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The *Pasteurella multocida nrfE* Gene Is Upregulated during Infection and Is Essential for Nitrite Reduction but Not for Virulence

David J. Boucher, Ben Adler,* and John D. Boyce

Australian Research Council Centre for Structural and Functional Microbial Genomics, Department of Microbiology, Monash University, Melbourne, Australia

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Pasteurella multocida **is the causative agent of a range of diseases with economic importance in production animals. Many systems have been employed to identify virulence factors of *P. multocida*, including in vivo expression technology (IVET), signature-tagged mutagenesis, and whole-genome expression profiling. In a previous study in which IVET was used with *P. multocida*, *nrfE* was identified as a gene that is preferentially expressed in vivo. In *Escherichia coli*, *nrfE* is part of the formate-dependent nitrite reductase system involved in utilizing available nitrite as an electron accepter during growth under anaerobic conditions. In this study, we constructed an isogenic *P. multocida* strain that was unable to reduce nitrite under either aerobic or anaerobic conditions, thereby demonstrating that *P. multocida nrfE* is essential for nitrite reduction. However, the *nrfE* mutant was still virulent in mice. Real-time reverse transcription-PCR analysis indicated that *nrfE* was regulated independently of *nrfABCD* by an independent promoter that is likely to be upregulated in vivo.**

Pasteurella multocida is a gram-negative bacterial pathogen that is responsible for a number of diseases that are prevalent worldwide, including bovine hemorrhagic septicemia, avian fowl cholera, porcine atrophic rhinitis, and lapine snuffles. The worldwide economic cost of these diseases in production animals is significant, but despite considerable research, safe and effective vaccines against pasteurellosis are still lacking. The molecular mechanisms of *P. multocida* pathogenesis are still largely unknown, and only a few virulence factors have been identified. These factors include toxins (8), capsule (5, 9), iron acquisition proteins (4, 13, 25), and hemagglutinins (25). Therefore, it is likely that many virulence factors remain uncharacterized. Identification of novel virulence factors could be used to identify new candidate vaccine antigens or targets for antimicrobial compounds.

Numerous methods have been utilized to identify genes expressed during *Pasteurella* infections, including in vivo expression technology (IVET) (21), signature-tagged mutagenesis (13, 16), and whole-genome expression profiling (6). In the *P. multocida* IVET study a number of genes that are upregulated in vivo in mice were identified (21). One of these genes, *nrfE*, was selected for further characterization.

The formate-dependent nitrite reductase (Nrf) system is present in a number of enteric bacteria, including *Escherichia coli* and *Salmonella* spp., and in the species closely related to *P. multocida*, *Haemophilus influenzae* and *Actinobacillus actinomycetemcomitans* (28). The Nrf system in *E. coli* is encoded by a seven-gene operon (*nrfABCDEFG*) and uses nitrite as an alternate electron acceptor for oxygen during anaerobic growth. *nrfA* encodes a 50-kDa cytochrome that utilizes nitrite as an electron acceptor, while *nrfBCD* encode proteins that are essential for electron transfer to the catalytic subunit, NrfA (22). *nrfEFG* have been proposed to encode proteins that form a heme lyase required for attachment of a heme group to the site of catalysis of NrfA (11, 14, 22). *nrfE* has been shown to be essential for formate-dependent nitrite reduction in *E. coli* (11) and has been identified in *P. multocida* as a gene that is upregulated in vivo during infection (21). For this reason it was of interest to determine what role *nrfE* plays in the metabolism of *P. multocida* during growth in vitro and during infection of the host.

In this study, the function of the *nrfE* gene of *P. multocida* was characterized by using a strain in which *nrfE* was inactivated; additionally, an analysis of transcriptional regulation of the *P. multocida nrf* operon was conducted.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are shown in Table 1. *P. multocida* and *E. coli* were grown with aeration at 37°C in brain heart infusion broth (BHI) and 2YT (Oxoid, Hampshire, England), respectively. Kanamycin (50 μg/ml) and tetracycline (2.5 μg/ml for *P. multocida* and 10 μg/ml for *E. coli*) were added to solid and liquid media when they were required.

Modified CDM for nitrite reduction studies. A modified version of the chemically defined medium (CDM) described by Jablonski et al. (24) was used for nitrite reduction studies. Unless specifically indicated otherwise, the concentrations of solutions were the concentrations described by Jablonski et al. (24). Briefly, the CDM was prepared as a 5× stock devoid of L-arginine, L-serine, L-glutamic acid, L-phenylalanine, L-leucine, L-isoleucine, L-aspartic acid, L-tyrosine, $MgSO_4$, and glucose but supplemented with 50 g of Casamino Acids (Sigma) per liter. The 5× stock was filter sterilized and diluted to a 1× working concentration by using one of three different filter-sterilized 2.5× base solutions. The first 2.5× base solution (normal CDM) contained L-aspartic acid, L-tyrosine, and $MgSO_4$, while the second base solution was identical except that it was supplemented to give 1× CDM working concentrations of 0.4% glycerol and 40 mM sodium fumarate (glycerol/fumarate CDM). The third solution was the same as the solution used for glycerol/fumarate CDM except that it was supplemented to give final concentrations of 2 mM sodium nitrite and 1 mM sodium nitrate (nitrite/nitrate CDM).

Recombinant DNA techniques. Genomic DNA was purified by using the cetyltrimethylammonium bromide method (1). Plasmid DNA was purified either by the alkaline lysis method (2) or by using anion-exchange columns (QIAGEN, Hilden, Germany). PCR amplification of DNA was carried out with *Taq* polymerase by using the reaction conditions specified by the manufacturer (Roche

* Corresponding author. Mailing address: Australian Research Council Centre for Structural and Functional Microbial Genomics, Department of Microbiology, Monash University, Victoria 3800, Australia. Phone: 61 3 9905 4815. Fax: 61 3 9905 4811. E-mail: Ben.Adler@med.monash.edu.au.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli DH5α	F^- *endA1 hsdR17*(r_k^- m_k^+) *thi-1* λ^- *recA1 gyrA96 relA1* Φ80d*lacZ*ΔM15	Bethesda Research Laboratories, Rockville, Md.
P. multocida strains		
X-73	Serotype A:1 wild-type strain	18
AL362	X-73 *nrfE*::*tet*(M) mutant	This study
AL464	*E. coli* DH5α harboring pAL263	This study
AL465	AL362 harboring pAL99	This study
AL466	AL362 harboring pAL263	This study
Plasmids		
pAL99	40-bp EcoRI fragment containing *P. multocida tpiA* promoter region cloned into pPBA1100 EcoRI site	17
pAL209	2.1-kb insert containing *nrfE* cloned into pWSK129, Kan^r	This study
pAL211	pWSK129 containing the *nrfE*::*tet*(M) cassette, Kan^r Tet^r	This study
pAL263	2-kb insert containing full *nrfE* gene in pAL99 (*nrfE* expression driven by *tpiA* promoter)	This study
pBA1100	*P. multocida-E. coli* shuttle vector, pUC18 derivative	19
pVB101	*tet*(M) gene from Tn*916* cloned into pBR322, Tet^r Amp^r	Vickers Burdett, Duke University, Durham, N.C.
pWSK129	Low-copy-number *E. coli* vector, Kan^r	29

Molecular Biochemicals, Basel, Switzerland). DNA was introduced into *E. coli* by the chemical transformation method of Hanahan (15) and into *P. multocida* by the electroporation method of Jablonski et al. (23). DNA sequencing was carried out by using BigDye Ready Reaction DyeDeoxy terminator cycle sequencing kits (Perkin-Elmer, Foster City, Calif.), and the reaction mixtures were analyzed with a 373A DNA sequencing system. Oligonucleotides used in this study are shown in Table 2. Prior to sequencing or cloning, PCR fragments were purified either by polyethylene glycol precipitation or by passage through a QIAGEN PCR purification kit. DNA *dam* methylation was conducted as specified by the manufacturer (Roche).

Construction of an *nrfE*::*tet*(M) mutant by allelic exchange. A mutagenesis construct containing the *P. multocida nrfE* gene disrupted by *tet*(M) was made (Fig. 1). Oligonucleotides 1914 and 1915 (Table 2) were used to amplify a 2.1-kb DNA fragment by PCR from *P. multocida* X-73 genomic DNA, and this fragment was then ligated into pWSK129 to generate pAL209 (Table 1). A 3.2-kb BamHI-digested fragment of pVB101 (Table 1) containing *tet*(M) was cloned into the unique BamHI site in pAL209 to form pAL211. The pAL211 sequence was verified by DNA sequencing, and the plasmid was *dam* methylated prior to electroporation into *P. multocida* X-73 (*dam* methylase; New England Biolabs). Allelic recombinants were selected on BHI agar with tetracycline (2.5 μg/ml). One putative mutant was identified, and the *nrfE*::*tet*(M) genotype was confirmed by PCR (Fig. 1C) and Southern hybridization by using an internal *nrfE* probe generated by PCR performed with the 1914 and 1915 oligonucleotides (data not shown). The *nrfE*::*tet*(M) mutant was designated AL362.

RNA isolation. Bacteria were harvested from four replicate cultures at an A_{600} of 0.5 (4×10^9 CFU/ml), added to 0.1 volume of ice-cold killing buffer (0.05 M Tris-HCl [pH 7.5], 15 mg of sodium azide per ml, 0.6 mg of chloramphenicol per ml), and pelleted by centrifugation. RNA was isolated from the bacteria by using Trizol (Gibco/BRL) as described by the manufacturer. Purified RNA was treated with DNase (20 U for 20 min at 37°C), and the RNA was further purified by using RNeasy columns (QIAGEN).

RT and real-time RT-PCR. Primers for real-time reverse transcription (RT)-PCR were designed with the Primer Express software (ABI) (Table 2). Reverse transcription reactions were performed at 42°C for 2 h, and the reaction mixtures contained 10 μg of total RNA, 15 μg of random hexamers, 5 U of Superscript II reverse transcriptase (Gibco/BRL), and each deoxynucleoside triphosphate at a concentration of 200 μM. Synthesized cDNA samples were diluted 80-fold prior to real-time RT-PCR, which was carried out by using an ABI PRISM model 7700 sequence detector with product accumulation quantified by incorporation of the fluorescent dye SYBR Green. Triplicate real-time RT-PCRs were performed by using 2.4 μl of cDNA with the SYBR Green PCR mixture (ABI) and each gene-specific primer at a concentration of 50 nM in a 20-μl (final volume) reaction mixture. Gene-specific standard curves were constructed from known concentrations of *P. multocida* X-73 genomic DNA and were used to determine relative template concentrations in each reaction mixture. *gyrB* was used as a normalizer for all reactions performed with primers 2106 and 2107 (Table 2). All RT-PCRs amplified a single product, as determined by melting curve analysis.

Nitrite reduction assay. The nitrite reduction assay was based on the method used for *E. coli* by Hussain et al. (22). For aerobic studies, 1 ml of normal CDM was inoculated with *P. multocida* and grown at 37°C overnight. Two hundred microliters of this preparation was used to inoculate 1 ml of nitrate/nitrite CDM in triplicate cultures, which were then incubated at 37°C overnight. The overnight cultures were diluted 1/10 in fresh glycerol/fumarate CDM and incubated for 24 h or until an optical density at 600 nm of >0.5 was reached. Aliquots (50 μl) of each test culture were mixed with 0.5 ml of 1% (wt/vol) sulfanilamide in 1 M HCl and 0.5 ml of 0.02% *N*-1-naphthylethylenediamine dihydrochloride. Formation of an intense pink color after 30 s indicated the presence of nitrite, and the absorbance of each sample was measured at 530 nm.

Disk diffusion SNAP susceptibility assay. An agar suspension of each *P. multocida* test strain was made by adding 1 ml of a suspension containing 10^8 CFU of bacteria per ml to 3 ml of nitrite/nitrate CDM containing 3% (wt/vol) Noble agar (Oxoid) and pouring the preparation onto plates. Whatman paper disks (diameter, 4 mm) were then soaked in 15 μl of a 250 mM solution of *S*-nitroso-*N*-acetyl-penicillamine (SNAP) (dissolved in methanol) and placed in the center of each plate. Disks soaked in methanol were used as controls, and the plates were incubated overnight under either aerobic or anaerobic growth conditions.

Mouse virulence assay. Individual strains of *P. multocida* X-73 were grown overnight in BHI, diluted 1:100 in 5 ml of fresh BHI, and incubated with shaking at 37°C for 4 to 6 h. The absorbance at 600 nm of each of the cultures was

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′-3′)[a]	Position[b]
683	CAATTTCAATCAATGCTCCCAC	NA
1914	GCACATGTCGACTGGATTGGGGTAG	23284-23308
1915	GGTAAATCTAGACATAAGCTTGGTTTT	25218-25190
2106	GCCCTTTCCGATAAATTGCAA	1670646-1670626
2107	ATCGCGGCTAATGGTGCTT	1670549-1670567
2123	AATGGCATGTTGGTCTTGTAAAAG-3′	20017-20040
2124	CCACCACTTGCCCATGTTG	20112-20094
2277	GCCGGTAGTGTTGATGGA	23172-23189
2278	AGCAAGGCGCCTAGTGCC	25411-25394
2283	TTGATAGAGCTCTTCAAATTT	23510-23530
2284	CATACTCATGAGCTCACCTGT	25494-25474
2363	CCATCGCCGTTTATTTGTTCTT	22553-22574
2364	TGCCGTACAACGTATCACATGA	22677-22656
2398	GATTTTTGGCTTTACTGATCGCTT	23560-23583
2399	CGCAGTACCAATCATATACGGC	23660-23639
2403	GGACAAATGACGCTAGCATAAATG	23449-23472
2404	ACATCGCAAAGGCTTTATGCTAC	23367-23389
2405	AGCGTCATTTGTCCTGCATAGA	23462-23441
2407	AAGCCAAAAAACCAAGTTCTGG	23571-23550
2442	CACTATGATCCCAGAACTTGGTTTT	23540-23564

[a] Underlined bases indicate engineered restriction sites.
[b] Position in the Pm70 genome (accession number NC_002663). NA, not applicable.

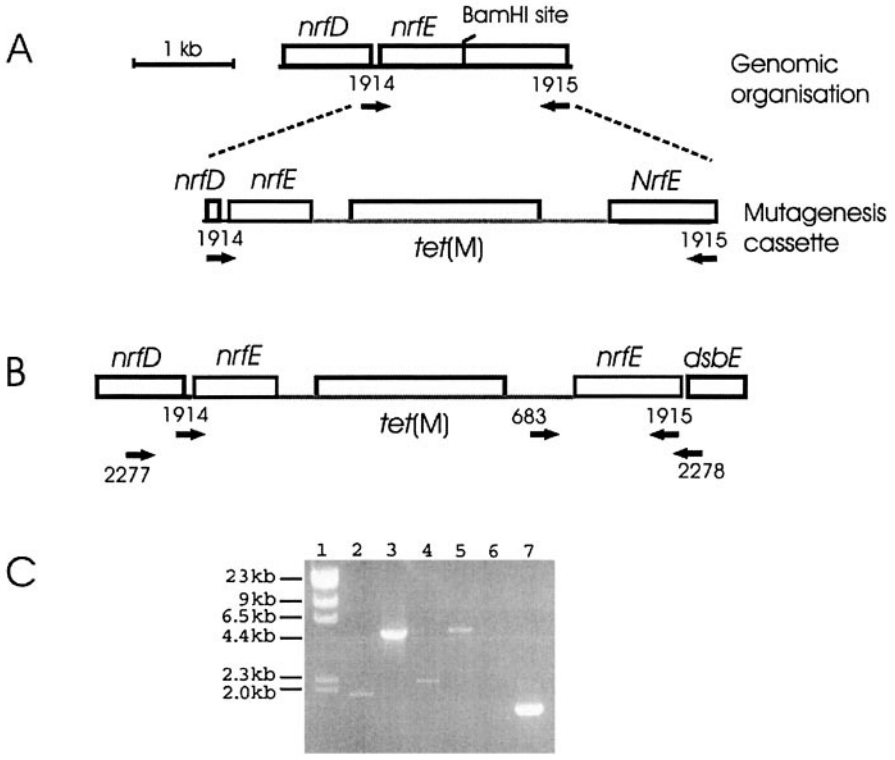

FIG. 1. Schematic diagram of the *P. multocida nrfE* mutagenesis construct and confirmation of the *nrfE* mutant by PCR. (A) A single 1.8-kb fragment containing *nrfE* was amplified by PCR by using primers 1914 and 1915 (indicated by the arrows labeled 1914 and 1915). This fragment was digested with BamHI to obtain two 900-bp fragments that were then ligated to either end of *tet*(M) to produce the mutagenesis cassette used for allelic exchange. (B) Schematic diagram of the genome organization around *nrfE* after insertion of the *tet*(M) cassette. The labeled arrows indicate primers used for PCR. (C) The genotype of AL362 was investigated by PCR. Genomic DNA from AL362 (lanes 3, 5, and 7) was compared to genomic DNA from wild-type strain X-73 (lanes 2, 4, and 6). Lanes 2 and 3, amplification with 1914 and 1915; lanes 4 and 5, amplification with 2277 and 2278; lanes 6 and 7, amplification with *tet*(M) primer 683 together with genomic primer 2278. Lane 1 contained λ DNA digested with HindIII.

determined, after which the cultures were diluted in sterile phosphate-buffered saline (pH 7.2) to obtain the required number of CFU. Groups of five 6-week-old female BALB/c mice were inoculated intraperitoneally with 100 μl of cells from appropriately diluted cell suspensions. The mice were monitored closely for the onset of symptoms and euthanized when they were moribund. Cell suspensions were plated onto BHI agar and counted after growth for 16 h at 37°C to determine the number of CFU.

Competition growth assays in mice. Competition growth assays were conducted as described previously (16). Briefly, 10^6 CFU of wild-type and mutant strains was injected into mice, and blood was recovered after 6 h. For the in vitro assay, a 100-fold dilution of the mixed bacterial preparation was grown for 6 h at 37°C, diluted appropriately, and plated onto nutrient agar. Bacteria were plated onto BHI agar and BHI agar containing tetracycline, and the relative competitive index was determined by dividing the percentage of tetracycline-resistant colonies (AL362) obtained in vivo by the percentage of tetracycline-resistant colonies obtained in vitro. Significance was determined by calculating a *P* value from an approximate *z* test for the difference between two proportions.

Sequence analysis and statistical programs. Sequences were aligned by using the lalign algorithm (20). Statistical analyses were conducted by using the InStat program (Graphpad Software Inc.)

RESULTS

Genetic organization of the *nrf* locus of *P. multocida*. The organization of the *nrf* locus in *P. multocida* strain Pm70 (25) was compared to the organization of the corresponding loci in *E. coli* K-12 (3) and *H. influenzae* Rd (12) (Fig. 2). The *P. multocida* Pm70 *nrf* locus comprised eight open reading frames, *nrfABCDE*, *dsbE_2*, *nrfF_1*, and *nrfF_2*. The *E. coli nrf* locus organization was very similar to the *P. multocida nrf* locus organization, except that there was no *dsb* homolog. Although *P. multocida* had two genes annotated as *nrfF* (*nrfF_1* and *nrfF_2*), *nrfF_2* exhibited higher identity to the *E. coli nrfG* gene than to the *nrfF* gene, indicating that *nrfF_2* is most likely an ortholog of *nrfG*. However, the organization of the *nrf* genes in *H. influenzae* was quite different; *nrfABCD* was organized as one locus, and *nrfEF* and *dsbE* were organized as a second locus 134 kb downstream from *nrfF*. *H. influenzae* contained a single *dsbE* homologue and a single *nrfF* gene but no *nrfG*. However, the *nrfF* gene of *H. influenzae* showed partial homology to *nrfF_1* (first 150 bp) and partial homology to *nrfF_2* (bp 150 to 270), indicating that the NrfF protein in *H. influenzae* may have two domains that perform the function of *nrfF* and *nrfG*. The *P. multocida nrfABCDEF* genes showed the highest levels of identity to the *nrf* genes of *H. influenzae*. This was not unexpected as *H. influenzae* and *P. multocida* are closely related and the presence and similar positions of the *dsbE* gene indicate that they may have originally had a similar *nrf* locus arrangement.

Construction and complementation of the *nrfE* mutant strain AL362. A *P. multocida nrfE*::*tet*(M) mutant designated AL362 was constructed by allelic exchange by using a *tet*(M)-disrupted copy of *nrfE* (Fig. 1) (see Materials and Methods). A plasmid containing an uninterrupted copy of *nrfE* was con-

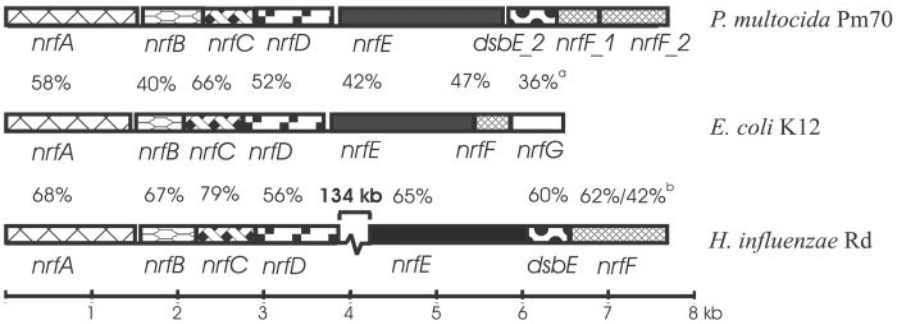

FIG. 2. Genomic organization of the formate-dependent nitrite reduction (*nrf*) locus in selected gram-negative bacteria. Genomic organizations shown are for the following strains: *P. multocida* Pm70 (GenBank accession number NC_002663), *H. influenzae* Rd (GenBank accession number NC_000907), and *E. coli* K-12 (GenBank accession number NC_000913). Open reading frames are indicated by labeled boxes. Cross-hatched boxes indicate predicted orthologs, and the percentage above each box indicates the level of protein sequence identity to the *P. multocida* Pm70 protein. The superscript *a* indicates homology to *nrfF_2*, and the superscript *b* indicates homology to *nrfF_1* and *nrfF_2*.

structed to complement AL362 by cloning *nrfE* into the vector pAL99, which contained the *P. multocida tpiA* promoter upstream of the cloning site. The complete *nrfE* coding fragment was amplified by PCR from *P. multocida* X-73 genomic DNA by using primers 2283 and 2284 (Table 2), digested with SacI, and cloned into the SacI site of pAL99 to generate pAL263 (Table 1). The *nrfE* insert was verified by DNA sequencing to be identical to wild-type *nrfE* and was in the same orientation as the *tpiA* promoter in pAL99. Plasmid pAL263 was transformed into strain AL362, yielding the complemented strain AL466. pAL99 was also transformed into AL362 as a vector control to construct strain AL465.

Nitrite reduction studies of the *P. multocida nrfE* gene. The ability of the *P. multocida nrfE* mutant to reduce nitrite was assessed by using a modified nitrite reduction assay (22). When grown both aerobically and anaerobically, wild-type strain X-73 was found to reduce nitrite (Nrf^+ phenotype), whereas the mutant strain AL362 was unable to do so (Nrf^- phenotype) (Fig. 3). The *nrfE*-complemented strain AL466 was able to reduce nitrite at wild-type levels, indicating restoration of the Nrf^+ phenotype (Fig. 3). Thus, the *P. multocida nrfE* gene is essential for nitrite reduction under both aerobic and anaerobic conditions. A higher level of nitrite was present in the AL362 strain cultures (and the anaerobic AL465 culture) than in the original CDM medium, indicating that during growth additional nitrite was produced. This may have been due to the actions of nitrate reductases (e.g., the product of the *nap* operon [periplasmic nitrate reductase]), which have been shown to be present in *P. multocida* strain Pm70 (25).

Nitric oxide reduction tests. *nrfE* mutant strains of *E. coli* have been shown in disk diffusion assays to be sensitive to nitric oxide (NO) released by SNAP, whereas the wild-type strains were resistant (27). Disk diffusion assays were used to compare the sensitivities of strains AL362, AL465, and AL466 and wild-type strain X-73 to SNAP under both aerobic and anaerobic conditions. However, none of the *P. multocida* strains tested showed growth inhibition by SNAP (data not shown), indicating that inactivation of *nrfE* in *P. multocida* did not affect reduction of nitric oxide.

Virulence in mice. To determine the virulence of the *nrfE* mutant AL362, 2×10^2 CFU of *P. multocida* X-73 and 10-fold dilutions of AL362 (range, 2×10^2 to 2×10^5 CFU) were injected intraperitoneally into mice (groups of five mice). There were no survivors in any of the test groups, indicating that *nrfE* is not required for *P. multocida* virulence in mice.

To quantitatively assess the growth rate of the *nrfE* mutant in vivo, competitive growth assays were used to compare the relative levels of survival of AL362 and X-73 in vitro and in vivo in mice. By using this method, mutants were identified as attenuated if the ratio of mutant to wild-type bacteria recovered after in vivo growth was significantly less than the ratio of mutant to wild-type bacteria recovered after in vitro growth.

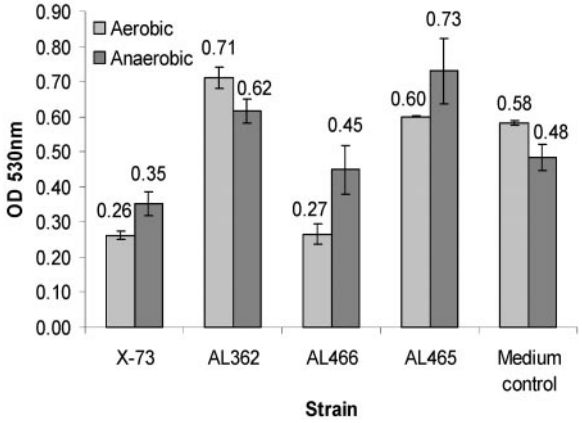

FIG. 3. Nitrite reduction by *P. multocida* strains grown either aerobically or anaerobically. Strains were grown in nitrate/nitrite CDM, and culture supernatants were tested for the presence of nitrite after 18 h (aerobic) or 72 h (anaerobic). The values are the means ± standard deviations for triplicate cultures. When grown both aerobically and anaerobically, wild-type strain X-73 reduced nitrite, whereas mutant strain AL362 was unable to reduce nitrite. Additionally, under both growth conditions, the *nrfE*-complemented strain AL466 was able to reduce nitrite at a level that was not significantly different from the level of nitrite reduction of wild-type strain X-73 ($P > 0.05$) but was significantly different from the levels of nitrite reduction of AL362 and AL465 ($P < 0.05$). The levels of nitrite in the vector control AL465 culture and the uninoculated medium control were significantly different from the levels for all of the other strains ($P < 0.001$) but not from each other in the aerobically grown cultures. In the anaerobic cultures, the medium control contained smaller amounts of nitrite than the amounts observed during the aerobic nitrite assays and was not significantly different from the wild-type X-73, AL362, and AL466 cultures ($P > 0.05$), but it was significantly different from AL465 cultures ($P < 0.01$). OD 530nm, optical density at 530 nm.

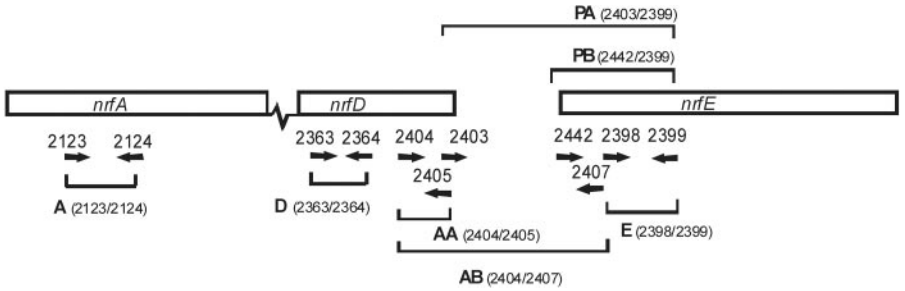

FIG. 4. Schematic diagram of the positions of primers used in real-time RT-PCRs to assess the levels of transcripts at different points between *nrfA* and *nrfE*. The PCR sets were designated A, D, E, AA, AB, PA, and PB. The arrows and the numbers 2123, 2124, 2363, 2364, 2404, 2405, 2403, 2442, 2407, 2398, and 2399 indicate primers 2123, 2124, 2363, 2364, 2404, 2405, 2403, 2442, 2407, 2398, and 2399, respectively.

Mutant AL362 was found not to be significantly attenuated for growth in vivo compared to X-73 in any of the mice tested ($P > 0.05$), which is consistent with the challenge results described above.

Transcriptional analysis of *nrfE*. Transcriptional regulation of the Nrf operon under various growth conditions was investigated by using quantitative real-time RT-PCR. Cultures of X-73 and AL362 were grown either aerobically or anaerobically in the same medium used for the nitrite reduction experiments (nitrate/nitrite CDM). Real-time RT-PCR was carried out by using primers that amplified internal regions within *nrfA* (region A), *nrfD* (region D), and *nrfE* (region E) from X-73 and AL362 cDNA (Fig. 4 and 5). *gyrB* was used as a normalizer for all reactions, as described previously (6), in order to determine relative expression from triplicate data sets. The expression of *nrfA*, *nrfD*, and *nrfE* was significantly higher (at least ninefold higher; $P < 0.001$, as determined by the Tukey-Kramer multiple-comparison test) during anaerobic growth than during aerobic growth in both X-73 and AL362 (Fig. 5). Thus, *nrfA*, *nrfD*, and *nrfE* were expressed at low levels during growth under aerobic conditions but were significantly upregulated during growth under anaerobic conditions. However, the *nrfE* expression in mutant AL362 increased 66-fold when this strain was grown anaerobically compared to when it was grown aerobically, which was significantly different ($P < 0.001$) from the increase in *nrfE* expression observed in X-73 (Fig. 5). Furthermore, during anaerobic growth the expression of *nrfE* was significantly different from the expression of *nrfA* and *nrfD* ($P < 0.001$) in both X-73 and AL362, whereas during aerobic growth the expression of *nrfA*, the expression of *nrfD*, and the expression of *nrfE* were not significantly different ($P > 0.05$) (Fig. 5). In addition, there were no statistically significant differences between the expression of *nrfA* and the expression of *nrfD* when either X-73 or AL362 was grown anaerobically. Taken together, these data indicated that *nrfA*, *nrfD*, and *nrfE* were all upregulated during anaerobic growth in both strains, but *nrfE* was expressed at much higher levels in strain AL362 (with *nrfE* inactivated), probably from an uncharacterized promoter between *nrfD* and *nrfE*. The level of expression of *nrfE* in X-73 grown anaerobically was also significantly lower than the levels of expression of *nrfA* and *nrfD*, suggesting that a transcriptional terminator or transcriptional attenuation sequence was present in this region. In *E. coli*, *nrfA* is under the control of FNR (10). A putative FNR recognition sequence (5′-TTG ATCAAGCGCAA-3′) was identified 128 bp upstream of *nrfA* in the Pm70 genome. However, no potential FNR recognition site was located upstream of *nrfE*, indicating that, unlike *nrfA*,

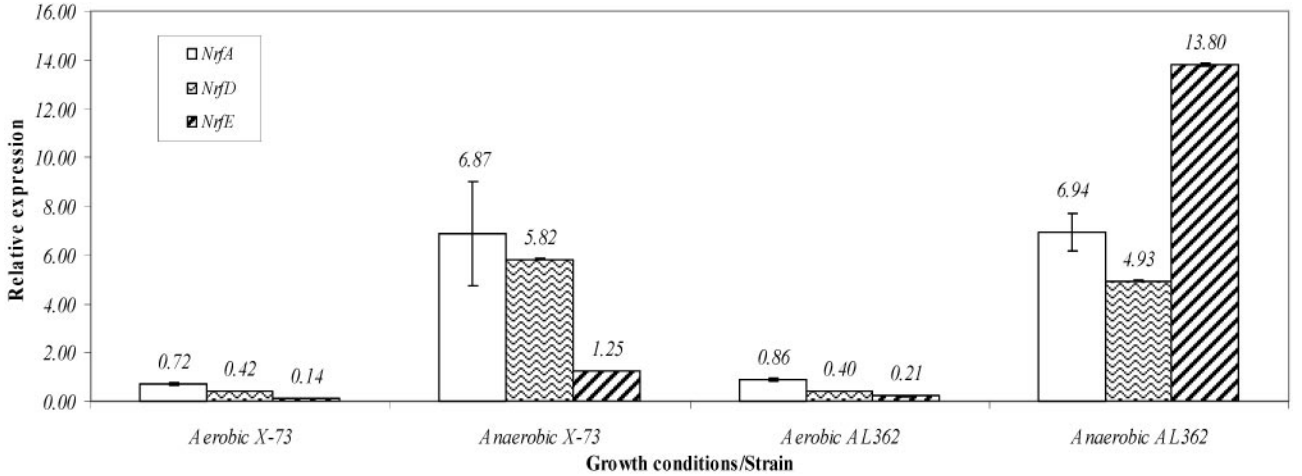

FIG. 5. Relative levels of *nrfA*, *nrfD*, and *nrfE* expression as determined by real-time RT-PCR (normalized with *gyrB*) during anaerobic growth of *P. multocida* X-73 and AL362. Cultures of X-73 and AL362 were grown simultaneously in conditions optimal for nitrite reduction. The values are means ± standard deviations for relative expression determined from a minimum of three reactions.

TABLE 3. Transcriptional regulation of expression of the *nrf* operon of *P. multocida*

P. multocida strain	Relative expression with the following primer sets[a]:						
	A	D	AA	AB	PA	PB	E
X-73	6.9 ± 2.14	5.8 ± 0.04	0.9 ± 0.01	0.3 ± 0.01	0.2 ± 0.01	1.2 ± 0.01	1.4 ± 0.01
AL362	6.9 ± 0.77	4.9 ± 0.03	0.8 ± 0.01	0.3 ± 0.004	0.3 ± 0.003	10.8 ± 0.11	13.3 ± 0.14

[a] The values are means ± standard deviations for relative expression determined from triplicate reactions.

the hypothesized *nrfE* promoter is not likely to be regulated by FNR.

The transcriptional regulation of *nrfE* in both wild-type strain X-73 and *nrfE* mutant AL362 grown anaerobically was investigated further by using real-time RT-PCR and a series of primer sets (Table 3 and Fig. 4 and 6). The relative level of transcripts was significantly less ($P < 0.001$) for primer set A than for primer set D (Table 3) in both X-73 and in AL362, indicating either that there was transcriptional attenuation within *nrfD* or there was instability at the 3′ end of the transcript. The relative level of transcripts dropped even further from primer set AA to primer set AB (Table 3), indicating that there was further transcript attenuation or degradation in the intergenic region between *nrfD* and *nrfE* that was independent of expression of *nrfE.* Importantly, there was a greater increase in the relative level of transcripts between primer sets PA and PB (Table 3) ($P < 0.001$) in AL362 than in X-73. These data indicated that there was a promoter between the binding sites of primers 2403 and 2442 (Fig. 6) which was regulated either directly or indirectly by the level of active NrfE. Thus, *nrfE* was expressed at low levels in X-73 but at much higher levels in AL362, in which no active NrfE was produced. Furthermore, these data indicated that a low level of transcription of *nrfE* probably occurred from the *nrfA* promoter, but most *nrfE* expression was a result of the *nrfE* promoter and this promoter was itself regulated by the expression levels of *nrfE*. Northern blot analysis was used in an attempt to confirm this proposal; however, no transcripts were detected from *nrfA*, *nrfD*, or *nrfE* (data not shown). A positive control (*tpiA*) simultaneously detected a transcript of the predicted size, suggesting that the inability to detect transcripts from the *nrf* locus was most likely due to low abundance and/or instability and/or the large size of the mRNA transcripts.

DISCUSSION

The *P. multocida* formate-dependent nitrite reduction (*nrf*) locus has not been characterized previously, nor has its role in virulence been assessed. The organization of the *nrf* operon in *P. multocida* is somewhat different from the organization of this operon in members of the family *Enterobacteriaceae* (Fig. 2), with the presence of two annotated *nrfF* homologues, no *nrfG*, and an additional gene, *dsbE_2*, although *nrfF_2* is most likely an ortholog of *nrfG.* In *E. coli nrfF* and *nrfG* (together with *nrfE*) have been proposed to be essential for nitrite reduction (11), although their role in *P. multocida* is still uncertain. The closely related bacterium *H. influenzae* Rd displayed an organization similar to that of *P. multocida*, although it had a single *nrfF* homologue and *nrfE*, *nrfF*, and *dsbE* were clustered 134 kb from *nrfABCD*. The predicted *H. influenzae nrfF*

FIG. 6. Locations of real-time PCR primers within *nrfD* and *nrfE* from Pm70 (GenBank accession number NC_002663). Primer sites and directions are indicated by arrows below the sequence. Translational start and stop codons are indicated by boldface type.

gene contains two domains with similarity to *nrfF_1* and *nrfF_2* in *P. multocida* and *nrfF* and *nrfG* in *E. coli*, indicating that its product may perform the function of both proteins. If the *nrf* system in *H. influenzae* is active, it is clear that the *nrfE*, *nrfF*, and *dsbE* genes must be transcribed from their own promoter due to their isolation from the rest of the *nrf* locus. As shown in this study, the *P. multocida nrfE* gene is regulated independently of the *nrfABCD* genes. It is therefore probable that the *P. multocida nrfF_1*, *nrfF_2*, and *dsbE_2* genes are also regulated separately from the *P. multocida nrfABCD* genes. The functions of DsbE_2 in *P. multocida* and DsbE in *H. influenzae* are unknown, although active DsbA, DsbD, and DsbB have been shown to be essential for Nrf activity and maturation of *c*-type cytochromes in *E. coli* (26). As there is a second *dsbE* gene (*dsbE_1*) in the *P. multocida* Pm70 genome, it is possible that DsbE_2 may play a specialized role in maturation of *nrfA* (c_{552}) in *P. multocida*, although at this stage we have no direct evidence for this.

In this work, *nrfE* was shown to be critical for nitrite reduction, which correlates with previous findings for *E. coli* (11). The *nrfE* mutant AL362 was unable to reduce nitrite either aerobically or anaerobically, whereas the wild-type X-73 strain was able to reduce nitrite under both conditions. In *E. coli*, formate-dependent nitrite reduction has been shown to be repressed under aerobic growth conditions and to be upregulated anaerobically (10). Unlike *E. coli*, *P. multocida* lacks any of the aerobic nitrate reductases (such as *nir*), so it is likely that the *nrf* locus has a dual function during both aerobic and anaerobic growth.

The reduction of nitric oxide by *E. coli* was investigated recently by Poock et al. (27), who found that strains deficient in Nrf activity were not able to reduce the nitric oxide released from SNAP. This property was investigated in the strain of *P. multocida* in which *nrfE* was inactivated (AL362), as the reduction of NO in the host might be an important virulence factor. However, AL362 was identical to wild-type strain X-73 in terms of resistance to the NO released by SNAP. Therefore, it is probable that *P. multocida* has alternative pathways for detoxifying NO that are not present in the *E. coli* strains.

The inactivation of *nrfE* in *P. multocida* did not result in attenuation in mice, as determined by either direct challenge experiments or competitive growth assays. These results indicated that although *nrfE* is essential for nitrite reduction in *P. multocida*, it is not essential for virulence despite being upregulated during a mouse infection (21). *P. multocida* has a number of predicted alternative electron acceptor systems (e.g., Nap, Dms, and Frd), and it is likely that when one of these systems is unavailable (such as Nrf), the other systems are utilized and hence *P. multocida* survival in vivo is not adversely affected.

Expression of the *nrf* operon in *E. coli* has been demonstrated to be regulated by FNR in response to anaerobic conditions and to be activated by the NarL or NarP proteins in response to nitrate or nitrite (7). We showed that the *P. multocida nrf* locus is also upregulated under anaerobic conditions, and a putative FNR recognition sequence was identified upstream of the *P. multocida nrfA* gene. However, there was also a low level of expression of the *nrf* locus during aerobic growth. Although nitrite reduction occurs under both aerobic and anaerobic conditions, high levels of nitrite reduction are not required during aerobic growth due to the availability of aerobic electron acceptors, and hence, *nrfE* is expressed only at very low levels. Correspondingly, it is likely that the upregulation of *nrfE* under anaerobic conditions is due to use of nitrite as an alternate electron acceptor by *P. multocida* when oxygen is unavailable.

Expression of the entire *nrf* operon in *E. coli* has been proposed to be driven by a single promoter upstream of *nrfA* (7). In this study, when X-73 was grown anaerobically, *nrfE* was expressed at lower levels than *nrfA* and *nrfD* (Fig. 5). However, when mutant AL362 was grown under anaerobic conditions, *nrfE* was expressed twofold more than *nrfA* and *nrfD*. Despite the differences in *nrfE* expression between X-73 and AL362 when the organisms were grown anaerobically, the levels of transcription of *nrfA* and *nrfD* were not significantly different for the two strains. These results indicate that expression of *nrfE* is not under the control of the *nrfA* promoter (*pnrfA*) and are consistent with the presence of an independent promoter between *nrfD* and *nrfE* and attenuation of the *nrfA* transcript in the same region. This is consistent with our previous work in which we identified *nrfE* as a gene that is downstream of an in vivo active promoter (21). To address this issue, we used real-time RT-PCR to show the presence of a potential *nrfE* promoter (*pnrfE*) within the *nrfD-nrfE* intergenic region. As mentioned previously, *nrfE* has been proposed to form part of a heme lyase that is responsible for attaching a heme group to the active site of NrfA (c_{552}) (14). As we found that *nrfE* was expressed at significantly higher levels in AL362 than in X-73, it is likely that *nrfE* is upregulated because the NrfE protein is nonfunctional. This upregulation is probably due to an as-yet-uncharacterized regulatory feedback loop.

The data presented here indicate that *P. multocida* X-73 has a functional Nrf system that is active both aerobically and anaerobically. This work also demonstrated that in *P. multocida nrfE* is essential for Nrf activity but is not required for virulence in mice. *nrfE* has also been shown to be upregulated independent of the *pnrfA* promoter by an unknown promoter that is active preferentially in vivo.

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