 600 nm [OD_{600}]).

High-performance liquid chromatography (HPLC) analysis. For anion analysis of mucus, we used a Dionex DX-500-Microbore system with a 10-μl injection volume, an IonPac AS11 column (at 30°C) with a 1 to 25 mM NaOH gradient as the eluent at a 0.5-ml/min flow rate, and a suppressed conductivity detector with the ASRS-ULTRA 2-mm AutoSuppression mode. The regression coefficient for the standard curve was used to demonstrate the linearity of the peak area with respect to concentration. The limit of detection of nitrate in this system was 3 μM. Mouse cecal mucus was isolated from the ceca of CD-1 male mice and lyophilized as described previously (72). Mucus samples from 5 mice were pooled and assayed in triplicate.

Mouse colonization experiments. Streptomycin-treated mice have been used since 1954 to overcome the colonization resistance that prevents colonization of conventional animals by experimentally introduced enteric bacteria (7). We have made extensive use of the streptomycin-treated mouse model to study nutritional factors that govern colonization of the mouse large intestine by *E. coli* and *Salmonella enterica* serovar Typhimurium (3, 8, 16, 18, 30, 31, 34, 35, 48, 49, 51, 52). Treatment of mice with streptomycin selectively removes facultatively anaerobic *E. coli* bacteria, enterococci, streptococci, lactobacilli, and anaerobic lactobacilli and bifidobacteria without changing the overall populations of anaerobes, including *Bacteroides* and *Eubacterium* (25). Therefore, the streptomycin-treated mouse model allows colonization by experimentally introduced *E. coli* strains and competition with large numbers of strict anaerobes and, thus, is our model of choice for studying competition among *E. coli* strains in the intestine. Stable colonization requires that streptomycin treatment be continued for the duration of the experiments. For *E. coli* EDL933, we emphasize that streptomycin-treated mice are used to model colonization, not pathogenesis (13). Briefly, CD-1 male mice, 6 weeks of age, are given drinking water containing streptomycin sulfate (5 g/liter) for 24 h, which by removing the native facultative anaerobes opens the niche for *E. coli* to colonize (50). Following 18 h of starvation for food and water, the mice are fed 1 ml of 20% (wt/vol) sucrose containing 10^5 CFU of *E. coli* strains grown overnight in Luria broth. After the bacterial suspension is ingested, both food (Teklad mouse and rat diet; Harlan, Madison, WI) and streptomycin-water are returned to the mice, and 1-g samples of feces are collected after 5 and 24 h and on odd-numbered days thereafter. Mice are housed individually in cages without bedding and are placed in clean cages at 24-h intervals. Fecal samples (1 g) are therefore no older than 24 h. Each fecal sample is homogenized in 10 ml of 1% Bacto tryptone (Difco), diluted in the same medium, and plated onto MacConkey agar plates containing either streptomycin (100 μg/ml) and nalidixic acid (50 μg/ml) to count wild type CFU or streptomycin and kanamycin (40 μg/ml) or chloramphenicol (30 μg/ml) to count CFU of the null allele mutants. Streptomycin sulfate, chloramphenicol, and nalidixic acid were purchased from Sigma-Aldrich (St. Louis, MO). The limit of detection for fecal plate counts is 10^2 CFU/g feces. Each colonization experiment was replicated at least twice, with essentially identical results. Pooled data from at least 2 independent experiments (a total of 6 mice) are presented in the figures as the log of fecal plate counts.

RESULTS

Anaerobic quinones contribute to respiration in the intestine. Previously, we showed that *E. coli* mutants lacking cytochrome *bd* oxidase, nitrate reductase (Nar), or fumarate reductase (Frd) are eliminated from the intestine by competition

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TABLE 2. Competitive colonization by *E. coli* quinone biosynthesis mutants and alternative anaerobic electron acceptor mutants[a]

Mutant genotype	Presence or absence[b] of:			Difference between *E. coli* EDL933 wild-type and mutant strains on:		Difference between *E. coli* MG1655 wild-type and mutant strains on:	
	UQ	MQ	DMQ	Day 1	Day 9	Day 1	Day 9
Δ(*ubiCA*)	−	+	+	0.21 ± 0.05	0.71 ± 0.14	0.78 ± 0.27	0.24 ± 0.22
Δ*ubiE*	−	−	+	**3.26 ± 0.13**	**5.80 ± 0.13**	**4.04 ± 0.09**	**5.03 ± 0.20**
Δ*menA*	+	−	−	**1.23 ± 0.16**	**5.15 ± 0.19**	1.00 ± 0.24	**5.66 ± 0.11**
Δ*dmsABC*	+	+	+	0.21 ± 0.11	−0.31 ± 0.25	0.21 ± 0.18	0.21 ± 0.17
Δ*ynfEFGH*	+	+	+	ND[c]	ND	0.16 ± 0.48	0.71 ± 0.25
Δ*dmsABC* Δ*ynfEFGH*	+	+	+	ND	ND	0.11 ± 0.17	0.62 ± 0.12
Δ*torCA*	+	+	+	0.52 ± 0.13	0.10 ± 0.11	0.18 ± 0.16	0.15 ± 0.26
Δ*torYZ*	+	+	+	ND	ND	0.47 ± 0.37	0.51 ± 0.17
Δ*torCA* Δ*torYZ*	+	+	+	ND	ND	0.61 ± 0.54	0.15 ± 0.58
Δ*dmsABC* Δ*torCA* Δ*torYZ*	+	+	+	ND	ND	0.38 ± 0.19	0.26 ± 0.23
Δ*nirBD*	+	+	+	0.30 ± 0.24	0.49 ± 0.46	0.05 ± 0.16	0.64 ± 0.30
Δ*nrfA*	+	+	+	ND	ND	0.02 ± 0.25	0.11 ± 0.21
Δ*nirBD* Δ*nrfA*	+	+	+	ND	ND	−0.21 ± 0.25	0.21 ± 0.11

[a] Mice were fed 10^5 CFU each of the mutant and its wild-type parent. The average differences between the wild-type strain and its isogenic mutant (among 6 mice) are represented as the log number of wild-type CFU per gram of feces minus the log number of mutant CFU per gram of feces ± the standard error of the mean on days 1 and 9 of the experiments. All values shown in bold are statistically significant: $P < 0.001$ (Student's t test).
[b] Presence (+) or absence (−) of UQ, MQ, or DMQ, in accordance with the indicated genotypes.
[c] ND, not determined.

with the wild-type parent strains (30). The relative contributions of aerobic and anaerobic respiratory processes to colonization are not known. The respiratory dehydrogenases are linked to the terminal reductases by a pool of quinones comprising ubiqinone (UQ), menaquinone (MQ), and demethylmenaquinone (DMQ). Theoretically, this allows formation of branched respiratory chains in which most dehydrogenases can interact with most terminal reductases (70). However, the midpoint potential of UQ is best suited for aerobic respiration, whereas MQ and DMQ are involved primarily in anaerobic respiration, with DMQ having a midpoint potential between those of UQ and MQ (70). In general, anaerobic terminal reductases have higher activity with MQ and DMQ, cytochrome bo_3 has the highest activity with UQ, and cytochrome *bd* apparently uses both UQ and MQ (23). Electron flow to fumarate, TMAO, and nitrite is via both MQ and DMQ, while electron flow to DMSO is primarily via MQ. The primary Nar enzyme (NarG) uses UQ and MQ but not DMQ (77). While all three quinones are present under both aerobic and anaerobic conditions, their relative concentrations vary with oxygen tension, with DMQ increasing rapidly and MQ increasing more slowly when oxygen is limited (6, 69, 75). Accordingly, while DMQ would be in the greatest abundance under the low oxygen tension in the mouse intestine (30), DMQ alone would not be sufficient for colonization since electron flow to nitrate and low oxygen would require MQ under these conditions. Thus, we predicted that respiratory electron flow via both MQ and DMQ supports colonization of the intestine by *E. coli*.

To confirm this hypothesis, we used mutants lacking one or more quinone biosynthetic pathways (46). Specifically, as shown in Table 2, *ubiCA* mutants cannot make UQ (33, 78, 80), *ubiE* mutants cannot make UQ or MQ (36, 77), and *menA* mutants cannot make MQ or DMQ (65, 79). The *ubiCA*, *ubiE*, and *menA* mutations were constructed in *E. coli* MG1655 and *E. coli* EDL933, the mutants were fed to streptomycin-treated mice together with the respective wild-type parent strains at 10^5 CFU of each strain, and the population of each strain was monitored by using fecal plate counts. The results of these experiments are shown in Table 2. The *ubiCA* mutants, lacking UQ, cocolonized with the wild type, which is evidence that MQ and DMQ are sufficient for colonization of the intestine by *E. coli*. The *menA* mutants, lacking both MQ and DMQ, were able to increase in population during the initiation stage of colonization (24 h), although not to the same extent as the wild type, but were completely eliminated (reaching levels of $<10^2$ CFU/g feces) by day 9 of the experiment. One interpretation of these results is that UQ helps support colonization initiation when MQ is unavailable. Given that the passage of electrons to oxygen is primarily via UQ when oxygen is not limiting, it may be that UQ is used during the initiation stage because oxygen levels are relatively high in animals that have been treated with streptomycin to remove the facultative anaerobes prior to colonization with *E. coli*. The *ubiE* mutants, which have only DMQ, failed to initiate colonization in competition with the wild type (in the first 24 h) and were eliminated from the mice (reaching levels of $<10^2$ CFU/g feces), which indicates that anaerobic electron flow via MQ in the intestine is more advantageous than that via DMQ and further supports the idea that UQ is used during colonization initiation. All four of the strains with competitive colonization defects (*E. coli* MG1655 *ubiE*, *E. coli* MG1655 *menA*, *E. coli* EDL933 *ubiE*, and *E. coli* EDL933 *menA*) colonized to yield between 10^7 and 10^8 CFU/g feces when fed alone to mice, indicating that the colonization defects were due to an inability to compete with the wild type rather than a general inability to grow in the intestine (data not shown). In summary, we conclude that respiratory electron flow via MQ and DMQ to cytochrome *bd* oxidase and anaerobic terminal reductases supports colonization of the intestine by *E. coli*.

Alternative electron acceptors DMSO, TMAO, and nitrite do not support colonization. In addition to its ability to respire nitrate and fumarate, *E. coli* possesses systems for reduction of DMSO, TMAO, and nitrite as terminal electron acceptors (11). To investigate these alternative respiratory pathways in the intestine, each of the redundant terminal reductase gene systems was knocked out in *E. coli* MG1655 by allelic replace-

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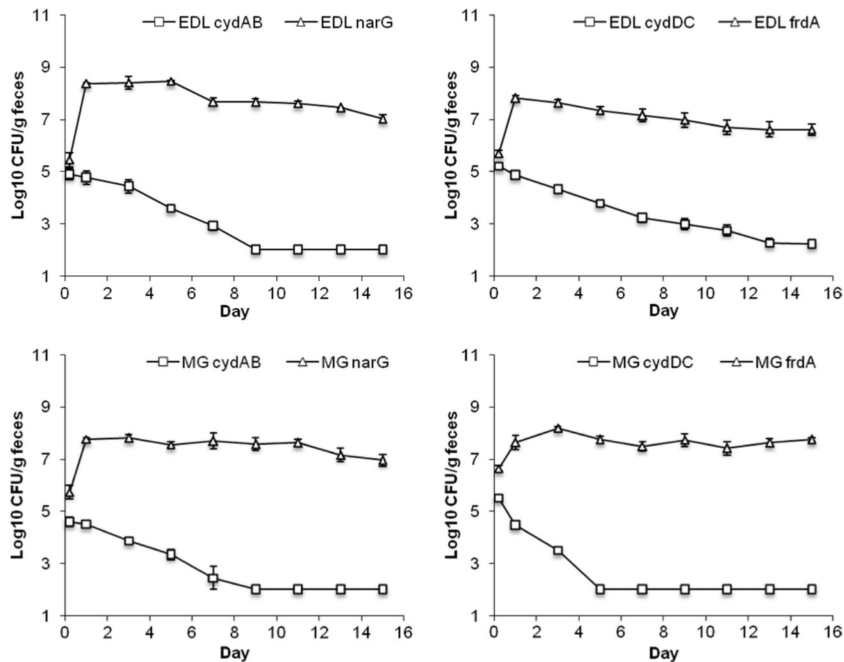

FIG. 1. Competition between mutants lacking cytochrome *bd* and mutants lacking either nitrate reductase or fumarate reductase. Upper left, *E. coli* EDL933 Δ*cydAB* (EDL cydAB) versus *E. coli* EDL933 Δ*narG* (EDL narG); upper right, *E. coli* EDL933 Δ*cydDC* (EDL cydDC) versus *E. coli* EDL933 Δ*frdA* (EDL frdA); lower left, *E. coli* MG1655 Δ*cydAB* (MG cydAB) versus *E. coli* MG1655 Δ*narG* (MG narG); lower right, *E. coli* MG1655 Δ*cydDC* (MG cydDC) versus *E. coli* MG1655 Δ*frdA* (MG frdA).

ment, the mutant strains were fed to mice together with their wild-type parents at 10^5 CFU of each strain, and the relative populations of the strains in fecal plate counts were determined. The DmsABC system reduces both DMSO and TMAO; a paralogous YnfEFGHI system for DMSO reduction (41) is now thought to reduce selenium (22). Mutants containing deletions of either or both of the corresponding gene systems were found to cocolonize with wild-type *E. coli* MG1655, indicating that reduction of DMSO does not provide a colonization advantage in the intestine (Table 2). The TorCAD and TorYZ systems, in addition to DmsABC, are responsible for TMAO reduction (9). Mutants lacking these TMAO reductase systems, either individually or collectively, cocolonized with the wild-type parent strain, indicating that reduction of TMAO does not contribute to the fitness of *E. coli* MG1655 in the intestine (Table 2). Lastly, to investigate the contribution of nitrite reductase to colonization fitness, we constructed mutants lacking either or both the cytoplasmic NirBCD and periplasmic NrfABCD nitrite reductase systems. We found that in competition experiments, each of these mutants cocolonized with wild-type *E. coli* MG1655 (Table 2), indicating that nitrite is not used in the intestine. Given that nitrate is used and that the product (nitrite) is potentially toxic (40), it is interesting that the loss of nitrite reductase does not result in a competitive disadvantage in the intestine. From these results we conclude that colonization of the intestine by *E. coli* is not supported by respiration of DMSO, TMAO, or nitrite, either because these electron acceptors are unavailable or because the terminal reductases are not functionally expressed. Together with results from the previous study (30), these new results lead us to conclude that respiration of nitrate and respiration of fumarate, in addition to fermentation, are the only anaerobic processes that contribute to the colonization fitness of *E. coli* in the mouse intestine.

Aerobic cytochrome *bd* is more advantageous than anaerobic respiration. We sought to evaluate the relative contributions of aerobic and anaerobic respiration to colonization of the intestine. Previously, we showed that the low-affinity, high-oxygen-tension cytochrome bo_3 oxidase does not contribute to colonization but that the high-affinity, low-oxygen cytochrome *bd* oxidase is essential for competition with wild-type *E. coli* in the intestine (30). The *E. coli* EDL933 and *E. coli* MG1655 *cydAB* mutants were competed against *E. coli* EDL933 and *E. coli* MG1655 *narG* mutants, respectively, by feeding the two strains together to mice at 10^5 CFU of each strain. In both experiments the *cydAB* mutants were eliminated from the intestine (Fig. 1). Likewise, the *E. coli* EDL933 and *E. coli* MG1655 *cydDC* mutants were eliminated from the intestine during competition against *E. coli* EDL933 and *E. coli* MG1655 *frdA* mutants, respectively (Fig. 1). We showed previously that *E. coli cydDC* mutants that cannot assemble cytochrome *bd* oxidase in the membrane (54) have colonization defects similar to those of *cydAB* mutants (30). These results indicate that aerobic respiration via cytochrome *bd* oxidase provides an advantage over anaerobic respiration of nitrate or fumarate in the intestine.

The primary Nar enzyme contributes a major colonization advantage. There are three nitrate reductase systems in *E. coli* (70). Previously, we showed that strains lacking the primary enzyme, NarG, have a significant colonization defect in competition with the wild type but that strains lacking the secondary cytoplasmic enzyme NarZ or Nap are as competitive as the

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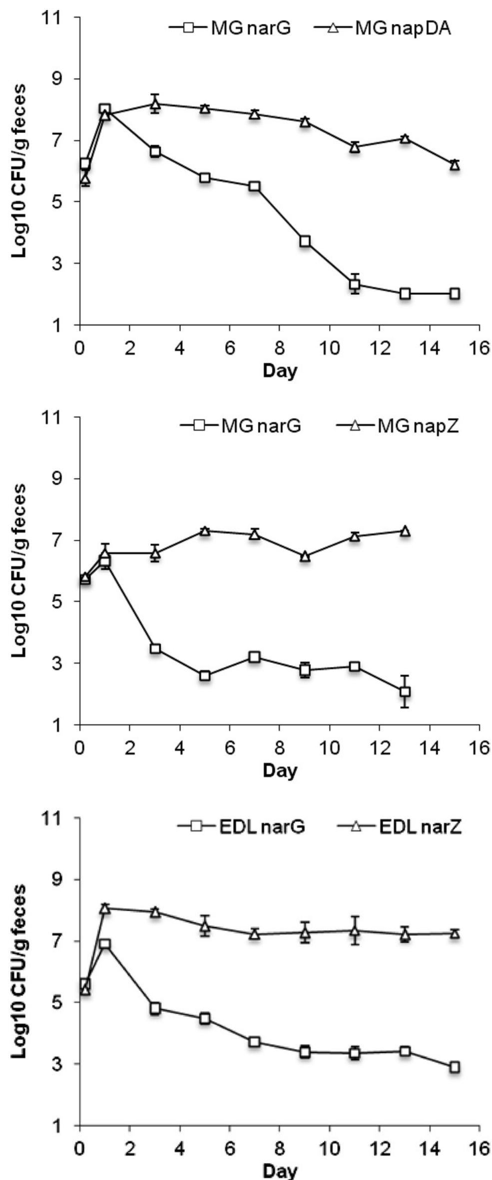

FIG. 2. Competition between nitrate reductase mutants. Top, *E. coli* MG1655 Δ*napDA* versus *E. coli* MG1655 Δ*narG*; middle, *E. coli* MG1655 Δ*narZ* versus *E. coli* MG1655 Δ*narG*; bottom, *E. coli* EDL933 Δ*narZ* versus *E. coli* EDL933 Δ*narG*.

wild type, which suggests that NarG is the major contributor to nitrate respiration in the intestine (30). Strains lacking all three systems had colonization defects up to 1 log more severe than the strain lacking NarG alone, which suggests that the other systems may contribute modestly to colonization in the absence of NarG (30). To further evaluate the relative importance of the Nar systems, we competed *narG* mutants individually against *narZ* and *napDA* mutants (Fig. 2). In competition with the *narZ* mutant, the *narG* mutants had a 4-log colonization defect. In competition with the *napDA* mutant, the *narG* mutant initially reached the same high population (10^8 CFU/g feces) before showing a 2-log defect at day 7 and then a more rapid decline to an undetectable number (10^2 CFU/g feces) by

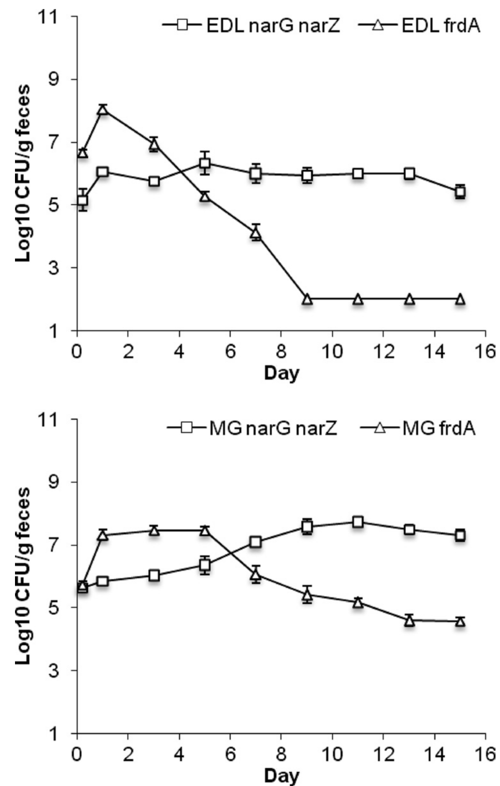

FIG. 3. Competition between nitrate reductase and fumarate reductase mutants. Top, *E. coli* EDL933 Δ*narG* Δ*narZ* versus *E. coli* EDL933 Δ*frdA*; bottom, *E. coli* MG1655 Δ*narG* Δ*narZ* versus *E. coli* MG1655 Δ*frdA*.

day 13 of the experiment. These results confirm that electron flow to nitrate occurs primarily via NarG.

Nitrate availability dictates competition for anaerobic electron acceptors in the intestine. To determine which of the two anaerobic electron acceptors used by *E. coli* in the intestine provides the superior colonization advantage, we competed *E. coli* EDL933 *narG narZ* with *E. coli* EDL933 *frdA* and *E. coli* MG1655 *narG narZ* with *E. coli* MG1655 *frdA* (Fig. 3). In both experiments the *frdA* mutants, which can respire nitrate but not fumarate, initially (at day 3 or day 5) had a colonization advantage but subsequently declined in numbers relative to the *narG narZ* mutants, which persisted at relatively high populations. Apparently, nitrate respiration provides an advantage during the initial stage of colonization, when the level of nitrate is expected to be high if no other members of the microbial community consume it. However, we conclude that the ability to respire fumarate provides a more significant colonization advantage than the ability to respire nitrate for long-term persistence of established populations of *E. coli*.

To determine the role of nitrate availability in the competition between Nar and Frd mutants, we measured the nitrate concentration in cecal mucus after association with 10^5 CFU each of *E. coli* EDL933 *narG narZ napDA* and *E. coli* EDL933 *frdA* (Table 3). In control experiments, mice colonized with *E. coli* EDL933 Str^r (the wild type) had undetectable levels of nitrate (<3 μM) in their cecal mucus on days 1, 5, and 11 following association. Mice colonized with *E. coli* EDL933

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TABLE 3. HPLC analysis of nitrate in mouse cecal mucus

Strain	NO_3^- concn[a] (mM) in mucus	Day of colonization
EDL933 Str[r]	ND	1
	ND	5
	ND	11
narG narZ napDA mutant	2.76 ± 0.27	1
	2.89 ± 0.33	5
	2.65 ± 0.49	11
narG narZ napDA frdA mutant	0.77 ± 0.31	1
	0.31 ± 0.25	5
	ND	11

[a] Nitrate was measured in samples of cecal mucus prepared from mice colonized with the indicated *E. coli* strains. Values are means ± standard errors of the means for samples from 5 mice. ND, not detected (<3 μM).

narG narZ napDA alone had an average of 2.8 mM nitrate. Following association with *E. coli* EDL933 *narG narZ napDA* and *E. coli* EDL933 *frdA*, nitrate in cecal mucus declined to a mean ± standard error of the mean of 0.77 ± 0.31 mM on day 1, reached 0.31 ± 0.25 mM on day 5, and was undetectable (<3 μM) on day 11 of the experiment (Table 3). In the colonization experiment (Fig. 3), the Nar$^+$ Frd$^-$ strain increased in population at 24 h, consistent with the availability of nitrate, whereas the Nar$^-$ Frd$^+$ strain was unable to increase in population and remained at 6 log CFU/g feces. By day 3 of the experiment, as the nitrate concentration decreased (presumably through respiratory metabolism by the competing Nar$^+$ strain), the Nar$^+$ Frd$^-$ strain declined in population whereas the Nar$^-$ Frd$^+$ strain persisted. Thus, the population of the Nar$^+$ Frd$^-$ strain declined as nitrate diminished, indicating that respiration of nitrate by *E. coli* in the intestine is limited by nitrate availability but that fumarate is more readily available and therefore more important for persistence in the intestine.

The NarX-NarL regulatory circuit contributes to colonization. Regulation of anaerobic respiration is thought to contribute to the success of *E. coli* in changing environments (10). Thus, it was of interest to determine the relative contributions to colonization of the two two-component systems that control anaerobic gene systems involved in nitrate respiration. NarG is activated by the NarX-NarL two-component system when nitrate is abundant, and NarX-NarL represses Nap, which is activated by the NarQ-NarP system when nitrate is 3 orders of magnitude lower (74, 76). To determine the relative contributions of the NarX-NarL and NarQ-NarP systems to colonization, mice were fed *E. coli* MG1655 *narXL* or *E. coli* MG1655 *narP* together with the wild-type parent at 10^5 CFU of each strain and the relative populations of the strains were determined (data not shown). The *narXL* mutant had a 1-log colonization defect ($P < 0.05$) in competition with the wild type, and the *narP* mutant cocolonized with the wild type. As discussed further below, it makes sense that the structural gene (*narG*) colonization defect is more severe than the regulatory gene (*narXL*) defect. These results confirm the importance of NarG for colonization, since the main function of NarX-NarL is to activate NarG.

Aerobic respiratory control is more important than aerobic respiration. Previously, we showed that Fnr and ArcA mutants are eliminated from the intestine in competition with the wild type (30). Since mutants lacking the high-affinity cytochrome *bd* oxidase have a colonization defect, we concluded that *E. coli* experiences microaerobic or alternately aerobic and anaerobic conditions in the intestine (30). Interestingly, the two-component ArcAB system is most active at low oxygen tension (1), i.e., at oxygen levels present in the large intestine (24). Thus, we considered the possibility that ArcAB contributes to colonization fitness because it activates transcription of the cytochrome *bd* oxidase genes (24). To test whether the loss of ArcAB activation of cytochrome *bd* was the sole reason for the previously observed colonization defect of the Δ*arcA* mutant in competition with the wild type (30), Δ*arcA::kan* and Δ(*cydAB*)::*cat* mutants of *E. coli* EDL933 and MG1655 were fed together to mice at a level of 10^5 CFU of each strain/mouse. The *arcA* mutants with either genetic background were quickly eliminated during competition with the *cydAB* mutants (Fig. 4). These results indicate that failure to activate *cydAB* under microaerobic conditions is not the sole reason for the colonization defect of the *arcA* mutant and imply that other aspects of ArcAB control, such as repression of cytochrome bo_3 oxidase genes and other genes that participate in aerobic processes (e.g., the tricarboxylic acid [TCA] cycle), are also important in the intestine.

DISCUSSION

Commensal *E. coli* MG1655 and pathogenic *E. coli* EDL933 must respire oxygen, nitrate, and fumarate to be competitive in

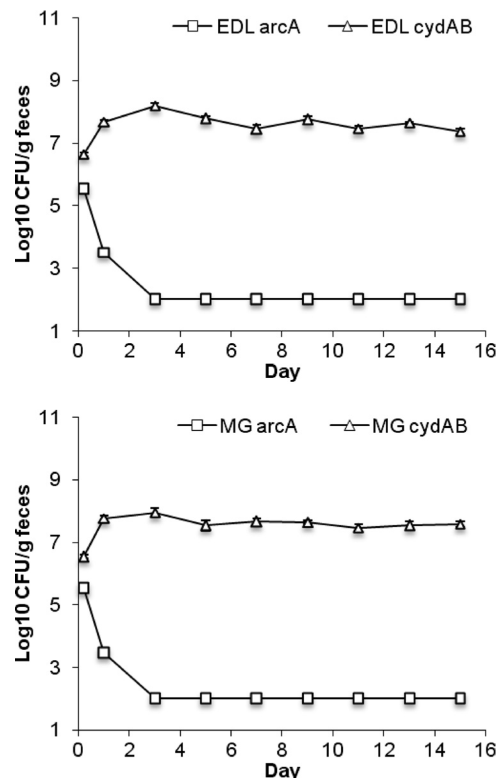

FIG. 4. Competition between *E. coli* EDL933 *arcA* and *E. coli* EDL933 *cydAB* (top) and *E. coli* MG1655 *arcA* and *E. coli* MG1655 *cydAB* (bottom).

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the intestine (30). Analysis of a series of quinone biosynthesis mutants (Table 2) demonstrated that electron flow in colonized *E. coli* is primarily via the naphthoquinone pool (MQ and DMQ), which is used by cytochrome *bd* oxidase and the anaerobic terminal reductases that use nitrate and fumarate. Competition experiments revealed that respiration of oxygen provides a greater colonization advantage than anaerobic respiration (Fig. 1). NarG is the most important of the three systems for nitrate respiration (Fig. 2). Similarly, a mutant lacking the NarX-NarL regulatory system, which activates NarG synthesis, had a colonization defect. Competition of mutants lacking nitrate reductase with mutants lacking Frd revealed an interesting interplay between the two strains, which can be explained by nitrate limitation in the cecum (Fig. 3 and Table 3). Lastly, competition between a mutant lacking the aerobic respiratory control system and a mutant lacking cytochrome *bd* oxidase indicated that the role of ArcA in colonization extends beyond simple activation of *cydAB* (Fig. 4).

The emerging picture is one in which *E. coli* generates the energy it requires to colonize the mammalian intestine by respiring oxygen, nitrate, and fumarate. As would be expected when considering the potential of these processes to generate energy (11), oxygen is favored over nitrate or fumarate. However, it is clear from published studies (24, 37) that oxygen is limiting in the intestine, consistent with our observations that anaerobic respiration also contributes to colonization. The studies described herein support the conclusion that nitrate, too, is limiting in the intestine. In this regard, *E. coli* can be thought of as a scavenger of available electron acceptors, using whichever one is available to maximize its competitiveness and achieve the highest possible population.

Availability and use of electron acceptors. Oxygen diffuses from the intestinal epithelium into the mucus layer (59). Fumarate is generated endogenously from carbohydrates via glycolysis and the reductive branch of the TCA cycle and also from available C_4-dicarboxylates and related compounds such as aspartate via metabolism (29). In humans, dietary nitrate, mostly from vegetables (42), as well as nitrate biosynthesis (21), contributes to the available nitrate in the body. While it is known that the availability of nitrate in the ferret intestine increases with antibiotic treatment to remove the microbiota (15), microbial nitrate metabolism in animals and humans remains largely unexplored.

We measured 2.8 mM nitrate in cecal mucus prepared from mice that were treated with streptomycin to remove native *E. coli* and subsequently colonized with an *E. coli* EDL933 mutant that is completely Nar deficient. Upon colonization of the streptomycin-treated mouse intestine with wild-type *E. coli* EDL933, the nitrate concentration was undetectable 24 h postassociation. This suggests, first, that *E. coli* is the only microorganism that consumes nitrate in the intestines of streptomycin-treated mice and, second, that at least in streptomycin-treated mice fed a controlled diet, intestinal nitrate is consumed as fast as it becomes available. Together with our previous results, our present results point to the intestinal environment as one containing limiting oxygen and limiting nitrate as the only available exogenous electron acceptors.

Terminal reductase synthesis is subject to hierarchical regulation such that electron acceptors with higher midpoint potentials are used preferentially (11, 26, 39, 60, 63, 70). The streptomycin-treated mouse model afforded the opportunity to test the hypothesis that this paradigm also operates *in vivo*. Our competition assays with streptomycin-treated mice revealed that mutants lacking cytochrome *bd* oxidase are outcompeted by mutants lacking either Nar or Frd, which is in keeping with the observed preference for oxygen over nitrate and for nitrate over fumarate. Given that nitrate has the potential to yield more energy than fumarate respiration, we expected a mutant lacking Nar to be outcompeted by a mutant lacking Frd. Instead, we found that Nar^+ Frd^- strains had an initial advantage but that after several days Nar^- Frd^+ strains gained an advantage. The population of Nar^+ Frd^- strains correlated with the availability of nitrate, i.e., the nitrate concentration in cecal mucus was reduced to submillimolar concentrations at the time when the Nar^- Frd^+ strains became dominant, presumably because they can use fumarate. Just as in a nitrate-limited chemostat (74), it appears that in the intestine the respiratory activity of *E. coli* decreases the low initial nitrate concentration to an undetectable level. Thus, in the streptomycin-treated mouse intestine it appears that nitrate is limiting and that fumarate, because it is generated from exogenous carbon sources, is more abundant. The observed competition between the Nar^+ Frd^- and Nar^- Frd^+ strains is what one would predict for an ecological succession based on competition for a single nutrient that becomes limiting (66).

Role of nitrate respiration and its regulation. Evidence suggests that NarG and NapA nitrate reductases function optimally at relatively high and low nitrate concentrations, respectively (55, 56). This predicts that the NapA enzyme would be more important than the NarG enzyme for intestinal colonization (11). However, our results support the opposite conclusion. As noted above, these results may reflect nitrate dynamics that are particular to the streptomycin-treated mouse intestine and colonization protocols employed in our studies. Certainly, the relative roles for these two forms of nitrate reductase in different natural environments deserve further study.

Dual nitrate-responsive two-component systems, NarX-NarL and NarQ-NarP, control terminal reductase synthesis. The NarX-NarL system activates synthesis of the NarG enzyme and represses synthesis of other terminal reductases, including NapA and FrdA (62). The NarQ-NarP system plays a more restricted role and is involved primarily in activating NapA and NrfA synthesis (12, 62). We found a colonization defect for the Δ(*narXL*) but not the Δ*narP* strain, congruent with the colonization defect of the Δ*narG* but not the Δ(*napDA*) strain.

However, whereas the Δ*narG* strain exhibited an approximately 5-log defect, the Δ(*narXL*) strain colonization defect was near the detection limit, approximately 1 log. Similarly, work with *Pseudomonas aeruginosa* demonstrated that a Δ(*narGH*) mutant is avirulent in *Caenorhabditis elegans* infection but that a Δ(*narXL*) mutant exhibits nearly wild-type virulence (71). We offer two hypotheses for why the Δ*narG* and Δ(*narXL*) mutant phenotypes differed in the streptomycin-treated mouse model. First, the Δ*narG* strain is devoid of the corresponding activity whereas the *narG* operon is expressed at low but detectable levels in the Δ(*narXL*) strain (64). Perhaps this low-level expression is sufficient to allow significant nitrate-dependent growth in this environment. Second, during

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growth with nitrate, the NarX-NarL system represses synthesis of enzymes for the manufacture and respiration of fumarate (12, 20, 27). Thus, the Δ*narG* mutant is defective not only for nitrate respiration but also for fumarate respiration, because of NarX-NarL-mediated repression. In contrast, the Δ(*narXL*) mutant is able to manufacture and respire fumarate even in the presence of nitrate. These hypotheses are not mutually exclusive, and further analysis will determine if either or both are likely to be correct.

In conclusion, our studies support the idea that *E. coli* in the intestine is poised for respiration of oxygen, nitrate, and fumarate as the availability of these electron acceptors dictates. Regulation of these respiration systems under low oxygen tension would allow simultaneous production of cytochrome *bd* oxidase and nitrate reductase (1, 5, 67). The low concentration of available nitrate (and oxygen) would also allow production of Frd (74, 76). Lastly, the flexibility of the quinone pool facilitates electron transfer from NADH dehydrogenase to whichever terminal reductase has a substrate available. It appears that the regulatory interplay of the ArcA, Fnr, and NarX-NarL (and perhaps other) systems is tuned to balance expression of the three terminal reductases that *E. coli* uses to colonize the intestine.

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