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Transcriptome of a *Nitrosomonas europaea* Mutant with a Disrupted Nitrite Reductase Gene (*nirK*)†

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Global gene expression was compared between the *Nitrosomonas europaea* wild type and a nitrite reductase-deficient mutant using a genomic microarray. Forty-one genes were differentially regulated between the wild type and the *nirK* mutant, including the *nirK* operon, genes for cytochrome *c* oxidase, and seven iron uptake genes. Relationships of differentially regulated genes to the *nirK* mutant phenotype are discussed.

Ammonia oxidizers are a widespread and ecologically important group of bacteria that catalyze the oxidation of ammonia to nitrite (NO_2^-) via hydroxylamine (NH_2OH) in terrestrial, aquatic, and marine environments (5). Ammonia oxidizers also produce nitrous oxide (N_2O) and nitric oxide (NO) either through the oxidation of NH_2OH (13) or the reduction of NO_2^- (9, 18). The gene for a homologue to dissimilatory nitrite reductase, *nirK*, is present at the end of a four-gene cluster in *Nitrosomonas europaea* (7). The four genes in this cluster have been separately disrupted by integration of antibiotic resistance gene cassettes to determine their physiological role (1–3). The loss of NirK activity in *N. europaea* resulted in increased aerobic production of N_2O and increased sensitivity of the bacteria to NO_2^- (1). Strains with mutations in each of the three genes preceding *nirK* were equally as sensitive as the *nirK* mutant to NO_2^- and became even more sensitive when NirK was introduced and expressed from a plasmid (3). In wild-type cells, expression of NirK increased with increasing NO_2^- concentration, but expression was not significantly changed by a decrease in O_2 concentration (2). Lastly, disruption of the NsrR repressor divergently encoded upstream of the *nirK* gene cluster resulted in constitutive expression of NirK (2). Together, these results suggest that *nirK* and the three genes in its cluster are negatively regulated as an operon by NsrR to aerobically detoxify NO_2^- produced by *N. europaea* during ammonia oxidation rather than to anaerobically reduce NO_2^- as is typical for NirK-like enzymes in denitrifying bacteria (6).

The goal of this study was to find genes linked to the *nirK* mutant phenotype, i.e., increased N_2O production and sensitivity to NO_2^-, by examining differences in global gene expression between wild-type and a *nirK*-deficient mutant of *N. europaea*.

***N. europaea* whole-genome microarray.** Construction of whole open reading frame (ORF) microarrays for *N. europaea* was done as described previously for *Shewanella oneidensis* MR-1 (11). Briefly, gene-specific fragments (<75% homology) were generated by PCR using individually designed primer sets (24) (see the supplemental material). All amplified products (8 to 16 reactions per primer set × 100 μl) were pooled together and purified using a robot (Biomek F/X Automated Workstation; Beckman). The range of product sizes was 100 to 1,200 bp, with most between 500 and 600 bp. Of the 2,603 total predicted genes, 2,318 ORFs were correctly amplified, and specific 50-mer oligonucleotide probes (74 ORFs) were synthesized for the remainder, representing 96.8% of the total predicted gene content of *N. europaea*. Diluted PCR products (50 ng μl^{-1}) were spotted onto CMT-GAPS slides (Corning, Corning, NY) using a BioRobotics Microgrid II microarrayer (Genomic Solutions, Ann Arbor, MI). Control spots were as previously described (11).

Differential gene expression in wild-type versus NirK-deficient *N. europaea*. The *nirK* mutant was generated from the wild type by integrating a kanamycin resistance gene cassette into the center of the *nirK* ORF via homologous recombination from an engineered plasmid introduced into *N. europaea* by conjugation (1). Batch cultures of wild-type and *nirK* mutant *Nitrosomonas europaea* (ATCC 19718) were grown in mineral medium to late exponential phase. Total cellular RNA was extracted from cells harvested from multiple cultures for each hybridization experiment (AquaPure RNA Isolation; Bio-Rad), treated with RNase-free DNase I (Ambion), and purified (RNeasy mini kit; QIAGEN). Fluorescently labeled cDNA libraries from total RNA were generated by incorporation of Cy3- or Cy5-dUTP by reverse transcriptase (SuperScript III; Invitrogen). Labeled cDNA was treated with NaOH (1 N, 65°C, 30 min), neutralized with Tris-Cl (1 M, pH 7.6), column purified (QIAGEN), and concentrated by vacuum centrifugation. Two sets of triplicate reactions were performed in which the fluorescent dyes were reversed during cDNA synthesis to minimize dye-specific effects that could influence analysis of hybridization signals to the microarrays. Labeled cDNA libraries (0.6 to 0.9 μg with 20 to 30% label incorporation frequency) were hybridized to *N. europaea* genomic microarrays in buffered solution (5× SSC [1× SSC is 0.15 M

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TABLE 1. Differential gene expression in *nirK* mutant relative to wild-type *N. europaea*

Gene ID[a]	Gene name[b]	Putative function[b]	Expression ratio[c]			
			Avg	SD	No. of replicates	Significance
Energy production						
(NE0684)	*coxB*$_2$	Cytochrome *c* oxidase (sub. II)	2.876	1.336	7	
(NE0683)	*coxA*$_2$	Cytochrome *c* oxidase (sub. I)	2.476	1.605	10	
(NE0927)	*pan1*	Multicopper oxidase	3.610	0.542	12	*
(NE0926)		Cytochrome *c*	2.542	0.394	9	*
(NE0925)	*cccA*	Cytochrome *c*	2.399	0.535	9	*
(NE2064)	*amoC2*	Ammonia monooxygenase (sub. C)	0.630	0.180	6	
(NE2063)	*amoA2*	Ammonia monooxygenase (sub. A)	0.730	0.187	5	
(NE2062)	*amoB2*	Ammonia monooxygenase (sub. B)	0.560	0.141	5	
(NE2303)	*nirB*	NAD(P)H nitrite reductase	0.301	0.058	10	*
(NE2304)	*ycaC*	Isochorismatase hydrolase	0.392	0.057	11	*
(NE2305)		Hypothetical	0.380	0.085	9	
Signal transduction						
NE1071	*fecI*	Iron uptake sigma factor	4.341	1.256	10	
NE1099	*fecI*	Iron uptake sigma factor	4.542	2.737	9	*
NE1101	*fecI*	Iron uptake sigma factor	3.352	1.118	10	
NE1217	*fecI*	Iron uptake sigma factor	6.711	3.680	8	
(NE2435)	*fecI*	Iron uptake sigma factor	5.557	3.345	10	
(NE2434)	*fecR*	Transmembrane receptor	2.934	1.994	7	
NE0534	*fecR*	Transmembrane receptor	0.515	0.070	8	
Biosynthesis						
NE1445	*nifU*	Iron-sulfur cluster biosynthesis	2.117	0.445	6	
NE2150	*trpE*	Anthranilate synthase	2.407	0.333	8	
(NE2300)	*bioB*	Biotin synthase	3.172	0.910	11	*
(NE2299)	*bioF*	Aminotransferase	2.806	1.306	6	
(NE2298)	*bioH*	Hydrolase	3.232	0.348	12	*
(NE2297)	*bioC*	SAM methylase	2.972	0.239	11	
(NE2296)	*bioD*	Dethiobiotin synthase	2.423	0.439	10	
NE1467		Fatty acid desaturase	0.610	0.050	8	
NE1826	*prsA*	Phosphoribosyl transferase	0.541	0.168	12	
Transport						
NE2124	*fiu*	TonB-dependent receptor for Fe	3.887	1.119	10	*
NE0345	*czcA*	Heavy metal efflux	0.460	0.183	8	
NE0577	*cysW*	Sulfate transporter	0.374	0.093	10	
NE2184	*ptsH*	Phosphocarrier HPr protein	0.470	0.044	7	
Miscellaneous						
NE0200	*atpB*	ATP synthase (subunit A)	1.969	0.339	8	
NE2143	*rpsD*	Ribosomal protein S4:S4	2.764	0.595	10	
NE2032	*amyA*	Glycosyl hydrolase	0.587	0.063	8	
Unknown function						
NE1542		Hypothetical	6.692	3.957	12	*
(NE2151)		Hypothetical	2.925	1.124	9	
(NE2152)		Hypothetical	3.266	1.312	10	
NE2154		Hypothetical	1.979	0.492	10	*
NE0314		Hypothetical	0.233	0.075	12	
NE2218		Hypothetical	0.379	0.099	12	*
NE2505		Hypothetical	0.554	0.096	10	

[a] Gene identification (ID) from *N. europaea* genome sequence (7). Genes grouped and in parentheses indicate operon structure based on presence of a single predicted sigma 70 promoter upstream of first ORF in each group (identified using BPROM; Softberry, Inc., Mt. Kisco, NY) and/or presence of overlapping ORFs.

[b] Gene names and putative functions were derived from multiple searches of gene sequences against nucleotide and polypeptide domain databases (http://genome.ornl.gov/microbial/neur/embl/).

[c] Average ratios of signal intensity are from hybridization of labeled *nirK* mutant/wild-type cDNA to *N. europaea* genomic microarrays. Standard deviations were calculated from number of indicated replicate spots out of a total of 12 hybridized positions. Spots with poor signal were removed from each data set. *, ratios that were significantly different at a *P* value of <0.05 across the indicated numbers of replicate spots. All other ratios were significantly different at a *P* value of <0.1.

NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate [SDS], 50% formamide, 0.1 mg · ml^{-1} salmon sperm DNA) in a chamber (Corning) under Hybrislips (Sigma-Aldrich) at 50°C for 12 to 16 h. The slides were washed with 2× SSC-0.1% SDS for 5 min at 42°C, 0.1× SSC-0.1% SDS for 10 min at 42°C, and four times with 0.1× SSC for 1 min at room temperature.

Scanned images (Scan Microarray Express, PerkinElmer) were analyzed using ImaGene version 5.6 (Biodiscovery). Spot grids were manually fitted to microarray images, and average signal intensity and local background were calculated for each microarray element. The quality of the hybridization signals was assessed for each microarray using the numbers of detected genes plus low and consistent background levels. Spots with poor signal quality, irregular shape, and/or high background were flagged and removed from each data set. Remaining data were transferred to Excel and Access 2001 (Microsoft Corp., Redmond, WA) and GeneSpring version 6.0 (Silicon Genetics). Normalization was done by scaling total fluorescence measured for both Cy3 and Cy5 to an equivalent averaged intensity across each microarray. Pearson correlation coefficients were calculated for 12 spots for each gene (duplicate spots across six microarrays), and genes showing statistically significant and greater-than-twofold differences in Cy3/Cy5 hybridizations from at least 5 spots were considered differentially regulated.

A total of 25 genes were up-regulated and 16 were down-regulated in *nirK* mutant relative to wild-type cells (Table 1). Twelve genes showed significant differences in expression between wild-type and *nirK* mutant *N. europaea* at a *P* value of <0.05 by Student's *t* test, and the remaining expression ratios were significant at a *P* value of <0.1. Genes were grouped into functional categories, i.e., energy production, signal transduction, biosynthesis, and transport, based on degrees of similarity to sequences and domains from multiple sequence databases (http://genome.ornl.gov/microbial/neur/embl/).

Confirmation of hybridization ratios. Ratios of *nirK* mutant/wild-type gene expression were confirmed by Northern blotting using three genes shown to be differentially expressed by the microarray analysis, NE2300, NE0927, and NE2303, and the *nirK* gene, NE0924. The same pools of RNA for making cDNA libraries were used for Northern hybridizations following standard protocols (17). Blots were probed with denatured, radiolabeled PCR products (Prime-a-Gene; Promega) generated with the same primer pairs for NE2300, NE0927, and NE2303 as those used for creating the microarrays and a primer pair targeting the 5′ end of NE0924 (*nirK*). Hybridized blots were exposed to a phosphor storage screen and hybridization signals were quantified (Typhoon phosphorimager; Amersham). Normalization of signal was accomplished by comparing levels of hybridization in three separate lanes to a probe specific for 23S rRNA of *N. europaea*, as described previously (20).

Calculated ratios of gene expression for *nirK* mutant/wild-type cells from the Northern blots were 4.7, 3.9, and −2.0 for NE2300, NE0927, and NE2303, respectively, which was comparable to the microarray hybridization ratios of 3.2, 3.6, and −3.3 for *nirK* mutant/wild-type cells for the same genes (Fig. 1 and Table 1). Although differential expression of the *nirK* gene could not be detected by microarray, expression of the *nirK* gene by Northern hybridization was about 2.4-fold higher in the *nirK* mutant relative to wild-type cells, a similar ratio for microarray hybridizations to NE0927, NE0926, and NE0925, which precede *nirK* in its cluster (i.e., 2.4 to 3.6). This result is consistent with a previous study suggesting that the four-gene *nirK* cluster is indeed expressed as an operon (3).

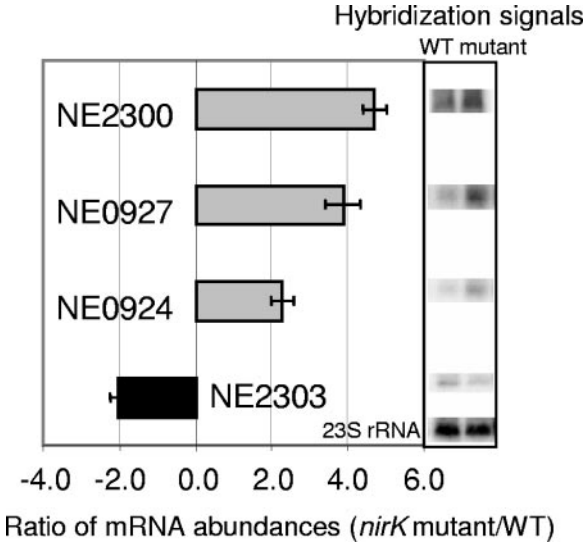

FIG. 1. Northern hybridizations. Intensities of hybridization to specific DNA probes for each gene were normalized between lanes by comparing signals to hybridization intensity from 23S rRNA. The bar graph on the left is a pictorial representation of levels of hybridization signal quantified from blots shown on the right. Error bars indicate standard deviations of the means from three replicate blots. NE2300 = *bioB*; NE0927 = *pan1*, the first ORF in the *nirK* operon; NE0924 = *nirK*; NE2303 = *nirB*. WT, wild type.

Relationships of genes to the *nirK* mutant phenotype. The main phenotypic characteristics of NirK-deficient *N. europaea* are elevated production of N_2O and sensitivity to NO_2^- (1), suggesting that the absence of aerobic nitrite reductase activity leads to increased NO production and likely nitrosative stress. Below, we discuss the regulatory regions and potential activities of the differentially regulated genes between *nirK* mutant and wild-type *N. europaea* to explain the *nirK*-deficient mutant phenotype.

One of two operons encoding cytochrome *c* oxidase, *coxBA*$_2$ (NE0683 and -0684), was up-regulated ~2.6-fold in *nirK* mutant/wild-type *N. europaea* (Table 1). The CoxA subunit of cytochrome *c* oxidase has similar domains to NorB nitric oxide reductase, and mitochondrial cytochrome *c* oxidase can catalyze oxidation of NO to NO_2^- (12, 22). Intriguingly, a conserved FNR binding motif, TTGTT(TaacA)AACAA (lowercase letters are not included in the inverted repeat sequence for recognition by FNR protein), was found 17 bp upstream of the translational start and 7 bp downstream of the −10 consensus sequence of NE0684, suggesting repression of *coxBA*$_2$ by Fnr (NE1719) (25). Besides its well-studied role in regulating O_2-responsive genes (19), Fnr also regulates genes in response to NO, such as the NO-detoxifying Hmp (flavohemoglobin) gene (10). Based on its catalytic potential and presumed control by Fnr, $CoxBA_2$ may be up-regulated by an excess of NO produced by NirK-deficient *N. europaea* and reduce it to N_2O. Although the source of substrate NO would not be from NirK activity, it may be from the chemical oxida-

tion of NH_2OH, which has been shown to accumulate in continuous cultures of *nirK* mutant *N. europaea* (18). A separate transcriptome experiment using the same whole-genome microarray showed that both $CoxBA_2$ and NorCB are up-regulated ca. 2.4-fold in growing versus NH_3/carbonate-starved *N. europaea* cells, indicating equal importance of these enzymes to ammonia-oxidizing metabolism (23). Also, NorCB was previously found to be inessential for N_2O production and tolerance to NO in batch cultures of *N. europaea*, and NorCB expression was not controlled by Fnr (4). Thus, $CoxBA_2$ is an excellent candidate to examine as the additional aerobic NO reductase that protects *N. europaea* from NO produced from ammonia and hydroxylamine oxidation.

Up-regulation of genes related to iron uptake, including three *fecI* sigma factors (NE1071, –1099, and –1217), an *fecIR* sigma factor/response regulator pair (NE2435 and -2434), and the gene for a TonB-dependent receptor (NE2124), indicated an increased need for iron by NirK-deficient cells (7). None of these genes showed significant differences in expression in the microarray experiment of growing/starved *N. europaea* (23), suggesting their specific regulation in response to stress. FeS clusters are excellent targets for inactivation by NO (14), which may explain the need for iron by *nirK* mutant *N. europaea* and also why NifU (NE1445), an FeS cluster biosynthesis gene, was more highly expressed in *nirK* mutant versus wild-type cells.

nirB (NE2303), a gene with similarity to NAD(P)H-dependent nitrite reductase, and its two associated ORFs (NE2304 and NE2305) were down-regulated ca. 2.8-fold in *nirK* mutant/wild-type *N. europaea*. Aside from N assimilation, NirB also detoxifies NO_2^- produced by cytoplasmic nitrate reduction in enteric bacteria (15). Thus, down-regulation of NirB in *nirK* mutant/wild-type *N. europaea* is perplexing as up-regulation of NO_2^- detoxification mechanisms was expected in NirK-deficient cells. However, assuming that *N. europaea* NirB functions as an NADH-linked NO_2^- reductase, its decreased expression may partially explain the sensitivity of *nirK* mutant *N. europaea* to NO_2^- (1). It should be noted that unlike in enteric bacteria, *nirB* of *N. europaea* is not associated with a *nirD* homologue and the *nirB* promoter region lacks FNR binding motifs (8). Thus, the activity and regulation of NirB in relation to ammonia oxidation should be defined experimentally.

Of the other genes, up-regulation of the biosynthetic *trpE* (NE2150) gene in *nirK* mutant/wild-type *N. europaea* is weakly connected to nitrosative stress, as tryptophan residues are highly reactive with NO (21). Up-regulation of the entire biotin synthesis operon in NirK-deficient cells is intriguing, as the structure and regulation of the *N. europaea* operon is similar to that of other eubacterial biotin biosynthesis operons (16), and there are no reports of an increased need for biotin in response to nitrosative stress. The remaining differentially regulated genes, including those for ammonia monooxygenase, do not possess activities easily relatable to N_2O production, NO_2^- sensitivity, or nitrosative stress, and none of the upstream regions of the remaining genes contained consensus sequences related to NO, such as Fnr or NsrR binding motifs. Of the hypothetical genes up-regulated in NirK-deficient *N. europaea*, NE2151 and NE2152 encode predicted membrane-spanning proteins and NE1542 is located immediately upstream of NE1543, which encodes another predicted multicopper oxidase.

Conclusion. This study identified genes in *N. europaea* that were regulated as a consequence of losing aerobic nitrite reductase activity. Not surprisingly, similar levels of expression of the four-gene *nirK* cluster in the *nirK*-deficient mutant of *N. europaea* indicated their coregulation, likely in response to increased NO_2^- toxicity. Up-regulation of $CoxBA_2$, iron uptake, and FeS cluster biosynthesis genes in *nirK* mutant/wild-type cells yielded clues for how *N. europaea* experiences and copes with nitrosative stress caused by lack of NirK activity. Further examination of specific physiological roles of differentially regulated genes in *nirK* mutant/wild-type *N. europaea* could further assist in revealing the significance of aerobic denitrification and nitrosative stress response in *N. europaea*. Microarray data analyzed in this study have been deposited in the Gene Expression Omnibus database with accession number GSE4517 (http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE4517).

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