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The role of denitrification genes in anaerobic growth and virulence of *Flavobacterium columnare*

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Abstract

Aims: Comparative genomics analyses indicated that the *Flavobacterium columnare* genome has unique denitrification genes relative to *Flavobacterium psychrophilum* and *Flavobacterium johnsoniae*, including *nasA* (nitrate reductase), *nirS* (nitrite reductase), *norB* (nitric oxide reductase), and *nosZ* (nitrous oxide reductase). The current study determines the roles of *nasA*, *nirS*, *norB*, and *nosZ* in anaerobic growth, nitrate reduction, biofilm formation, and virulence.

Methods and Results: Four in-frame deletion mutants in virulent *F. columnare* strain 94–081 were constructed by allelic exchange using pCP29 plasmid. Compared with parent strain 94–081, *Fc*Δ*nasA, Fc*Δ*nirS*, and *Fc*Δ*nosZ* mutants did not grow as well anaerobically, whereas the growth of *Fc*Δ*norB* strain was similar to the parent strain (*Fc*WT). Exogenous nitrate was not significantly consumed under anaerobic conditions in *Fc*Δ*nasA*, *Fc*Δ*nirS*, and *Fc*Δ*nosZ* compared to parent strain 94–081. Under anaerobic conditions, *Fc*Δ*nasA*, *Fc*Δ*norB*, and *Fc*Δ*nosZ* formed significantly less biofilm than the wild type strain at 24 and 96 hours, but *Fc*Δ*nirS* was not significantly affected. The nitrite reductase mutant *Fc*Δ*nirS* was highly attenuated in catfish, whereas *Fc*Δ*nasA*, *Fc*Δ*norB*, and *Fc*Δ*nosZ* had similar virulence to *Fc*WT.

Conclusions: These results show, for the first time, that denitrification genes enable *F. columnare* to grow anaerobically using nitrate as an electron acceptor, and nitrite reductase contributes to *F. columnare* virulence.

Significance and Impact of the Study: These findings indicate potential for *F. columnare* to grow in nitrate-rich anaerobic zones in catfish production ponds, and they suggest that a *Fc*Δ*nirS* strain could be useful as a safe live vaccine if it protects catfish against columnaris disease.

Keywords

Flavobacterium columnare; denitrification; anaerobic respiration; catfish; columnaris disease; aquaculture; virulence

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Author contributions

ML and SN conceived and designed the experiments. SN constructed and characterized the mutants. HA performed the catfish immersion challenge. HA and SN analyzed the data. HA, SN, ML, and AK wrote the manuscript.

Conflict of interest

The authors have declared no conflicts of interest.

Introduction

Flavobacterium columnare is a long Gram-negative rod causing columnaris disease, which affects numerous fish species worldwide (Wagner et al. 2006). Six genomovar types have been described among *F. columnare* isolates (I, II, II-B, III, I/II, and II-A) (LaFrentz et al. 2014; Garcia et al. 2018). More recently, *F. columnare* isolates were reclassified into four genetic groups based on 16S rRNA gene sequences (LaFrentz et al. 2018). Genetic group 2 is the most virulent for channel catfish (*Ictalurus punctatus*) (Triyanto and Wakabayashi 1999; Shoemaker et al. 2008). In the United States, *F. columnare* is responsible for significant economic losses in channel catfish aquaculture (Wagner et al. 2002). Infected catfish often exhibit external lesions on the body surface, gills, and fins. *F. columnare* may cause chronic infection with low-level mortalities, or it may cause acute infection with mortalities occurring within a few days (Declercq et al. 2013; Mohammed and Arias 2014). Host stress (e.g., low oxygen, high nitrite and ammonia, elevated water temperature, mechanical injury, or crowding) enhances the occurrence and severity of columnaris disease. Minimizing fish stress helps prevent columnaris disease outbreaks, but the ubiquitous presence of this pathogen in aquatic environments makes eradication of the disease in aquaculture systems difficult.

F. columnare is capable of surviving for extended periods in pond water, sediments, and mud slurry (Declercq et al. 2013; Mohammed and Arias 2014). It is common for pond sediment and water at the bottom of ponds to be anaerobic. It is also common for commercial production ponds to be rich in nitrogenous compounds due to stocking density and feeding. Also, in natural habitats, *F. columnare* often grow as biofilms, which create oxygen-limited conditions (Cai et al. 2013). In addition, during infection and ulcer formation in fish tissue, bacteria encounter microenvironments of reduced oxygen concentration. When oxygen is not available, alternative electron acceptors, including nitrate, nitrite, or nitrous oxide, can be used by some bacteria (Filiatrault et al. 2006).

Comparison of the *F. columnare* genome with *F. psychrophilum* and *F. johnsoniae* showed that *F. columnare* encodes four enzymes capable of reducing nitrate to nitrogen gas through the denitrification pathway (Figure 1). In sequential order, the four enzymes are nitrate reductase (encoded by *nasA*), which reduces nitrate to nitrite; nitrite reductase (*nirS*), which reduces nitrite to nitric oxide (Kawasaki et al. 1997); nitric oxide reductase (*norB*), which reduces nitric oxide to nitrous oxide (Schreiber et al. 2007); and nitrous oxide reductase (*nosZ*) for reduction of nitrous oxide to inert gaseous nitrogen (Zumft et al. 1990). *F. columnare* is capable of anaerobic growth in the presence of sodium nitrate (Tekedar et al. 2017). This suggests that *F. columnare* is capable of using nitrogenous compounds as alternate electron acceptors for anaerobic respiration, which would enable *F. columnare* to replicate in the nitrogen-rich anaerobic pond sediments of catfish production ponds, potentially serving as an environmental source of infection in catfish ponds.

Some pathogenic bacteria are capable of denitrification (Philippot 2005). The denitrification pathway has been linked to virulence of several species such as *Mycobacterium bovis*, *Brucella melitensis*, *Neisseria meningitidis*, and *Pseudomonas aeruginosa* (Weber et al. 2000). Furthermore, inactivation of the denitrification pathway hindered biofilm formation

in *P. aeruginosa* and *Neisseria gonorrhoeae* (Van Alst et al. 2007; Falsetta et al. 2009). In *N. meningitidis*, active denitrification was linked to modulation of host cytokine responses, enhanced intracellular survival, and inhibition of apoptosis in a macrophage model (Stevanin et al. 2005; Stevanin et al. 2007), and denitrification prevented the establishment of anti-inflammatory nitric oxide steady-state levels (Barth and Clark 2008).

Therefore, the current study was undertaken to determine the role of four genes (*nasA*, *nirS*, *norB*, and *nosZ*) in *F. columnare* anaerobic growth, nitrate respiration, and biofilm formation. We also determined whether these genes are required for *F. columnare* virulence in catfish fingerlings. *F. columnare* denitrification may be physiologically relevant to its survival and growth in aquaculture ponds or during infection of fish tissue.

MATERIALS AND METHODS

Ethics statement

All fish disease challenges were conducted in compliance with protocol #17–288 approved by the Mississippi State University Institutional Animal Care and Use Committee (IACUC). The approved protocol included humane endpoints, and when morbid fish met established criteria, they were immediately euthanized by immersion in tricaine methane sulfonate (MS-222). Criteria for euthanasia were loss of balance, hanging at the water surface, or non-responsiveness to external stimuli. Some of the fish died during the study as a result of the experimentally induced systemic infection due to its rapid progression. All personnel on this experiment received IACUC-approved training in animal care and welfare by the University Laboratory Animal Veterinarian.

Bacterial strains, growth conditions, and plasmid

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strain DH5αMCR was used for cloning, and *E. coli* BW19851 was used for transfer of pCP29 into *F. columnare* strain 94–081 (genetic group 2) (Kumru et al. 2016). *E. coli* strains were cultured in Luria Broth (LB) broth or agar at 37 °C. *F. columnare* strain 94–081 was grown at 30 °C in *Flavobacterium columnare* growth medium (FCGM) broth [tryptone (8.00 g), yeast extract (0.80 g), MgSO4 7 H2O (1.00 g), CaCl2 2 H2O (0.74 g), NaCl (5.00 g), and sodium citrate (1.50 g) per liter] or on FCGM agar (Dumpala et al. 2010). *F. columnare* strains were cultured aerobically with 5% CO_2 or anaerobically in a jar with GasPak system (Fisher Scientific, PA, USA) at 30 °C. The pCP29 plasmid was modified and used to construct in-frame gene deletions by allelic exchange (Kempf and McBride 2000). Plasmid pCP29 carries cefoxitin (*cfxA*) and erythromycin (*ermF*) resistance genes that function in *Flavobacterium* (Kempf and McBride 2000). It also carries a beta lactamase gene (*bla*) that is used to maintain the plasmid in *E. coli*. Ampicillin was used at 100 μg/ml for plasmid maintenance in *E. coli* DH5αMCR during cloning steps. For selection of *F. columnare* transconjugants, cefoxitin was used at 10 μg/ml. Colistin (12.5 μg/ml) was used for counterselection against *E. coli* BW19851 following conjugation. pCP29 was converted into a homologous recombination-deletion mutagenesis vector by removal of the pCP1 fragment containing the origin that allows the plasmid to replicate in *Flavobacterium* species (Staroscik et al. 2008). This was accomplished by digesting with a combination of two

restriction endonucleases (either *Sma*I and *Sph*I or *Sma*I and *Pst*I). The resulting 8 kb fragment was isolated by gel purification and used for ligation with overlap extension PCR fragments (described in the next section).

Construction of *F. columnare nasA*, *nirS*, *norB*, and *nosZ* mutants

Four in-frame deletion strains were constructed using the overlap extension PCR method as previously described (Horton et al. 1989). In brief, the nucleotide sequences of *nasA* (AWN65_05950), *nirS* (AWN65_04985), *norB* (AWN65_04995), and *nosZ* (AWN65_09420) from the *F. columnare* strain 94–081 genome (GenBank accession: CP013992.1) (Kumru et al. 2016) were used to design four primers (A, B, C, D) for each gene using Primer3 (Table 2). Restriction endonuclease recognition sequences were added to A and D primers for cloning, and the reverse complement of primer B was added to the 5' end of primer C to enable overlap extension PCR. Amplicons flanking the target genes using AB and CD primers were amplified by PCR from *F. columnare* 94–081 genomic DNA. Then both fragments were mixed equally and used as a template in subsequent overlap extension PCR using A and D primers. The resulting fusion fragments for each individual gene were cleaned, digested with restriction endonucleases (*Sma*I and *Sph*I for *nasA* and *norB*, and *Sma*I and *Pst*I for *nirS* and *nosZ*), ligated into the 8 kb fragment isolated from pCP29, and transformed into *E. coli* DH5αMCR to generate pCP29S-*nasA*, pCP29S-*nirS*, pCP29S-*norB*, and pCP29S-*nosZ*.

These four plasmids were transferred from donor *E. coli* BW19851 into recipient *F. columnare* strain 94–081 by conjugation (Alvarez et al. 2004). Briefly, donor *E. coli* and recipient *F. columnare* were grown to mid-log phase, concentrated by centrifugation at 8,000 rpm for 10 min, and washed twice with FCGM medium. Concentrated donor and recipient bacteria were mixed at a ratio of 1:4 (based on pellet weight) and placed on a sterile nitrocellulose filter (Fisher Scientific) on an FCGM agar plate. After incubation overnight at 30 °C, the bacterial mixture was washed off the filter and resuspended in FCGM broth with a 1 ml syringe. The resuspended bacteria mixture was spread on FCGM agar containing 10 μg/ml of cefoxitin to select *F. columnare* transconjugants and 12.5 μg/ml colistin for counterselection. Isolated colonies were propagated in FCGM broth and incubated with shaking at 30°C overnight, and 1 ml was spread on FCGM agar with colistin to allow for a second allelic exchange event. Colonies were screened for cefoxitin sensitivity, and putative cefoxitin sensitive mutant colonies were tested by PCR using A and D primers to discriminate between mutant and wild type alleles. PCR amplicons with size consistent with a gene deletion were confirmed by DNA sequencing. *Fc*Δ*nasA* had a deletion of 2,085 bp out of 2,214 bp (94.17%), *Fc*Δ*nirS* had a deletion of 1,374 bp out of 1,437 bp (95.54%), *Fc*Δ*norB* had a deletion of 1,263 bp out of 1,329 bp (95.03%), and *Fc*Δ*nosZ* had a deletion of 1,806 bp out of 1,971 bp (91.62%) (Table 3).

Growth of mutants

Anaerobic and aerobic growth of wild-type *F. columnare* (*Fc*WT) and mutants (*FcΔnasA, FcΔnirS, FcΔnorB,* and *FcΔnosZ*) were compared in FCGM supplemented with 10 mM sodium nitrate (FCGM-N). Starter cultures were prepared by inoculating FCGM broth from agar plates and aerobic incubation at 30°C with shaking (220 rpm). After reaching stationary

phase, cultures were added to anaerobically preconditioned FCGM-N broth in a 10% v/v proportion. FCGM-N broth without bacteria was included as a negative control. Cultures were incubated under anaerobic conditions at 30°C using a GasPak anaerobic system in a standard anaerobic jar. Bacterial growth was measured by monitoring optical density (OD_{600}) in triplicate at 1, 7, and 14 days post-inoculation. Aerobic growth was compared using FCGM-N broth with shaking.

Addition of extracellular nitrate and nitrite under anaerobic conditions

Nitrate and nitrite concentrations were measured during anaerobic growth of the *Fc*WT and mutant strains to determine the amount of anaerobic respiration that had occurred. For each strain, triplicate anaerobic cultures were inoculated in FCGM-N as described above. FCGM-N broth without bacterial inoculation was included as control. For each sample, both nitrate and nitrite were determined using a commercial kit (Colorimetric Nitrite/Nitrate Assay Kit, Sigma Aldrich) that is based on the Griess reaction (Griess 1879) with some modification. Serially diluted sodium nitrite was used to obtain a standard curve.

To determine nitrate concentration, the nitrate+nitrite concentration was determined, and nitrite concentration was then subtracted. For nitrate+nitrite concentration, supernatant was collected from cultured *Fc*WT and mutants (*FcΔnasA, FcΔnirS, FcΔnorB,* and *FcΔnosZ*) following centrifugation, and 80 μl was transferred to a 96-well plate in triplicate (Corning, NY, USA). After adding culture supernatants, 10 μl of enzyme cofactor and 10 μl of nitrate reductase (Cayman chemical) were added to each sample and incubated for one hour at room temperature (protected from light). Then 50 μl of sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) was added to each well and incubated in the dark for 10 min at room temperature. Following incubation, 50 μl of NED solution (0.1% N-1-naphthyl ethylenediamine dihydrochloride in water) was dispensed to each well and incubated for an additional 10 min at room temperature without light. Absorbance was measured at 540 nM using a SpectraMax M5 ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

For nitrite concentration, 100 μl of diluted *Fc*WT and mutant strain culture supernatant was added to triplicate wells, and 50 μl of sulfanilamide solution and 50 μl of NED solution was subsequently dispensed to each well. Color was allowed to develop for 10 min at room temperature, and absorbance at 540 nm was measured using a SpectraMax M5 ELISA reader.

Quantification of biofilm formation

The ability of *FcΔnasA, FcΔnirS, FcΔnorB,* and *FcΔnosZ* to form biofilm was compared to *Fc*WT using a crystal violet staining technique as previously described with minor modifications (Cai et al. 2013). Briefly, *Fc*WT and the mutant strains were grown overnight in FCGM at 30 °C before being subcultured by diluting at 1:100 into the wells of a microtiter plate (Costar, USA) under aerobic and anaerobic conditions for 24 and 96 hours. Wells with uninoculated FCGM medium were included as a negative control. Plates were incubated at 30 °C for 24 or 96 hours. Then wells were gently washed two times with PBS to remove unbound bacteria and subsequently stained with 0.01% crystal violet (Sigma-

Aldrich) for 10 min at room temperature. After incubation, plates were rinsed with PBS and dried at room temperature followed by elution with 70% ethanol. The absorbance at OD_{538} nm was measured using a spectrophotometer (Biotek Synergy Mx, USA). The level of the biofilm formation was determined by subtracting the mean OD_{538} value of the negative control from the value of the test samples. The experiment was performed in four replicates for each strain and repeated two independent time.

Virulence of *FcΔnasA, FcΔnirS, FcΔnorB,* and *FcΔnosZ* in catfish

Approximately 480 eight-month-old specific pathogen free (SPF) channel catfish fingerlings (20 to 25 g) were stocked into 24 40 L flow-through tanks (flow rate: 1-l/ min) with a stocking density of 20 fish/tank and acclimated for one week. Fingerlings were obtained from the Mississippi State University College of Veterinary Medicine SPF Laboratory. Catfish in the SPF Laboratory are free from known obligate pathogens and parasites. Fish are hatched from disinfected eggs and maintained in indoor facilities with appropriate biosecurity protocols and monitoring for pathogens and parasites. Fish were fed to satiation twice daily and monitored three times daily for morbidity and mortality. Chlorine, dissolved oxygen, and temperature were monitored daily. Each treatment had four replicate tanks. Treatments consisted of *FcΔnasA, FcΔnirS, FcΔnorB,* and *FcΔnosZ*, *Fc*WT (positive control), and FCGM (sham control). Bacterial strains were cultivated in FCGM broth overnight at 30°C. Absorbance at 600 nm (OD_{600}) was measured, and culture densities were adjusted with sterile medium as needed to a standard absorbance. Water volume in tanks was adjusted to 10 L, and then 100 ml of appropriate bacterial culture was added to each tank (final dose of 2.45×10^7 CFU/ml of water). Channel catfish were infected by immersion for 6 hours, followed by gradual removal of bacteria. Mortalities were recorded daily for 21 days following challenge. *F. columnare* was confirmed as cause of death based on clinical signs and by culturing gills and skin swabs on FCGM agar from each mortality.

Statistical analysis

Linear regression analysis in PROC MIXED of SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC, USA) was used for the anaerobic growth experiment, extracellular nitrate concentration, and biofilm formation assays where mutant strains were compared to *Fc*WT. Separate models were developed for each analytical test using manual forward selection, and each included mutant type and time point as fixed effects. Replicates were included as a random effect. Adjustment for multiple comparisons were made using the SIMULATE option. Results are reported as least square means ± standard error. In the fish challenge experiments, mean percent mortality data were arcsine transformed, and analysis of variance (ANOVA) was applied using PROC GLM in SAS for Windows v9.4 (SAS Institute, Inc., Cary, NC) to assess significance. An alpha level of 0.05 was used in all analyses.

Results

Anaerobic growth characteristics of *FcΔnasA, FcΔnirS, FcΔnorB,* and *FcΔnosZ*

The growth of *F. columnare* wild-type and four mutant strains in FCGM broth supplemented with nitrate was compared under anaerobic conditions at 1, 7, and 14 days post-inoculation (Fig. 2). At all tested time points, significantly lower growth was observed in *FcΔnasA,*

FcΔnirS, and *FcΔnosZ* as compared with *Fc*WT. By contrast, anaerobic growth of *Fc*Δ*norB* was not significantly decreased compared to *Fc*WT strain with nitrate supplementation. There was no significant difference in the growth between mutant and wild type strains under aerobic condition (data not shown).

Nitrate accumulation by *FcΔnasA, FcΔnirS, FcΔnorB,* and *FcΔnosZ*

To determine whether *F. columnare* uses nitrate as an electron acceptor during anaerobic growth, we measured nitrate consumption during anaerobic growth. We also compared the ability of denitrification mutants to utilize nitrate during anaerobic growth (Fig. 3). At both 7 days and 14 days of anaerobic growth, *Fc*Δ*nasA*, *Fc*Δ*nirS*, and *Fc*Δ*nosZ* had significantly ($P < 0.0001$) higher nitrate concentration compared with *Fc*WT. In contrast, the *Fc*Δ*norB* mutant (encoding nitric oxide reductase) showed no significant difference in nitrate concentration compared to *Fc*WT at 7 and 14 days.

Biofilm assay

Biofilm formation of *FcΔnasA, FcΔnirS, FcΔnorB,* and *FcΔnosZ* was examined using a static biofilm assay at 24 and 96 hours. Under aerobic conditions, no significant ($P > 0.05$) differences in biofilm formation between mutants and *Fc*WT were observed (data not shown). Under anaerobic conditions, *FcΔnasA, FcΔnorB,* and *FcΔnosZ* formed significantly less biofilm than *Fc*WT at 24 and 96 hours (Fig 4). There was no significant difference in biofilm formation between *Fc*Δ*nirS* and *Fc*WT.

Virulence of *FcΔnasA, FcΔnirS, FcΔnorB,* and *FcΔnosZ* in catfish

Catfish fingerlings challenged with *Fc*Δ*nirS* exhibited significantly fewer mortalities (6.67%) ($P < 0.0001$) compared to those challenged with *Fc*WT (83.33%). However, mortalities in catfish challenged with *Fc*Δ*nasA* (80%), *Fc*Δ*norB* (86.67%), and *Fc*Δ*nosZ* (100%) were similar to that of *Fc*Wt challenged catfish (Fig 5 A and B).

Discussion

Denitrification is a microbial process that reduces nitrate (NO_3^-) or nitrite (NO_2^-) to nitrogen (N_2) via two obligate intermediates, nitric oxide (NO) and nitrous oxide (N_2O), through anaerobic respiration. This process is linked to the respiratory electron transport chain, and denitrifying bacteria can use nitrate or nitrite as alternative electron acceptors to support respiratory growth (Zumft 1997). Each step within the denitrification pathway is catalyzed by independent metalloenzymes that are usually induced sequentially under anaerobic conditions (Zumft 1997; Tavares et al. 2006). The *F. columnare* genome encodes four enzymes that are likely to be involved in denitrification: nitrate reductase (encoded by *nasA* gene), nitrite reductase (encoded by *nirS* gene), nitric oxide reductase (encoded by *norB* gene), and nitrous oxide reductase (encoded by *nosZ* gene) (Tekedar et al. 2012). We have previously shown that *F. columnare* strain 94–081 can grow anaerobically with nitrate supplementation (Tekedar et al. 2017).

Bacterial species capable of complete denitrification such as *Paracoccus denitrificans* and the denitrifying pseudomonads contain up to 40 genes involved in synthesis of the

denitrification apparatus (De Boer et al. 1996). On the other hand, other bacterial species that are capable of catalyzing part of the denitrification pathway such as *N. meningitidis* possess only a few denitrifying genes (*aniA, norB, fnr, narP, narQ,* and *azu*) (Anjum et al. 2002; Barth et al. 2009). The *nasA, nirS, norB,* and *nosZ* genes of *F. columnare* have significant identity with denitrification genes from other bacteria such as *P. aeruginosa* and *N. meningitidis.*

We reasoned that *nasA, nirS, norB,* and *nosZ* of *F. columnare* might be involved in anaerobic growth and nitrate reduction to nitrogen gas. To test this hypothesis, four mutants were constructed using plasmids derived from pCP29, which is an *E. coli-F. johnsoniae* shuttle vector (Kempf and McBride 2000). This vector combines the pCU19-based suicide vector pLYL03 with a cryptic plasmid (pCP1) isolated from *F. psychrophilum* strain D12. It carries genes encoding cefoxitin resistance and erythromycin resistance that function in *F. columnare* and other *Bacteroidetes* (Kempf and McBride 2000; Alvarez et al. 2004). pCP29 also carries a gene encoding β-lactamase that confers resistance to ampicillin in *E. coli*. To avoid the problem of plasmid replication in *F. columnare*, pCP1 was removed from pCP29, and the resulting plasmids were transferred by conjugation from *E. coli* to successfully construct deletion mutants in *F. columnare* strain 94–081.

As expected, there was significant anaerobic growth deficiency in the *Fc*Δ*nasA, Fc*Δ*nirS,* and *Fc*Δ*nosZ* mutants with nitrate supplementation, indicating that nitrate reductase, nitrite reductase, and nitrous oxide reductase are needed for optimal anaerobic respiration. However, no significant decrease in anaerobic growth was observed between *Fc*Δ*norB* and *Fc*WT, indicating that nitric oxide reductase is not essential for anaerobic respiration in *F. columnare*. By contrast, in *N. gonorrhoeae*, *norB* gene was required for anaerobic growth, but the absence of *norB* did not dramatically decrease anaerobic survival (Householder et al. 2000). Similarly, mutagenesis of *Paracoccus denitrificans norC, norB, norQ,* and *norD* resulted in bacteria being unable to grow anaerobically (De Boer et al. 1996). However, *Alcaligenes eutrophus* mutants bearing single-site deletions in *norB* or *norZ* were shown to have no anaerobic growth defects using nitrate or nitrite. However, a mutant with double deletion of both *norB* and *norZ* failed to grow anaerobically on nitrate. Anaerobic growth was restored in the double mutant by introducing either *norB* or *norZ* on a broad-host-range plasmid (Cramm et al. 1997). Thus, it is possible that *F. columnare* encodes a second nitric oxide reductase that has not been identified. Indeed, the *F. columnare* genome has three genes currently annotated as nitric oxide reductases: *norB* (AWN65_04995) and two other genes (AWN65_05000 and AWN65_04960).

To further investigate if the growth defects observed in *Fc*Δ*nasA, Fc*Δ*nirS,* and *Fc*Δ*nosZ* were a result of an inability to reduce nitrate, the abilities of four mutants to consume extracellular nitrate were analyzed and compared with *Fc*WT. Consistent with the reduced growth under anaerobic conditions, *Fc*Δ*nasA, Fc*Δ*nirS,* and *Fc*Δ*nosZ* failed to reduce nitrate to nitrite even after incubation for 14 days, as indicated by significantly more residual nitrate in the medium than was found for the wild-type. By contrast, significant residual nitrate in medium was not observed in the *Fc*ΔnorB mutant compared to *Fc*WT, which correlates with the ability of this mutant to grow anaerobically in the presence of nitrate. Mutants of *norB,*

norC, and *nirS* in *P. aeruginosa* are defective in nitrate and nitrite reduction activity (Borrero-de Acuna et al. 2016).

Taken together, results from the anaerobic growth experiment and nitrate consumption experiment indicate that *F. columnare* anaerobic growth is linked to its ability to reduce nitrate, suggesting that *F. columnare* can use nitrate as an alternative electron acceptor for the electron transport system during anaerobic growth. The inability of the nitrate reductase mutant to reduce nitrate was expected. Results showed that nitrite reductase and nitrous oxide reductase activities are also essential for this process, indicating that capability of the complete denitrification process to molecular nitrogen is necessary to allow use of nitrate in *F. columnare*. The conflicting nitric oxide reductase results are explainable by the possible presence of three genes encoding nitric oxide reductases in the *F. columnare* genome. In many bacterial species, reduction of either nitrate or nitrite is essential for anaerobic growth (Berks et al. 1995). Interestingly, denitrification also promotes the growth of *N. meningitidis*, a strictly aerobic human pathogen, under oxygen-limited conditions (Rock et al. 2005).

Biofilm formation is considered an essential feature in the pathogenicity of *F. columnare* (Staroscik and Nelson 2008). In the environment and during infection*, F. columnare* demonstrates capacity to form thick, multilayered biofilms under static and flow conditions (Cai et al. 2013). In *P. aeruginosa*, anaerobic respiration via denitrification is important for its formation of biofilms during anaerobic growth (Hassett et al. 2002; Worlitzsch et al. 2002). Because respiratory mucus of cystic fibrosis patients is an anaerobic environment rich in nitrate and nitrite, induction of biofilm in this environment is beneficial to the pathogen. Gene expression studies have revealed that both *nirS* and *norCB* are highly expressed in biofilms compared with planktonic *P. aeruginosa* (Wagner et al. 2003; Sauer et al. 2004). A *P. aeruginosa norBC* insertion mutant forms virtually no biofilm under anaerobic growth conditions, while a *nir* mutant forms biofilms similar to those of the wild-type (Yoon et al. 2002). A *N. gonorrhoeae norB* mutant had a more pronounced biofilm-deficient phenotype than an *aniA* mutant (Falsetta et al. 2009). Therefore, we investigated whether the deletion of *F. columnare nasA, nirS, norB,* and *nosZ* genes would reduce biofilm formation. Our result indicates that *nasA*, *norB*, and *nosZ* are necessary for biofilm formation under anaerobic growth. The role of *norB* in biofilm formation is noteworthy because this gene is not essential for anaerobic growth. Despite the potential existence of similar nitric oxide reductases encoded in the *F. columnare* genome with overlapping functions in anaerobic respiration, it appears that *norB* plays a unique role in biofilm formation. Taken together, *F. columnare* is similar to other denitrifying bacteria in that anaerobic respiration appears to be linked to biofilm formation, suggesting that biofilm formation may be a beneficial phenotype for *F. columnare* in anaerobic environments.

A significant contribution of anaerobic nitrate respiration to virulence has been reported in *M. bovis* in immunodeficient mice (Weber et al. 2000). A *P. aeruginosa* membrane nitrate reductase mutant was avirulent in the surrogate model host *Caenorhabditis elegans*, whereas nitrate sensor-response regulator mutants were fully virulent (Van Alst et al. 2007). In *Brucella suis*, transposon inactivation of the gene encoding nitrate reductase affected growth inside the macrophage (Kohler et al. 2002). A *B. melitensis norB* mutant (nitric oxide

reductase deficient) is attenuated in activated macrophages but only slightly attenuated in mice at 4 weeks postinfection (Haine et al. 2006). However, in *F. columnare*, we found that nitrate reductase, nitric oxide reductase, and nitrous oxide reductase activities are not linked with virulence.

However, deletion of the *nirS* gene (nitrite reductase) significantly reduced *F. columnare* virulence. In *Neisseria*, nitrite and nitric oxide reductase are required for anaerobic growth and have a role in mitigating nitric oxide toxicity in macrophages (Anjum et al. 2002). Therefore, it is possible that reduced virulence of the *F. columnare nirS* mutant is due to reduced ability to inactivate reactive nitrogen species (RNS) such as nitric oxide, which is a host defense mechanism. However, this does not seem likely because nitrate reductase contributes equally to detoxification of RNS, and *Fc*Δ*nasA* is not attenuated. An alternative explanation is that *nirS* contributes to regulation of *F. columnare* virulence genes. In *P. aeruginosa*, a *nirS* knockout mutant was deficient in swarming motility, which involves both flagella and pili (de la Fuente-Nunez et al. 2013). Swarming motility using flagella and pili is important for initial cell-cell and cell-surface interactions in *P. aeruginosa* (O'Toole and Kolter 1998; Köhler et al. 2000). Furthermore, *nirS* expression in *P. aeruginosa* is required to induce the type III secretion system (T3SS) components that translocate effector proteins such as extracellular protease and elastase into host cells (Van Alst et al. 2009). Extracellular protease activity and chondroitin AC lyase activity contribute to *F. columnare* virulence (Stringer-Roth et al. 2002; Suomalainen et al. 2006; Li et al. 2017); it is possible that *F. columnare nirS* is involved in regulating expression of these or other yet-unidentified virulence factors during infection. It is interesting to note that *nirS* is also the only gene that is not required for biofilm formation, indicating that the ability to form biofilm anaerobically is not essential for virulence.

In summary, we determined the role of denitrification genes *nasA, nirS, norB,* and *nosZ* in anaerobic growth, nitrate reduction, biofilm formation, and virulence in *F. columnare* strain 94–081. Deletion of *nasA, nirS*, and *nosZ* genes, which encode nitrate reductase, nitrite reductase, and nitrous oxide reductase made *F. columnare* incapable of growing anaerobically and reducing nitrate to nitrite. The *nirS* gene, encoding nitrite reductase, has an additional role in *F. columnare* virulence and may contribute to regulating expression of factors important in virulence. The *Fc*Δ*nirS* mutant could be useful as a safe, live vaccine against columnaris disease if it protects catfish aginst *Fc*WT infection.

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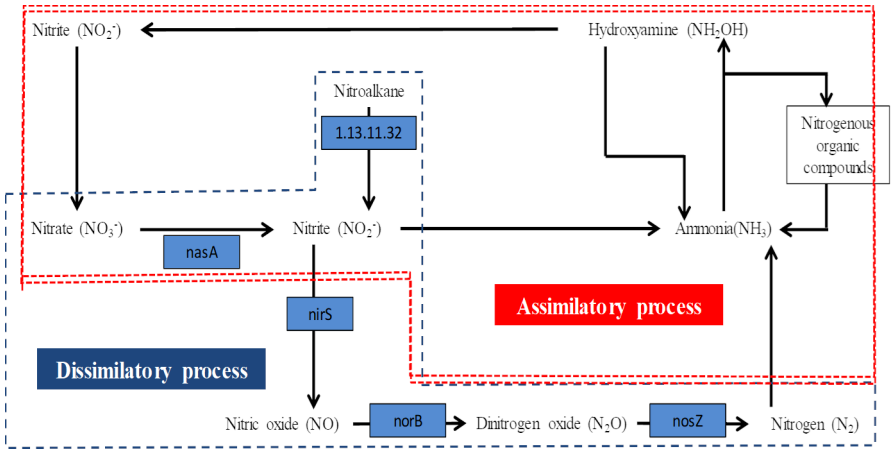

Figure 1.

Scheme for denitrification pathway from nitrate to nitrogen. Genes encoding enzymes that mediate the denitrification steps of *F. columnare* 94–081 include those for nitrate reductase (*nasA*), nitrite reductase (*nirS*), nitric oxide reductase (*norB*), and nitrous oxide reductase (*nosZ*). Blue highlights show enzymes encoded by *F. columnare* 94–081 strain 94–081 genes.

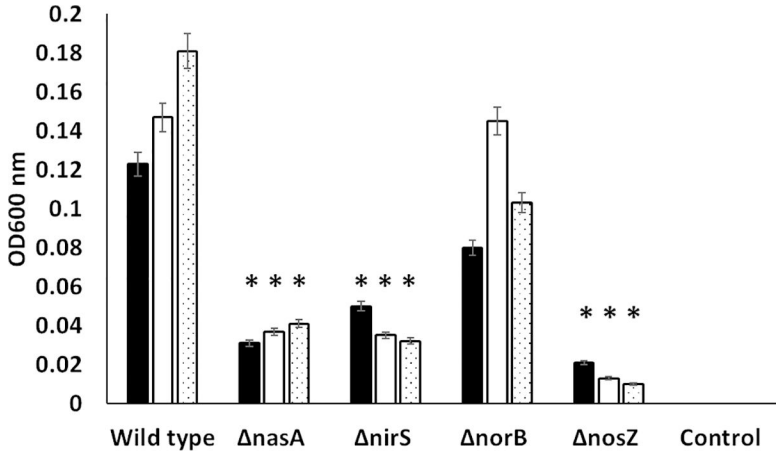

Figure 2.

Growth of *FcΔnasA, FcΔnirS, FcΔnorB,* and *FcΔnosZ* compared to *Fc*WT in FCGM supplemented with 10 mM potassium nitrate (FCGM-N) under anaerobic conditions. Anaerobic conditions were generated in a jar using a standard anaerobic gas pack, and growth was measured on days 1, 7, and 14. Data points represent the mean of three replicates. Significant differences are indicated with asterisks ($P < 0.05$). Control group includes FCGM-N broth without bacterial inoculation.

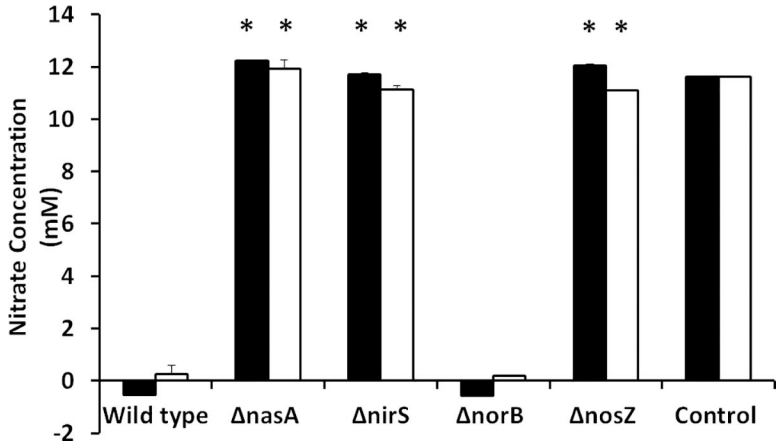

Figure 3.

Concentration of extracellular nitrate in *Fc*WT, *FcΔnasA, FcΔnirS, FcΔnorB,* and *FcΔnosZ* cultures grown anaerobically in FCGM-N medium supplemented with 10 mM nitrate (measured at 7 and 14 days post-inoculation). Data points represent the mean of six replicates. Significant differences are indicated with asterisks ($P < 0.05$). Control group includes FCGM-N broth without bacterial inoculation.

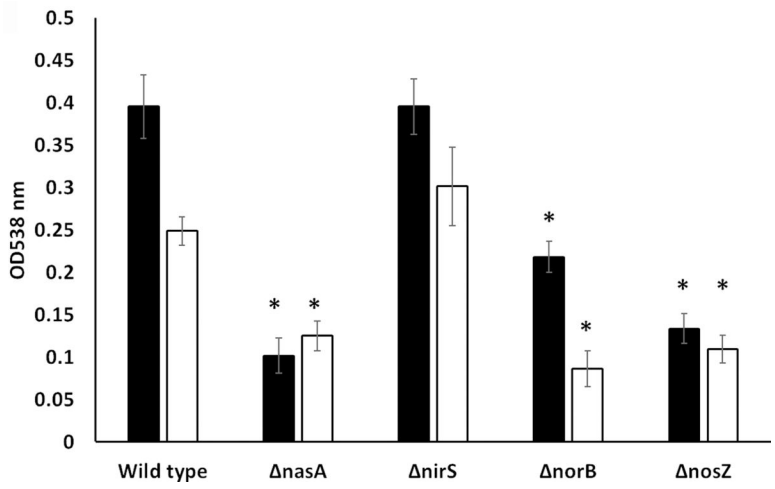

Figure 4.

Quantification of biofilm formation in *Fc*WT, *FcΔnasA, FcΔnirS, FcΔnorB,* and *FcΔnosZ* using crystal violet staining and measured by absorbance at 538 nm after 24 and 96 hours under anerobic conditions. The data represent means of four replicates plus or minus standard error.

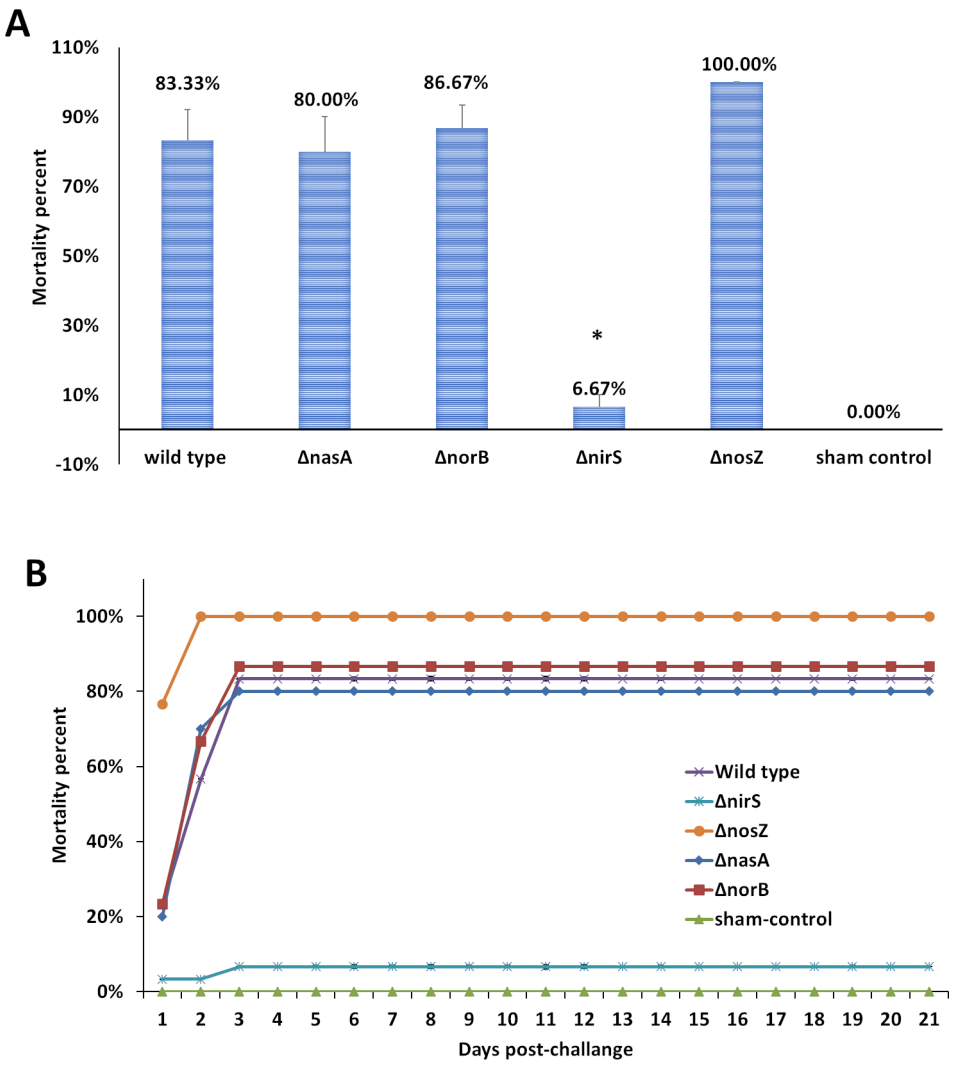

Figure 5.

Percent mortality (A) and cumulative mortalities (B) of catfish fingerlings immersion challenged with *Fc*WT, *FcΔnasA, FcΔnirS, FcΔnorB,* and *FcΔnosZ*. Data points represent the mean of four tanks for each treatment. Significant differences are indicated with asterisks ($P < 0.05$).

Table 1

Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference
Strains		
E. coli strain		
DH5αMCR	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA mcrA* Δ(*mrr-hsdRMS-mcrBC*)	(Grant et al. 1990)
BW19851	*RecA* Tra_{RP4}^{+} Tp^{r} Str^{r} Pir^{+}	(Metcalf et al. 1994)
F. columnare strain		
94–081	Wild-type	(Soto et al. 2008)
FcΔnasA	94–081 derivative, *ΔnasA*	This study
FcΔnirS	94–081 derivative, *ΔnirS*	This study
FcΔnorB	94–081 derivative, *ΔnorB*	This study
FcΔnosZ	94–081 derivative, *nosZ*	This sutdy
Plasmid		
pCP29	*E. coli-F. columnare* shuttle plasmid; Ap^{r} (Cf^{r} Em^{r})	(Kempf and McBride 2000)
pCP29S	Suicide plasmid derived from pCP29; Ap^{r} (Cf^{r} Em^{r})	This study
pCP29S-*nasA*	pCP39S, *ΔnasA*	This study
pCP29S-*nirS*	pCP39S, *ΔnirS*	This study
pCP29S-*norB*	pCP39S, *ΔnorB*	This study
pCP29S-*nosZ*	pCP39S, *ΔnosZ*	This study

[a]Antibiotic resistance phenotypes: Ap^{r}, ampicillin resistant; Cf^{r}, cefoxitin resistant; Em^{r}, erythromycin resistant. The antibiotic resistance phenotypes in parentheses are those expressed in *F. columnare* but not in *E. coli.*

Table 2

Primers used in this study.

Primer ID		Primer Sequence (5' to 3')[a]	RE[b]
nasAEF	A	CA**CCCGGG**AAACGGCACAAGATAAATGG	*Sma*I
nasAIR	B	TCAGCGTGCAAACTAGAACC	
nasAIF	C	GGTTCTAGTTTGCACGCTGATAGATGGGGACCAAAATCTTC	
nasAER	D	CA**GCATGC**GGAATTGTGTATCGCACCTC	*Sph*I
nasASeq		GCATCGGCTGGACATACATTT	
nirSEF	A	AA**CCCGGG**ACTGGACAATGCAGGCTAAA	*Sma*I
nirSIR	B	GGCTAAAGAGGCCGAGAATA	
nirSIF	C	TATTCTCGGCCTCTTTAGCCATGGTTGCAGCTGTTAGAGC	
nirSER	D	AA**CTGCAG**TCCATTGGCTGGTTAGGTTA	*Pst*I
nirSSeq		GGCTAGACGTTTGCCTTGAC	
norBEF	A	CA**CCCGGG**CAAAAAGTTCTGGGTGCTTC	*Sma*I
norBIR	B	AAACCAATAGGCTACTTTTTGTG	
norBIF	C	CACAAAAAGTAGCCTATTGGTTTGGTAAAACGAACGATGAGG	
norBER	D	CA**GCATGC**TTATGATTTGTTCCATACAACGG	*Sph*I
norBSeq		AATACCTCCCATGATTAATTC	
nosZEF	A	AA**CCCGGG**TTTCCATTCATTGGGCTTTT	*Sma*I
nosZIR	B	GTATCTCCCGCCAATAGTAAAG	
nosZIF	C	CTTTACTATTGGCGGGAGATACATCACCACTTACAGCATCGC	
nosZER	D	AA**CTGCAG**CAAGCATTAAATGAAGACCCTG	*Pst*I
nosZSeq		AGGGTACTGAGGGGCATCTA	

[a]Bold letters at the 5' end represent restriction enzyme (RE) recognition sequences. Bases preceding the RE recognition sequences were added to increase the RE efficiency. Underlined bases in primer C is the reverse complement of primer B.

[b]RE stands for restriction enzyme recognition sequence sites incorporated into the 5' end of the primer sequence.

Table 3

The sizes of upstream (AB), downstream (CD), and deletion fragments.

Gene	Locus tag	Protein name	UF (AB) (bp)[a]	DF (CD) (bp)	DR (bp / aa)	5' UD (bp / aa)	3' UD (bp / aa)
nasA	AWN65_05950	Nitrate reductase	1101	969	2085 / 695	72 / 24	57 / 19
nirS	AWN65_04985	nitrite reductase, copper-containing	951	1029	1374 / 458	36 / 12	27 / 9
norB	AWN65_05000	Nitric oxide reductase	1035	975	1263/421	36/12	30/10
nosZ	AWN65_09420	Nitrous oxide reductase	1062	1092	1806 / 602	66/ 22	99/ 33

[a]UF = Upstream fragment; DF = Downstream fragment; DR = Deleted region; UD = Undeleted region; bp = base pair; aa = amino acid.