AN ABSTRACT OF THE DISSERTATION OF

Sunhwa Park for the degree of Doctor of Philosophy in Biological and Ecological Engineering presented on May 1, 2008.

Title: Physiological, Proteomic, and Whole-Genome Transcriptional Responses of *Nitrosomonas europaea* to Heavy Metals (Zn^{2+},Cd^{2+}, Cu^{2+}, and Hg^{2+}) and Cyanide: Identification of Heavy Metals and Cyanide Stress Response Genes.

Abstract approved:

Roger L. Ely

Nitrosomonas europaea (ATCC19718) participates in the biological removal of nitrogen from wastewater by oxidizing ammonia to nitrite, the first step in nitrification. This bacterium is sensitive to heavy metals (Zn^{2+}, Cd^{2+}, Cu^{2+}, and Hg^{2+}) and cyanide, compounds commonly encountered in wastewater treatment plants. Therefore, we characterized physiological and transcriptional responses of *N. europaea* cells exposed to these inhibitors in batch reactors to identify stress-induced genes. *N. europaea* exposed to 3.4 μM $ZnCl_2$ showed about 61% inhibition in nitrification within 30 minutes. In microarrays of *N. europaea* exposed to 3.4 μM $ZnCl_2$ for 60 minutes, 27 genes were observed to be up regulated more than 2-fold, of the total of 2460 genes, while 30 genes were seen to be down regulated. Up-regulated genes included mercury resistance genes (*merTPCAD*), inorganic ion transport genes, oxidative stress genes, toxin-antitoxin genes (TA). *merTPCAD* was the highest up regulated operon (46-fold). Down-

regulated genes included RubisCO (*cbbO*), biosynthesis (*mrsA*), and amino acid transporters. *N. europaea* exposed to 1 μM $CdCl_2$, 6 μM $HgCl_2$, or 8 μM $CuCl_2$ showed about 90% inhibition in nitrification within 30 minutes. In microarrays of *N. europaea* exposed to 1 μM $CdCl_2$ for 60 minutes, 66 genes were up regulated while 50 genes were down-regulated. *merTPCADE* showed the highest up regulation (277-fold) under cadmium stress. Interestingly, *merA* also showed 250-fold up regulation in *N. europaea* cells exposed to 6 μM $HgCl_2$. Therefore, *merTPCADE* appear to be common stress response genes to zinc, cadmium, and mercury exposure. *N. europaea* cells were very sensitive to low concentrations of cyanide; nitrification decreased about 50% in 30 minutes after exposure to 1 μM NaCN. In microarrays of *N. europaea* exposed to 1 μM NaCN for 60 minutes, 35 genes showed up regulation while 29 genes showed down regulation. A gene cluster that included *moeZ* (NE2353), encoding a rhodanese homologue, thought to be involved in detoxification of cyanide, showed the highest up regulation (7-fold). The down-regulated genes included genes encoding proteins involved in the sulfate reduction pathway, signal transduction mechanism, carbohydrate transport, energy production, and coenzyme metabolism.

Physiological, Proteomic, and Whole-Genome Transcriptional Responses of *Nitrosomonas europaea* to Heavy Metals (Zn^{2+},Cd^{2+}, Cu^{2+}, and Hg^{2+}) and Cyanide: Identification of Heavy Metals and Cyanide Stress Response Genes

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APPROVED:

Major Professor, representing Biological and Ecological Engineering

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Sunhwa Park, Author

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Chapter 1

General Introduction

Nitrogen in the environment

Nitrogen concentrations in the hydrosphere have increased as a result of increases in population, industrialization, and urbanization. The excess nitrogen in aquatic environments not only limits uses of natural water but also brings toxicity to aquatic ecosystems and human health. One major effect of excessive accumulation of nitrogen in the hydrosphere is eutrophication of the water and the nitrogen-driven bacterial growth then depletes water oxygen (24). The low amounts of dissolved oxygen can adversely affect the respiration of aquatic animals, fish, and aquatic plants (24). Since nitrogen and phosphorous are the key targets to diminish eutrophication, the regulatory trend in the U.S. has been to require more restrictive nitrogen and phosphorus discharge limits according to the National Pollution Discharge Elimination System (NPDES). To remove nitrogen efficiently and economically in wastewater treatment plants (WWTPs), biological nitrogen removal (BNR) processes are generally favored over chlorination, ion exchange, and air striping processes (24) . The first step in BNR in WWTPs and in the global nitrogen cycle is the conversion of ammonia or organic nitrogen to nitrite, and the most commonly recognized genus of bacteria that carries out this first step is *Nitrosomonas*. The nitrite is then oxidized to nitrate by *Nitrobacter*, which is the predominant genus of NO_2^- oxidizers. Finally, the nitrate formed may be used in synthesis to promote plant growth, or it may be subsequently reduced by

denitrification to nitrogen gas.

Ammonia oxidizing bacteria (AOB)

Ammonia oxidizing bacteria (AOB), such as *N. europaea*, complete the first step of nitrification (14, 24) via two processes carried out by the ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) enzymes, respectively, as follows:

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$$

The four electrons derived from hydroxylamine oxidation support both AMO activity and ATP synthesis. As chemoautotrophs, *N. europaea* utilize inorganic carbon (CO_2) as their sole carbon source and thus have relatively low yields; this bacterium has an 8-12 hr doubling time under ideal conditions (13). In addition, AOB are generally regarded as the most sensitive microorganisms to a variety of potential inhibitors commonly encountered in the WWTPs, including heavy metals (25, 39, 40) and cyanide (49). Therefore, the ammonia oxidation step is often the rate-determining step in the BNR process (24). Although the slow growth rates and the high sensitivities to environmental toxic compounds are well documented (24), relatively little is known of toxicity effects on ammonia-oxidizing bacteria at the molecular level. It also is hard to determine what is causing a problem in an ammonia oxidation process. Therefore, the U.S. EPA has emphasized the need to develop methodologies that can detect and identify nitrification inhibition from various inhibitors. The major goal of this work was to understand the physiological and molecular responses of *N. europaea* exposed to various toxic compounds that inhibit the nitrification process. A future goal based on this work is the development of a biosensor to detect nitrification inhibition in

WWTPs and identify the source of the inhibition in a timely manner.

Inhibition of *N. europaea*

To determine short-term physiological responses of *N. europaea* to the selected inhibitors, the activities of cells in batch reactors were periodically monitored by measuring nitrite production rates, via a colorimetric method (22), in the presence and absence of the inhibitory compounds. The ammonia-dependent (AMO-SOUR) and hydrazine-dependent (HAO-SOUR) oxygen uptake rates were examined for a more detailed characterization of the cellular injuries or inhibition as previously described (22). The results of the specific oxygen consumption rates were helpful in identifying the nature of the inhibition of *N. europaea*, such as whether the inhibitor targeted AMO directly or affected other "downstream" oxygen-dependent cellular functions. In our experiments, *N. europaea* were placed in batch reactors containing either 50 mM HEPES buffer (for metal experiments) or growth medium (for CN experiments) both of which contained 5 mM total ammonia. The cells achieved a metabolic steady state within 1 hour and the steady state was continuous for the remainder of the 4-hour experiment. The metabolic steady state was confirmed by constant nitrite production rates over three consecutive time points (81). Oxygen and ammonia were not limited during the experiments. Once *N. europaea* reached metabolic steady state, the potential inhibitors, such as heavy metals or cyanide, were injected into the batch reactor. The toxicity of each inhibitor to *N. europaea* was determined by the concentration at which it decreased ammonia oxidation rates by about 50%. Each inhibitor was checked to see if the inhibition was reversible or irreversible.

Monitoring stress response genes

Stress proteins were first mentioned as gene products induced by heat and other sources of stress (61). Since then, over 12,000 references have shown that stress proteins are inducible by several agents, including environmental conditions such as temperature and pH (8, 26). To identify "stress response genes" or "sentinel genes", which are expressed only in the presence of particular inhibitors or toxic compounds, the global gene expression of *N. europaea* was analyzed using Affymetrix microarrays. Microarrays were designed to determine the levels of all mRNA transcripts present in the cells via the hybridization between cDNA, transcribed from mRNA, and the microarrays. Whole-genome cDNA microarrays of *N. europaea* were designed and constructed in previous research to find sentinel genes under starvation conditions (95). Affymetrix microarrays were constructed with gene-specific fragments that were amplified with primers designed by PRIMEGENS software (97) and covered 97.2% of the whole genome of *N. europaea* (95). Gene-specific fragments were used because full-length genes on microarrays can cause cross hybridization (97). In addition, to detect the stress proteins, proteins were compared between stressed and unstressed *N. europaea* cells by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The proteins in 2D-PAGE are separated by their isoelectric points in the horizontal direction (the pH at which a protein's net charge is zero) and are then further separated by their molecular weight in the vertical direction.

The first paper in this dissertation describes the physiological, proteomic, and transcriptional responses of *N. europaea* to zinc in a metabolic steady-state batch reactor. Zinc has been used in a variety of fields and applications such as

wood preservation, catalysis, galvanization, batteries, and paints. Since zinc cannot be degraded by microorganisms, zinc can accumulate in the environment from car emissions, deicing salts, and fuel and oil (81), and thus inhibit nitrification processes (55). Although zinc is an essential metal for bacteria, excessive amounts of zinc inhibited the nitrification process; 3.4 μM $ZnCl_2$ caused 61% inhibition of nitrification in batch reactors. In addition, 3.4 μM $ZnCl_2$ changed the transcriptional and proteomic levels, as determined by the Affymetrix microarrays and 2D-PAGE set-ups described above. In the 2D-PAGE experiments with the soluble protein fraction of *N. europaea*, two putative stress proteins (outer membrane proteins and S1 RNA binding domain) were up regulated in cells exposed to zinc. The time-dependent transcriptional levels of genes encoding the proteins were determined using reverse transcriptase quantative PCR (qRT-PCR). The transcriptional levels of these genes started to increase within 1 h after exposure to zinc. Based on this finding, we decided to harvest mRNA samples for Affymetrix microarrays after 1 h of incubation. In the microarray experiments with *N. europaea* exposed to zinc for 1 hour, mercury resistance genes (*merTPCADE*) were the highest up-regulated (46-fold) genes, and genes encoding membrane transporter and signal transduction also were up regulated. Down-regulated genes involved biosynthesis, amino acid transport, and transcription. The up- or down-regulated genes under zinc stress provided important information about how *N. europaea* responded to zinc inhibition.

The second paper presents physiological, proteomic, and transcriptional responses of *N. europaea* to cadmium. Heavy metals such as cadmium (27, 52), mercury, and copper (57) are extensively used in industry (*i.e.* in the fabrication of pigments, batteries, electronics, etc.) and improper disposal of the metals or their

by-products causes the metals to contaminate the environment (79). These metals may inhibit nitrification in the reclamation of wastewater (99). In our work, 1 μM $CdCl_2$ inhibited the ammonia oxidation process about 90%. In the microarray results with *N. europaea* exposed to 1 μM $CdCl_2$ for 1 hour, *merTPCADE* again showed the highest up regulation (277-fold). Based on this finding, it seems that *merTPCADE* may be common stress genes for heavy metals. Therefore, time-dependent transcriptional levels of *merA* were also determined by qRT-PCR with cells exposed to copper and mercury. The *merA* gene showed significant up-regulation (250-fold) under mercury stress but only showed 1.7-fold up-regulation under copper stress. Interestingly, the cells were able to recover under mercury stress. This is thought to be because *merA* can reduce toxic Hg^{2+} to non-toxic Hg^{0}. The recovery of *N. europaea* from mercury stress is a unique inhibition pattern compared to other heavy metals such as cadmium, zinc, copper. Genes down-regulated in response to cadmium exposure included RubisCO, transcription related genes, and genes involved in amino acid transport. The down-regulation of these genes suggests that the cells may conserve energy for NH_3^+ metabolism under stress conditions.

Finally, the third paper deals with how cyanide exposure influences nitrification and transcriptional responses of *N. europaea*. Cyanide is a known inhibitor of the respiratory processes in nitrifying activated sludge (35, 49) and in pure cultures of *N. europaea*. Cyanide inhibits respiration by binding to heme P460 of the HAO enzyme in *N. europaea* (62). Cyanide-transforming organisms can work together with nitrifying bacteria to clean-up cyanide contaminated water (21), where the cyanide-transforming organisms produce ammonia from free cyanide (HCN, CN^-) and nitrifiers use the ammonia as an electron donor. However, because

of toxicity to the nitrifiers, nitrifying bacteria cannot participate in the process if too much cyanide is present (21).

In our batch reactor experiments, 1 μM NaCN inhibited nitrification by about 50%, and identified several genes that were up regulated or down regulated under cyanide stress. When cells were exposed to 1 μM NaCN for 1 h, *moeZ* showed the highest up regulation (7-fold) in microarray results, which were confirmed by qRT-PCR. Up regulation of *moeZ* (homologue of rhodanases) suggests a possible detoxification process for cyanide in *N. europaea*. In addition, down regulation of NE0852/0853/0857 encoding proteins involved in the sulfate reduction pathway may indicate that *N. europaea* can use the final product (SCN^-) of the detoxification process as an alternative sulfur source for cysteine biosynthesis, instead of using the final product of the sulfate reduction pathway. The detoxification process also can decrease free cyanide concentrations in batch reactors.

Chapter 2

Genome-Wide Transcriptional Responses of *Nitrosomonas europaea* to Zinc

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ABSTRACT

Nitrosomonas europaea, a Gram-negative obligate chemolithoautotroph, participates in global nitrogen cycling by carrying out nitrification and derives energy for growth through oxidation of ammonia. In this work, the physiological, proteomic, and transcriptional responses of *N. europaea* to zinc stress were studied. The nitrite production rate and ammonia-dependent oxygen uptake rate of the cells exposed to 3.4 μM $ZnCl_2$ decreased about 61% and 69% within 30 minutes, respectively. Two proteins were notably up regulated in zinc treatment and the mRNA levels of their encoding genes started to increase by one hour after the addition of zinc. A total of 27 genes were up regulated and 30 genes were down regulated. Up-regulated genes included mercury resistance genes (*merTPCAD*), inorganic ion transport genes, oxidative stress genes, toxin-antitoxin genes (TA) and two-component signal transduction systems genes. *merTPCAD* was the highest up regulated operon (46-fold). Down-regulated genes included the RubisCO operon (*cbbO*), biosynthesis (*mrsA*) and amino acid transporter.

INTRODUCTION

Nitrosomonas europaea (ATCC19718) is an obligate chemolithoautotroph that obtains energy and reductant for growth from the oxidation of ammonia to nitrite through a two-step process (43). The two steps are carried out by ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO), respectively, as follows:

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-.$$

The four electrons derived from hydroxylamine oxidation support both AMO activity and ATP synthesis. *N. europaea* belongs to the betaproteobacteria and contains a single circular chromosome of 2,812,094 bp with 2460 predicted genes. Most of the genes in the 285 open reading frames (ORFs) related to active transport in *N. europaea* encode transporters of inorganic ions (14).

Zinc is a vital catalytic component of seven enzymes, such as zinc metallopeptidase and zinc transporters, and of two zinc-based domains (termed "zinc fingers") in *N. europaea.* However, industrial developments have elevated zinc concentrations in some environments and zinc is regarded as a permanent contaminant because of its non-degradability (78). Excessive zinc in wastewater can accumulate inside cells and may cause zinc cytotoxicity in two ways: 1) by binding with SH groups of proteins involved in electron transport systems and 2) by generating reactive oxygen species ($HO^{\bullet}$ or $HOO^{\bullet\bullet}$) (70, 74).

Heavy metals can enter into the cytosol of prokaryotes through specific or non-specific transporters (70). The specific transporters import the metal ions into the cells under requirement, starvation or specific metabolic conditions. In contrast, the non-specific transporters bring metals into the cells by a diffusion gradient across the cytoplasmic membrane. In this case, metal ions can be imported even after the required micronutrient concentrations are met, causing toxic effects in the cells (9, 16).

Bacteria display several resistance mechanisms against metal toxicity. For example, permeable walls allow metals to diffuse out of the cells and active export systems can remove metal ions via efflux pumps. In addition, metal ions can be physically sequestered by periplasmic proteins, cytoplasmic proteins, or ligands like polyphosphate granules (16, 30). Although physiological and transcriptional

responses of *N. europaea* under starvation and toxic conditions by chloroform and chloromethane have been determined (33, 94, 95) the responses of the cells under zinc stress have not been studied. In this work, we determined the whole-genome transcriptional change of *N. europaea* exposed to 3.4 μM $ZnCl_2$ for one hour using cDNA microarrays and by quantitative reverse transcriptase-PCR (qRT-PCR).

MATERIALS AND METHODS

Cell growth and Experimental design (Batch reactor).

N. europaea cells were cultured in medium containing 25 mM $(NH_4)_2SO_4$ as previously described (23, 86) and harvested in mid to late exponential phase. At harvest, 1 L of cells ($OD_{600} \cong 0.07$) was washed twice with 40 mM $NaHPO_4$ (pH 7.8) to remove residual ammonia and resuspended in 1 L of 50 mM HEPES buffer (pH 7.8). The cells ($OD_{600} \cong 0.07$) were equally separated into two gas-tight 1.67 L reactor vessels (Wheaton Double-sidearm Cell stir). HEPES buffer was used to prevent zinc precipitates and complexes that can form in phosphate or carbonate buffers. $(NH_4)_2SO_4$ (2.5 mM) was injected in the reactor vessels and the cultures were stirred with a magnetic stirring bar rotating at 700 rpm. Results from preliminary experiments (data not shown) were used to establish desired zinc concentrations, a preferred monitoring strategy, and appropriate time points and intervals for the physiological, genomic, and proteomic observations (described below). Preliminary experiments also compared performance of cells in HEPES buffer with their performance in the phosphate/carbonate-buffered *N. europaea* growth media, showing their performance in HEPES buffer to be indistinguishable from their performance in phosphate/carbonate-buffered growth media over the 4-

hour observation periods used in these experiments (data not shown).

Oxygen uptake measurement and nitrite assay.

Nitrite concentration and oxygen uptake rate were determined every 30 min for 4 h. A 1 ml aliquot of the cells was centrifuged immediately and 10 μl of supernatant was analyzed to determine nitrite concentration spectrophotometrically (43). Ammonia- and hydrazine- dependent specific O_2 uptake activities were measured in a 1.8 ml glass, water-jacketed reaction vessel at 30℃ using a heated circulating water bath (22). The hydrazine-dependent oxygen uptake rate was determined by blocking ammonia-dependent oxygen uptake with 100 μM allylthiourea (ATU), followed by the addition of 750 μM hydrazine, an alternative substrate for HAO. The specific oxygen uptake rate (SOUR) of the cells was calculated based on the saturated oxygen concentration in the water (22). After a constant nitrite production rate and SOUR were detected over three consecutive time points, where the cells were considered to have reached quasi-steady state (44), the zinc solution was added to the treatment reactor. In separate experiments to determine the recovery of *N. europaea* activities after zinc stress, cells that had been incubated for 1 h with 3.4 μM $ZnCl_2$ were washed 6 times with HEPES buffer. The residual zinc concentration was determined by inductively coupled plasma-mass spectrometry (ICP-MS). The washed cells were then resuspended in HEPES buffer with $(NH_4)_2SO_4$ (5 mM) and nitrite production rate was determined. ICP-MS measurements were also used to confirm that zinc concentrations did not change substantially during the quasi-steady-state reactor experiments.

2-D gel electrophoresis.

After 3 h of zinc exposure, total proteins, including membrane proteins, were extracted from the cells using a ReadyPrep Sequential Extraction Kit (BIO-RAD, Hercules, CA). Extracted protein (100 μg) was treated with Benzonase (Sigma-Aldrich, St. Louis, MS, USA) and incubated for 60 min to remove residual nucleic acid. The solution was then homogenized for 5 min for complete lysis and centrifuged (10 min at 8,000 x g). ReadyStrip Immobilized pH Gradient (IPG) Strips (BIO-RAD), with non-linear pH gradient from 3 to 10, were used for isoelectric focusing (IEF). After IEF, the IPG strips and 3 μl unstained protein standard (BIO-RAD) were placed on 12.5% SDS-polyacrylamide gel (BIO-RAD) to separate the proteins. Proteins were fixed in 30% methanol/7.5 % acetic acid and stained with 150 ml SYPRO Ruby protein gel stain (Cambrex Bio science, Rockland, ME) overnight in the dark. The proteins were viewed under fluorescent light and protein spots between control- and zinc-treated conditions were compared. Proteins appearing to be related to zinc stress were excised using a pipette tip. The proteins were identified by nano/LC/MS/MS from Midwest Bio Services, LLC (Overland, KS).

Total RNA preparation.

After 60 min of zinc exposure, 180 ml of *N. europaea* (OD_{600} ≅ 0.07) was harvested and washed with sterilized 40 mM $NaHPO_4$ (pH 7.8). Total cellular RNA was extracted using 1 ml of Trizol (Ambion Inc., Austin, TX) following the manufacturer's instructions. The extracted RNA was purified with an RNeasy Mini

kit (Qiagen Inc., Valencia, CA.) and treated with RNase-free Dnase I (Qiagen) to digest residual chromosomal DNA. The concentration of purified RNA was determined by using a Nanodrop spectrophotometer (Nanodrop Technologies, Rockland, DE) and RNA quality was checked by the A_{260}/A_{280} ratio and RNA 6000 Nano LabChip kit on the Agilent Bioanalyzer 2100 (Agilent Technologies Inc., Palo Alto, CA.).

DNA microarray construction and microarray experiment.

The custom Nimble express™ Made-to-Order array for *N. europaea* was manufactured by NimbleGen Systems, Inc. for Affymetrix based on the published genome sequence (AL954747) (14). Identified genes in *N. europaea* are represented on the array by the probe sets with 24 pairs of perfect match/mismatch oligo probes. Microarray analyses were performed in triplicate (three controls and treatments), using RNA samples extracted from independent experiments. cDNA synthesis, labeling, and hybridization were performed by the Center for Genome Research and Biocomputing Core Lab (CGRB) at Oregon State University, Corvallis OR. GeneSpring software was used to analyze gene expression data obtained from Affymetrix GeneChip Operating Software (GCOS) by applying filters to identify the genes that matched with user defined criteria, and evaluating triplicate samples. GC-RMA (Robust Multi-chip Average, with GC-content background correction) was applied to normalize and summarize probe-level intensity measurements from Affymetrix GeneChips (46). The two normalized groups were filtered using a detected 2-fold change as a minimum for up or down regulation. The variation of filtered up- or down-regulated genes was evaluated by using unpaired two sample t-tests with a cutoff p-value of 0.05. Biological

functions of up-regulated or down-regulated hypothetical genes were searched by BLASTx.

Reverse transcriptase quantative -PCR (qRT-PCR).

The total RNA from control and treatment samples was used to synthesize cDNA with the Iscript^{TM} cDNA Synthesis kit (BIO-RAD, Hercules, CA). The synthesized cDNA was amplified with primers (Invitrogen, Carlsbad, CA) designed by using Primer 3 software (82) and IQ^{TM} SYBR Green Supermix (BIO-RAD). qRT-PCR was performed with an ABI 7500 instrument (Applied Biosystems, Foster City, CA). The qRT-PCR efficiency for each gene was checked through standard curves created by serial dilution of RNA samples. The fold changes of genes showing ideal amplification efficiency were calculated using the formula $2^{-\Delta\Delta Ct}$ (77). Using the $2^{-\Delta\Delta Ct}$ method, the data are presented as fold changes in gene expression normalized to a housekeeping gene and relative to the control condition. Twelve genes representing the most up regulated genes from each of 12 operons were selected for qRT-PCR validation of the change of mRNA expression level detected from Affymetrix microarrays.

ArrayExpress accession number.

The microarray data for this research is available at the Gene Expression Omnibus database (http://www.ncbi.nih.gov/geo) under accession number GSE7552.

RESULTS

Physiological responses to zinc.

N. europaea in quasi-steady state exposed to 3.4 μM $ZnCl_2$ showed a 61% decrease in the rate of nitrite production (Fig. 2-1a) and a 69% decrease in the ammonia-dependent specific oxygen uptake rate (AMO-SOUR) (Fig. 2-1b) within 30 min of exposure and remained at these levels of activity throughout the experiment. Cells in quasi-steady state exposed to 5 μM $ZnCl_2$ showed a 96% decrease in their nitrite production rate (Fig. 2-1a) and a 94% decrease in the AMO-SOUR (Fig. 2-1b). The AMO-SOUR remained constant ($\cong$0.35 mM O_2/ min-OD_{600}) before injection of zinc and in the control condition, but the uptake rate was reduced after 30 min of exposure to zinc. However, the hydrazine-dependent specific O_2 uptake rate (HAO-SOUR) did not change after exposure to zinc (data not shown), suggesting that zinc inhibition of ammonia oxidation targets AMO specifically rather than HAO or other metabolic processes. The inhibition pattern of zinc in *N. europaea* for nitrite production rate and AMO activity was consistent with that of *Nitrosococcus mobilis*, a halophilic ammonia oxidizing bacteria (80). Exposure of batch-cultured *N. mobilis* to 10 μM $ZnCl_2$ resulted in 100% inhibition in nitrite production, 71% inhibition in AMO-SOUR, and 32% inhibition in HAO-SOUR in 30 min. Also, in our experiments the decreases in nitrite production rate and AMO activity of the cells exposed to 3.4 μM $ZnCl_2$ for 1 h were not recovered when cells were washed to remove residual zinc and placed in fresh HEPES buffer with 5 mM ammonia; 50% inhibition continued for the 4 h observation period (data not shown). ICP-MS analyses confirmed that residual zinc

concentration in cell suspensions washed and resuspended, as described above, was essentially zero.

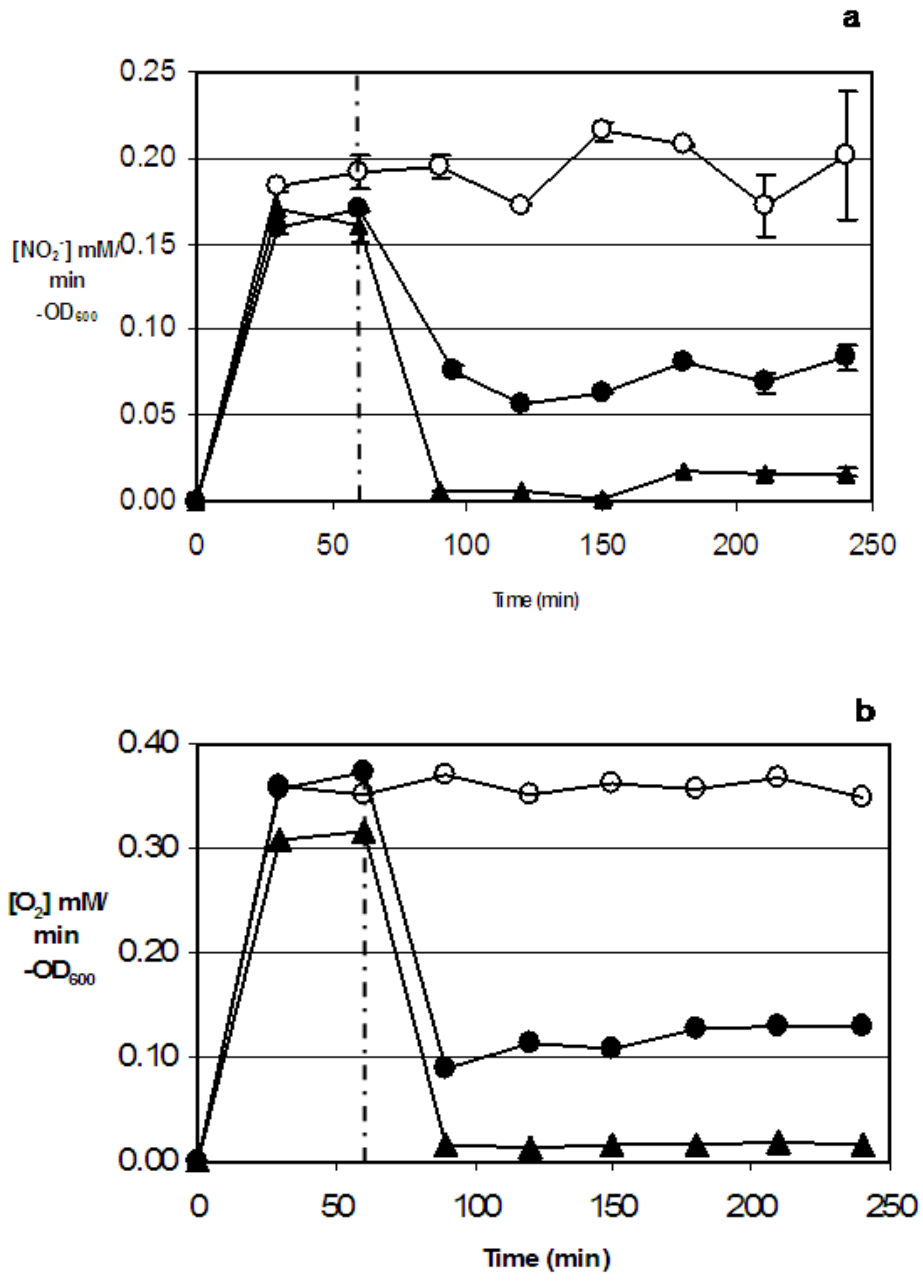

Fig. 2-1. Physiological responses of *N. europaea* exposed to 3.4 μM $ZnCl_2$ or 5 μM $ZnCl_2$ for 4 hours. Nitrite production rate normalized by cell density (a). Ammonia-dependent oxygen uptake rate normalized by cell density (b). Open circles represent no zinc; filled circles represent 3.4 μM $ZnCl_2$; filled triangles represent 5 μM $ZnCl_2$; Lines connect data points. Vertical dashed lines indicate the injection time (60 min) of zinc.

Analysis of protein expression changes.

2-D SDS-PAGE of the soluble protein fractions of *N. europaea* treated with 3.4 μM $ZnCl_2$ for 3 h showed two notably up-regulated proteins (Fig. 2-2). The proteins were identified as an outer membrane protein (NE2548) and a S1 RNA binding domain (NE0760) (Fig. 2-2). The transcription levels of the proteins were determined by using qRT-PCR before the injection of zinc and at 20, 60, and 180 min after the injection of zinc. No substantial up regulation in corresponding genes of these two proteins, as indicated by mRNA levels, was apparent after 20 min of zinc exposure but major up regulation was evident in the 60-min RNA samples (data not shown). Based on this observation RNA samples for corresponding microarray analyses were taken after 60 min of incubation with 3.4 μM $ZnCl_2$**.**

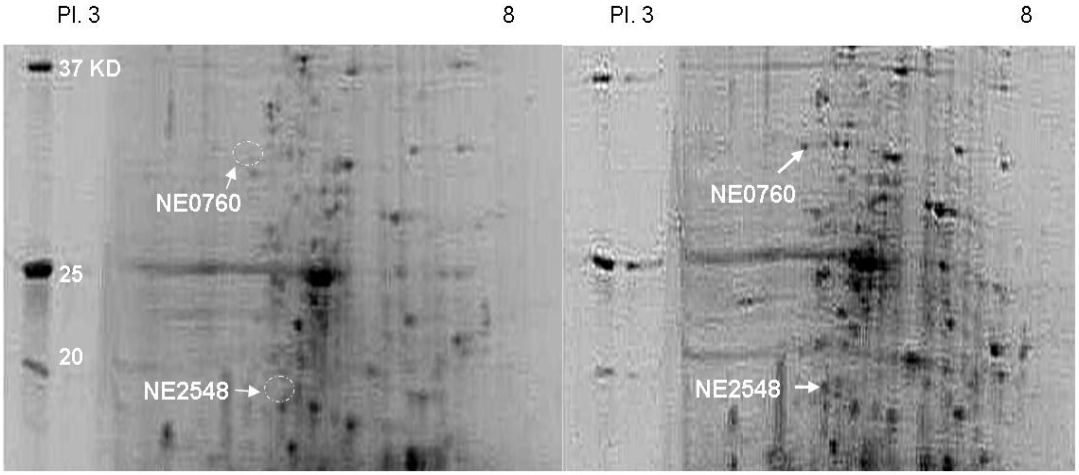

Fig. 2-2. Differences in gels of the soluble protein fraction of *N. europaea* without (left) and with (right) 3.4 μM $ZnCl_2$. Circles indicate absent proteins under control condition. Arrows indicate protein spots up regulated in zinc-exposed cells.

Transcriptional changes.

In total, of the 2460 predicted genes of the *N. europaea* genome, 27 genes showed statistically significant (>2-fold) up regulation while 30 genes displayed

statistically significant (>2-fold) down regulation following treatment with zinc. The up- and down-regulated genes were categorized by function according to the *N. europaea* genome database (http://genome.ornl.gov) (Fig. 2-3). The up-regulated genes were involved in various functions such as mercury resistance, inorganic ion transport, oxidative stress, toxin-antitoxin (TA) functions, two-component signal transduction system, DNA repair, and translation. The largest group of up-regulated genes (11 genes) was related to inorganic transport, while only three were related to DNA repair or translation. When cells in exponential growth are exposed to a high concentration of zinc, the cells import zinc non-specifically into the periplasm, where it causes heavy metal toxicity (70). Therefore, genes that encode inorganic transporters and heavy metal binding proteins may be up regulated to sequester the free zinc imported non-specifically. The down-regulated genes in our experiments are involved in transcription (*cspD2*, NE1312), multicopper oxidase (*mnxG*, NE0315), biosynthesis (*mrsA*, NE0530), and amino acid transport (*gcvH1*, NE0608). In addition, genes for signal transduction mechanisms (*cheYZ*, NE1923/4) and universal stress protein (*yxiE*, NE2292) showed statistically significant down regulation. NE1918/9 (*cbbOQ*, Ribulose bisphosphate carboxylase/oxygenase (RubisCO)) and NE0668/0670 (outermembrane efflux protein (MtrC)) were also down regulated. Since transcript of genes in the RubisCO operon have been observed to increase under low CO_2 and decrease under higher CO_2, it might indicate that cells in our system were not carbon-limited (93). The RubisCO operon for *N. europaea* also has been shown to be expressed only under non-limited energy conditions (93). Hence, the down regulation of *cbbOQ* may suggest that *N. europaea* under zinc stress may conserve energy by down regulating transcription of the RubisCO operon

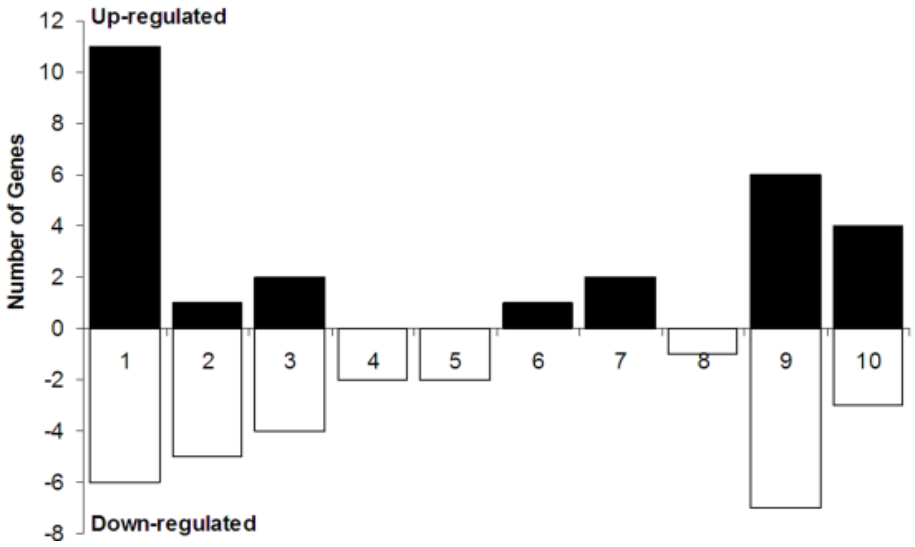

Fig. 2-3. Differentially expressed genes grouped by functional classification according to the *N. europaea* genome database (http://genome.ornl.gov). Columns: 1, inorganic transport and membrane permeability; 2, general function prediction; 3, signal transduction system; 4, carbohydrate transport; 5, amino acid transport; 6, DNA repair; 7, translation; 8, transcription; 9, hypothetical proteins; 10, intergenic region.

DISCUSSION

Mercury-resistance operon.

Mercury scavenger-like transporters that might be involved in heavy-metal tolerance of *N. europaea* (14) were members of the highest up-regulated operon detected under zinc stress. The operon (Fig. 2-4) includes *merTPCAD*, with *merE* being adjacently located and *merR* regulating expression of the operon. (87). These genes may play an important role in the zinc resistance pathway of *N. europaea*; that is, once zinc is imported into the periplasm through unspecific transport proteins under a zinc-ample condition, *merTPC* may transport zinc into the cytoplasm to prevent toxic effects in the periplasm (70). The *merA* is related to glutathione reductase and may reduce the oxidized bisglutathione (GS-SG) formed by the metal cations (70). Transcriptional levels of the five genes in *merTPCAD*

were different as shown in Table 1, perhaps because of premature termination of transcription, rapid mRNA processing or mRNA degradation. In a BLASTx search, full-length alignments of NE0837 yielded a best hit (E value, 10^{-127}) to *urf*-2 (a putative mercury resistance gene) of *Pseudomonas* sp. A19-1. NE2575 (*merE*), located between NE0837 and NE0838, may have caused the signal observed from NE0837, which encodes a transposition function (TnpM), possibly due to the immediate proximity of the two genes or read-through of the region (87).

Fig. 2-4. Organization of *merR, merTPCADE* and transposase-related genes (NE0837/0836/0835) in *N. europaea*. Arrows indicate the orientation of the genes.

Up regulation of genes involved in metal transport and membrane permeability.

NE2124, encoding TonB-dependent outer membrane receptor, was up regulated 2.1-fold under zinc treatment. Gram-negative bacteria scavenge Fe^{3+} ions by employing chelators and express substrate-specific proteins to recognize the ions. Three outer membrane proteins (TonB, ExbB, and ExbD) are required to transport these ferric chelators into the periplasm of the cells and play an important role in the transport of heavy metals such as iron (10, 67, 85). Under Fe-limiting conditions, the TonB-dependent outer membrane receptor gene was up regulated (94). In contrast, the TonB-dependent outer membrane receptor gene of *Caulobacter crescentus* was up regulated under excessive concentrations of cadmium, chromium, and uranium (38). Therefore, it can be suggested that the

TonB-dependent outer membrane receptor is involved in adaptation of the cells in both heavy metal-limited or heavy metal-ample conditions. The transporter genes of Gram-negative bacteria are responsible for the regulation of heavy metal levels between the cytoplasm and the periplasm. Gram-negative bacteria exposed to excessive heavy metals generally express genes encoding transporters for heavy metal resistance (90). In our research, NE1898, 1899 and 1900, encoding the ABC transporter, were up regulated linking this transporter to heavy metal stress. NE1029, encoding a putative soluble binding metallo-chaperon, was up regulated. The metallo-chaperones sequester metal ions and regulate the metal-dependent gene expression under excess zinc conditions (72). Therefore, this gene might be up regulated to reduce free zinc ions which can cause toxic effects. The outer membrane proteins of Gram-negative bacteria provide a permeability barrier to protect the cell from toxic agents. Outer membrane protein genes, NE2563 (encoding OmpC) and NE2548 (encoding OmpA), showed statistically significant up regulation (Table 2-1). OmpA also showed up regulation in 2-D SDS-PAGE of the soluble protein fraction extracted from zinc-exposed cells as shown in Fig. 2-2, thus confirming the microarray data. The Tol protein in *E.coli* is related to the stability of the outer membrane by transporting crucial outer membrane components such as LPS, Lpp or Pal (53). It can be suggested that NE0920 (TolB) was also induced to maintain the function of the outer membrane as a barrier under heavy metal toxicity.

Table 2-1. Up regulation of genes involved in the mercury resistance pathway, inorganic ion transport and membrane permeability

Locus tag	Description	Fold change[1]	P value (<0.05)	Fold change[2]
Mercury resistance pathway				
NE0838/ *merD*	Bacterial regulatory protein (MerR family)	6.8	1.9×10^{-4}	
NE0839/ *merA*	Mercuric reductase	46.2	1.9×10^{-5}	48.9
NE0840/*merC*	Mercury transport protein	22.8	1.7×10^{-4}	
NE0841/*merP*	Mercury scavenger protein	14.3	1.9×10^{-4}	
NE0842/*merT*	Mercuric transport protein	13.1	6.9×10^{-6}	
Inorganic ion transport and membrane permeability				
NE2124	TonB-dependent receptor protein	2.3	2.5×10^{-2}	2.1
NE1029	Solute metal-binding protein	2.0	1.8×10^{-2}	
NE1898	Unknown function	2.4	2.6×10^{-2}	
NE1899	ATPase component ABC-type transport system	2.4	3.3×10^{-2}	1.8
NE1900	Permeases and putative transmembrane proteins	2.5	2.0×10^{-3}	
NE0902	Periplasmic component of the Tol biopolymer transport system	2.3	8.9×10^{-3}	
NE1176	Peptidoglycan binding	2.1	4.2×10^{-2}	2.1
NE2563	General diffusion protein in Gram-negative bacteria (OmpC)	2.7	1.1×10^{-2}	2.3
NE2548	Peptidoglycan-associated Protein (OmpA)	3.2	2.2×10^{-3}	

1 and 2 represent the fold change in microarray and qRT-PCR, respetively

Up regulation of putative oxidative stress genes.

Aerobic bacteria such as *Bacillus subtilis* have enzymes that can remove reactive oxygen species (HO· or HOO··), superoxide dismutase (SODs) or hydroperoxidases (HP). *N. europaea* has genes for several comparable enzymes such as *katA, katG,* and *ahpC* (14) and thioredoxin is a disulfide reductase that removes the protein disulfide bonds produced by reactive oxygen species (38). NE1319 encoding thioredoxin showed up regulation when *N. europaea* were exposed to 3.4 μM $ZnCl_2$ suggesting that 3.4 μM $ZnCl_2$ may lead to oxidative stress in *N. europaea*. RNA polymerase sigma (σ) factors have been shown to be up-regulated to cope with environmental stresses such as oxidative stress (90). In the current research, NE1071 and NE1096, encoding two extra cytoplasmic function (ECF) σ-factors, showed greater than 1.9-fold up regulation and the microarray data of NE1096 was confirmed by qRT-PCR (Table 2-2). NE1071 (*fecI*) and NE2143 (*rpsD*) that also showed up regulation under chloroform stress (33) and nitrosative stress (15) might be general stress genes. *yvqQ* (Nitrite and sulfite reductase) showed up regulation in our research and it was categorized as an oxidative stress gene in the presence of heavy metals such as cadmium and chromium in previous research (38).

Table 2-2. Up regulation of oxidative stress and Toxin-antitoxin (TA) genes

Locus tag	Description	Fold change[1]	P value (<0.05)	Fold change[2]
Oxidative stress				
NE1319	Thioredoxin	2.0		3.4
NE1071	Sigma ECF(extra cytoplasmic-Function) family	1.9	3.7×10^{-2}	
NE1096	Sigma ECF(extra cytoplasmic-Function) family	1.9	2.8×10^{-2}	1.6
NE0852/*yvgQ*	Nitrite and sulfite reductase	1.9	3.0×10^{-2}	2.1
Toxin-antitoxin (TA) genes				
NE0974/*mazEF-2*	PemK-like protein	2.2	4.6×10^{-2}	2.1
0975	Unknown function	2.4	4.8×10^{-2}	2.1

1 and 2 represent the fold change in microarray and qRT-PCR, respectively

Toxins-antitoxins, two-component signal transduction systems and DNA repair genes.

Once bacteria are exposed to harmful environment conditions such as nutrient limitation, starvation stress, and chlorinated hydrocarbons stress (33), toxins-antitoxins (TA) loci that are ubiquitous in free-living bacteria regulate the global levels of translation and replication. The *mazEF-2* is a representative TA of *N. europaea* and the PemK belongs to the *mazEF* family (29). NE0974 and NE0975, encoding PemK, was up regulated in zinc stress and they were also up regulated under chloroform stress (33). Transcripts for Helix-turn-helix (HTH) protein (GopG) that belongs to *mazEF-3* (73) also showed up regulation (>1.5-fold change). Two-component signal transduction systems (TCS), consisting of sensor

histidine kinases and phosphorylated cognate target regulators, respond to changes in metal concentrations in the environment to maintain the proper concentration of the metals in the cell (16). NE0728 and NE0729 encoding TCS were observed to be up regulated in this research. TCS-related genes were also up regulated under uranium and copper stress in previous research (38, 71). Formamidopyrimidine-DNA glycosylase encoded by NE2552 (*mutM*), an enzyme for DNA repair, requires zinc for its activity. DNA repair genes were up regulated in response to the toxic conditions caused by TCE oxidation (98). Therefore, the up regulation of this gene in this work may indicate that 3.4 μM $ZnCl_2$ may damage DNA replication in *N. europaea.* However, the genes may also be up regulated to take up free zinc to fulfill requirements for the functional enzyme.

Table 2-3. Up regulation of two-component signal transduction systems genes and DNA repair genes

Locus tag	Description	Fold change[1]	P value (<0.05)	Fold change[2]
Two-component signal transduction systems				
NE0728	Sensory transduction histidine kinase	2.3	8.9×10^{-3}	
NE0729	Response regulators consisting of CheY-like receiver domain and a winged helix DNA-binding domain	3.3	6×10^{-3}	1.8
DNA repair				
NE2552/*mutM, fpg*	Formamidopyrimidine-DNA glycolase	2.2	2.5×10^{-2}	2.4
Translation				
NE0389	Rnase P protein component	2.4	2.5×10^{-2}	2.4
NE2143/*rpsD*	Ribosomal protein S4	2.1	8.9×10^{-3}	2.5

1 and 2 represent the fold change in microarray and qRT-PCR, respetively

Validation of microarray trends using qRT-PCR.

The fold changes of twelve genes obtained by qRT-PCR corresponded with values obtained by Affymetrix microarray analyses, as shown in Table 2-1, 2-2, and 2-3.

SUMMARY

This research suggests that although zinc is an essential metal for seven enzymes and two-zinc based domains of *N. europaea*, 3.4 μM $ZnCl_2$ can cause batch-cultured cells about 60% decrease in nitrite production rate and ammonia-dependent oxygen uptake rate without recovery. In addition, 3.4 μM $ZnCl_2$ changes the transcriptional expression levels of several groups of genes that allow the cells to resist unfavorable environmental conditions. The mercury resistance genes, *merTPCAD*, showed the highest up regulation (46.2-fold) after 60 min exposure to 3.4 μM $ZnCl_2$, suggesting that the resistance pathway of *N. europaea* in zinc-ample conditions is comparable to the mercury resistance pathway. In addition, the genes involved in inorganic transport, membrane transport, oxidative stress, toxin-antitoxin genes, two-component signal transduction systems, and DNA repair were greater than 2.0-fold up regulated. Down-regulated genes were related to RubisCO, transcription, and amino acid transport.

ACKNOWLEDGEMENTS

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Chapter 3

Candidate Stress Genes of *Nitrosomonas europaea* to Monitor Inhibition of Nitrification by Heavy Metals

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ABSTRACT

Heavy metals have been shown to be strong nitrification inhibitors in wastewater treatment plants. In this research, the effects of cadmium, copper, and mercury on *Nitrosomonas europaea* were studied in quasi-steady state batch reactors. When cells were exposed to 1 μM $CdCl_2$, 6 μM $HgCl_2$, or 8 μM $CuCl_2$, ammonia oxidation rates were decreased by about 90%. Whole-genome transcriptional and proteomic responses of *N. europaea* to cadmium were used to identify heavy metal stress response genes. When cells were exposed to 1 μM $CdCl_2$ for 1 hour, 66 genes were up regulated and 50 genes were down regulated more than 2-fold of the total of 2460 genes. Of these, the mercury resistance genes (*merTPCADE*) showed 277-fold up regulation under 1 μM $CdCl_2$. *merA* (mercuric reductase) showed 250-fold up regulation in *N. europaea* cells exposed to 6 μM $HgCl_2$ and a 1.7-fold up regulation in cells exposed to 8 μM $CuCl_2$. Cells showed the ability to recover quickly from Hg^{2+}-related toxic effects, apparently associated with up regulation of the mercury resistance genes and *amoA*, but no such recovery was evident in Cd^{2+}-exposed cells even though the *merTPCADE* were highly up regulated. We suggest that the up regulation of *merA* in response to $CdCl_2$ and $HgCl_2$ exposure may provide a means to develop an early warning indicator for inhibition of nitrification by these metals.

INTRODUCTION

Nitrifying bacteria, such as *Nitrosomonas europaea* (ATCC19718), are important in the removal of nitrogen in wastewater reclamation plants. *N. europaea* obtain essential reductant for energy and biosynthesis from the oxidation of ammonia (NH_3^+) to nitrite (NO_2^-) and use CO_2 as their carbon source (4). The oxidation of NH_3^+ by *N. europaea* is a two-step reaction catalyzed by ammonia monooxygenase (AMO, a membrane-bound enzyme) and hydroxylamine oxidoreductase (HAO, a periplasmic protein), generating NO_2^- as the final product (4). Nitrifying bacteria are sensitive to various environmental contaminants and generally have slow growth rates, with doubling times of about 8-12 hr (92), making them the critical step in biological nitrogen removal. Heavy metals such as Cd^{2+} (27, 52), Hg^{2+}, and Cu^{2+} (57) are extensively used in industry (*i.e.* in the fabrication of pigments, batteries, electronics, etc) and improper disposal of the metals or their by-products tends to contaminate the environment (79). These metals may inhibit nitrification in the reclamation of wastewater (99).

The inhibition of nitrification has been best documented in the model bacterium *N. europaea*. *N. europaea* is sensitive to inorganic compounds such as Cd^{2+} (64), organic compounds such as chlorinated aliphatic hydrocarbons (44), and to pH shifts (32), among other factors. With the sequencing of the *N. europaea* genome, transcriptomics studies to test the effect of inhibitors now are possible to help identify stress genes that can be used as early warning toxicity indicators. Exposure to Zn^{2+} caused the inhibition of ammonia oxidation and, concomitantly, the expression of specific genes encoding membrane transporter and putative metal resistance proteins (76). Exposure to chlorinated aliphatic hydrocarbons increased the expression of genes encoding heat shock proteins, sigma-factors of the

extracytoplasmic function subfamily, and toxin-antitoxin loci (34). Such genes may be used as indicators to prevent the inhibition of nitrification and may be of value in the biological wastewater treatment processes.

This work characterized the responses of *N. europaea* to the heavy metals Cd^{2+}, Hg^{2+} and Cu^{2+} and identified metal-specific stress response genes. Whole-genome transcriptional changes of *N. europaea* exposed to 1 μM $CdCl_2$ were monitored by microarrays. The metal Cd^{2+} has no known biological function, and it has been suggested that Cd^{2+} is toxic to the cell by interfering generally with zinc metabolism and by causing membrane damage (70). *N. europaea* exposed to Cd^{2+} in a batch reactor showed dynamic changes in the expression of the mercury resistance genes (*merTPCADE*). There is precedent for up regulation of *merTPCADE* upon exposure to Zn^{2+} (76), although to a lesser extent than seen with Cd^{2+} in this work. We further tested whether these genes might be used as general sensors to heavy metals toxicity (*i.e.* Hg^{2+} and Cu^{2+}). The metals Hg^{2+} and Cu^{2+} are toxic to *N. europaea*, perhaps through unspecific compounds that are generated by the cells (70). Up regulation of *merTPCADE*, followed by up regulation of *amoA*, appeared to provide relatively fast recovery from the effects of Hg^{2+} exposure, but the same was not true for cells exposed to Cd^{2+}. Over expression of specific stress response genes may be used as an early warning signal to help prevent failure of nitrification processes in wastewater treatment plants and to monitor the health of nitrifying bacteria such as *N. europaea*.

MATERIALS AND METHODS

Batch reactor experiments.

N. europaea were grown in batch cultures with 25 mM $(NH_4)_2SO_4$ as described previously (76) and harvested in mid to late exponential phase ($OD_{600} \cong 0.07$). The cells were washed two times with 40 mM NaH_2PO_4 (pH 7.8) and resuspended in 1 L of 50 mM HEPES buffer (pH 7.8) containing $(NH_4)_2SO_4$ (2.5 mM). The cell suspension was evenly divided into two, gas-tight, 1.67 L reactor vessels (Wheaton Double-sidearm Cell stir). Medium buffered with 50 mM HEPES (pH 7.8) and containing 5 mM total NH_4 sustained a quasi-steady state condition during 4 hr incubations comparable to *N. europaea* batch reactor experiments previously reported using phosphate buffered medium (34). For the experiments, cells were stirred for 1 hr to allow them to reach quasi-steady state, at which point, either 1 μM $CdCl_2$, 6 μM $HgCl_2$ or 8 μM $CuCl_2$ was injected into the treatment reactor vessels.

Oxygen uptake measurement and nitrite assay.

The ammonia-dependent oxygen uptake rate (AMO-SOUR), hydrazine dependent oxygen uptake rate (HAO-SOUR) and NO_2^- production rate were tested every 30 min during the incubations as described (44). An aliquot of 1 ml drawn from a batch reactor was centrifuged immediately and analyzed to determine NO_2^- concentration as described (43). AMO-SOUR was measured in a 1.8 ml glass water-jacketed reaction vessel at 30℃ using a heated circulating water bath (22). HAO-SOUR was then determined by blocking ammonia-dependent oxygen uptake with 100 μM allylthiourea, followed by the addition of 750 μM hydrazine, an

alternative substrate for HAO. The specific oxygen uptake rate (SOUR) of the cells was calculated based on the saturated oxygen concentration in water (22).

Affymetrix microarray experiments.

A volume of 180 ml of *N. europaea* culture ($OD_{600} \cong 0.07$) was obtained from three independent experiments (three control reactors and three Cd^{2+}-treatment reactors). Trizol (Ambion Inc., Austin, TX) was used to extract total RNA from the suspension, following the manufacturer's instructions. After purifying with an RNeasy Mini kit (Qiagen Inc., Valencia, CA), the quality and quantity of the RNA samples were measured using a RNA 6000 Nano LabChip kit on an Agient Bioanlyzer 2100 (Agilent Tech. Inc., Palo Alto, CA). All of the 2460 annotated *N. europaea* genes were represented on the custom Nimble express™ Made-to-Order arrays (NimbleGen Systems, Inc.). cDNA synthesis, labeling, and hybridization were carried out by the Center for Genome Research and Biocomputing Core Laboratories at Oregon State University. GeneSpring software (version 7.2, Silicon Genetics) was used to identify genes that were up or down regulated greater than 2-fold under Cd^{2+}, applying the unpaired two sample t-test with a cutoff p-value of 0.05. The microarray data are available at Gene Expression Omnibus database (http://www.ncbi.nih.gov/geo, accession number GSE9221)

Quantitative reverse transcription-PCR (qRT-PCR).

qRT-PCR was used to evaluate the transcriptional levels of selected up- or down-regulated genes identified in the microarrays and to determine transcriptional levels of *merA* in *N. europaea* cells exposed to Cu^{2+} and Hg^{2+}. cDNA was synthesized using the Iscript™ cDNA synthesis kit (BIO-RAD Laboratories, Inc.

Hercules, CA) with total RNA extracted from cells treated with or without Cd^{2+}, Cu^{2+}, or Hg^{2+} as described (76). qRT-PCR was performed on an ABI 7500 instrument (Applied Biosystems, Foster city, USA) and IQ™ SYBR Green Supermix (BIO-RAD). Primers were designed using Primer3 software and manufactured commercially (Invitrogen, Carlsbad, CA). The qRT-PCR efficiency was determined using standard curves created by serial dilution of RNA samples. The fold changes to show ideal amplification efficiency were calculated using the formula $2^{-\Delta\Delta Ct}$ (77).

2D SDS-PAGE.

To find proteins that were differentially expressed under Cd^{2+} stress, total proteins, including membrane-bound proteins, were prepared from *N. europaea* cells after 3 h incubation with and without 1 μM $CdCl_2$ using a ReadyPrep Sequential Extraction Kit (BIO-RAD). The extracted total proteins were then compared by 2D SDS-PAGE as described (76). ReadyStrip IPG Strips (13 cm; BIO-RAD) with non-linear pH gradient from 3 to 10 were used for isoelectric focusing. SDS-PAGE was carried out on 12.5% precast SDS-PAGE gels (BIO-RAD) at 200 V for 45 min. The up-regulated proteins in Cd^{2+}-exposed cells were excised from SDS-PAGE gel stained with SYPRO Ruby (Cambrex Bio science, Rockland, ME) and identified by nano/LC/MS/MS from Midwest Bio Services, LLC (Overland, KS).

RESULTS

Physiological response to heavy metals.

To test the toxicity of the heavy metals in this study *N. europaea* cells in lag phase were placed in two batch reactors (a treatment reactor and a control reactor). In the control reactors, the cells consistently reached pseudo-steady state in about one hour (constant NO_2^- production rate of about 0.2 mM/min-OD_{600}) and were stable for up to 4 hr (Fig. 3-1a). In the treatment reactors, 1 μM $CdCl_2$, 6 μM $HgCl_2$ or 8 μM $CuCl_2$ was added at 1 hr in pseudo-steady state. After 1 hr of further incubation with Cd^{2+} the cells showed approximately 90% decrease in NO_2^- production rate (Fig. 3-1a) and 79% decrease in AMO-SOUR (Fig. 1b). *N. europaea* exposed to 6 μM $HgCl_2$ or 8 μM $CuCl_2$ showed about 100% and 76% inhibition of the NO_2^- production rate respectively (Fig. 3-1b). AMO-SOUR of the cells exposed to Hg^{2+} and Cu^{2+} decreased to about 89% and 82% respectively.

Determinations of HAO-SOUR showed that hydrazine-dependent activity of HAO remained unaffected in *N. europaea* cells exposed to 1 μM $CdCl_2$ and 6 μM $HgCl_2$, but hydrazine-dependent activity decreased to 71% when exposed to 8 μM $CuCl_2$ (data not shown). The decrease in HAO-SOUR under 8 μM $CuCl_2$ suggests that Cu^{2+}-related inhibition was not confined only to AMO. Interestingly, 6 μM $HgCl_2$ almost completely stopped NO_2^- production (Fig. 3-1a) as indicated by AMO-SOUR (Fig. 3-1b), but the cells started to accumulate nitrite again after 90 min of further incubation, suggesting that the cells were able to recover from the Hg^{2+} exposure. Our results indicate that the inhibitory effects of Cd^{2+} and Hg^{2+} were confined mainly to AMO while apparently causing little or no damage to other elements of the electron transport chain.

We showed previously in batch reactors that another metal, Zn^{2+} (3.4 μM $ZnCl_2$), caused 50% nitrification inhibition (76). Compared to Zn^{2+}, Cd^{2+} and Hg^{2+} were more toxic to nitrifiers, but Cu^{2+} was less toxic. Other studies have shown the inhibition of nitrification by Cd^{2+} in wastewater sludge (64), and as our results show, it is likely due to the sensitivity of ammonia oxidizers (e.g. *N. europaea*) to Cd^{2+}.

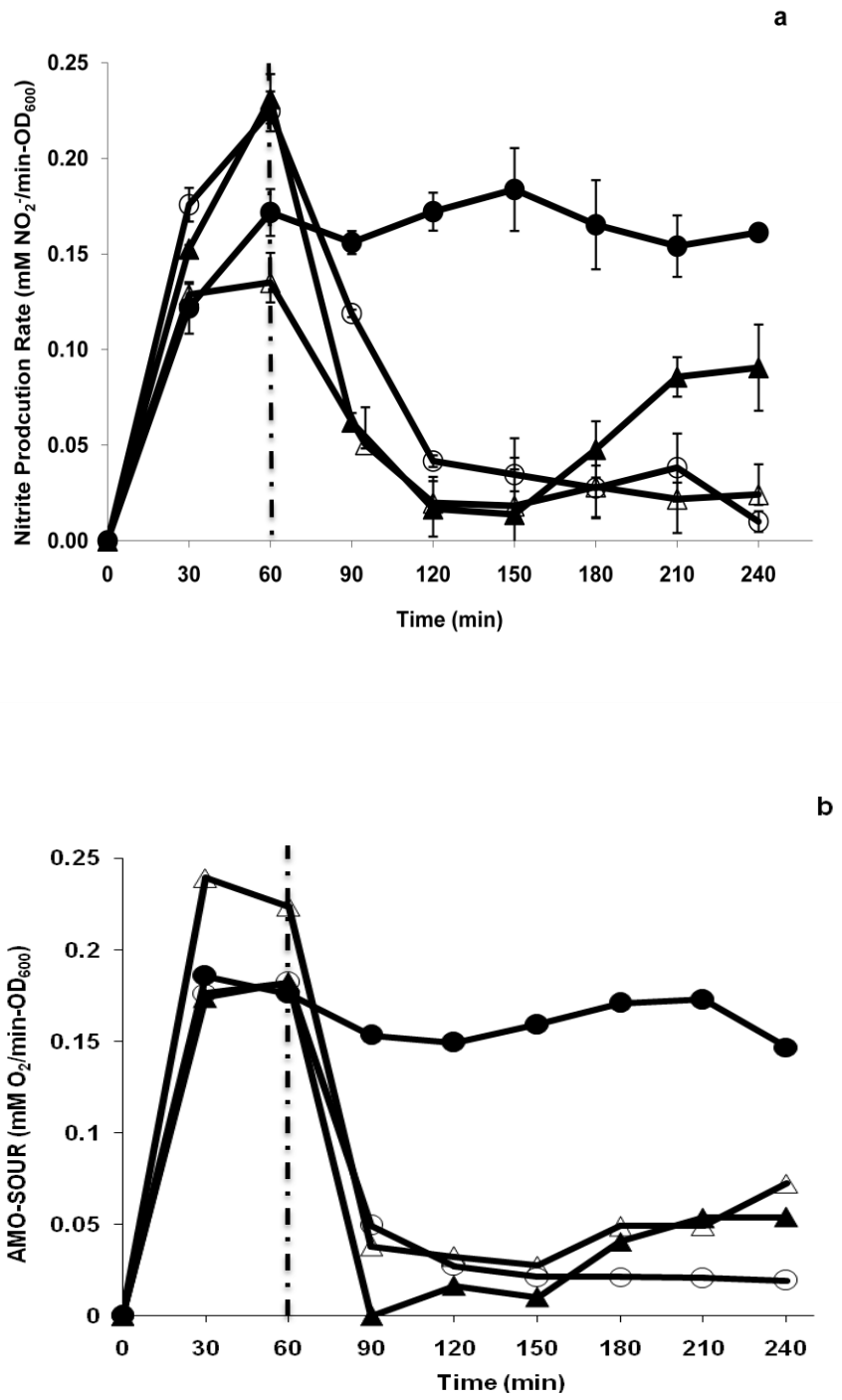


Fig. 3-1. *N. europaea* in nitrite production rate (a) and AMO activity (b) under 1 μM $CdCl_2$, 6 μM $HgCl_2$, or 8 μM $CuCl_2$ treatments for 4 hours. Nitrite production rate and AMO activity were normalized by cell density. Closed circles, open circles, closed triangles, and open triangles represent control condition, 8 μM $CuCl_2$, 6 μM $HgCl_2$, and 1 μM $CdCl_2$, respectively. The error bar indicates 95% confidence interval.

Transcriptomic responses to Cd^{2+}.

Using microarrays, we determined the global transcriptional changes of *N. europaea* when exposed to Cd^{2+}. The analysis revealed that 1 μM $CdCl_2$ caused significant changes (>2-fold) in the transcript levels of 116 genes. The genes included 66 genes with known functions (39 up-regulated and 27 down-regulated), 21 open reading frames with no known functions (12 up-regulated and 9 down-regulated), and 29 intergenic regions (15 up-regulated and 14 down-regulated). The up- or down-regulated genes with known function were grouped by functional classification based on the *N. europaea* genome database (http://genome.ornl.gov) (Tables 3-1 and 3-2). Among these, the mercury resistance genes and two genes upstream of that operon were up regulated more than 100-fold. Various genes involved in coenzyme metabolism, translation, DNA replication, and nucleotide transport showed intensities slightly above 2-fold over the control. The arrays also showed 50 down-regulated genes slightly above 2-fold and include genes with function in signal transduction mechanisms and cell processes. Interestingly, the membrane-bound metallopeptidase encoded by NE2218 showed about 80-fold down regulation in response to Cd^{2+} toxicity exposure, perhaps to prevent cytotoxicity by self-digestion when the cells slow down their metabolism.

To confirm the expression changes observed in the microarrays selected genes were analyzed by qRT-PCR. The fold changes measured by qRT-PCR were consistent and in agreement with the fold changes in the microarrays (Fig. 3-2).

Table 3-1. Selected up-regulated genes under Cadmium stress

NE gene no./name	Gene function	Fold change	P value (<0.05)
Mercury resistance pathway			
NE0838/ *merD**	Bacterial regulatory protein (MerR family)	107.4	1.9×10^{-5}
NE0839/ *merA**	Mercuric reductase	296.7	9.1×10^{-6}
NE0840/*merC**	Putative mercury transport protei	171.8	9.2×10^{-5}
NE0841/*merP**	Mercury scavenger protein	438.6	9.6×10^{-7}
NE0842/*merT**	Mercuric transport protein	370.3	4.8×10^{-5}
Inorganic ion transport mechanism			
NE0852/*yvgQ*	Nitrite and sulfite reductase	2.0	2.1×10^{-3}
Efflux pumps			
NE1640/*czcC*	Outer membrane efflux protein	2.4	1.4×10^{-3}
Oxidative stress			
NE1034/*trxA*	Thioredoxin domain-containing Protein	2.1	2.6×10^{-2}
ABC transporter			
NE1899*	ATPase component ABC-type transport system	2.4	2.5×10^{-3}
Coenzyme metabolism			
NE0856	FAD biosynthesis	2.0	4.9×10^{-3}
NE0634/*cobO*	Cobalamine biosynthesis	2.7	2.8×10^{-4}

NE0636	Outer membrane cobalamin receptor protein	2.0	2.6×10^{-4}
Posttranslational modification, protein turnover, chaperones			
NE0221	Organic radical activating enzymes	2.1	3.3×10^{-4}
NE2206/*ppiD*	peptidyl-prolyl isomerase	2.0	8.0×10^{-3}
Cell evelope biosynthesis			
NE0378	Sugar transferases involvec lipopolysaccharide synthesis	2.1	4.7×10^{-4}
NE2279/*yccZ*	Periplasmic protein involve polysaccharide export	2.0	2.6×10^{-4}
Translation, ribosomal structure and biogenesis			
NE2072/*gatA*	Amidase:Glutamyl-Trna (Gln) Amidotransferase A subunit	2.9	3.0×10^{-4}
NE2073/*gatB*	gatB: glutamyl-Trn amidotransferase, B subunit	2.1	4.7×10^{-4}
NE0389/*rnpA**	Rnase P protein component	2.4	2.6×10^{-4}
NE1457	Ribonucleases G and E	2.4	9.2×10^{-5}
NE2363/*glnS*	Glutamyl- and glutaminyl-Trna Synthetases	2.1	3.3×10^{-4}
DNA replication, recombination and repair			
NE0835/*tnpA*	Transposase and inact derivatives TnpA family	25.5	9.1×10^{-6}

NE0836/*tnpR*	Site-specific recombinases DNA	36.7	3.0×10^{-6}
NE0837*	Domain of unknown function 2	157.7	1.4×10^{-5}
NE2207/*hupB*	Bacterial histone-like DNA b protein	2.1	2.5×10^{-5}
Transcription			
NE2324/*rnc*	dsRNA-specific ribonuclease	2.4	1.4×10^{-4}
NE1035	Transcription termination factor	2.6	2.5×10^{-3}
NE0854/*cysB*	Transcriptional regulator	2.2	5.0×10^{-3}
NE0951	Predicted transcriptional Regulators (MerR family)	2.0	1.2×10^{-3}
Amino acid transport and metabolism			
NE1005/*argB*	Acetylglutamate kinase	2.7	3.4×10^{-4}
NE0872/*hisD*	Histidinol dehydrogenase	2.1	5.0×10^{-3}
Nucleotide transport and metabolism			
NE0277	Xanthosine tripho pyrophosphatase	2.2	1.4×10^{-3}
Carbohydrate transport and metabolism			
NE1691	phosphogluconolactonase/ Glucosamine-6-phosphate isomerase/deaminase	3.8	6.2×10^{-4}
Fatty acid biosynthesis			
NE1646	Fatty acid synthesis	2.1	3.1×10^{-4}
Signal transduction mechanism			
NE0848	Phosphoglycerate mutase family	2.1	3.1×10^{-4}

Others

NE1176*	Peptidoglycan binding	2.6	3.0×10^{-4}
NE0090	Predicted ATPase	2.0	2.6×10^{-4}
NE2325	Transmembrane protein	2.4	5.1×10^{-5}
NE2326/*lepB*	Signal peptidase I	2.0	3.0×10^{-4}
NE0218/*tolB*	Periplasmic component of the Tol biopolymer transport system	2.0	5.3×10^{-4}

* Commonly up-regulated genes under Cd^{2+} and Zn^{2+} treatment.

Table 3-2. Selected down-regulated genes under Cadmium stress

NE gene no./name	Gene function	Fold change	P value (<0.05)
Inorganic ion transport mechanism			
NE0730	Ferric uptake regulator family	2.6	4.7×10^{-2}
NE0731	TonB-dependent receptor protein	2.4	4.4×10^{-2}
NE0999	phosphate transport system permease protein	3.5	2.4×10^{-2}
NE1000	ABC-type phosphate transport system permease component	2.9	4.6×10^{-2}
NE1001/*pstB*	phosphate transport system ATP-binding protein	2.7	2.4×10^{-2}
NE1531*	TonB-dependent receptor proteir	2.6	2.4×10^{-2}
NE0345	Acriflavin resistance protein: Heavy metal efflux pump CzcA	5.9	2.4×10^{-2}
RubisCO			
NE1918/*cbbO**	von Willebrand factor type A domain	7.3	2.4×10^{-2}
NE1919*	nitric oxide reductase NorQ protein	5.7	2.4×10^{-2}
Cell processes			
NE2290	Bacterial type II secretion systen protein E:GAF domain	2.0	4.0×10^{-2}

NE1298*	TPR (tetratricopeptide) repeat	2.2	4.0×10^{-2}
NE2315/*pilN*	putative type 4 fimbrial biog protein	2.2	2.7×10^{-2}
NE2488/*flhA*	Bacterial export FHIPEP family	2.1	2.4×10^{-2}
NE0346	possible cation transporter transmembrane protein	5.2	2.4×10^{-2}
NE2218*	Membrane-bound metallo-peptidase	79.78	2.7×10^{-2}
NE1538	Chromosome segregation ATPases	2.2	2.4×10^{-2}
Transcription			
NE2435/ *fecI*	Specialized sigma subunits of RNA polymerase	2.2	2.4×10^{-2}
NE1217	sigma-70 factor, ECF subfamily	2.4	2.4×10^{-2}
NE0533	sigma-70 factor, ECF subfamily	2.3	2.4×10^{-2}
NE1452	Transcriptional regulator	2.1	4.7×10^{-2}
NE0787*	Response regulator containing a CheY-like receiver domain and an HTH DNA-binding doma	2.1	2.7×10^{-2}
Signal transduction mechanism			
NE1923/*cheY**	Response regulator receiver domain	3.0	2.7×10^{-2}
NE0534	transmembrane sensor	2.2	2.7×10^{-2}
Posttranslational modification, protein turnover, chaperones			

NE1529	signal peptide protein	2.0	2.7×10^{-2}
Others			
NE0315/*mnxG**	possible multicopper oxidase	2.1	4.0×10^{-2}
NE2038*	Myeloperoxidase, thyroid peroxidase, cyclooxygenase catalytic domain	2.2	4.4×10^{-2}
NE0353/*exbB1*	MotA TolQ ExbB proton channel family	2.3	2.7×10^{-2}
NE1545	Pirin-related protein	3.0	2.4×10^{-2}

* Commonly down-regulated genes under Cd^{2+} and Zn^{2+} toxicity.

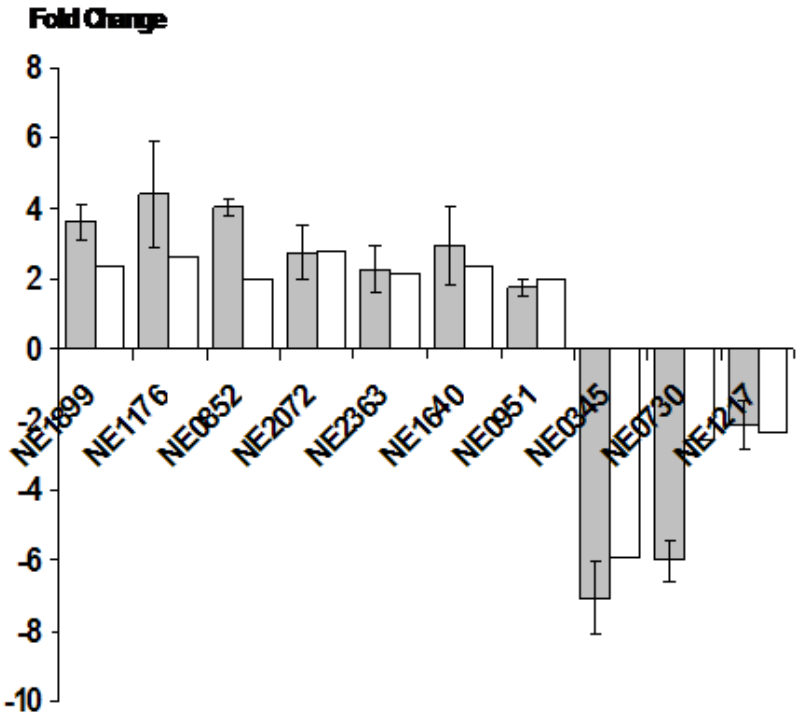


Fig. 3-2. Comparison of mRNA expression level of selected up- or down-regulated genes determined by qRT-PCR (black bars) and microarray (white bars). The positive value represents up regulation and negative value represents down regulation. Error bars represent standard error of the mean.

Expression of *merA* and *amoA* under Cu^{2+} and Hg^{2+} toxicity.

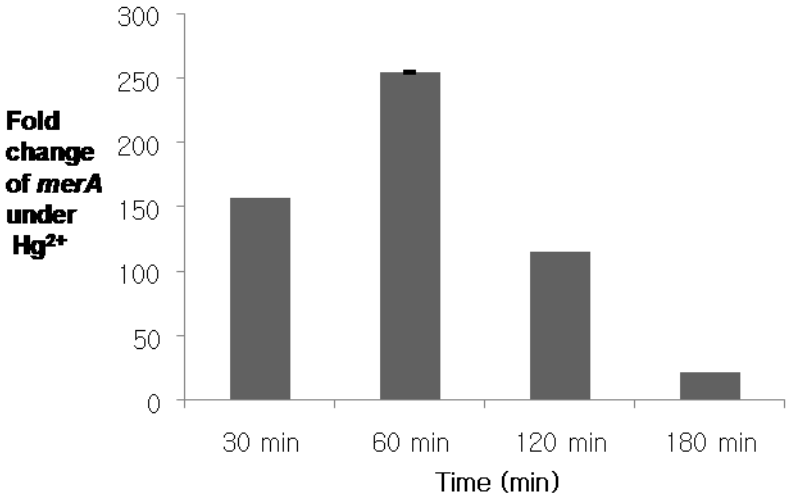
Because 297-fold up regulation of *merTPCADE* was observed in the Cd^{2+} treatment in this work and 46-fold up regulation was observed in similar work with Zn^{2+} (76), we examined whether *merTPCADE* is commonly up regulated when *N. europaea* is exposed to other heavy metals. We tested the time-dependent transcriptional response of *merA* to 6 μM $HgCl_2$ and 8 μM $CuCl_2$ using qRT-PCR. The transcript level of *merA* increased 150-fold in the first 30 min in response to Hg^{2+} and continued to increase up to 250-fold up regulation (Fig. 3-3a). Consistent with detoxification, during the recovery of nitrification activity the transcript levels of *merA* decreased to 21-fold up regulation in the span of 3 hr (Fig. 3-3a). In

contrast, the transcript level of *merA* did not show a significant change in response to Cu^{2+} (Fig. 3-3b) at levels that inhibited nitrification (Fig. 3-1), perhaps because *merA* cannot confer resistance to this metal by transforming it to a less toxic form.

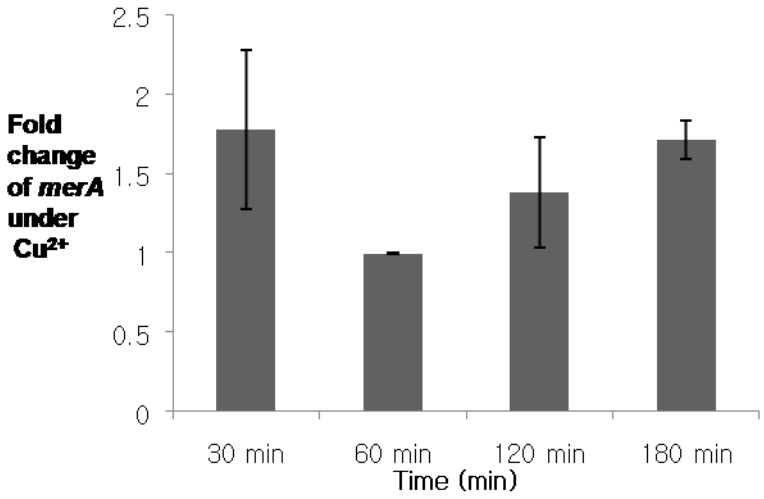
We also examined the time-dependent transcriptional response of *amoA* under Cu^{2+} stress (Fig. 3-3d). The transcript level of *amoA* increased 2-fold, possibly uptaking some of the Cu^{2+} in the first 60 min, but decreased 2-fold by 180 min. Excessive amounts of Cu^{2+} increased the transcript level of *amoA* in the early stages of the incubation, but this did not translate into more AMO activity. This indicates that Cu^{2+}, although essential for AMO activity (23), can be detrimental even at relatively low concentrations.

We also tested the transcriptional response of *amoA* under Hg^{2+} stress in a similar experimental design. *N. europaea* exposed 30 min to 6 μM $HgCl_2$ almost lost all AMO activity and, consequently, NO_2^- production (Fig. 3-1b), but as the transcript level of *merA* increased by 1 hr, the AMO activity and nitrite activities started to increase. This was reflected in the transcript level of *amoA*, which did not change during the first 30 min of the incubation, but the *amoA* transcript level increased 4-fold once the cells started to recover (Fig. 3-3c). This suggests that *merA* is involved in detoxification of Hg^{2+}, likely by helping to reduce the cation to the less toxic Hg^0, allowing the cells to recover (5, 36).

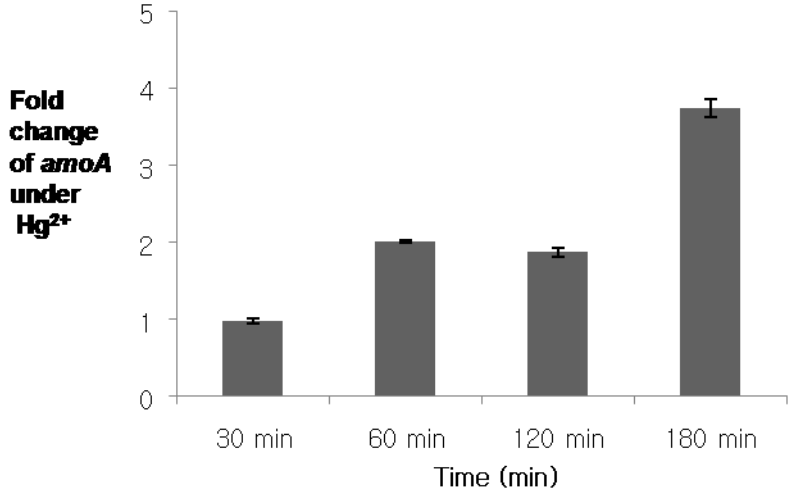
a


Fold change of merA under Hg2+
300
250
200
150
100
50
0
30 min
60 min
120 min
180 min
Time (min)

b


Fold change of merA under Cu2+
2.5
2
1.5
1
0.5
0
30 min
60 min
120 min
180 min
Time (min)

c


Fold change of amoA under Hg2+
5
4
3
2
1
0
30 min
60 min
120 min
180 min
Time (min)

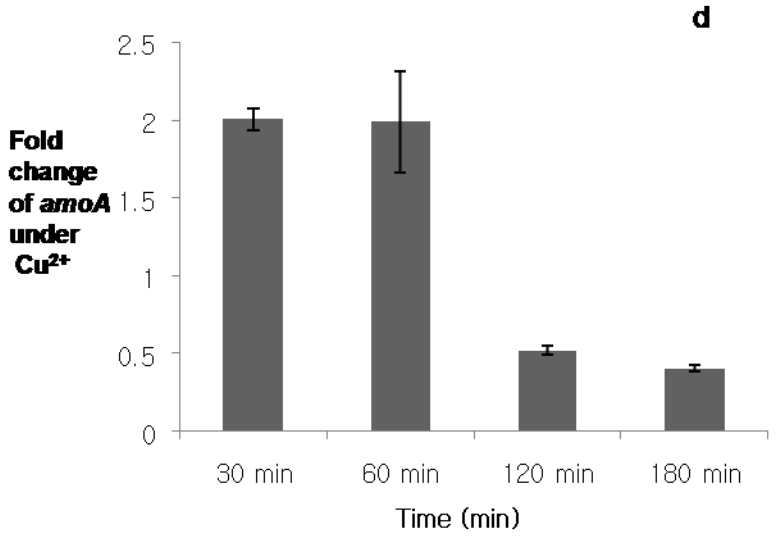


Fig. 3-3. Expression change of *merA* and *amoA* determined by qRT-PCR under Hg^{2+} and Cu^{2+} treatment. Error bars indicate 95% confidence interval.

Proteomic responses to Cd^{2+}.

To determine the changes in protein expression we used 2D SDS-PAGE, using protein extracts from control and Cd^{2+}-exposed cells (1 μM $CdCl_2$) taken after a 3 hr incubation. Several proteins showed higher intensity in the Cd^{2+}-exposed cells than in control cells. The differentially expressed proteins were excised from the gels for identification by nano/LC/MS/MS and to deduce the genes that encode them. Proteins over expressed were nitrite reductase, encoded by NE0924, Rieske iron-sulfur protein, encoded by NE1503, and two hypothetical proteins encoded by NE2057 and NE1752 (Fig. 4). Interestingly, in microarray experiments (1 hr incubation), the transcript levels of the genes NE0924, NE1503, NE2057, and NE1752 corresponding to the over expressed peptides (3 hr incubation) did not show detectable increases. Similar discrepancies between transcript and protein levels have been seen in studies with human cells (12) and with *Plasmodium falciparum* (54). Post-transcriptional splicing and post-translation modifications have been suggested as possible reasons for different

responses in transcription and translation (12, 54). In our experiments, microarray data showed up regulation of the genes that encode posttranslational modification proteins, NE0221 and NE2206, consistent with the possibility that posttranslational modifications may have been important in cellular responses to Cd^{2+} toxicity.

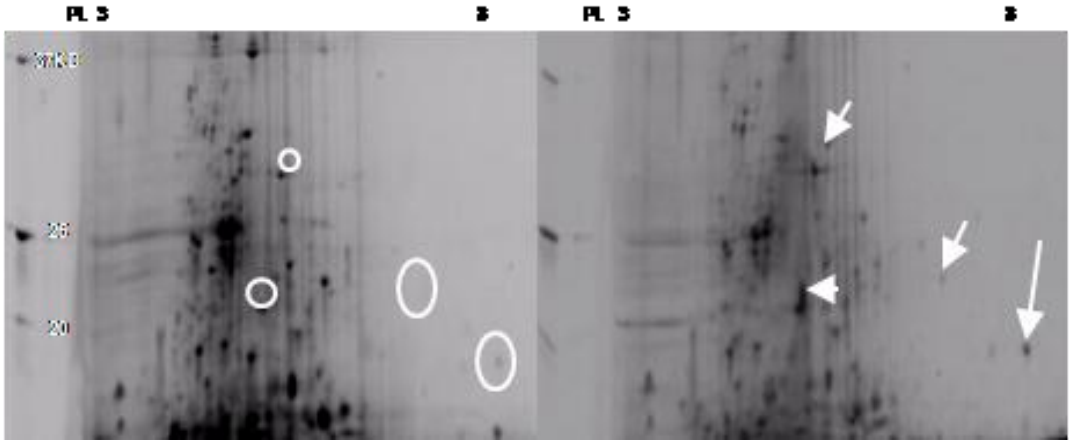


Fig. 3-4. Comparison in 2-D SDS-PAGE of soluble protein fraction of *N. europaea* treated without (left) and with 1 μM $CdCl_2$ (right) for 3 hours. Circles represent absent or low translation under control condition. Arrows represent protein spots up regulated under cadmium stress.

DISCUSSION

Expression of *merTPCADE* under Cd^{2+} toxicity.

Of the 66 up-regulated genes detected in the microarrays of cells exposed to 1 μM $CdCl_2$, the highest up-regulated genes (more than 100-fold) were those encoding mercury resistance proteins (*merTPCADE*). This observation is consistent with higher transcript levels of the *merTPCADE* operon seen previously in Zn^{2+}-exposed cells (76), suggesting that the mercury resistance operon in *N. europaea* may play an important role in protecting the cell from toxic heavy metals. In addition, the transposase encoded by *tnpA* (NE0836) and the resolvase encoded by *tnpR* (NE0836) were up regulated under Cd^{2+} (31-fold) and Hg^{2+} (54-fold) stress,

but not under Zn^{2+} and Cu^{2+} stress (data now shown). The transposase and resolvase are thought to form a transposase-related protein, known as mercury resistance transposon (65, 66), that may increase antibiotic resistance in gram-negative facultative bacteria (58). In *N. europaea*, *tnpA* and *tnpR* are located upstream of the operon *merTPCADE* (Fig. 3-5). The role of the mercury resistance transposon with respect to heavy metal toxicity in *N. europaea* remains unclear, as we did not observe increased metal tolerance or adaptation in our experiments. However, we can suggest that the up regulation of *merTPCADE* and *tnpAR* may act in concert when exposed to highly toxic heavy metals (e.g., Cd^{2+} and Hg^{2+}), but not when exposed to less toxic metals (e.g., Zn^{2+} and Cu^{2+}). Cd^{2+} may enter into the periplasm through unspecific transport proteins. The *merTPC* then could transport Cd^{2+} into the cytoplasm to reduce toxicity effects in the periplasm (70). The common function of the protein encoded by *merA* (glutathione reductase) is thought to be a reductase for Hg^{2+}. When the glutathione reductase in the cell cannot reduce the metal cations (e.g. Cd^{2+}), the enzyme then would likely reduce the oxidized bis-glutathione (GS-SG) that is formed by the metal and that is toxic to the cell, thus affording some protection from the metal (70). We also observed 2.4-fold up regulation of NE1640, which putatively encodes CzcC, the outer membrane protein of the CzcCBA efflux pump thought to be involved in Cd^{2+} detoxification in *Alcaligenes eutrophus* and other Gram negative bacteria (70). However, because *N. europaea* did not recover from Cd^{2+} treatment as they did from Hg^{2+} treatment, a role for this gene in the removal of Cd^{2+} seems unlikely. The accumulation of Cd^{2+} in the cytoplasm would eventually become deleterious to the cell and inhibit nitrification completely.

Fig. 3-5. Organization of *merR, merTPCADE* and transposase-related genes (NE0837/0836/0835) in *N. europaea*. Arrows indicate the orientation of the genes.

Shared up- or down-regulated genes in Cd^{2+} and Zn^{2+} treatment.

Several genes that may be related to detoxification of heavy metals showed transcript level changes with Cd^{2+} or Zn^{2+} exposure. NE1176, encoding peptidoglycan binding protein, known to interact with integral outer membrane proteins (31), was up regulated 2.4 to 2.6-fold. A similar observation has been reported with *Caulobacter crescentus* exposed to the Cd^{2+} and Cr^{2+} (38). Other up-regulated genes included NE1899, encoding an ATPase component ABC-type metal transporter or an arsenite resistance protein (70), and NE0389, encoding an Rnase P protein that processes a precursor of transfer RNA (37). Genes that were down regulated included NE2218, encoding a membrane-bound metallopeptidase (80-fold under Cd^{2+} and 8-fold under Zn^{2+}), which requires a low amount of a transition metal such as Zn^{2+} or Co^{2+} in its active site (47). The genes *cbbQO* (NE1919/1918), encoding ribulose bisphosphate carboxylase/oxygenase (RubisCO), were down regulated in both Zn^{2+} and Cd^{2+} treatments. Carbon sequestration by RubisCO is an energy intensive process (93). Under starvation conditions in *N. europaea* (95) and under Cu^{2+} stress in *Nitrosococcus mobilis* (81), genes encoding RubisCO were down regulated, probably to conserve energy for NH_3^+ metabolism.

Heavy metals cause oxidative stress in Gram-negative bacteria (70), and in this work, the microarrays detected changes in transcript levels of genes

encoding proteins associated with oxidative stress. Myeloperoxidase (MPO), encoded by NE2038, is thought to be a reactive oxygen species (ROS)-generating enzyme (45) and was down regulated, perhaps to reduce oxidative stress under heavy metal toxicity. NE0315 (*mnxG),* encoding a multicopper oxidase known to catalyze Mn^{2+} oxidation, was down regulated under the Zn^{2+} and Cd^{2+} treatments, again perhaps to reduce oxidative stress (20). Genes that were also down regulated under both Zn^{2+} and Cd^{2+} stress include NE1298, encoding tetratricopeptide repeat (TPR), involved in protein-protein interactions (59), NE1531, encoding TonB-dependent protein, and NE0787, encoding CheY protein. NE1923, encoding CheY (a flagellar protein) in *N. europaea*, was previously seen to be down regulated in response to NO, as NO promoted the formation of biofilm and mobility was no longer necessary (83). It could be that the expression of CheY decreases during metal stress to promote the formation of cell agglomerates to protect some of the cells from further exposure.

Table 3-3. Up regulation of two-component signal transduction systems genes and DNA repair genes

Locus tag	Description	Fold Change[1]	P value (<0.05)	Fold change[2]
Two-component signal transduction systems				
NE0728	Sensory transduction histidine kinase	2.3	8.9×10^{-3}	
NE0729	Response regulators consisting of CheY-like receiver domain and a winged helix DNA-binding domain	3.3	6×10^{-3}	1.8
DNA repair				
NE2552/*mutM, fpg*	Formamidopyrimidine-DNA glycolase	2.2	2.5×10^{-2}	2.4
Translation				
NE0389	Rnase P protein component	2.4		
NE2143/*rpsD*	Ribosomal protein S4	2.1	8.9×10^{-3}	2.5

1 and 2 represent the fold change in microarray and qRT-PCR, respectively

Candidate genes to detect Cd^{2+} stress.

Of the *N. europaea* genes that were up or down regulated under Cd^{2+} treatment, some potentially may serve as specific indicators of Cd^{2+} exposure. Because Cd^{2+} causes oxidative stress by producing reactive oxygen species that deplete glutathione and protein-bound sulfhydryl groups (70), the up regulation of NE1034, encoding thioredoxin (disulfide reductase) (38) could help the cell to resist oxidative stresses. NE1005 (*argB)* and NE0872 (*hisD*), encoding an amino acid transport, were up regulated under Cd^{2+}. Biosynthesis of amino acids would use more energy than their uptake through an amino acid transport, therefore these

genes might be up regulated to conserve energy in a toxic environment (91). NE0221, encoding an organic radical-activating enzyme, and NE2206 (*ppiD*), encoding a peptidyl-prolyl isomerase that belongs to a posttranslational modification protein, showed up regulation under Cd^{2+} treatment (*ppiD* was also up regulated under chloroform treatment (34)). NE2324 (*rnc*), NE1035, NE0854 (*cysB*), and NE0951, which relates to transcription, showed up regulation, while NE2435 (*fecI*), NE1217, NE0533, NE1452, and NE0787 showed down regulation (Table 3-2). Transcript of NE1217, that belongs to the ECF family, was also increased in response to chloroform toxicity (34). NE0835, NE0836, NE0837 and NE2207 (*hupB*), involved in DNA replication, recombination, and repair, showed up regulation. NE0378 and NE2279 (*yccZ*), involved in cell envelope biosynthesis, were up regulated, while six genes in the groups were down regulated to statistically significant degrees (Table 3-2). Although the role(s) of these genes in heavy metal stress was/were not clear, they could be important for finding heavy metal toxicity mechanisms or general stress genes in the future.

SUMMARY

In this work, *N. europaea* showed decreasing toxicity effects in the order: $Cd^{2+} > Hg^{2+} > Zn^{2+} > Cu^{2+}$. In addition, we confirmed that mercury resistance genes (*merTPCADE*) were strong stress response genes of *N. europaea* exposed to Zn^{2+}, Cd^{2+}, and Hg^{2+}, but not to Cu^{2+}. We also observed that *N. europaea* cells could recover quickly from Hg^{2+} exposure, but that the same was not true for cells exposed to Cd^{2+}, even though both Hg^{2+} and Cd^{2+} exposure led to very high up regulation of the mercury resistance genes. Since this is the first report of *merA* of

N. europaea as a common stress gene under Zn^{2+}, Cd^{2+}, and Hg^{2+}, the promoter related to expression of mercury resistance genes may provide a basis to design biosensors to monitor *N. europaea* exposure to these heavy metals in biological wastewater treatment processes and to reflect the overall nitrification process.

ACKNOWLEDGEMENTS

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Chapter 4

Whole-Genome Transcriptional and Physiological Responses of *Nitrosomonas europaea* to Cyanide: Identification of Cyanide-Stress Response Genes

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ABSTRACT

Nitrosomonas europaea (ATCC 19718) participates in the biological removal of nitrogen from wastewater by oxidizing ammonia to nitrite, the first step in nitrification. Because nitrifying bacteria are particularly sensitive to cyanide, a compound often encountered in wastewater treatment plants, we characterized the physiological and transcriptional responses of *N. europaea* cells to cyanide. The cells were extremely sensitive to low concentrations of cyanide, with NO_2^- production and ammonia-dependent oxygen uptake rates decreasing by 50% within 30 min of exposure to 1 μM NaCN. Whole-genome transcriptional responses of cells exposed to 1 μM NaCN were examined using Affymetrix microarrays to identify stress-induced genes. The transcript levels of 35 genes increased more than 2-fold while transcript levels of 29 genes decreased more than 20-fold. A gene cluster that included *moeZ* (NE2353), encoding a rhodanese homologue and thought to be involved in detoxification of cyanide, showed the highest up regulation (7-fold). The down-regulated genes included genes encoding proteins involved in the sulfate reduction pathway, signal transduction mechanisms, carbohydrate transport, energy production, coenzyme metabolism, and amino acid transport.

Keywords: *Nitrosomonas europaea*, cyanide, microarray, qRT-PCR, *moeZ*, sulfate reduction pathway

INTRODUCTION

Cyanide is a known inhibitor of the respiratory processes in nitrifying activated sludge (35, 49) and to pure cultures of *Nitrosomonas europaea* (62). Cyanide inhibits respiration by binding to heme P460 of the hydroxylamine oxidoreductase (HAO). Free cyanide, in the form of HCN or CN^-, is more toxic to nitrification processes than thiocyanate and metal-cyanide complexes (50, 51). Cyanide is used in electroplating, mining, steel industries, and chemical industries (51). Potassium and sodium cyanide are mainly used in industrial operations and in the recovery of gold and silver from ores (17). Cyanide can also be formed in flue gas scrubber water in wastewater treatment plants (WWTPs) that incinerate sludge (24). When the scrubber water is routed to the WWTPs for treatments, a potentially problematic cyanide loading can occur. Although cyanide is extremely toxic to nitrification, relatively little is known of its effects on ammonia-oxidizing bacteria at the molecular level.

Ammonia oxidizing bacteria (AOB), such as *N. europaea*, obtain all reductant for energy and biosynthesis through oxidizing ammonia to nitrite and play a key role in the global nitrogen cycle. Autotrophic AOB use CO_2 as their sole carbon source (13). Ammonia oxidation is a two-step reaction catalyzed by ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) (4).

N. europaea is extremely sensitive to various environmental conditions such as pH shifts (49, 88) and ammonia concentration (3). The slow growth rate and sensitivity to various inhibitors of *N. europaea* are considered crucial factors in the effectiveness of the nitrification processes (60). The inhibition of nitrification in WWTPs can result in high ammonia discharge and eutrophication of receiving bodies of water (24). Therefore, major attention has been aimed at developing a

sensitive and rapid method to detect and identify the source of nitrification inhibition (60). “Specific-stress genes” expressed in the presence of specific inhibitors have the potential to serve as diagnostic and prognostic tools in water reclamation schemes. Mercury resistance genes expressed under heavy metal stress (75) and NE2571, encoding for metallo-β-lactamase, expressed under aliphatic hydrocarbons stress (33), are examples of specific-stress genes in *N. europaea.*

In this work, we examined the impact of cyanide on nitrite production rates and oxygen uptake rates by *N. europaea* in batch reactors. We also examined global transcriptomic responses of *N. europaea* exposed to cyanide at concentrations causing 50% inhibition of NO_2^- production rates. Based on the known toxicity mechanism of cyanide on cytochrome c-554, the electron transporter of HAO to cytochrome c-552 in *N. europaea* (1), the transcriptional changes in genes encoding HAO were monitored using quantitative real-time PCR (qRT-PCR). In this study a gene cluster that included *moeZ*, encoding a rhodanese, showed the highest up regulation, suggesting a role in cyanide detoxification (19). In addition, 8 genes encoding TonB-receptor proteins and 3 genes encoding ECF $\tilde{\sigma}$factors showed up regulation under cyanide stress. Genes encoding proteins for sulfate reduction, ribosomal subunit proteins, and carbohydrate transport were down-regulated.

MATERIALS AND METHODS

Cyanide inhibition studies in batch reactors.

The cyanide inhibition studies were performed in batch reactors with *N. europaea* cells in the presence of 5 mM ammonia with and without 1 μM NaCN. *N.*

europaea cells were grown in batch cultures in media containing 25 mM $(NH_4)_2SO_4$, as previously described (42), and harvested in mid to late exponential phase ($OD_{600} \cong 0.07$). The cells were washed two times with 40 mM NaH_2PO_4 (pH 7.8) and evenly divided into two 2 L glass Kimax media bottles, one treatment and one control, containing 1 L of fresh growth media with 2.5 mM $(NH_4)_2SO_4$ (pH 7.8). The bottles were sealed tightly with stainless steel caps fitted with stainless steel tubing and a rubber septum for sampling from the liquid phase. The cell suspensions were aerated by stirring with magnetic bars at 700 rpm on a Wheaton Biostir plate (Wheaton Science Products, Millville, NJ). When the cells reached pseudo-steady state (~1 h), cyanide was injected into the treatment reactor bottle. The ammonia-dependent oxygen uptake rate (AMO-SOUR), hydrazine-dependent oxygen uptake rate (HAO-SOUR) and NO_2^- production rate were tested every 30 min during the 4 h incubation (76). The cell density (OD_{600}) was monitored every 30 min to normalize the data and did not show a significant increase during the 4 h experiment. All experiments were carried out in triplicate.

Determination of NO_2^- concentration and respiration rate.

The NO_2^- concentration was determined from a 1 ml aliquot drawn from the batch reactors, after centrifugation to sediment the cells, as previously described (43, 76). AMO-SOUR was measured using a YSI 5331 Oxygen Probe (Yellow Springs Instruments, Yellow Springs, OH) in a 1.8 ml glass water-jacketed reaction vessel at 30^oC connecting a heated circulating water bath (22). HAO-SOUR was detected by inhibiting AMO-SOUR with 100 μM allylthiourea, followed by the addition of 750 μM hydrazine, an alternative substrate for HAO. The specific oxygen uptake rates of the cells were calculated based on the saturated oxygen

concentration (7.54 mg/l at 30^{o}C) in water (22).

Determination of CN^- concentration.

Free cyanide concentrations was measured using Cyanide vacu-vials® (CHEMetrics, Inc., Calverton, VA) following the manufacturer's instructions. The equation for the regression line (standard curve) derived with 0.5, 1, 2, 4 μM NaCN was y=0.079x, R^2=0.999 (x= μM of free cyanide, y= OD_{600}). This equation was used to determine the concentrations of CN^- in the batch reactor. A 10-ml cell suspension was passed through a 2 μm filter before determining the free CN^- concentrations. The pH of the standard NaCN solution and the samples was adjusted to 11 to displace the equilibrium to the free cyanide form. In the reactors at pH 7.8 more than 95% of NaCN exists as HCN. HCN is readily volatile but it is also totally miscible in water. The HCN Henry's constant (K_H) of $1.7x10^{-3}$ (mg HCN/L air)/(mg HCN/L water) (51) was used to calculate the aqueous phase cyanide concentration.

Affymetrix microarray experiments.

For the microarrays, three control and three cyanide treatment reactors were prepared. *N. europaea* cell cultures were harvested (180 ml at $OD_{600} \cong 0.07$) from the reactors after a 1 h incubation. The cells were washed twice with PBS buffer (0.1 M phosphate buffered saline (pH 7.4)) and resuspended in Trizol (Ambion Inc., Austin, TX) following the manufacturer's instructions to prevent RNA degradation. Total RNA was extracted and purified with an RNeasy Mini kit (Qiagen Inc., Valencia, CA). The quality and quantity of the RNA samples were measured using a RNA 6000 Nano LabChip kit on an Agient Bioanlyzer 2100 (Agilent Tech. Inc.,

Palo Alto, CA). Nimble express™ Made-to-Order arrays (NimbleGen Systems, Inc.) with all 2460 annotated *N. europaea* genes represented were used. cDNA synthesis, labeling, and hybridization were carried out by the Center for Genome Research and Biocomputing Core Laboratories at Oregon State University. The microarray data were analyzed by "log of ratio" mode in GeneSpring software (version 7.2, Silicon Genetics). The treatment/control ratios of transcript levels were used to identify genes that were up regulated or down regulated more than 2-fold under cyanide treatment, applying the unpaired two sample t-test with a cutoff p-value of 0.05. The microarray data are available at the Gene Expression Omnibus database (http://www.ncbi.nih.gov/geo, accession number GSE10664).

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was used to evaluate the transcriptional levels of selected up- or down-regulated genes identified from the microarray data. The primers were designed using Vector NTI Advance 10 and manufactured commercially (Invitrogen, Carlsbad, CA). cDNA was synthesized from 1 μg of total RNA extracted from control cells and cells exposed to 1 μM NaCN using the IScript™ cDNA synthesis kit (BIO-RAD, Hercules, CA). qRT-PCR was performed in triplicate on an ABI 7500 instrument (Applied Biosystems, Foster city, USA). A 25 μl of iTaq SYBR Green Supermix with ROX (BIO-RAD), 5 μM forward/reverse primers, and 60 ng of cDNA were mixed and filled to 50 μl with nuclease water. The following cycle conditions were used for qRT-PCR; 2 min at 95°C, then 50 cycles at 95°C for 30 s, 55°C for 45 s and 72°C for 45 s. To monitor quality of the reaction, dissociation curves of the qRT-PCR-products were generated (starting temperature at 60°C, then raising the temperature by 0.5°C every 20 s to a final

temperature of 95^{o}C). qRT-PCR efficiency was determined using standard curves created by serial dilution of RNA samples. The fold changes to show ideal amplification efficiency were calculated using the formula $2^{-\Delta\Delta Ct}$, normalizing with 16S RNA (77).

RESULTS AND DISCUSSION

Ammonia oxidation inhibition by cyanide.

N. europaea cells in batch-reactors with fresh media containing 5 mM total ammonia reached a pseudo-steady state of about 0.18 mM NO_2^-/min-OD_{600} in 1 h (Fig.4-1a). In the control reactors, the pseudo-steady state continued for 4 h without limitation of ammonia or oxygen. After 4 h, based on 2.5 mM accumulated NO_2^-, the residual ammonia concentration in the reactors was 2.5 mM, 50% of the initial ammonia concentration. When *N. europaea* were exposed for 3 h to 1 or 2 μM NaCN, the cells showed 58% and 88% inhibition in NO_2^- production rates (Fig.1a), respectively. In this condition, the cells showed 39% or 67% inhibition in AMO-SOUR, respectively (Fig.4-1b) while no significant inhibition was observed in HAO-SOUR (data not shown). The inhibited NO_2^- production rates and AMO-SOUR did not recover during the experiment. A previous work (62) showed that purified HAO of *N. europaea* was inhibited in *in vitro* activity tests, and it was suggested that the inhibition occurred via binding of heme P460 of HAO with free cyanide. In the current work, however, inhibition of HAO activity in whole cells was not observed.

During the incubations the total free cyanide concentration in the treatment batch reactors decreased from 0.95 μM to 0.67 μM. The inhibited NO_2^- production

rate and AMO-SOUR could not recover as the cyanide decreased in the incubations. The experiments show that cyanide is extremely toxic to *N. europaea* and are in agreement with the literature. It is known that levels of free cyanide above 4.23 µM (69) and 7.69 µM (50) inhibit nitrification in activated sludge.

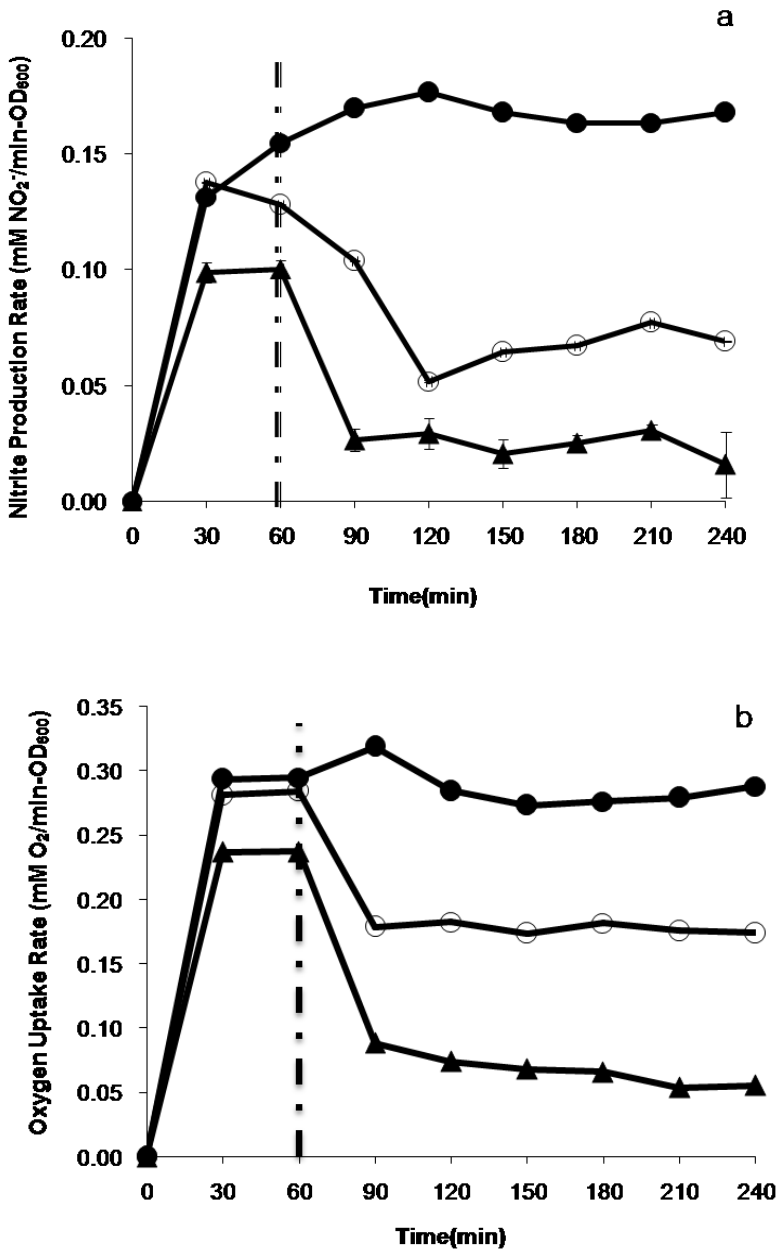

Fig. 4-1. (a) Nitrite production rate of *N. europaea* control cells (●) and treatment cells with 1 µM (○) or 2 µM (▲) NaCN. (b) AMO-SOUR of *N. europaea* control cells (●) and treatment cells with 1 µM (○) or 2 µM (▲) NaCN.

Transcriptomic response of whole-genome of *N. europaea* to cyanide.

Transcriptomic responses of *N. europaea* to heavy metals (76) and chlorinated aliphatic hydrocarbons inhibitors of nitrification (33) showed that the most significant transcriptional changes occurred after a 1 h incubation. To identify general-stress response genes commonly up regulated here and in the previous work, transcriptomic responses in *N. europaea* cells were also evaluated after 1 h of exposure to 1 μM NaCN.

Whole-genome microarrays were used to analyze the mRNA pools from cells exposed to 1 μM NaCN. Of the total 2460 genes, the transcript levels of 35 genes increased more than 2-fold while the transcript levels of 29 genes decreased more than 2-fold. Up-regulated genes included 27 genes that encode proteins with known functions, such as TonB-receptor proteins and σ-factors. The up-regulated genes also included 8 genes that encode proteins with unknown functions and 6 intergenic regions. Down-regulated genes included 25 genes that encode proteins with known functions, such as ribosomal, amino acid transport proteins and energy production. Also down regulated were 3 genes that encode proteins with unknown functions and 10 intergenic regions. In microarray experiments with *N. europaea* cells exposed to Zn^{2+} (76) or Cd^{2+} (75), the transcriptional levels were changed 2.1% and 4.7% in a total of 2460 genes, respectively. In this work, the cells exposed to cyanide showed 3.2% transcriptional change. Therefore, although the functions of the up- or down-regulated genes differed in cells exposed to heavy metals or cyanide, the percentage of transcriptional change was approximately the same.

Validation of the microarray data.

To corroborate the transcript levels observed in the microarray data, 15

genes were selected and analyzed by qRT-PCR. For each of the selected genes the observed fold change using qRT-PCR was consistent and in agreement with the fold change observed in the microarrays (Fig. 4-2). Overall, the fold change of these genes showed relatively higher ratio in qRT-PCR data than in microarray data and all followed the same trend. Therefore, the fold changes seen in the microarrays are approximately correct and in agreement with similar work (33, 76).

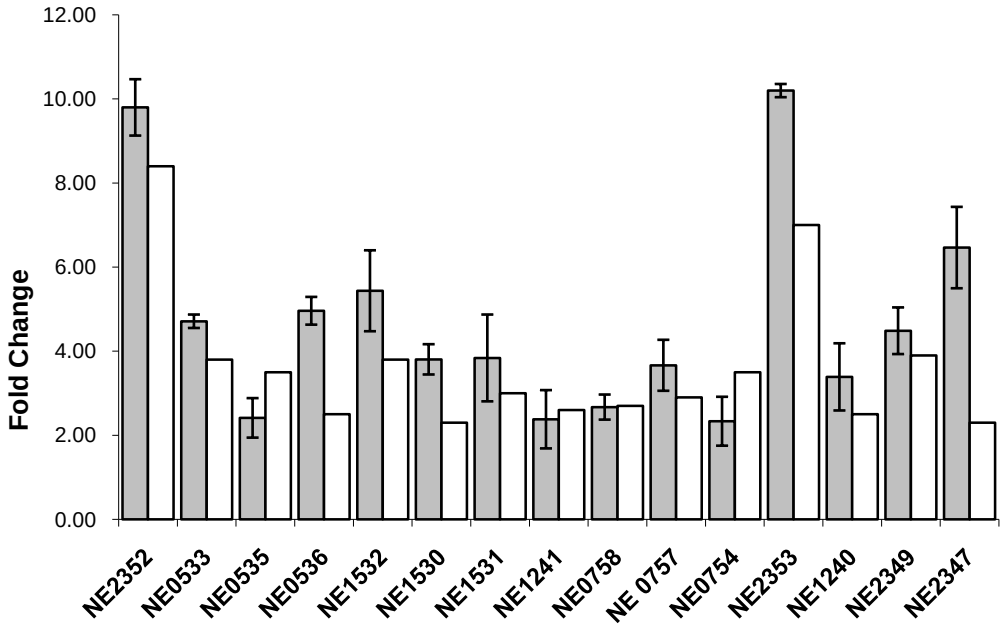

Fig. 4-2. Evaluation of the expression of selected genes by qRT-PCR (black bars) and by microarray (white bars). Error bars represent 95% confidence interval.

Up regulation of *moeZ, amo,* and *hao* genes.

In the microarray analysis of *N. europaea* cells exposed to 1 μM NaCN, the highest up-regulated gene (7-fold) was *moeZ* (NE2353), encoding a rhodanese homologue (19). *moeZ* encodes a c-terminal that contains the active site and is similar to other rhodanese homologues. Rhodanese catalyzes the transfer of sulfur

to free cyanide, the electron acceptor, reducing the free cyanide (CN^-) to thiocyanate (SCN^-) by binding with reduced a sulfur source (17). Since SCN^- is less toxic than CN^-, this mechanism is considered a general detoxification process of cyanide in bacteria and higher organisms (96).

To confirm whether the protein encoded by *moeZ* participates in this detoxification process or not, the time-dependent concentration of cyanide and transcript levels of *moeZ* were measured over the course of the experiment. The cyanide concentration was monitored every 30 min and the transcript levels were determined at 30, 60, 90, 120, and 180 min after exposure to cyanide using qRT-PCR (Fig. 4-3). The transcript level of *moeZ* showed a 12-fold up regulation at 60 min of exposure, comparable to the fold change measured in microarray experiments (7-fold). The transcript of the gene was up regulated 35-fold after 90 min of exposure to cyanide while the cyanide concentration was decreasing from 0.95 μM to 0.68 μM during the experiment. The decreased cyanide concentration and increased *moeZ* expression is consistent with *moeZ*-catalyzed formation of SCN^- from CN^-. In addition, it suggests that *moeZ* is an attractive candidate stress gene to monitor cyanide toxicity in *N. europaea*. However, though the gene likely catalyzes the formation of SCN^-, removing the free cyanide, it was not helpful in recovering from cyanide toxicity. The gene cluster NE2347/48/49/50/51/52, including *moeZ* (NE2353) (Fig. 4-4a), showed up regulation of more than 2-fold upon exposure to cyanide. In the cluster, NE2347 encodes a general substrate transporter, and NE2348 (*fadE1*) and NE2349 (*ydiD*) encode acyl-CoA dehydrogenase and AMP-dependent synthetase/ligase, respectively. Although these genes showed up regulation, possibly because they are in same cluster, their functions under cyanide stress are still unclear.

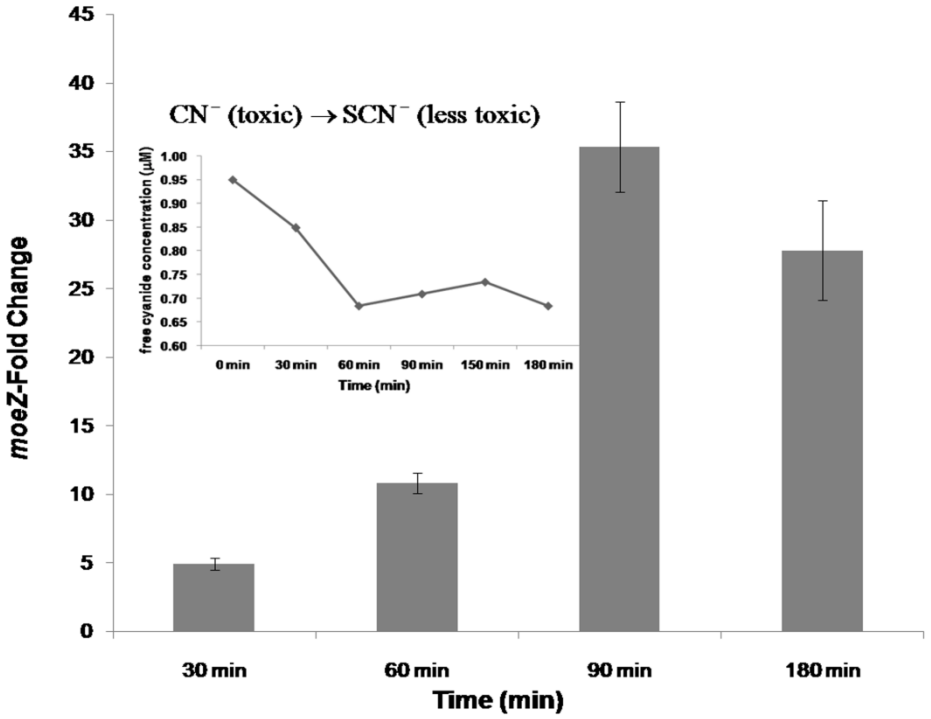

Fig. 4-3. Total free cyanide concentration (μM) (a) and transcriptomic response of *moeZ* (b). Error bars represent 95% confidence interval.

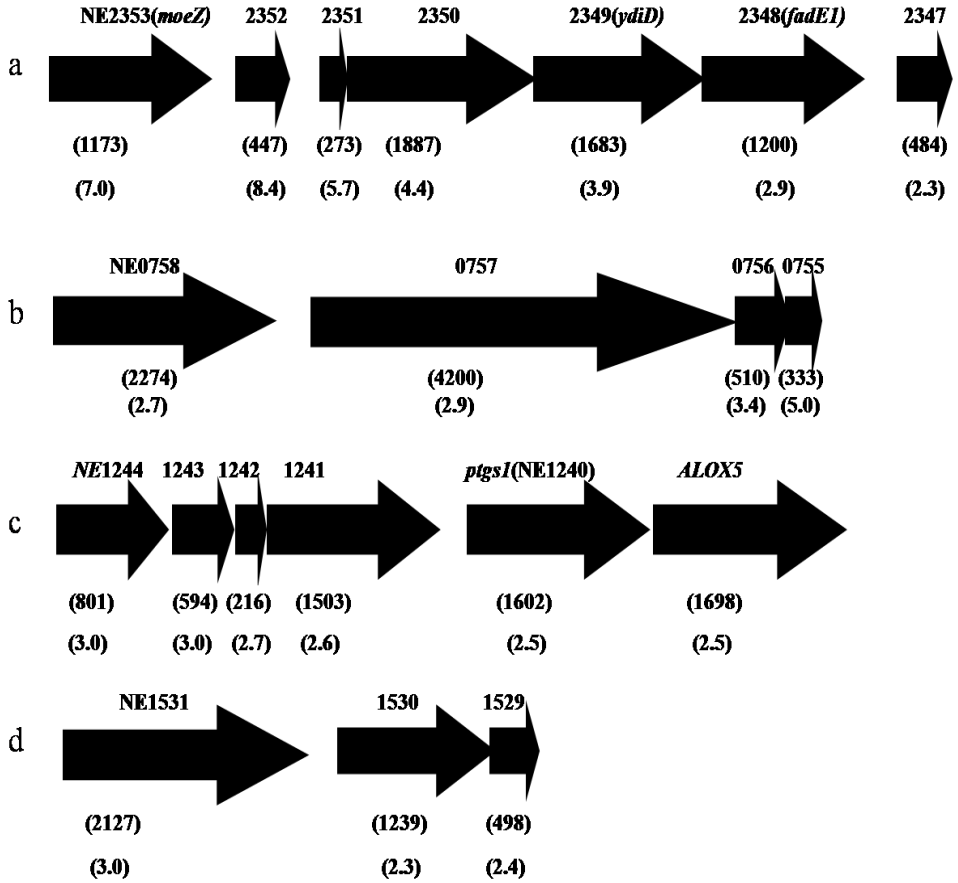

Fig. 4-4. Up-regulated genes in the same gene cluster or in same operon. (a and b represent genes in same cluster, and c and d represent in same operon). The upper and lower numbers in parentheses below each gene indicate the size (bp) and the fold-change of up regulation, respectively, for that gene.

The mRNA levels of *amoA* and *hao* under cyanide stress did not appear to change in the microarrays. To test whether this was a valid observation, the transcriptional levels of *amoA* and *hao* were measured at 30, 60, 90, and 180 min after exposure to 1 μM NaCN using qRT-PCR (Fig. 4-5). Cyanide is known as an inhibitor of respiratory processes in nitrification (69). Specifically, a single molecule of cyanide bound to hemeP460 of HAO inhibited HAO activity in *N. europaea* (62). In agreement with the microarrays, the *amoA* gene expression did not show significant change (Fig. 4-5), however, the mRNA for *hao* was increased slightly from 1.2 to 2.4-fold up regulation upon cyanide exposure. These results show that qRT-PCR can be more sensitive in detecting small changes in mRNA

levels and that HAO is up regulated in response to cyanide toxicity. Therefore, it may be that decreases in HAO-SOUR were not detected because the cells were successful in overcoming cyanide toxic effects.

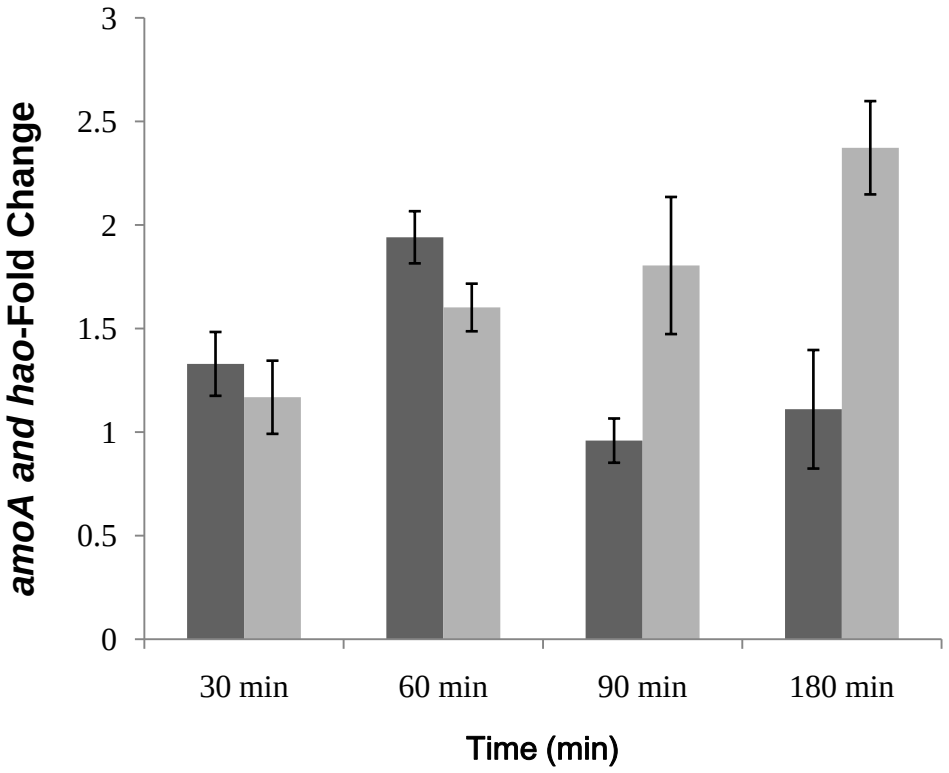

Fig. 4-5. Transcriptional level of *amoA* (black) and *hao* (gray) genes encoding AMO and HAO, respectively. RNA samples were extracted at 30, 60, 90, 180 min after exposure to cyanide. Error bars represent 95% confidence interval.

Up regulation of genes encoding TonB-receptor proteins and σ-factors.

Seven genes encoding TonB-receptor proteins (TRPs) and a gene encoding a transmembrane sensor showed significant up regulation in *N. europaea* exposed to 1 μM NaCN (Table 4-1). Transcript levels of three genes (NE0533/NE0541/0547) out of 29 genes annotated as σ-factors in *N. europaea* significantly increased under cyanide treatment. All three belong to a subfamily of 23 extracytoplasmic function (ECF) proteins. The genes encoding TRPs and σ-factors showed up regulation in *N. europaea* cultured in Fe-limiting conditions (94). Since *N. europaea* has a high iron requirement (94), growth medium in the batch reactor experiments contained 10 μM iron. Iron is an essential cofactor for the transfer of electrons in many enzymes involved in energy production pathways in bacteria (2). However, in the presence of free cyanide, iron can be come limited because the free cyanide can form cyanide complexes with heavy metals such as iron, cobalt, and platinum (21). Under iron-limited conditions, the genes encoding proteins involved in iron acquisition (e.g. TonB-receptor proteins and σ-factors) were up-regulated to capture iron (94). Whether up regulation of σ-factors is a specific response to iron limitation or a more general stress response is unclear because genes encoding σ-factors of *N. europaea* showed up regulation in chloroform treatment (33) and those genes are known to encode essential proteins in the response to stress conditions (33).

Genes, encoding membrane proteins, were up regulated. Two up-regulated genes (NE0755/NE0756) encoding proteins with unknown function were located between two TRPs (NE0754/NE0758). BLAST analysis of the proteins encoded by NE0755 and NE0756 produced matches with an E-value of e^{-12} for

MotA/TolQ/ExbB protein and with E-value e^{-11} for a putative membrane protein. NE0757 encoding magnesium chelatase was up regulated. NE0757 is in an operon with NE0755 and NE0756. NE1179 encoding a transmembrane protein also showed up regulation. Up-regulated NE0755/56/57 are in same cluster with NE0758, but the specific function of NE0755/56/57 under cyanide stress are not clear.

Table 4-1. Up-regulated genes encoding TonB-dependent receptor proteins and ECF σ-factors in cyanide-treated *N. europaea* cells

gene number /name	Description	Fold change	p-value(<0.05)
NE0321	TonB-dependent receptor protein	2.0	1.3×10^{-2}
NE0617/*oprC*	TonB-dependent receptor protein	2.8	1.2×10^{-3}
NE1531	TonB-dependent receptor protein	3.0	1.2×10^{-3}
NE1532	TonB-dependent receptor protein	3.8	6.8×10^{-3}
NE0758	TonB-dependent receptor protein	2.7	1.3×10^{-3}
NE0754	TonB-dependent receptor protein	3.5	1.1×10^{-3}
NE0535	TonB-dependent receptor protein	2.5	4.7×10^{-3}
NE0534	Transmembrane sensor	3.2	2.1×10^{-3}
NE0533	σ-factors, ECF family	3.8	1.1×10^{-3}
NE0541	σ-factors, ECF family	2.7	6.6×10^{-4}
NE0547	σ-factors, ECF family	2.1	6.1×10^{-4}

Other up-regulated genes under cyanide.

A gene cluster composed of NE1241/42/43 (Fig. 4-4c) showed significant up regulation. NE1242/43 encode proteins of unknown function and NE1241 encodes a tyrosinase. In β-proteobacteria several microorganisms contain tyrosinase homologous to *N. europaea* (63). In vitro, monophenolhydroxylase, diphenolhydroxylase, and tyrosinase have been used for the decontamination of phenol-containing wastewaters and polluted soils (18), but in vivo, the role of tyrosinase is not clear. During starvation, genes encoding tyrosinase in *N. europaea* showed up regulation (95) and a role in the adaptation to environmental stress was hypothesized. Tyrosinase is a Cu^{2+}-containing enzyme (18) and metals such as iron, cobalt, and zinc, cannot replace Cu^{2+} to maintain enzymatic activity (18). Tyrosinase isolated from plants or insects treated with cyanide lost activity due to cyanide binding Cu^{2+} (56). It may be that the transcript level of NE1241 increased in *N. europaea* exposed to 1 μM NaCN only to compensate for the loss of tyrosinase activity.

NE1239, encoding lipoxygenase, and NE1240 (Fig 4c), encoding cyclooxygenase, were expressed at significantly higher levels under cyanide stress. mRNA levels of NE1239 also increased during starvation conditions to cope with oxidative stress due to reductant limitation (95). The mRNA levels of NE1529 and NE1530 (Fig 4d), encoding a signal peptide protein, were higher in cyanide-exposed cells. NE0536 and NE1537, encoding BNR (bacterial neuraminidase repeat), showed up regulation. Up-regulated NE0511, encoding recombination association protein (rdgC), is involved in bacterial DNA recombination. NE2343 and NE2376, encoding proteins with unknown function, also showed up regulation

under cyanide treatment.

Down regulation of sulfate reduction pathway.

The genes NE0852/53/57 encoding the proteins involved in the sulfate reduction pathway in *N. europaea* showed significant down regulation. Sulfate reduction has two steps (7): 1) sulfate is reduced to sulfite and 2) sulfite is reduced further to sulfide for cysteine biosynthesis. The sulfate reduction pathway (Fig. 4-6), which is found in the *N. europaea* Pathway/Genome database in the BioCyc collection of databases, has been computationally predicted based on the MetaCyc pathway (11).

NE0857 (*cysN*) encodes a protein that participates in the first step of the sulfate reduction pathway. NE0852 (*yvgR*) and NE0853 (*yvgQ*) encode proteins that then contribute to the second step of the pathway. These genes might be down regulated because thiocyanate (SCN^-) is formed by the protein encoded by *moeZ*. The SCN^- could be used in cysteine biosynthetic pathway directly, possibly at the sulfide level. In *Pseudomonas aeruginosa* the use of thiocyanate for the cysteine biosynthesis was suggested (41). Therefore, the sulfate reduction pathway might be down regulated due to a lower need of sulfides for cysteine biosynthesis.

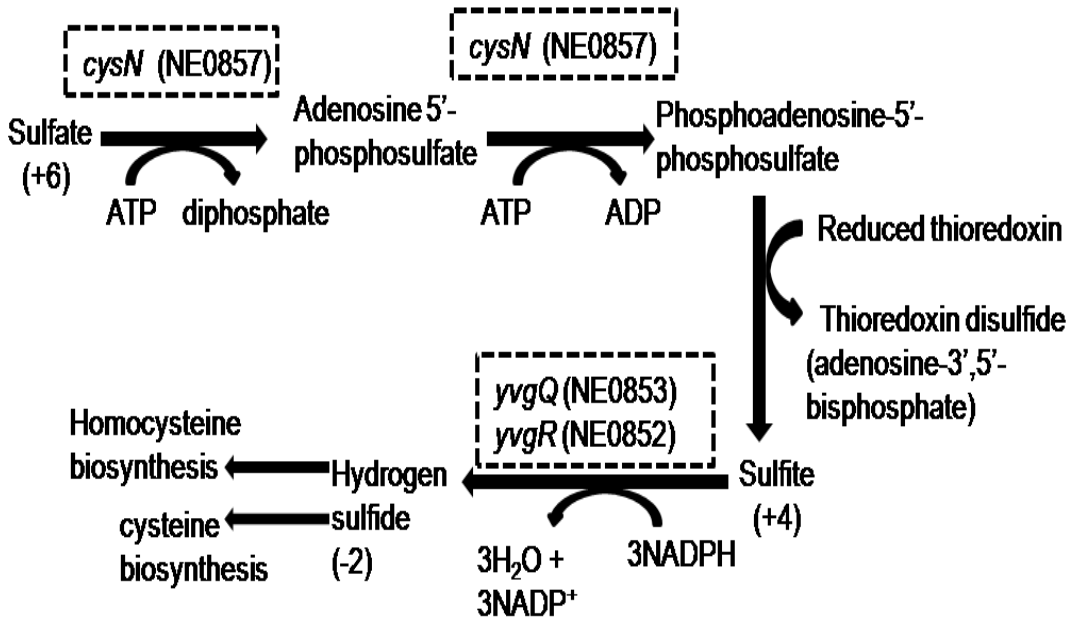

Fig. 4-6. Sulfate reduction Ⅰ pathway of *N. europaea* in the BioCyc collection of databases. Down-regulated genes (*cysN*, *yvgQ*, *yvgR*) that encode the proteins involved in sulfate reduction pathway in *N. europaea* exposed to 1 μM NaCN for 1 hour. The number in parentheses is oxidative state of sulfur.

Other down-regulated genes.

The down-regulated genes included genes encoding signal transduction mechanisms, carbohydrate metabolism, energy production, coenzyme metabolism, amino acid metabolism, DNA-replication, and nucleotide metabolism (Table 4-2). NE1644 (*rpmF*) and NE0389 (*rnpA*) encode ribosomal proteins and were down regulated under cyanide treatment. In *N. europaea* this gene group also showed down regulation when exposed to chlorinated aliphatic hydrocarbons stress (33). Transcript levels of NE2363 (*glnS*), encoding glutaminyl-tRNA synthetase (GlnRS) evolved from glutamyl-tRNA synthetase (GluRS) (84), and NE2072 (*gatA*), encoding the amidotransferase A subunit, were significantly decreased. The proteins encoded by *glnS* and *gatA* are required for protein synthesis in bacteria (28). Therefore, down regulation of them might indicate cyanide impact on translation and ribosomal biosynthesis in *N. europaea.*

Table 4-2. Down-regulated genes in cyanide-treated *N. europaea* cells

NE gene no. /name	Description	Fold change	P value(<0.05)
Signal transduction mechanism			
NE0579	Domain of unknown function 2 (FOG EAL domain)	2.1	2.5×10^{-2}
NE0580	Domain of unknown function 2 (FOG EAL domain)	2.1	2.5×10^{-2}
Translation, ribosomal structure and biogenesis			
NE1644/*rpmF*	probable 50S ribosomal subunit protein L32	2.2	2.4×10^{-2}
NE2363/*glnS*	Glutamyl-tRNA synthetase:Glutaminyl-tRNA synthetase GlnS	2.1	1.4×10^{-2}
NE2072/*gatA*	Amidase:Glutamyl-tRNA(Gln) amidotransferase A subunit	2.6	1.4×10^{-2}
NE0389/*rnpA*	Bacterial ribonuclease P protein	2.3	1.4×10^{-2}
Carbohydrate transport and metabolism			
NE1691	Glucosamine galactosamine-6-phosphate isomerase	2.1	2.0×10^{-2}
NE1809	probable beta subunit of citrate lyase	2.8	1.4×10^{-2}
NE0328/*cbb*	Transketolase	2.3	1.4×10^{-2}
Energy production and conversion			

NE1874	putative ferredoxin 2fe-2s protein	2.5	2.0×10^{-2}
NE1766/*nuoL*	probable nuoL; transmembrane NADH dehydrogenase I (chain L) oxidoreductase protein	2.0	1.8×10^{-2}
Coenzyme metabolism			
NE0634/*cobO*	ATP:corrinoid adenosyltransferase BtuR CobO CobP	2.2	1.4×10^{-2}
NE0636	TonB-dependent receptor protein	2.3	1.9×10^{-2}
NE0660/*ahcY*	S-adenosyl-L-homocysteine hydrolase	2.1	3.9×10^{-2}
Amino acid transport and metabolism			
NE0607/*gcvT*	Glycine cleavage T-protein (aminomethyl transferase)	2.0	1.4×10^{-2}
NE1957	Orn DAP Arg decarboxylases family 2	2.1	1.4×10^{-2}
NE1662/*carA*	carA; carbamoyl-phosphate synthase (small chain) protein	2.2	1.4×10^{-2}
DNA replication, recombination and repair			
NE2453/*ssb*	Single-strand binding protein family	2.1	3.6×10^{-2}
NE0197	hypothetical protein (Primosomal replication protein N)	2.1	1.4×10^{-2}
Nucleotide transport and metabolism			

NE0087/*purN*	purN; phosphoribosylglycinamide formyltransferase protein	2.2	1.4×10^{-2}
Cell envelope biogenesis, outer membrane			
NE0922/*cphA*	putative cyanophycin synthetase	2.2	4.0×10^{-2}
Others			
NE1487	hypothetical protein	2.1	2.0×10^{-2}
NE0581	possible predicted diverged CheY-domain	2.1	5.0×10^{-2}
NE1143/*glnS*	SecD SecF SecDF export membrane proteins	2.0	1.4×10^{-2}
NE0583	hypothetical protein	2.0	1.7×10^{-2}

SUMMARY

In this work, we characterized the response of *N. europaea* to cyanide at the physiological and transcriptional levels. Cyanide was extremely inhibitory to ammonia oxidation activity in batch reactor experiments. Concentrations as low as 1 μM NaCN inhibited nitrite production rates and AMO-SOURs more than 50%. Although inhibition was not detectable by monitoring the HAO-SOUR, the transcriptional level of the *hao* gene was 2.4-fold up regulated, likely to compensate for the inhibitory conditions. In microarray results, up regulation of *moeZ* (encoding a homologue of rhodanases) suggested a possible cyanide detoxification mechanism of cyanide in *N. europaea*. In addition, the down regulation of genes encoding proteins involved in sulfate reduction possibly

suggest that *N. europaea* can use the SCN^- produced in the detoxification process as an alternative source for cysteine biosynthesis. The decreased free cyanide concentration in batch reactors treated with cyanide also may support the detoxification of cyanide. The highest transcriptional change was observed in the *moeZ* gene cluster, suggesting that *moeZ* may be the best candidate for reporter constructs to diagnose or predict inhibition of AOB by cyanide. However, it also must be determined whether the *moeZ* cluster is up regulated in response to other toxic compounds.

Chapter 5

Conclusion

Putative toxic mechanism of heavy metal ions such as Zn^{2+}, Cd^{2+}, Hg^{2+}, and Cu^{2+}.

Although heavy metal ions play critical roles in catalysis, organometallic reactions, and biochemical reactions in prokaryotes (6), their chemical behaviors inside the cells are often unclear. When heavy metal ions exceed natural levels inside the cells, they can act as potent disrupters of various biological systems by competing with essential metal ions for binding sites in enzymes or by other means. For example, Cu^{2+} or Cd^{2+} can replace Zn^{2+},an essential metal, in many proteins and enzymes (6). In addition, heavy metals can inhibit cells by binding to active groups: carboxylate ($-COO^-$), amine ($-NH_2$), and thiol (-SH) (70).

Heavy metals (*i.e.* Zn^{2+}, Cd^{2+}, Hg^{2+}, and Cu^{2+}) have been shown to be strong nitrification inhibitors in wastewater treatment plants (39, 48). In this research, the effects of the heavy metals on *N. europaea* were studied in quasi-steady state batch reactors. Through the batch reactor experiments, we confirmed that the nitrification process is very sensitive to the heavy metals tested. 0.5 μM $CdCl_2$, 3.3 μM $HgCl_2$, 3.4 μM $ZnCl_2$, and 5 μM $CuCl_2$ decreased ammonia oxidation rates and ammonia-dependent oxygen uptake rates (AMO-SOUR) of *N. europaea* by 50% after a 1 h incubation. Transcriptional changes incurred in *N. europaea* cells after 1 h of exposure to the selected heavy metals were determined by microarrays or reverse transcriptase quantative-PCR (qRT-PCR). In the microarray and qRT-PCR experiments, *merA* (a gene that encodes mercuric reductase) showed the highest up regulation; 42-fold, 277-fold, or 250-fold up

regulation in *N. europaea* cells exposed to Zn^{2+}, Cd^{2+}, or Hg^{2+}, respectively.

The ammonia oxidation process of *N. europaea* cells exposed to Zn^{2+}, Cd^{2+}, or Cu^{2+} could not recover from the toxic conditions in a short 3h time-period. In contrast, the ammonia oxidation rates in cells exposed to Hg^{2+} did recover from 0.02 mM NO_2^-/min-OD_{600} to 0.09 mM NO_2^-/min-OD_{600} in 90 minutes. The recovery of *N. europaea* from Hg^{2+} toxicity was thought to be related to mercury resistance genes (*merTPCAD*), in particular to *merA*, because the *merA* gene product can reduce Hg^{2+} to a volatile form (Hg^0) (70), as follows:

$$Hg^{2+} + NADPH \text{ ----------}merA\text{----------> } Hg^0 + NADP^+ + H^+$$

Hg^0 then can be easily volatilized by the cells (68).

To observe how the *merA* gene responds to mercury toxicity, the transcriptional levels of *merA* in the cells exposed to Hg^{2+} were measured using qRT-PCR after 30, 60, 120, 180 min of exposure to Hg^{2+}. When the ammonia oxidation process of *N. europaea* was completely stopped 30 min after exposure to Hg^{2+}, *merA* showed a 150-fold up regulation. The transcript levels increased to 250-fold up regulation at 60 min (the highest fold change observed). Finally, once the ammonia oxidation rates had recovered (at 120 min), the transcript level of the gene decreased to 120-fold up regulation and then decreased to 20-fold up regulation at 180 min. These observations are consistent with the postulate that *N. europaea* is able to recover from Hg^{2+} toxicity by controlling the expression of *merA*. Interestingly, although the transcript levels of the *merA* gene were highly up regulated in both Zn^{2+}- and Cd^{2+}-treated *N. europaea* cells, the cells could not recover from Zn^{2+} or Cd^{2+} toxicity.

Heavy metals such as Zn^{2+}, Cd^{2+}, Hg^{2+}, and Cu^{2+} may enter into the periplasm via identical pathways through unspecific transport proteins (70). However, although zinc, cadmium, mercury, and copper are all divalent metal ions, the metals may invoke different resistance mechanisms by the cell as they may use different toxicity mechanisms to inhibit the cells. The toxicity mechanisms of heavy metals could possibly depend on their affinity to bind with active sites of certain enzymes and their overall chemical/physical properties (6). For example, to resist heavy metal toxicity, many microorganisms and plants synthesize enzymatic peptides, called phytochelatins, which contain a high amount of cysteine (6). In addition, to combat the toxicity induced by non-essential metals such as Cd^{2+} and other soft transition metals, metallothionein proteins have an important role in the detoxification of the excessive metals (89). The sequestration of heavy metal ions by phytochelatins, and/or metallothioneins is related to the affinity of the sulfur atom in the cysteine side-chain. In a study of interactions of cysteine with the closed-shelled Zn^{2+}, Cd^{2+}, and Hg^{2+} and the open-shelled Cu^{2+}, Cu^{2+} was shown to have the highest affinity to cysteine followed by Zn^{2+}, Cd^{2+}, and Hg^{2+} (6). This might explain why Cu^{2+} was less toxic to *N. europaea* compared to Zn^{2+}, Cd^{2+}, and Hg^{2+} in our work.

Cu^{2+}, Zn^{2+}, and Cd^{2+} are often removed from Gram-negative bacteria by efflux-pumps (70). The genes that encode efflux-pumps in *N. europaea* showed a 2.4-fold up regulation in microarray studies of *N. europaea* cells exposed to 1 μM $CdCl_2$ for 1 h. However, the small change in gene expression might not be enough to diminish Cd^{2+} toxicity. The *merA* gene did not show up-regulation in *N. europaea* exposed to Cu^{2+}. This may suggest that Cu^{2+} invokes Cu^{2+}-specific stress response genes that do not include *merA*.

General-stress response genes under heavy metal and cyanide stress.

There were 10 common up-regulated genes with one intergenic region (IGR) and 13 common down-regulated genes with three IGRs in both Zn^{2+} and Cd^{2+} treatments (Table 5-1 and Table 5-2). However, there was no common up- or down-regulated gene between the cells exposed to heavy metals (*i.e.* zinc and cadmium) and cells exposed to cyanide. Since *merTPCAD* were the highest up-regulated genes in both Zn^{2+} and Cd^{2+} treatments, *merTPCAD* appear to be the best candidate genes to detect Zn^{2+} and Cd^{2+} stress in *N. europaea*. The commonly up-regulated genes in both Zn^{2+} and Cd^{2+} treatments also include genes that encode ABC-type transport, peptidoglycan binding domain, and three hypothetical proteins. However, they would not be strong stress response genes because the genes showed limited up regulations (2.5-fold).

Table 5-1. The commonly up-regulated genes under Zn^{2+} and Cd^{2+} stress

NEgene no./name	**Gene function**	**Fold-change inCd^{2+} stress**	**Fold-change inZn^{2+} stress**
NE0841/*merP*	Mercury scavenger protein	439.5	14.3
NE0842/*merT*	Mercuric transport protein	370.4	13.1
NE0839/*merA*	Mercuric reductase	296.7	46.2
NE0840/*merC*	Mercury transport protein	171.8	22.8
NE0837	Unknown function	157.7	19.8
NE0838	Bacterial regulatory protein	107.41	6.8
NE0512	Unknown function	2.7	3.2

NE1176	Peptidoglycan binding domain	2.6	2.1
NE1899	ATPase component ABC-type transport system	2.4	2.4
NE1283	Unknown function	2.2	2.1

Among the 13 commonly down-regulated genes, genes encoding RubisCO and CheY provide crucial information about a general resistance mechanism that *N. europaea* uses to save energy, possibly for major metabolism pathways, under toxic conditions. The down regulation of genes that encode RubisCO (NE1918/1919) indicated that *N. europaea* cells may want to conserve energy for NH_3^+ metabolism under stress conditions because carbon sequestration by RubisCO is an energy intensive process (93). Under starvation conditions in *N. europaea* (95) and under Cu^{2+} stress in *Nitrosococcus mobilis* (81), genes encoding RubisCO were down regulated, probably also to conserve energy for NH_3^+ metabolism. NE1923, encoding CheY (a flagellar protein) in *N. europaea*, was previously seen to be down regulated in response to NO, as NO promoted the formation of biofilms and mobility was no longer necessary (83). It could be that the expression of CheY decreases during metal stress to promote the formation of cell agglomerates to protect some of the cells from further exposure. Therefore, since RubisCO and CheY of *N. europaea* showed down regulation in various inhibition conditions, the down-regulation of these genes may be considered a general stress response.

Table 5-2. The commonly down-regulated genes under Zn^{2+} and Cd^{2+} stress

NEgene no./name	**Gene function**	**Fold-change in Cd^{2+} stress**	**Fold-change in Zn^{2+} stress**
NE2039	Unknown function	2.5	3.4
NE2038	Myeloperoxidase, thyroid peroxidase, cyclooxygenase catalytic domain	2.2	2.8
NE0315/*mnxG*	multicopper oxidase	2.1	2.3
NE1298	TPR repeat	2.2	2.1
NE0787	Unknown function	2.1	2.2
NE1923/cheY	Response regulator receiver domain	3.0	2.6
NE2218	Unknown function	79.8	7.7
NE1919/*cbbQ*	nitric oxide reductase NorQ protein	5.7	5.1
NE1918/*cbbQ*	von Willebrand factor type A domain	7.3	5.3
NE1531	TonB-dependent receptor protein	2.6	2.1

Putative cyanide stress response genes of *N. europaea.*

The most important finding in the microarray experiments of *N. europaea* cells exposed to 1 μM NaCN for 1 h was the identification of a putative cyanide stress response gene, *moeZ*. The *moeZ* gene (NE2353) showed the highest up-

regulation (7-fold) in the microarray experiments. It encodes a c-terminal that contains an active site that is similar to other rhodanese homologues (19). Rhodanese catalyzes the transfer of sulfur from a reduced sulfur source, the electron donor, to free cyanide (CN^-), the electron acceptor, resulting in thiocyanate (SCN^-) (17). Since SCN^- is less toxic than CN^-, this mechanism is considered to be a general detoxification process of cyanide in bacteria and other organisms (96). To confirm whether *moeZ* participated in this detoxification process or not, the time dependent-transcript levels of *moeZ* and the concentrations of cyanide were measured over the course of the experiment. The transcript levels of *moeZ* showed a 12-fold up regulation after 60 min of exposure and increased to 35-fold after 90 min of exposure to cyanide while the cyanide concentration decreased from 0.95 μM to 0.68 μM during the period. The decreased cyanide concentration and increased *moeZ* expression suggests that *moeZ* catalyzed the formation of SCN^- from CN^-. In addition, the high up regulation response of *moeZ* suggests that *moeZ* may be the best candidate as a cyanide stress response gene in *N. europaea*.

Another crucial finding in microarrays of *N. europaea* exposed to 1 μM NaCN was the down regulation of genes that encode the sulfate reduction pathway. The genes NE0852/0853/0857 encode proteins that are involved in the sulfate reduction pathway in *N. europaea* and showed significant down regulation upon exposure to cyanide. The sulfate reduction pathway has two steps; 1) sulfate is reduced to sulfite and 2) sulfite is reduced further to sulfide for cysteine biosynthesis (7). NE0857 (*cysN*) participates in the first step of the sulfate reduction pathway. NE0852 (*yvgR*) and NE0853 (*yvgQ*) then participate in the second step of the pathway. These genes may be down regulated because the postulated SCN^- formed by *moeZ* could be used in the cysteine biosynthetic

pathway directly as a sulfide source. Use of thiocyanate for the cysteine biosynthesis was show in *Pseudomonas aeruginosa* (41). Therefore, the sulfate reduction pathway was down regulated due to a lower need of sulfides for cysteine biosynthesis.

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APPENDIX- Primer Sequence for qRT-PCR

Primer name	Primer sequence
16S For	5’-GGCTTCACACGTAATACAATGG-3’
16S Rev	5’-CCTCACCCCAGTCATGACC-3'
NE0533 For	5’-TTTCAGATTCTATTTCTCAAAGGCC-3’
NE0533 Rev	5’-ACATAAGCAACCAGCGATGA-3’
NE0535 For	5’-TCTCGGCCAATGTTGCTGCG-3’
NE0535 Rev	5’-AACGCCGATGAGCAAACCTG-3’
NE0536 For	5’-CCCGGTCGGGTTGCTTGTAT-3’
NE0536 Rev	5’-CGTCATCCGTTTTCGCCAAA-3’
NE0754 For	5’-CTATACAGCCAATTGAGTGC-3’
NE0754 Rev	5’-GGTAATATCAATAGCGGGAATA-3’
NE0755 For	5’-CTGATCGCAGTGATGGAAAA-3’
NE0755 Rev	5’-GATCTGTCCCGTGGATTTGT-3’
NE0757 For	5’-AGTTTCAAGCCGGAGAATGC-3’
NE0757 Rev	5’-GGAATTATGCCTGGCTGCTC-3’
NE0758 For	5’-TCAGAATCGCAAATTGACTT-3’
NE0758 Rev	5’-TATACCTGGAACAAAATGGC-3’
NE0760 For	5’-GATTCTGGAAATCAAAGCAG-3’
NE0760 Rev	5’-AAAGCATTTACGACGAATGT-3’
NE0836 For	5’-CAGGGTATTCACCGACAA-3’
NE0836 Rev	5’-TCAAACTCTCCTTGACGAAC-3’
NE0837 For	5’-AGTATCTGGAAATCGAGCTG-3’
NE0837 Rev	5’-CGATTGGTCGATCTTGAG-3’
NE0839 Rev	5’-GCTTTATCAAGCTGGTCATC-3’
NE0839 For	5’-ACATCCTTGTTGAAGGTCTG-3’
NE0853- For	5’-TCAATAAACATCGCGCTGAT-3’
NE0853-Rev	5’-GGTCACCGTTATTGAAGAAC-3’
NE0854 For	5’-ATCCAGCTACTGGAACAAGA-3’
NE0854 Rev	5’-TGTGTGTGAGTAGTCGCAAT-3’
NE0857 For	5’-CTCGAGCATGCCGAGTTACTTCGAT-3’
NE0857 Rev	5’-TGGAGGTCTTCGCAATGGCA-3’
NE0924 For	5’-GCAATTACTGAGGGGCATGT-3’
NE0924 Rev	5’-CTTCCCCTATGCCACCTTAA-3’
NE 0951 For	5’-CAGGAGTTTACCCAACTGAG-3’
NE0951 Rev	5’-GGACTGACAAGATTGACCAC-3’
NE1176 For	5’-CTTGGCCTTGATCAGATAAC-3’
NE1176 Rev	5’-AGATACTTGGTCTGCCTCTG-3’
NE1232 For	5’-TGGAAACCCGTTTTTACCTG-3’
NE1232 Rev	5’-ACGCGGATCAAATCAAAATC-3’
NE1239 For	5’-ATGACATGGCCAATAGTCTC-3’

NE1239 Rev	5’-TGGTGTGTTGTTATGGAATG-3’
NE1240 For	5’-AGCAGAGGTTCTGAAACTTG-3’
NE1240 Rev	5’-AAAAGACCTTCTCGTCCGTC-3’
NE1241For	5’-CGTCACCCGAACAGAATAAG-3’
NE1241 Rev	5’-GATGATTCAGGCATCTCCTT-3’
NE1243 For	5’-GCATGAAGAATGCGCTATCA-3’
NE1243 Rev	5’-AGGTTTGCGGTAACGATGAC-3’
NE1503 For	5’-CGTGACATTAACCGGGATCT-3’
NE1503 Rev	5’-TATCGAACTTACCCTTAAGC-3’
NE1530 For	5’-TCCCCCTCTGCATTAAAGAA-3’
NE1530 Rev	5’-ATCGTGGCGATACCTGGTCA-3’
NE1531 For	5’-TCCTGAAGAACACCCGCATT-3’
NE1531 Rev	5’-CGCTGCTGGGTTTCAAAATC-3’
NE1532 For	5’-GGGAAGAACAAGGCCGAGTT-3’
NE1532 Rev	5’-AGCTGGGTAAGCGCAACAAA-3’
NE1640 For	5’-GGAACGGATGATACTACTGC-3’
NE1640 Rev	5’-TAACACCCTGATTTCATTCC-3’
NE1752 For	5’-CATGAGCGTATCAAACAAAA-3’
NE1752 Rev	5’-GATCTGTTCGGGAGTCAATA-3’
NE1899 Rev	5’-ATGAACATACTCGGCTGTCT-3’
NE1899 For	5’-GGCTGTAACCTGCGTATAGT-3’
NE2057 For	5’-TCAGGTAAGGCAAATGTTT-3’
NE2057 Rev	5’-TCTTCATCTTCAGCCTTAG-3’
NE2072 For	5’-AGTGGAATCAAACCCACTTA-3’
NE2072 Rev	5’-TTTTTCCAGATCTTGCGTAT-3’
NE2124 For	5’-TAACGCTTGCCTTTTACTTC-3’
NE2124 Rev	5’-TTCACTGTGATGGATTCAGA-3’
NE2143 For	5’-GTGGTAAGTCCTGCAATGTT-3’
NE2143 Rev	5’-CGGCTCGATAATGTAGTGTT-3’
NE2347 For	5’-AACCGATACATGCGAGCGAC-3’
NE2347 Rev	5’-ATCGCTTGCGTGGAGTCTGT-3’
NE2348 For	5’-ATGCCATAGCAGGGTAATGC-3’
NE2348 Rev	5’-TGGTGGAAAAATTGCCTCTC-3’
NE2349 For	5’-TCCTTGCTGAAAAATCCACTCTAC-3’
NE2349 Rev	5’-TATTTGAGACAACTGCCGCT-3’
NE2352 For	5’-CAAGCCACCATCATCTGGAA-3’
NE2352 Rev	5’-TAAGGAAGAAATCCGCAGTT-3’
NE2353 For	5’-AGATCGGCAGCGATTGGTCG-3’
NE2353 Rev	5’-TTCACGTGATGTGCTGCTCG-3’
NE2363 For	5’-ACATCATCGAGGAAGACAAC-3’
NE2363 Rev	5’-ATATTCCTGGGCTTCTTTTT-3’
NE2548 For	5’-CTGATCTGGTCAAACAACCT-3’

NE2548 Rev	5’-CAGCAATGATCAAGTCGTAA-5’
NE2552 For	5’-GAGCACTTCCTTGATACTGG-3’
NE2552 Rev	5’-GATCATTTCGATCTGCAAGT-3’
NE2563 Rev	5’-GTATGAGCGTGTGTACGATG-3’
NE2563 For	5’-GGTATTGCCAATGGTGTAGT-3’