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The Periplasmic Nitrate Reductase NapABC Supports Luminal Growth of *Salmonella enterica* Serovar Typhimurium during Colitis

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The food-borne pathogen *Salmonella enterica* serovar Typhimurium benefits from acute inflammation in part by using host-derived nitrate to respire anaerobically and compete successfully with the commensal microbes during growth in the intestinal lumen. The *S.* Typhimurium genome contains three nitrate reductases, encoded by the *narGHI*, *narZYV*, and *napABC* genes. Work on homologous genes present in *Escherichia coli* suggests that nitrate reductase A, encoded by the *narGHI* genes, is the main enzyme promoting growth on nitrate as an electron acceptor in anaerobic environments. Using a mouse colitis model, we found, surprisingly, that *S.* Typhimurium strains with defects in either nitrate reductase A (*narG* mutant) or the regulator inducing its transcription in the presence of high concentrations of nitrate (*narL* mutant) exhibited growth comparable to that of wild-type *S.* Typhimurium. In contrast, a strain lacking a functional periplasmic nitrate reductase (*napA* mutant) exhibited a marked growth defect in the lumen of the colon. In *E. coli*, the *napABC* genes are transcribed maximally under anaerobic growth conditions in the presence of low nitrate concentrations. Inactivation of *narP*, encoding a response regulator that activates *napABC* transcription in response to low nitrate concentrations, significantly reduced the growth of *S.* Typhimurium in the gut lumen. Cecal nitrate measurements suggested that the murine cecum is a nitrate-limited environment. Collectively, our results suggest that *S.* Typhimurium uses the periplasmic nitrate reductase to support its growth on the low nitrate concentrations encountered in the gut, a strategy that may be shared with other enteric pathogens.

Pathogenic bacteria are supremely adept at infiltrating barriers to colonization in order to thrive in specific niches. For intestinal pathogens, the challenges include surviving passage through the gastrointestinal tract, neutralizing host defenses, and competing with the commensal microflora for space and nutrients. While it is clear that many bacterial virulence proteins help pathogens by thwarting host immunity, how bacteria have adapted metabolically to the host environment is more nebulous.

The food-borne pathogen *Salmonella enterica* serovar Typhimurium is well equipped for survival and proliferation in the harsh environment of the gut. Once ingested, *S.* Typhimurium traverses the intestinal tract and localizes to the epithelium in the distal regions of the gut (1, 2). Here, an invading population initiates translocation through the epithelium using a type 3 secretion system (T3SS) encoded on *Salmonella* pathogenicity island 1 (SPI-1), termed T3SS-1 (1, 3). T3SS-1 subsequently injects a cocktail of effector proteins that promote epithelial invasion, allowing *S.* Typhimurium to traverse into the underlying lamina propria (4). Patrolling macrophages and dendritic cells detect the invading bacteria and, upon activation of microbial pattern recognition receptors, release proinflammatory cytokines to induce a robust immune response (summarized in references 5, 6, and 7). This response includes chemokine production to recruit neutrophils (8) that, upon migration to infected intestinal sites, discharge antimicrobial reactive oxygen species (9). Additionally, increased inducible nitric oxide synthase (iNOS) production leads to the release of nitric oxide (NO) into the intestinal lumen (10–12). This host response to *S.* Typhimurium invasion is purported to eliminate the pathogen from tissue, but several studies have indicated beneficial effects for the bacterial population residing in the lumen.

S. Typhimurium encodes multiple metabolic pathways that enable it to adapt quickly to the altered luminal landscape caused by the host inflammatory response. A flexible metabolic network is particularly evident in *S.* Typhimurium nitrate respiratory pathways (Fig. 1A). *S.* Typhimurium encounters nitrate as a by-product of inflammation of the gut, where iNOS-derived NO and reactive oxygen species react to form nitrate (11). The pathogen is capable of chemotaxis toward, and respiration of, nitrate, thereby enhancing its growth in the intestinal lumen (11, 14). *S.* Typhimurium encodes three nitrate reductases, which are also present in the closely related species *Escherichia coli* and support growth in the lumen of the inflamed gut (11, 15, 16). Most studies on the regulation and functional characterization of these nitrate reductases have been performed with *E. coli*. This body of work suggests that nitrate reductase A, encoded by *narGHI*, is the major nitrate reductase. Nitrate reductase Z, encoded by *narZYV*, is a homologue synthesized constitutively at low levels and may function during the transition to anaerobic environments, before *narGHI* expression is induced by anaerobiosis (17). Nitrate reductases A and Z are oriented to the cytosol and are capable of proton translocation across the cytoplasmic membrane, thereby generating a net proton gradient during respiration (17–20). The periplasmic nitrate reductase, encoded by *napABC*, on the other hand, is ori-

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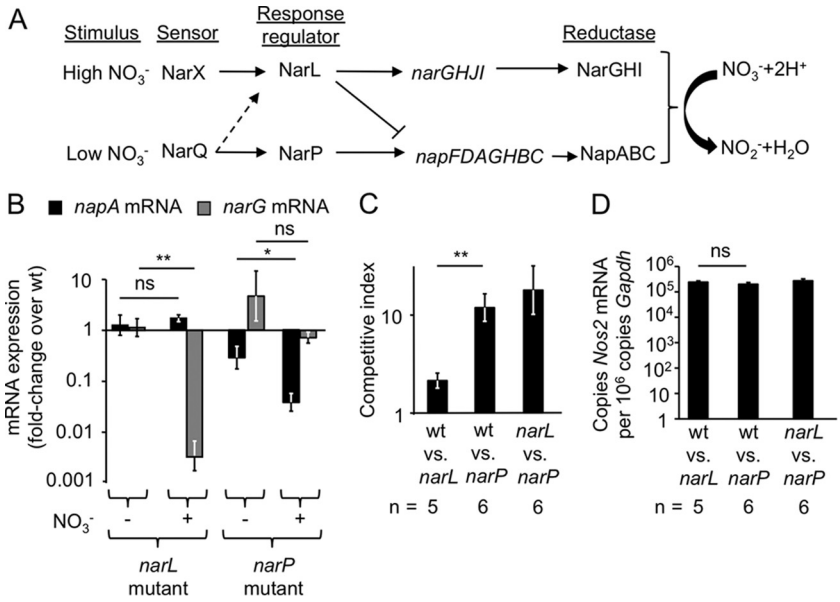

FIG 1 Functional NarP contributes to the growth of *S.* Typhimurium in the intestine during infection. (A) Schematic of nitrate sensing and regulation. Environmental nitrate concentrations are sensed by the 2-component histidine kinases NarX and NarQ, which subsequently phosphorylate the response regulators NarL and NarP, respectively, although some cross talk exists between response regulator pairs. NarL and NarP alter the transcription of the nitrate reductases NarGHI and NapABC, which function to catalyze the reduction of nitrate to nitrite for respiration. (B) Levels of *napA* and *narG* mRNA transcripts in a *narL* (left) or *narP* (right) mutant were determined relative to the transcript levels detected in wild-type *S.* Typhimurium grown anaerobically in the presence (+) or absence (−) of nitrate (0.4 mM). Experiments were performed in triplicate. **, $P < 0.01$; *, $P < 0.05$; ns, not statistically significantly different. (C) Mice were infected with a mixture of the indicated *S.* Typhimurium strains in equal amounts. Four days after infection, mice were euthanized, and the competitive index was determined by dividing the output ratio by the input ratio. (D) *Nos2* mRNA expression in the cecal mucosa was determined by quantitative real-time PCR.

ented to the periplasm, and there is no evidence that this enzyme mediates proton translocation (21, 22). The physiological role of NapABC remains unclear, since it does not appear to be a major player during growth on nitrate *in vitro*. For instance, batch culture experiments show that *E. coli* expressing *narGHI* exhibits a strong growth advantage over a strain with a *narGHI* mutation, while a strain expressing *napABC* has no growth advantage over the respective single mutant (23). These results are corroborated by *in vivo* work showing that *E. coli* strains lacking NarGHI, but not *E. coli* strains lacking NapABC, have growth defects relative to the wild type (wt) in the mouse intestine (24).

The synthesis of nitrate reductase A (NarGHI) and the periplasmic nitrate reductase (NapABC) is controlled by two 2-component systems, NarXL and NarQP, respectively, and is repressed under aerobic conditions (25). NarX senses high nitrate concentrations, leading to phosphorylation of the response regulator NarL, which induces *narGHI* transcription while inhibiting *napABC* transcription. NarQ responds to low nitrate concentrations, phosphorylating NarP, which, in turn, leads to the upregulation of *napABC* transcription (25, 26). As a result, the periplasmic nitrate reductase is synthesized optimally under anaerobic conditions in the presence of low levels of nitrate, while its synthesis is repressed when concentrations of nitrate are high. In contrast, nitrate reductase A is synthesized optimally in anaerobic environments containing high concentrations of nitrate (Fig. 1A). Neither two-component system affects *narZYV* transcription, since the encoded nitrate reductase Z is synthesized constitutively at low levels, thereby ensuring that enzymatic activity is available when bacteria transit from an aerobic to an anaerobic environment.

While the molecular mechanisms governing the regulation and enzymatic function of nitrate reductases are well studied in *Escherichia coli*, understanding of the physiological relevance of these reductases during *S.* Typhimurium infection is lacking (17, 23, 24, 27, 28). Characterization of nitrate reductases in *E. coli* would predict that NarGHI is the major player conferring a nitrate respiration-dependent benefit during *S.* Typhimurium infection. In this study, we tested this prediction by investigating the contributions of individual *S.* Typhimurium nitrate reductases to the intestinal lifestyle of this pathogen.

MATERIALS AND METHODS

Bacterial strains and culture conditions. For the *E. coli* and *S.* Typhimurium strains used in this study, see Table 1. Strains were routinely grown in Luria-Bertani (LB) broth (catalog no. 244620; BD Biosciences, San Jose, CA) or on LB plates. Antibiotics in bacterial cultures were used at the following concentrations: for carbenicillin (Carb), 0.1 mg/ml; for chloramphenicol (Cm), 0.015 mg/ml; for kanamycin (Kan), 0.1 mg/ml; and for streptomycin (Strep), 0.1 mg/ml.

Mutant construction. To construct an *S.* Typhimurium SL1344 wild-type strain carrying a selectable Cm resistance marker, we generated a phage lysate of SW284 (11), an *S.* Typhimurium strain carrying a Cm^r cassette inserted into the *phoN* gene, using the methods described previously (11). Transfer of this cassette using P22 phage transduction into *S.* Typhimurium SL1344 generated strain CAL128, which is referred to below as the wild-type strain.

To construct an *S.* Typhimurium *narP* mutant, an internal *narP* region was amplified via PCR, inserted into the cloning vector pCR2.1 to generate pCAL32, and propagated in *E. coli* TOP10 (Invitrogen, Carlsbad, CA). After the insert was sequenced to confirm that there were no mutations in the amplified region, pCAL32 and plasmid pEP185.2 were digested with XbaI and SacI (New England BioLabs, Ipswich, MA). The

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
S. Typhimurium		
SL1344	ST4/74 *his*	48, 49
SW284	Strain IR715; Δ*phoN*::Cm^r	50
CAL51	Δ*narZ*	11
CAL55	Δ*napA* Δ*narZ narG*::pCAL5	11
CAL63	Δ*phoN*::Kan^r	11
CAL64	Δ*napA* Δ*narZ narG*::pCAL5 Δ*phoN*::Kan^r	11
CAL65	*narG*::pCAL5	11
CAL128	Δ*phoN*::Cm^r	This study
CAL139	Δ*phoN*::Kan^r *narP*::pCAL35	This study
CAL156	Δ*narL*::Kan^r	This study
CAL160	Δ*narL*	This study
CAL173	Δ*phoN*::Kan^r Δ*narL*	This study
CAL277	Δ*phoN*::Kan^r Δ*napA*	This study
CAL315	Δ*narZ* Δ*phoN*::Cm^r	This study
E. coli		
DH5α λ*pir*	F$^-$ *endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169* ϕ80*lacZ*ΔM15 λ*pir*	51
TOP10	ϕ80*lacZ*ΔM15 *lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL endA1 nupG*	Invitrogen
S17-1 λ*pir*	C600:RP4 2-(Tet::Mu) (Kan::Tn7) λ*pir recA1 thi pro hsdR* (r_K^- m_K^+)	51a
Plasmids		
pCR2.1	Cloning vector	Invitrogen
pGP704	*ori*(R6K) *mobRP4* Carb^r	52
pRDH10	*sacRB ori*(R6K) *mobRP4* Tet^r Cm^r	53
pEP185.2	*ori*(R6K) *mobRP4* Cm^r	54
pUC4 KSAC	*ori*(pMB1) Carb^r Kan^r	Pharmacia (GE Healthcare)
pCAL5	pGP704 with internal region of *narG*	11
pCAL32	pCR2.1 with *narP* internal region inserted	This study
pCAL35	pEP185.2 with *narP* internal region inserted	This study
pCAL36	pCR2.1 with regions upstream and downstream of *narL*	This study
pCAL39	pRDH10 with regions upstream and downstream of *narL*	This study
pCAL42	pRDH10 with regions upstream and downstream of *narL*; Kan^r cassette inserted between flanking regions	This study

XbaI/SacI-digested insert from pCAL32 was ligated into pEP185.2 to generate pCAL35. This plasmid was propagated first in *E. coli* DH5α λ*pir* and then in *E. coli* S17-1 λ*pir* for conjugation with *S.* Typhimurium CAL63 (Δ*phoN*::Kan^r). Single homologous recombination events were selected for by growth on Cm and Kan plates. Insertion into the *narP* locus was confirmed via PCR, and this strain was designated CAL139.

To construct an *S.* Typhimurium *narL* mutant, approximately 1 kb regions upstream and downstream of *narL* were PCR amplified and digested with XbaI. The two flanking regions were ligated and PCR amplified to obtain an approximately 2 kb region that was inserted into pCR2.1 to generate pCAL36. After the insert was sequenced for accuracy, pCAL36 and the suicide plasmid pRDH10 were digested with SalI (New England BioLabs, Ipswich, MA). The *narL* flanking region fragment from pCAL36 was ligated into pRDH10 to generate pCAL39, which was propagated in *E. coli* DH5α λ*pir*. A Kan^r cassette from pUCKSAC was inserted into the XbaI restriction site between the *narL* flanking regions to generate pCAL42. *E. coli* S17-1 λ*pir* was transformed by pCAL42 and conjugated with *S.* Typhimurium SL1344. Single homologous recombination events were selected for by growth on Strep and Kan plates. To select for strains that had lost the integrated plasmid, leaving the Kan^r cassette in place of *narL*, we used sucrose selection as described in reference 11. The strain that successfully replaced *narL* with the Kan^r cassette was designated CAL156. To remove the Kan^r cassette and generate a nonpolar mutant, CAL156 was conjugated with *E. coli* S17-1 λ*pir* containing pCAL39. After selecting for plasmid integration on Cm and Kan plates, we performed sucrose selection to identify a strain that had lost both the integrated plasmid and the Kan^r cassette. After confirming the nonpolar mutation via PCR, we designated the strain CAL160. To provide a selection marker for the *narL* mutant strain, CAL160 was transduced with a phage lysate from CAL63 to transfer the Δ*phoN*::Kan^r mutation. This *narL* mutant strain with Kan^r at the *phoN* locus was designated CAL173.

We had previously constructed single nonpolar *napA* and *napZ* mutants (11). To provide these strains with a selective marker, CAL67 (11) was transduced with a phage lysate from CAL63 to transfer the Δ*phoN*::Kan^r mutation, while CAL51 was transduced with a phage lysate from CAL128 to transfer the Δ*phoN*::Cm^r mutation. These strains were designated CAL277 and CAL315, respectively.

Mouse experiments. The Institutional Animal Care and Use Committee at the University of California, Davis, approved all animal experiments. We used the streptomycin model of gastroenteritis to reproducibly colonize mice with *S.* Typhimurium. In this model, mice first received a single dose of streptomycin sulfate (20 mg/mouse) (Calbiochem, San Diego, CA) intragastrically (i.g.). Mice were then inoculated i.g. with 100 μl of 1×10^8 *S.* Typhimurium CFU per mouse or, for mock-treated animals, with LB broth. In competitive infections, each strain was provided at a concentration of 5×10^7 CFU per mouse. At either 3 or 4 days postinfection, mice were euthanized. Colon contents were removed and stored in phosphate-buffered saline (PBS) on ice before being homogenized and plated on selective media for the enumeration of *S.* Typhimurium CFU. For RNA analysis, we collected the middle cecal section, removed fecal matter, and flash froze samples in liquid nitrogen for storage at −80°C.

TABLE 2 Primers used in this study

Gene	Organism	Primer sequence[a]
narP	*S.* Typhimurium	5′-CCTGGAGCTCGTATTCGTCAATTACTG-3′
		5′-ACGGTCTAGAGTAGATTACGAATGTGC-3′
narL (upstream)	*S.* Typhimurium	5′-ATTTGTCGACCGCAGAAGATGTCACG-3′
		5′-CCACTCTAGACACCGTTGCTGGCTTCTCC-3′
narL (downstream)	*S.* Typhimurium	5′-CTGCTCTAGACGAAAGTACGGTCAAAGTG-3′
		5′-TACGTCGACTGGTCTTACGCAACAG-3′
napA (qPCR)[b]	*S.* Typhimurium	5′-TGAAAGAGAAAGGACCAGAAGCG-3′
		5′-TTGTTAGAGCGGAAACCAGCC-3′
narG (qPCR)	*S.* Typhimurium	5′-GAAGTGGAGTGGCGTGACAATG-3′
		5′-AGCGGATGAATAAACGGATGC-3′
16S rRNA	*S.* Typhimurium	5′-TGTTGTGGTTAATAACCGCA-3′
		5′-GACTACCAGGGTATCTAATCC-3′
Nos2	Mouse	5′-CCTCTTTCAGGTCACTTTGGTAGG-3′
		5′-TTGGGTCTTGTTCAGCCACGG-3′
Gapdh	Mouse	5′-TGTAGACCATGTAGTTGAGGTCA-3′
		5′-AGGTCGGTGTGAACGGATTTG-3′

[a] Underlined sequences indicate engineered restriction sites.
[b] qPCR, quantitative PCR.

Isolation of RNA from the mouse. For the isolation of murine RNA, colon tissue sections were homogenized in a Mini-Beadbeater (BioSpec Products, Bartlesville, OK), and RNA was isolated by the TRI-Reagent method (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. Contaminating DNA was removed using the DNA-free kit (Applied Biosystems, Waltham, MA), and RNA was stored at −80°C.

For the isolation of bacterial RNA from the mouse, intestinal colon contents and the mucus layer (gently scraped from the colonic epithelial cells) were collected and were stored in RNAlater (Ambion, Carlsbad, CA) overnight at 4°C. Excess solution was subsequently removed from the pellet, and TRIzol (Invitrogen, Carlsbad, CA) was added. Next, chloroform was added, and the solution was transferred to 2-ml Phase Lock tubes (5 Prime, Hilden, Germany) and was centrifuged at 14,000 × g. The upper phase was removed, and RNA was precipitated with 100% ethanol at −20°C overnight. RNA was collected after centrifugation and was washed with 70% ethanol. Contaminating DNA was removed as described above.

***In vitro* RNA expression.** To quantify *napA* and *narG* induction *in vitro* in response to nitrate, *S.* Typhimurium was grown in M9 minimal medium (2 mM $MgSO_4$, 0.2 mM $CaCl_2$, M9 minimal salts [29], 0.4% glucose) supplemented with 40 μg/ml histidine. Overnight cultures of the strains to be tested were diluted 1:100 into fresh medium, and bacteria were grown in an anaerobic chamber (Shel Lab, Cornelius, OR) (atmosphere of 5% CO_2, 5% H_2, and 90% N_2) at 37°C until the optical density reached 0.1 to 0.2. At this time, a 1 M sodium nitrate solution was added to the medium to reach a final concentration of 0.1 mM or 0.4 mM. The bacterial cultures were incubated for 5 min and were then immediately placed on ice to halt growth.

For the isolation of RNA, 1 ml of bacterial culture was processed by using the Aurum Total RNA minikit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Contaminating DNA was removed as described above.

Quantitative PCR. Isolated RNA was reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (Applied Biosystems, Waltham, MA). Quantitative real-time PCR was performed using SYBR green (Applied Biosystems, Waltham, MA) PCR mix and the appropriate primer sets (Table 2) at a final concentration of 0.25 mM. Absolute values were calculated using a series of standards and a standard curve. To generate standards, the primer sets mentioned above were used in standard PCR to amplify the target genes, which were subsequently inserted into pCR2.1 using the TOPO cloning kit (Life Technologies, Carlsbad, CA). The resulting plasmids were sequenced for accuracy and were quantified to create a set of standards ranging from 10^8 to 10^1 copies/ml diluted in a 0.02-mg/ml yeast RNA (Sigma, St. Louis, MO) solution.

Cecal nitrate measurements. Mouse ceca were removed and were divided along their sagittal planes. Solid fecal matter was carefully removed from the lumen, while the mucus layer was gently scraped off the tissue. This layer was homogenized in 200 μl PBS and was placed immediately on ice. Samples were next centrifuged at 5,000 × *g* for 10 min at 4°C to remove remaining solid particles. The supernatant was removed and was filtered using a 0.2-μm Acrodisc syringe filter (Pall Life Sciences, Port Washington, NY). Measurement of cecal nitrate followed an adaptation of the Griess assay. In this assay, nitrate was first reduced to nitrite via vanadium(III) chloride by adding 50 μl of the cecal sample with 50 μl of Griess reagent 1 (0.5 M HCl, 0.2 mM VCl_3, 1% sulfanilamide) and incubating the mixture at room temperature for 10 min. Next, 50 μl of Griess reagent 2 [0.1% (1-naphthyl)ethylenediamine dichloride] was added. Absorbance at 540 nm was measured immediately after the addition of Griess reagent 2. Nitrite immediately reacts with the added reagents, and the initial color change indicates available nitrite in the samples. The reduction of nitrate to nitrite via VCl_3 occurs slowly, requiring incubation for 8 h at room temperature. Measurements of absorbance at 540 nm, along with a standard curve of known nitrate concentrations, were taken after the incubation. The initial absorbance (immediately after the addition of Griess reagent 2) was subtracted from the final absorbance to determine nitrate concentrations in the cecal mucus layer. Samples were tested in duplicate, and all measurements were standardized to the initial sample weight.

Histopathology. The distal segment of the cecum was fixed in 10% buffered formalin phosphate and was stained with hematoxylin and eosin (H/E). A veterinary pathologist scored histopathological changes using blinded scoring of sections by a scheme described previously (30).

Statistical analysis. Geometric mean values were determined throughout the study. Data on ratios were converted logarithmically to obtain a normal distribution prior to analysis. We used Student's *t* test to assess significance and considered a *P* value of 0.05 or less to be significant.

RESULTS

NarP, but not NarL, contributes to the growth of *S.* Typhimurium in the intestinal lumen. Transcription of *E. coli* nitrate reductases is largely controlled through nitrate-dependent activation of the NarXL and NarQP two-component systems (Fig. 1A). To determine the relative contributions of the homologous two-

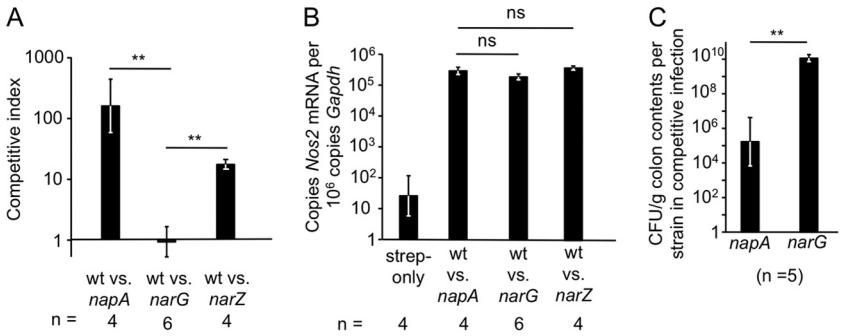

FIG 2 The periplasmic nitrate reductase NapABC provides a fitness advantage during competitive infection. (A) Mice were infected with mixtures of the indicated *S.* Typhimurium strains in equal amounts. Four days after infection, mice were euthanized, and the competitive index (output ratio over input ratio) was determined in colon contents. *, $P < 0.05$; **, $P < 0.01$; ns, not statistically significantly different; wt, the wild-type strain CAL128; *napA*, the *napA* mutant CAL277; *narG*, the *narG* mutant CAL65; *narZ*, the *narZ* mutant CAL315. (B) *Nos2* mRNA levels in colonic tissues from the animals used in the experiment for which results are shown in panel A and in control animals treated with streptomycin and inoculated 1 day later with a sterile medium. (C) Mice were infected with a mixture of a *napA* mutant and a *narG* mutant in equal amounts. Four days after infection, mice were euthanized, and the CFU counts of both strains in colon contents were determined.

component systems to *S.* Typhimurium fitness, we constructed *S.* Typhimurium mutant strains with defects in the response regulator *narL* or *narP*. *In vitro* analysis of these strains cultured anaerobically showed that neither mutant exhibited any significant difference in *narG* or *napA* mRNA expression from the wild-type (wt) strain when no nitrate was added to the medium (Fig. 1B). In the presence of nitrate (0.4 mM), *narG* expression was reduced only in the *narL* mutant, whereas *napA* expression was reduced only in the *narP* mutant. These data suggested that inactivation of *narL* and *narP* specifically affected the transcription of *narG* and *napA*, respectively, a finding consistent with previous reports on the function of these regulatory proteins in *E. coli* (Fig. 1A) (25, 26).

To test the significance of NarL and NarP for the growth of *S.* Typhimurium in the intestinal lumen during infection, we used a mouse colitis model (31), where mice were provided a single dose of streptomycin 1 day prior to infection with *S.* Typhimurium. Streptomycin pretreatment disrupts the resident microbiota, enabling *S.* Typhimurium to elicit acute cecal inflammation (reviewed in reference 32). Using this model for human gastroenteritis, we infected mice with 1:1 ratios of wild-type *S.* Typhimurium (CAL128) and either the *narL* mutant (CAL173) or the *narP* mutant (CAL139). Four days postinfection, mice were euthanized, and we quantified the ratios of the bacterial strains recovered. Interestingly, similar numbers of wild-type *S.* Typhimurium and a *narL* mutant were recovered from colon contents (Fig. 1C), suggesting that NarL was not necessary for *in vivo* growth in this model. In contrast, wild-type *S.* Typhimurium was recovered from the colon contents of mice in >10-fold-higher numbers than the *narP* mutant (Fig. 1C). Transcript levels of *Nos2*, the gene encoding iNOS, in the cecal mucosa were similar for the two groups (Fig. 1D), suggesting that differences in host-derived NO production were not responsible for the phenotypes observed. Collectively, these data indicated that, surprisingly, NarP contributed to *S.* Typhimurium growth in the intestinal lumen, while NarL was dispensable. To test this conclusion further, mice were infected with a mixture of equal parts of the *narL* and *narP* mutant strains. Higher recovery of the *narL* mutant (which encodes a functional *narP* gene) than the *narP* mutant (encoding a functional *narL* gene) further supported the conclusion that NarP played a more substantial role than NarL in the growth of *S.* Typhimurium in the intestinal lumen.

The periplasmic nitrate reductase NapABC boosts the growth of *S.* Typhimurium in the colon. Next, we wanted to test whether the fitness advantage conferred by NarP arose from its role in regulating the transcription of genes important for anaerobic respiration, specifically the genes encoding the periplasmic nitrate reductase NapABC. To explore this notion, mice were infected with a mixture of equal parts of wild-type *S.* Typhimurium (CAL128) and an isogenic mutant carrying a mutation in either *narG* (CAL65) or *napA* (CAL277). In competitive infections, wild-type *S.* Typhimurium was recovered from colon contents in higher numbers than the *napA* mutant, while the wild type and the *narG* mutant were recovered in similar numbers (Fig. 2A). Infections with these different strain mixtures caused similar increases in *Nos2* expression levels in the cecal mucosae (Fig. 2B). When mice were inoculated with a mixture of equal parts of a *napA* mutant and a *narG* mutant, the strain with a functional periplasmic nitrate reductase (*narG* mutant) grew to significantly higher levels in colon contents (Fig. 2C). We also sought to determine the contribution of NarZYV to the growth of *S.* Typhimurium in the intestinal lumen. In competitive infections with wild-type *S.* Typhimurium (CAL63) and a *narZ* mutant (CAL315), we recovered higher numbers of the wild-type strain than of the mutant (Fig. 2A).

A growth defect caused by the *napA* mutation was also observed when mice were infected with individual bacterial strains. Mice were infected with either wild-type *S.* Typhimurium, a *narG* mutant, a *narZ* mutant, or a *napA* mutant. Interestingly, 4 days after infection, the wild type, the *narG* mutant, and the *narZ* mutant were recovered from colon contents in approximately 10-fold higher numbers than the *napA* mutant (Fig. 3A), although cecal *Nos2* transcript levels were elevated by similar magnitudes during infections with the different *S.* Typhimurium strains (Fig. 3B). Differences in the luminal *S.* Typhimurium population did not affect host pathology: mice in all infected groups experienced moderate to high levels of intestinal inflammation (Fig. 3C), with neutrophil influx into the gut lumen and massive thickening of the mucosa and submucosa (Fig. 3D). In agreement with the observed differences in growth in the intestinal lumen between the *napA* and *narG* mutants, we also noted significantly higher expression of the *napA* gene than of the *narG* gene in RNAs extracted from the cecal contents of mice infected with wild-type *S.* Typhimurium

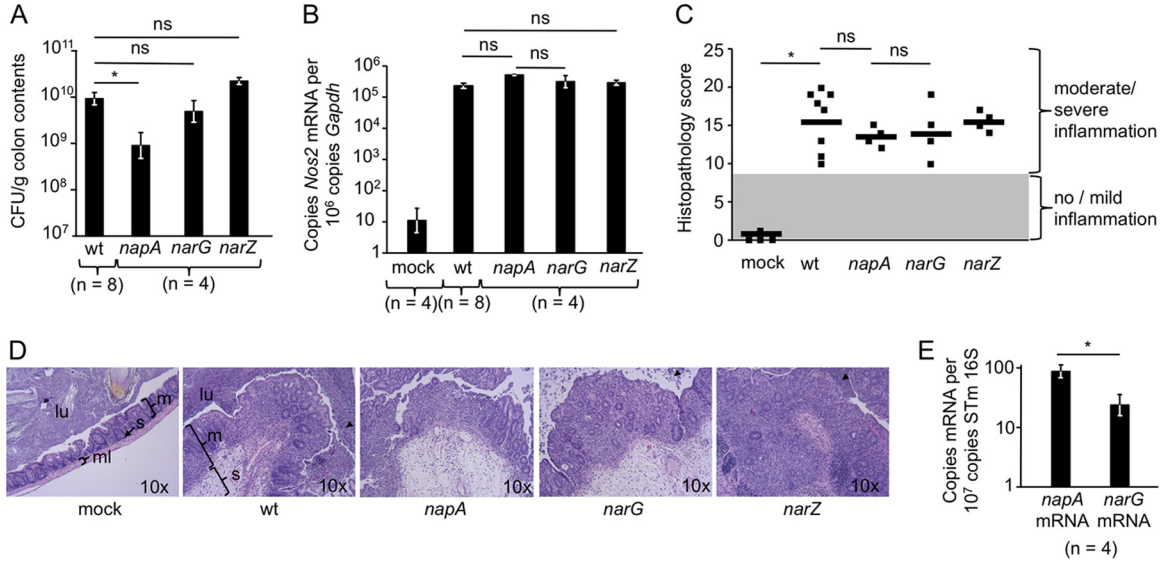

FIG 3 The periplasmic nitrate reductase NapABC confers a luminal growth benefit during single-infection experiments. (A) Mice were infected with either the wild-type *S.* Typhimurium strain CAL128 (wt), the *napA* mutant CAL277, the *narG* mutant CAL65, or the *narZ* mutant CAL315. Four days after infection, mice were euthanized, and *S.* Typhimurium CFU counts in colon contents were determined. *, $P < 0.05$; **, $P < 0.01$; ns, not statistically significantly different. (B) *Nos2* mRNA levels in the cecal mucosae of the animals used in the experiment for which results are shown in panel A and in those of a control group inoculated with sterile medium (mock). (C) Histopathology scores for the mice used in the experiments for which results are shown in panels A and B. Each data point represents an individual mouse. (D) Representative images of H/E-stained cecal sections scored in panel C. All images were taken at the same magnification. m, mucosa; s, submucosa; ml, muscle layer; lu, lumen. Arrowheads indicate luminal neutrophils. (E) Levels of *narG* and *napA* mRNA transcripts were determined in RNAs isolated from the cecal contents of mice 4 days after infection with wild-type *S.* Typhimurium.

(Fig. 3E). No expression of either gene was detected in RNA extracted from the cecal contents of mock-infected control mice (data not shown). Together, our results support the idea that periplasmic nitrate reductase provides the primary benefit during the growth of *S.* Typhimurium in the host gut by nitrate respiration.

The murine colon is a low-nitrate environment. *Salmonella* nitrate reductase regulation is largely dependent on nitrate concentrations, and on the basis of our results showing that NapABC-deficient *S.* Typhimurium exhibits a growth defect *in vivo*, we predicted that the intestine is a nitrate-limited environment. To test this prediction, we determined cecal nitrate concentrations 3 days after infection. This time point was chosen because it coincided with the peak of *Nos2* expression observed in a previous study using this model (11). Mice either were mock treated (streptomycin treatment followed 1 day later by inoculation with a sterile medium) or were infected with wild-type *S.* Typhimurium or with a nitrate respiration-deficient *S.* Typhimurium mutant (*napA narG narZ* mutant strain CAL55). Mock-treated mice produced small but measurable amounts of nitrate (0.03 mM) in the cecal mucus layer. In contrast, nitrate was recovered at a 2- to 3-fold-higher level (0.29 mM) in mice 3 days after infection with the *S.* Typhimurium *napA narG narZ* mutant. Nitrate concentrations were also elevated in mice infected with wild-type *S.* Typhimurium (0.21 mM) (Fig. 4A). The two strains were recovered from the colon contents in equivalent numbers at this time point (Fig. 4B), since nitrate-dependent outgrowth is not observed before day 4 after infection in this model (Fig. 3A). Enhanced nitrate

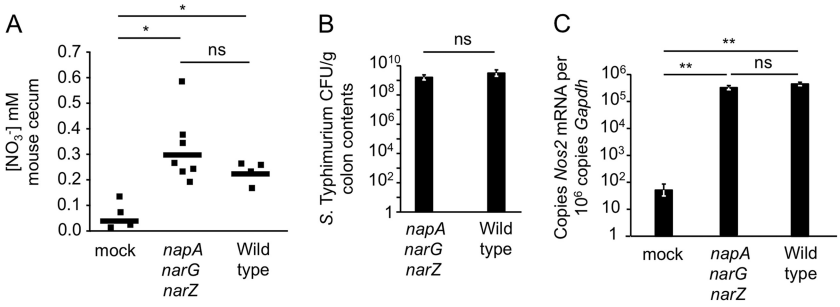

FIG 4 Nitrate levels in the murine cecum. (A) Nitrate concentrations were determined in the cecal mucus layers of mice receiving either streptomycin followed 1 day later by inoculation with a sterile medium (mock), an *S.* Typhimurium nitrate reductase mutant (*napA narG narZ* mutant CAL64), or the wild-type *S.* Typhimurium strain CAL63. Measurements were taken 3 days after mice had received bacteria or sterile medium. Values were normalized to initial sample weights. Each data point represents an individual mouse. *, $P < 0.05$; **, $P < 0.01$; ns, not statistically significantly different. (B) *S.* Typhimurium CFU counts 3 days after infection in the colon contents of the mice for which results are shown in panel A. (C) Cecal *Nos2* mRNA expression in the mice for which results are shown in panel A.

production correlated with increased expression of murine *Nos2* in infected mice (Fig. 4C). These data suggest that *S.* Typhimurium encounters, on average, low nitrate concentrations during colonization of the distal gastrointestinal tract.

DISCUSSION

Like many pathogenic bacteria in the family *Enterobacteriaceae*, *S.* Typhimurium encodes a variable and highly regulated respiratory network that provides flexibility in responding to changes in the availability of electron donors and acceptors. We are only beginning to understand the environmental conditions that *S.* Typhimurium experiences during infection and the mechanism by which the resulting changes in central metabolism contribute to successful intestinal colonization. We previously found nitrate respiration to be an important factor for the growth of *S.* Typhimurium during acute inflammation (11), and here we examined the individual roles of the inducible nitrate reductases (NarGHI and NapABC) in order to understand their contributions to the intestinal lifestyle of *S.* Typhimurium.

S. Typhimurium directly detects nitrate with the two-component systems NarXL and NarQP, and we sought to determine if loss of the response regulator led to a fitness defect *in vivo*. Remarkably, inactivation of *narL* did not reduce growth in the intestinal lumen, although NarL regulates a variety of metabolic genes, and this regulation includes activation of *fdnGHI* (encoding formate dehydrogenase) and repression of *frdABCD* (encoding fumarate reductase), *dmsABC* (encoding dimethyl sulfoxide [DMSO] reductase), *dcuSR* (encoding a two-component system), and *nrfABCDEFG* and *nirBDC* (encoding two nitrite reductases) (reviewed in reference 33). To the best of our knowledge, no evidence exists supporting a role in *S.* Typhimurium virulence or luminal growth for formate dehydrogenase-N, DMSO reductase, or nitrite reduction via *nirBDC*. While conclusive data on the individual contributions of these NarL-regulated genes *in vivo* are lacking, at this point we cannot discount the possibility that NarL-regulated genes other than the nitrate reductases are important in intestinal colonization by *S.* Typhimurium.

Mutation of *narP* resulted in an *in vivo* growth defect, highlighting the importance of the encoded response regulator for the growth of *S.* Typhimurium in the intestine. NarP positively regulates the *napABC* genes, encoding a periplasmic nitrate reductase, as well as *nrfABCDEFG* and *nirBDC*, two gene clusters encoding nitrite reductases (34). These three gene clusters are thus oppositely regulated by the NarXL and NarQP two-component systems. A *napA* mutant exhibited a marked luminal growth defect in the mouse colitis model, suggesting that the phenotype of a *narP* mutant was, at least in part, due to reduced expression of NapABC. In contrast, strains lacking the cytosolic nitrate reductase A exhibited little difference from the wild-type strain in intestinal growth. The third nitrate reductase, NarZYV, also appeared to contribute to the growth of *S.* Typhimurium, although the benefit was seen only in competition with wild-type *S.* Typhimurium, not when mice were infected with individual bacterial strains.

Previous work with *E. coli* would predict that nitrate reductase A contributes most heavily to nitrate-dependent growth in the gut (24, 35). There are technical reasons that may help explain the apparently contradictory results from previous work with *E. coli* and our *in vivo* analysis with *S.* Typhimurium. Early *in vitro* experiments that probed the functional characteristics of nitrate reductases used batch cultures in which high nitrate levels (as high as 40 mM) were added to the medium (21, 23). This ensured that ample nitrate was available for respiration and growth, but it also biased expression toward nitrate reductase A, which is synthesized optimally with nitrate concentrations of 10 mM or higher (36). In contrast, when nitrate is provided at low concentrations (<1 mM), a condition that favors expression of the periplasmic nitrate reductase, the electron acceptor is consumed rapidly, before growth phenotypes can be recorded (37). Later work confirmed that continuous cultures with chemostats providing nitrate at a constant concentration were needed in order to detect a contribution of the periplasmic nitrate reductase to *in vitro* growth (23, 36). The high affinity of the periplasmic nitrate reductase for nitrate, coupled with its maximal expression at nitrate concentrations around 1 mM (21), suggest that during acute inflammation, the niche in which *S.* Typhimurium resides is nitrate limited. In agreement with the hypothesis that the inflamed gut is a nitrate-limited environment, we detected low average nitrate levels in murine ceca, even after infection with an *S.* Typhimurium mutant that cannot consume nitrate (0.29 mM). A low nitrate concentration in the gut may also explain why the growth of *S.* Typhimurium in the intestinal lumen requires energy taxis to seek out spatial niches containing this limited electron acceptor (14). Taken together, our data support a model where pathogen-induced inflammation generates low concentrations of nitrate that support the luminal growth of *S.* Typhimurium using the periplasmic nitrate reductase NapABC.

S. Typhimurium presents a prime example of a bacterial pathogen well adapted to proliferation in an environment greatly altered during infection. Gut inflammation results in shifts in resident microbial populations, disrupting the relative stability of the normally dominant phyla *Firmicutes* and *Bacteroidetes* (38, 39), while the reactive oxygen and nitrogen species released by infiltrating immune cells change the availability of key nutrients, such as tetrathionate (40) and ethanolamine (30). Other catabolized nutrients available for the growth of *S.* Typhimurium include fructose-asparagine (41), hydrogen (42), fucose (13), and sialic acid (13). This ever-changing environment requires the pathogen to exhibit metabolic flexibility in order to maximize its growth by responding to alterations in nutrient availability. This appears to be true with regard to changes in the availability of host-derived nitrate, since regulated expression of the appropriate nitrate respiratory enzymes can significantly impact the fitness of *S.* Typhimurium by reinforcing its growth via enhanced ATP production, redox balance, or carbon source utilization.

An important role for the periplasmic nitrate reductase may reveal a common metabolic strategy for intestinal bacterial pathogens. The species *S. enterica* contains two pathovars, one associated with gastrointestinal disease (to which *S.* Typhimurium belongs) and the other associated with extraintestinal disease (to which *Salmonella enterica* serovar Typhi belongs) (43). Comparison of the genomes of serovars belonging to the gastrointestinal pathovar with those belonging to the extraintestinal pathovar identifies genes important for intestinal survival, because they are undergoing decay in the latter group (43). That is, extraintestinal serovars accumulate mutations affecting genes important only for luminal growth, since no selective pressure exists to preserve their function; the result is pseudogene formation (43–45). Analysis of the nitrate reductases and their regulators found that only the *narQP* two-component system was rendered nonfunctional in extraintestinal serovars due to mutations (43). This suggests that

NarQP, or genes regulated by NarQP, such as *napABC*, are critical for gastrointestinal serovars but not for extraintestinal serovars. This hypothesis is consistent with our results showing that maintenance of the periplasmic nitrate reductase strongly supports the enteropathogenic lifestyle of *S.* Typhimurium. The benefit provided by the periplasmic nitrate reductase is also relevant to bacteria in lineages outside the *Enterobacteriaceae*. For instance, *Vibrio cholerae*, the causative agent in the diarrheal disease cholera, multiplies to large numbers in the intestines during infection. *V. cholerae* encodes a homologue of the periplasmic nitrate reductase, while homologues of cytosolic nitrate reductases are absent. Interestingly, a study examining genes upregulated by *V. cholerae* after passage through a human host found markedly increased expression of the periplasmic nitrate reductase complex (46). Similarly, *Campylobacter jejuni*, a frequent cause of food-borne intestinal illness, respires nitrate during infection (47). *C. jejuni* encodes a periplasmic nitrate reductase but lacks homologues of the cytosolic nitrate reductase. Therefore, a high-affinity periplasmic nitrate reductase may be a common mechanism to assist pathogen growth in a highly competitive intestinal environment.

Our work highlights an important contribution of the periplasmic nitrate reductase to the growth of *S.* Typhimurium in the intestines during infection and may indicate a common mechanism to support the fitness of intestinal pathogens in diverse lineages. This adds to the small but growing body of literature deciphering the critical role of metabolism in the survival and proliferation of bacterial pathogens.

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We declare no conflicts of interest.

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