Candidate Stress Genes of *Nitrosomonas europaea* for Monitoring Inhibition of Nitrification by Heavy Metals[▽]

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Heavy metals have been shown to be strong inhibitors of nitrification 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 h, 66 genes (of the total of 2,460 genes) were upregulated, and 50 genes were downregulated more than twofold. Of these, the mercury resistance genes (*merTPCADE*) averaged 277-fold upregulation under 1 μM CdCl$_2$, with *merA* (mercuric reductase) showing 297-fold upregulation. In *N. europaea* cells exposed to 6 μM HgCl$_2$ or to 8 μM CuCl$_2$, *merA* showed 250-fold and 1.7-fold upregulation, respectively. Cells showed the ability to recover quickly from Hg^{2+}-related toxic effects, apparently associated with upregulation of the mercury resistance genes and *amoA*, but no such recovery was evident in Cd^{2+}-exposed cells even though *merTPCADE* were highly upregulated. We suggest that the upregulation 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.

Nitrifying bacteria, such as *Nitrosomonas europaea* (ATCC 19718), are important in the removal of nitrogen in wastewater reclamation plants. *N. europaea* obtains essential reductants for energy and biosynthesis from the oxidation of ammonia (NH$_3^+$) to nitrite (NO$_2^-$) and uses CO$_2$ as its carbon source (1). 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 (1). Nitrifying bacteria are sensitive to various environmental contaminants and generally have low growth rates, with doubling times of about 8 to 12 h (46), making them the critical step in biological nitrogen removal (BNR). Heavy metals, such as Cd^{2+} (12, 28), Hg^{2+}, and Cu^{2+} (30), are extensively used in industry (e.g., in the fabrication of pigments, batteries, and electronics), and improper disposal of the metals or their by-products tends to contaminate the environment (40). These metals may inhibit nitrification in the reclamation of wastewater (4, 5, 27, 44).

Inhibition of nitrification has been best documented in the model bacterium *N. europaea*. *N. europaea* is sensitive to inorganic compounds such as Cd^{2+} (7, 21) and organic compounds such as chlorinated aliphatic hydrocarbons (23) and to pH shifts (14), among other factors. With the sequencing of the *N. europaea* genome, transcriptomics studies to characterize *N. europaea* stress responses to inhibitors are now possible. Exposure to Zn^{2+} caused inhibition of ammonia oxidation and, concomitantly, expression of specific genes encoding membrane transporter and putative metal resistance proteins (38).

Exposure to chlorinated aliphatic hydrocarbons increased the expression of genes encoding heat shock proteins, sigma factors of the extracytoplasmic function (ECF) subfamily, and toxin-antitoxin loci (15). Such genes potentially may be useful as early-warning toxicity indicators to prevent excessive inhibition of nitrification and in BNR processes.

Failure or inhibition of BNR processes caused by heavy metals, such as Cd^{2+}, Hg^{2+}, and Cu^{2+}, can lead to high ammonia discharges and contribute to eutrophication of water bodies (44). Because current methods to monitor nitrification are laborious and cannot identify causes of inhibition, quick and sensitive methods are required for early detection of nitrification inhibition and for identification of its causes. Such methods could rely on "sentinel genes" that are highly upregulated in the presence of a certain heavy metal or group of heavy metals. In a previous study, *merTPCAD* were identified as putative sentinel genes in *N. europaea* exposed to Zn^{2+} (38). The main objective of the current study was to look for similar sentinel genes by examining whole-genome transcriptional changes in *N. europaea* exposed to Cd^{2+}. For comparison, transcriptional changes of selected genes were also determined in cells exposed to Hg^{2+} and Cu^{2+}.

MATERIALS AND METHODS

Batch reactor experiments. *N. europaea* cells were grown in batch cultures with 25 mM (NH$_4$)$_2$SO$_4$ as described previously (38), harvested in mid- to late-exponential phase (optical density at 600 nm [OD$_{600}$] of ≅0.07), and washed two times with 40 mM NaH$_2$PO$_4$ (pH 7.8). Washed cells were resuspended in 1 liter of 50 mM HEPES buffer (pH 7.8) containing (NH$_4$)$_2$SO$_4$ (2.5 mM). The 1-liter cell suspension was evenly divided into two, gas-tight, 1.67-liter reactor vessels (Wheaton double-sidearm cell stir), and experiments were conducted without further additions of either buffer or ammonia. Cell suspensions prepared in this way sustained a quasi-steady-state condition during 4-h incubations comparable to *N. europaea* batch reactor experiments previously reported using phosphate-buffered medium (15). For the experiments, cells were stirred for 1 h 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.

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Oxygen uptake measurement and nitrite assay. The ammonia-dependent specific oxygen uptake rate (A-SOUR), hydrazine-dependent specific oxygen uptake rate (H-SOUR), and NO_2^- production rates were tested every 30 min during the incubations, as described previously (23). An aliquot of 1 ml drawn from a batch reactor was centrifuged immediately and analyzed to determine the NO_2^- concentration (22). The A-SOUR was measured in a 1.8-ml glass water-jacketed reaction vessel at 30°C using a heated circulating water bath (10). The H-SOUR was then determined by blocking ammonia-dependent oxygen uptake with 100 μM allylthiourea and adding 750 μM hydrazine as an alternative substrate for HAO. The SOUR of the cells was calculated based on the saturated oxygen concentration in water (10).

Affymetrix microarray experiments. A volume of 180 ml of *N. europaea* culture (OD_{600} of $\cong 0.07$) 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 purification with an RNeasy Mini kit (Qiagen Inc., Valencia, CA), the quality and quantity of the RNA samples were measured using an RNA 6000 Nano LabChip kit on an Agilent Bioanlyzer 2100 (Agilent Technologies, Inc., Palo Alto, CA). All of the 2,460 annotated *N. europaea* genes were represented on the custom Nimble Express made-to-order arrays (Nimble-Gen 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 upregulated or downregulated more than twofold under Cd^{2+}, applying an unpaired two-sample t test with a cutoff P value of 0.05.

qRT-PCR. Quantitative reverse transcription-PCR (qRT-PCR) was used to evaluate the transcriptional levels of selected up- or downregulated 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 an 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 previously (38). qRT-PCR was performed on an ABI 7500 instrument (Applied Biosystems, Foster City, CA) with 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 relative changes to show ideal amplification efficiency were calculated using the formula $2^{-\Delta\Delta C_T}$ (where C_T is cycle threshold) (39).

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 a 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 two-dimensional (2D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (38). ReadyStrip immobilized pH gradient strips (13 cm; Bio-Rad) with a nonlinear 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 upregulated proteins in Cd^{2+}-exposed cells were excised from SDS-PAGE gels stained with Sypro Ruby (Cambrex Bioscience, Rockland, ME) and identified by nano-liquid chromatography-tandem mass spectrometry (Midwest Bio Services, LLC, Overland, KS).

Microarray data accession number. The microarray data are available at the Gene Expression Omnibus database under accession number GSE9221 (http://www.ncbi.nih.gov/geo).

RESULTS

Nitrite production and SOURs. To test the toxicity of the heavy metals in this study, *N. europaea* cells were placed in two batch reactors (a treatment reactor and a control reactor). In the control reactors, the cells consistently reached quasi-steady state in about 1 h (constant NO_2^- production rate of about 0.2 mM/min · OD_{600}) and were stable for up to 4 h (Fig. 1a). In the treatment reactors, 1 μM $CdCl_2$, 6 μM $HgCl_2$, or 8 μM $CuCl_2$ was added at 1 h in quasi-steady state. After 1 h of further incubation with Cd^{2+}, the cells showed about an 88% decrease in the NO_2^- production rate (Fig. 1a) and an 82% decrease in A-SOUR (Fig. 1b). *N. europaea* exposed to 6 μM $HgCl_2$ or 8 μM $CuCl_2$ for 1 h showed about 90% and 82%
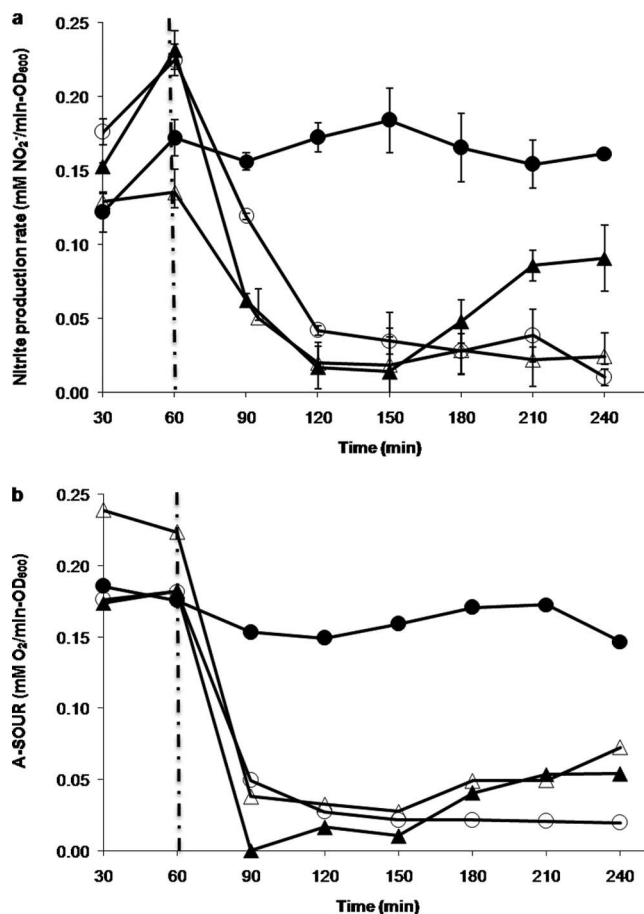

FIG. 1. *N. europaea* nitrite production rate (a) and A-SOUR (b) under 1 μM $CdCl_2$, 6 μM $HgCl_2$, or 8 μM $CuCl_2$ treatments for 4 h. Nitrite production rate and AMO activity were normalized by cell density. Closed circles, open circles, closed triangles, and open triangles represent control condition, 8 μM $CuCl_2$, 6 μM $HgCl_2$, and 1 μM $CdCl_2$, respectively. The vertical dashed lines indicate the injection time of inhibitors. The error bars indicate 95% confidence intervals.

inhibition, respectively, of A-SOUR (Fig. 1b), while their corresponding nitrite production rates decreased by about 88% and 76%, respectively.

Determinations of H-SOUR showed that hydrazine-dependent oxygen uptake activity remained unaffected in *N. europaea* cells exposed to 1 μM $CdCl_2$ and 6 μM $HgCl_2$ but that it decreased by 71% in cells exposed to 8 μM $CuCl_2$ (data not shown).

Interestingly, 6 μM $HgCl_2$ almost completely stopped NO_2^- production (Fig. 1a) and ammonia-dependent oxygen uptake (Fig. 1b), but both nitrite production and A-SOUR began to rebound within 60 min of further incubation (Fig. 1a and b), suggesting that the cells were able to recover from the Hg^{2+} exposure.

Transcriptomic responses to Cd^{2+}. Using microarrays, we determined the global transcriptional changes of *N. europaea* exposed to Cd^{2+}. The analysis revealed that 1 μM $CdCl_2$ caused significant changes (greater than twofold) in the transcript levels of 116 genes. The genes included 66 genes with known functions (39 upregulated and 27 downregulated), 21 open reading frames

TABLE 1. Selected upregulated genes under cadmium stress[a]

Gene function and *N. europaea* locus tag	Gene name	Description	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 protein	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 pump, 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		Flavin adenine dinucleotide 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 envelope biosynthesis				
NE0378		Sugar transferases involved in lipopolysaccharide synthesis	2.1	4.7×10^{-4}
NE2279	*yccZ*	Periplasmic protein involved in polysaccharide export	2.0	2.6×10^{-4}
Translation, ribosomal structure, biogenesis				
NE2072	*gatA*	Amidase:glutamyl-tRNA (Gln), amidotransferase A subunit	2.9	3.0×10^{-4}
NE2073	*gatB*	GatB:glutamyl-tRNA(Gln) 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, repair				
NE0835	*tnpA*	Transposase and inactivated derivatives TnpA family	25.5	9.1×10^{-6}
NE0836	*tnpR*	Site-specific recombinase 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 binding protein	2.1	2.5×10^{-5}
Transcription				
NE2324	*rnc*	Double-stranded-RNA-specific RNase	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		XTP 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}

[a] Commonly upregulated genes under Cd^{2+} and Zn^{2+} treatment are indicated by an asterisk.

TABLE 2. Selected downregulated genes under cadmium stress[a]

Gene function and *N. europaea* locus tag	Gene name	Description	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 protein	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 system protein E; GAF domain	2.0	4.0×10^{-2}
NE1298*		Tetratricopeptide repeat	2.2	4.0×10^{-2}
NE2315	*pilN*	Putative type 4 fimbrial biogenesis 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 metallopeptidase	79.78	2.7×10^{-2}
NE1538		Chromosome segregation ATPases	2.2	2.4×10^{-2}
Transcription				2.4×10^{-2}
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 a helix-turn-helix DNA-binding domain	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, chaperone, 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}

[a] Commonly downregulated genes under Cd^{2+} and Zn^{2+} toxicity are indicated with an asterisk.

with no known functions (12 upregulated and 9 downregulated), and 29 intergenic regions (15 upregulated and 14 downregulated). The up- or downregulated genes with known function were grouped by functional classification based on the *N. europaea* genome database (http://genome.ornl.gov) (Tables 1 and 2). Among these, the mercury resistance genes and two genes upstream of that operon were upregulated more than 100-fold. Various genes involved in coenzyme metabolism, translation, DNA replication, and nucleotide transport showed intensities slightly above twofold over the control. The arrays also showed 50 downregulated genes slightly above twofold and included genes that function in signal transduction mechanisms and cell processes. Interestingly, the membrane-bound metallopeptidase encoded by NE2218 showed about 80-fold downregulation in response to Cd^{2+} toxicity exposure, perhaps to prevent cytotoxicity by self-digestion when the cells slow down their metabolism.

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

Expression of *merA* and *amoA* under Cu^{2+} and Hg^{2+} toxicity. The *merTPCADE* cluster was upregulated 297-fold in cells exposed to Cd^{2+} and 46-fold in cells exposed to Zn^{2+} (38). To examine whether *merTPCADE* respond similarly to other heavy metals, we used qRT-PCR to examine time-dependent transcriptional responses of *merA* to 6 μM $HgCl_2$ and 8 μM $CuCl_2$. The transcript level of *merA* increased 150-fold in the first 30 min in response to Hg^{2+} and continued to increase up
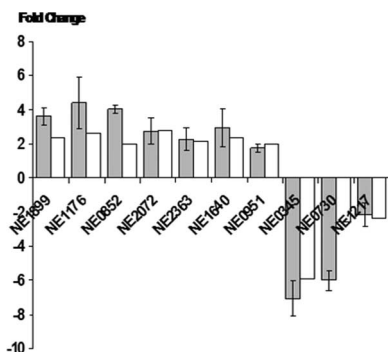
FIG. 2. Comparison of mRNA expression levels of selected up- or downregulated genes determined by qRT-PCR (black bars) and microarray (white bars). The positive value represents upregulation, and the negative value represents downregulation. Error bars represent standard errors of the means.

to 250-fold upregulation (Table 3). Consistent with detoxification, during the recovery of nitrification activity, the transcript level of *merA* decreased to 21-fold upregulation in the span of 3 h (Table 3). In contrast, the transcript level of *merA* did not show a significant change in response to Cu^{2+} (Table 3) at levels that inhibited nitrification (Fig. 1).

We also examined the time-dependent transcriptional responses of *amoA* under Cu^{2+} and Hg^{2+} stress (Table 3). The transcript level of *amoA* increased twofold in Cu^{2+}-exposed cells, which may have caused uptake of a small amount of the Cu^{2+} in the first 60 min, but decreased twofold by 180 min. *N. europaea* exposed 30 min to 6 μM $HgCl_2$ lost almost all AMO activity and, consequently, NO_2^- production (Fig. 1b). However, as the transcript level of *merA* increased within 1 h, AMO transcript also increased, and nitrite production rates began to rebound.

2D SDS-PAGE. To determine 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-h 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-liquid chromatography-tandem mass spectrometry and to deduce the genes that encode them. Identified proteins included nitrite reductase, encoded by NE0924, Rieske iron-sulfur protein, encoded by NE1503, and two hypothetical proteins encoded by NE2057 and NE1752 (Fig. 3).

DISCUSSION

Physiological responses to Cd^{2+}, Hg^{2+}, or Cu^{2+}. Our results (Fig. 1) suggest that the inhibitory effects of Cd^{2+} and Hg^{2+} exposure were confined mainly to AMO while apparently causing little or no damage to other elements of the electron transport chain. However, the decrease in H-SOUR with 8 μM $CuCl_2$ suggests that Cu^{2+}-related inhibition was not confined only to AMO. Cu^{2+} has been reported to induce cytotoxicity by producing hydroperoxide and by causing losses of intracellular K^+, an indication of loss of membrane integrity, in nitrifying autotrophic bacteria (20; also T. S. Radniecki and R. L. Ely, unpublished data). Decreases in both A-SOUR and H-

SOUR, as observed in this study, would be consistent with compromised membrane integrity associated with Cu^{2+} exposure.

We showed previously in batch reactors that Zn^{2+} caused 50% nitrification inhibition at a concentration of 3.4 μM (38). Compared to Zn^{2+}, Cd^{2+} and Hg^{2+} were more toxic to ammonia oxidizers, but Cu^{2+} was less toxic, with 1 μM Hg^{2+} or 6 μM Cu^{2+} causing 50% inhibition (data not shown) and 1 μM Cd^{2+} causing 90% inhibition. Other studies have shown the inhibition of nitrification by heavy metals in wastewater sludge (27, 33), consistent with our results showing the sensitivity of ammonia oxidizers (e.g., *N. europaea*) to Cd^{2+}, Hg^{2+}, or Cu^{2+}.

Proteomic responses to Cd^{2+}. In microarray experiments (1-h incubation), transcript levels of the genes NE0924, NE1503, NE2057, and NE1752, corresponding to the overexpressed peptides (3-h incubation) (Fig. 3), did not show detectable increases. Similar discrepancies between transcript and protein levels have been seen in studies with human cells (6), *Plasmodium falciparum* (29), iron-regulated genes of *Vibrio anguillarum* (8), and with *Escherichia coli* (25). Posttranscriptional splicing and posttranslation modifications have been suggested as possible reasons for different responses in transcription and translation (6, 29). In our experiments, microarray data showed upregulation 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. Overexpression of NirK (nitrite reductase) in *N. europaea* exposed to Cd^{2+} might support the metal tolerance hypothesis in soil microorganisms (16, 43). It has been reported that NirK is involved in heavy metal tolerance in denitrifying bacteria (16, 43). Therefore, in addition to conferring nitrite tolerance in *N. europaea* (3), perhaps NirK could play a role in Cd^{2+} tolerance as well.

Expression of *merTPCADE* under Cd^{2+} toxicity. Of the 66 upregulated genes detected in the microarrays of cells exposed to 1 μM $CdCl_2$, the highest upregulated 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 (38), suggesting that the mercury resistance operon in *N. europaea* may play an important role in protecting the cells from toxic heavy metals. In addition, the transposase encoded by *tnpA* (NE0835) and the resolvase encoded by *tnpR* (NE0836) were upregulated under Cd^{2+} (31-fold) and Hg^{2+} (54-fold) stress but not under Zn^{2+} and Cu^{2+}

TABLE 3. Expression change of *merA* and *amoA* determined by qRT-PCR under Hg^{2+} and Cu^{2+} treatment

Treatment and gene	Fold change in expression after incubation for[a]:			
	30 min	60 min	120 min	180 min
Hg^{2+} treatment				
merA	157.1 ± 0.22	254.5 ± 0.72	115.7 ± 0.01	21.6 ± 0.26
amoA	1.0 ± 0.02	2.0 ± 0.02	1.9 ± 0.05	3.7 ± 0.12
Cu^{2+} treatment				
merA	1.8 ± 0.50	0.9 ± 0.01	1.4 ± 0.35	1.7 ± 0.12
amoA	2.0 ± 0.07	2.0 ± 0.33	0.5 ± 0.03	0.4 ± 0.02

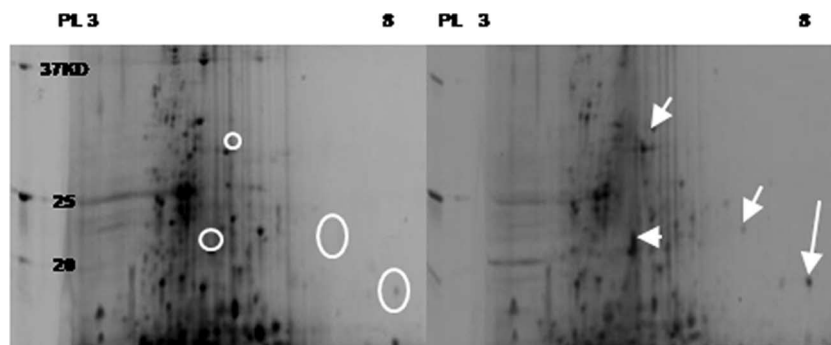[a] The error (± value) indicates the 95% confidence interval.

FIG. 3. Comparison in 2D SDS-PAGE of the soluble protein fraction of *N. europaea* treated without (left) and with 1 μM CdCl$_2$ (right) for 3 h. Circles represent absent or low translation under control conditions. Arrows represent protein spots upregulated under cadmium stress.

stress (data not shown). The transposase and resolvase are thought to form a transposase-related protein, known as the mercury resistance transposon (34, 35), that may increase antibiotic resistance in gram-negative facultative bacteria (31). In *N. europaea*, *tnpA* and *tnpR* are located downstream of the operon *merTPCADE* (Fig. 4). The role of the mercury resistance transposon with respect to Cd^{2+} toxicity in *N. europaea* remains unclear, as we did not observe increased Cd^{2+} tolerance or adaptation in our experiments. However, we can suggest that the upregulation of *merTPCADE* and *tnpAR* may occur 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+}). We also observed 2.4-fold upregulation of NE1640, which putatively encodes CzcC, the outer membrane protein of the CzcCBA efflux pump thought to be involved in Cd^{2+} detoxification in *Ralstonia eutropha* and other gram-negative bacteria (37). However, because *N. europaea* cells 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.

Transcriptional responses of *merA* and *amoA* in cells exposed to Hg^{2+} and Cu^{2+}. In cells exposed to Zn^{2+} (38), Cd^{2+}, or Cu^{2+}, nitrite production and ammonia-dependent oxygen uptake rates did not rebound from heavy metal toxicity within the 3-h time period used in this study even though *merTPCAD* were upregulated 277-fold in Cd^{2+}-exposed cells, perhaps due to severe oxidative stress (7, 12). In contrast, cells exposed to Hg^{2+} did recover, with nitrite production rates rebounding from 0.02 mM NO$_2^-$/min · OD$_{600}$ to 0.09 mM NO$_2^-$/min · OD$_{600}$ in 90 min (Fig. 1), accompanied by increased expression of *merA* (Table 3). Recovery of *N. europaea* from

Hg^{2+} toxicity is thought to be related to the mercury resistance genes (*merTPCAD*), in particular, to *merA* because the *merA* gene product can reduce Hg^{2+} to a volatile form (Hg^0) (2, 17, 37), as follows: Hg^{2+} + NADPH → Hg^0 + NADP$^+$ + H$^+$ (36). Hg^0 then can be volatilized by the cells (36).

Transcript levels of *amoA* increased in the early stages of Cu^{2+}-induced toxicity, but they did not translate into increases in A-SOUR. This indicates that Cu^{2+}, although essential for AMO activity (11), can be detrimental even at relatively low concentrations.

Increases in the A-SOUR in cells exposed to Hg^{2+} were reflected in the transcript levels of *amoA* (Table 3). While it did not change appreciably during the first 30 min of Hg^{2+} exposure, *amoA* transcription increased fourfold during the time that cells showed recovery. These observations suggest also that monitoring *amoA* expression can be valuable for tracking cellular responses to toxicity.

Shared up- or downregulated 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 (13), was upregulated 2.4- to 2.6-fold. A similar observation has been reported with *Caulobacter crescentus* exposed to Cd^{2+} and Cr^{2+} (19). Other upregulated genes included NE1899, encoding an ATPase component ABC-type metal transporter or an arsenite resistance protein (37), and NE0389, encoding an RNase P protein that processes a precursor of tRNA (18). Genes that were downregulated included NE2218, encoding a membrane-bound metallopeptidase (80-fold under Cd^{2+} and 8-fold under Zn^{2+} treatments), which requires a small amount of a transition metal such as Zn^{2+} or Co^{2+} in its active site (26). The genes *cbbQO* (NE1919/1918), encoding ribulose bisphosphate carboxylase/oxygenase (RubisCO), were downregulated in both Zn^{2+} and Cd^{2+} treatments. Carbon sequestration by RubisCO is an energy-intensive process (47). Under starvation conditions in *N. europaea* (48) and under Cu^{2+} stress in *Nitrosococcus mobilis* (41), genes encoding RubisCO were downregulated, possibly to conserve energy for NH$_3^+$ metabolism.

Heavy metals cause oxidative stress in gram-negative bacteria (37), and in this work, the microarrays detected changes in transcript levels of genes encoding proteins associated with oxidative stress. Myeloperoxidase, encoded by NE2038, is

FIG. 4. Organization of *merR*, *merTPCADE*, and transposase-related genes (*tnpMRA*) in *N. europaea*. Arrows indicate the genes and their orientation. The numbers in parentheses indicate the relative changes of upregulated *merTPCADE* and *tnpMRA* under 1 μM CdCl$_2$ treatments.

thought to be a reactive oxygen species-generating enzyme (24) and was downregulated, perhaps to reduce oxidative stress under heavy metal toxicity. NE0315 (*mnxG*), encoding a multicopper oxidase known to catalyze Mn^{2+} oxidation, was downregulated under the Zn^{2+} and Cd^{2+} treatments, again perhaps to reduce oxidative stress (9). Genes that were also downregulated under both Zn^{2+} and Cd^{2+} stress include NE1298, encoding tetratricopeptide repeat, involved in protein-protein interactions (32); NE1531, encoding a TonB-dependent protein; and NE0787, encoding CheY protein. NE1923, encoding CheY (a flagellar protein) in *N. europaea*, was previously seen to be downregulated in response to NO, as NO promoted the formation of biofilm, and mobility was no longer necessary (42). 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.

Candidate genes to detect Cd^{2+} stress. Of the *N. europaea* genes that were up- or downregulated 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 (37), the upregulation of NE1034, encoding thioredoxin (disulfide reductase) (19), could help the cell to resist oxidative stresses. NE1005 (*argB*) and NE0872 (*hisD*), encoding an amino acid transport, were upregulated under Cd^{2+}. Biosynthesis of amino acids would use more energy than their uptake through an amino acid transport; therefore, these genes might be upregulated to conserve energy in a toxic environment (45). NE0221, encoding an organic radical-activating enzyme, and NE2206 (*ppiD*), encoding a peptidyl-prolyl isomerase that belongs to a posttranslational modification protein, showed upregulation under Cd^{2+} treatment (*ppiD* was also upregulated under chloroform treatment) (15). NE2324 (*rnc*), NE1035, NE0854 (*cysB*), and NE0951, which relates to transcription, showed upregulation, while NE2435 (*fecI*), NE1217, NE0533, NE1452, and NE0787 showed downregulation (Table 2). Transcript of NE1217, belonging to the ECF family, was also increased in response to chloroform toxicity (15). NE0835, NE0836, NE0837, and NE2207 (*hupB*), involved in DNA replication, recombination, and repair, showed upregulation. NE0378 and NE2279 (*yccZ*), involved in cell envelope biosynthesis, were upregulated, while six genes in the groups were downregulated to statistically significant degrees (Table 2). Although the role(s) of these genes in heavy metal stress was not clear, they could be important for finding heavy metal toxicity mechanisms or general stress genes in the future.

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