Survival and Growth in the Presence of Elevated Copper: Transcriptional Profiling of Copper-Stressed *Pseudomonas aeruginosa*†

Gail M. Teitzel,¹ Ashley Geddie,² Susan K. De Long,³ Mary Jo Kirisits,³
Marvin Whiteley,² and Matthew R. Parsek^{4*}

Department of Civil and Environmental Engineering, Northwestern University, Evanston, Illinois 60208¹; Department of Periodontics, The University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104²; Department of Civil, Architectural and Environmental Engineering, University of Texas at Austin, Austin, Texas 78712³; and Department of Microbiology, University of Iowa, 500 Newton Road, Iowa City, Iowa 52242⁴

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Transcriptional profiles of *Pseudomonas aeruginosa* exposed to two separate copper stress conditions were determined. Actively growing bacteria subjected to a pulse of elevated copper for a short period of time was defined as a “copper-shocked” culture. Conversely, copper-adapted populations were defined as cells actively growing in the presence of elevated copper. Expression of 405 genes changed in the copper-shocked culture, compared to 331 genes for the copper-adapted cultures. Not surprisingly, there were genes identified in common to both conditions. For example, both stress conditions resulted in up-regulation of genes encoding several active transport functions. However, there were some interesting differences between the two types of stress. Only copper-adapted cells significantly altered expression of passive transport functions, down-regulating expression of several porins belonging to the OprD family. Copper shock produced expression profiles suggestive of an oxidative stress response, probably due to the participation of copper in Fenton-like chemistry. Copper-adapted populations did not show such a response. Transcriptional profiles also indicated that iron acquisition is fine-tuned in the presence of copper. Several genes induced under iron-limiting conditions, such as the siderophore pyoverdine, were up-regulated in copper-adapted populations. Interesting exceptions were the genes involved in the production of the siderophore pyochelin, which were down-regulated. Analysis of the copper sensitivity of select mutant strains confirmed the array data. These studies suggest that two resistance nodulation division efflux systems, a P-type ATPase, and a two-component regulator were particularly important for copper tolerance in *P. aeruginosa*.

Human activity has produced elevated levels of heavy metals in the environment (13). The U.S. Environmental Protection Agency has placed 13 heavy metals, including copper (Cu), on its priority pollutants list of 129 compounds (33). Unlike some organic contaminants, heavy metals cannot be degraded by microorganisms and, thus, are some of the most persistent pollutants.

Many heavy metals, such as Cu, iron (Fe), and zinc (Zn), are required in trace amounts for bacterial growth yet are toxic when present in excess. Toxicity in biological systems occurs through a variety of mechanisms. Heavy metals bind to free thiol groups, disrupting protein structure or function (8, 19). They can also displace essential metal cofactors in proteins (e.g., Fe in cytochromes). Some heavy metals, such as Cu, also generate reactive oxygen species (ROS) through auto-oxidation or Fenton-like reactions (52, 61). Hydrogen peroxide can be produced intracellularly through the oxidation of NADPH (equation 1) and subsequent activity of the enzyme superoxide dismutase (equation 2). Hydrogen peroxide can then interact with copper to produce ROS (equations 3 and 4).

Cu-induced oxidative stress can damage the cell membrane through lipid peroxidation, leading to membrane permeability and cell death (4, 36).

Bacteria have several mechanisms for coping with heavy metal stress, allowing them to thrive in ecosystems contaminated with toxic levels of heavy metals (49, 58, 68). Active efflux is a key aspect of resistance, transporting metal cations out of the cytosol and periplasmic space of gram-negative species (43, 56). Previous studies indicate that a network of different families of transporters coordinates efflux (38, 43, 56). One family of transporters is soft metal P-type ATPases (55). ATP hydrolysis drives transport of heavy metal cations from the cytoplasm into the periplasmic space, such as the well-characterized CopA of *Escherichia coli* (which effluxes Cu^{+1}) (54). Also contributing to metal efflux across the inner membrane are cation diffusion facilitator (CDF) transporters (25, 43, 51). The CDF family has been primarily implicated in resistance to heavy metals such as Zn, Cd, and Co (3). Finally, some members of the CBA family mediate proton-driven efflux of heavy metals from the periplasm (and possibly cytoplasm) (20, 22, 43), such as the CusABC system in *E. coli* (16, 22, 42,

* Corresponding author. Present address: Department of Microbiology, University of Washington, Box 357242, 1959 NE Pacific St., Seattle, WA 98195-7242. Phone: (206) 221-7871. Fax: (206) 543-8297. E-mail: parsem@u.washington.edu.

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48). Many CBA systems consist of an inner-membrane-associated resistance nodulation cell division (RND) transporter, a membrane fusion protein, and an outer membrane protein, forming a complex that spans the inner and outer membranes.

Some species can also reduce or oxidize heavy metals to less toxic forms. *Pseudomonas aeruginosa* reduces cytosolic mercury (Hg) to Hg⁰, which then passively diffuses out of cells (71, 73). The periplasmic multicopper oxidase of *E. coli*, CueO, has been hypothesized to protect the cell by converting Cu⁺¹ to its less toxic form Cu⁺² in an oxygen-dependent reaction (21). Alternatively (or additionally), CueO may protect *E. coli* by oxidizing catecholate siderophores, such as enterobactin, which can then sequester free Cu (23). The plasmid-encoded Pco system of *E. coli* has a multi-Cu oxidase, PcoA, that may function in a similar manner (30).

Several recent studies have explored the global responses of the paradigm enteric species, *E. coli*, to elevated levels of required heavy metals using transcriptional profiling (12, 34, 37, 74). Different approaches were taken to subject bacterial populations to the stress. In some cases, the heavy metal was applied to growing cultures as a "shock," and transcriptional profiles were assayed after a short period of exposure. In other cases, profiles were determined after longer periods of exposure, i.e., after bacterial populations had adapted to the stress and were actively growing. Both approaches were informative, indicating how *E. coli* responds and ultimately adapts to a heavy metal stress.

However, little is known in general about the global transcriptional responses of environmental bacterial species to heavy metal stress. Other than a recent report on the transcriptional response of *Bacillus subtilis* to different heavy metals, little has been published on environmental species (41). Unlike *E. coli*, *P. aeruginosa* is primarily an environmental organism commonly found in soil and water. Its genome has been sequenced, and commercially available DNA microarrays make it an excellent model organism for studying global transcriptional responses to environmental stimuli (64). Indeed, this approach has been successfully used to define transcriptional profiles of *P. aeruginosa* cultured under conditions ranging from elevated oxidative stress to denitrifying metabolism (14, 46, 50, 59, 60, 70).

In this study we examine the transcriptional profiles of *P. aeruginosa* exposed to a brief pulse of excess Cu (Cu shock) and cells growing continuously in the presence of excess Cu (Cu-adapted culture). Cu shock up-regulated expression of several genes involved in active transport functions as well as genes involved in oxidative and general stress responses. In contrast, transcriptional profiles of Cu-adapted populations suggested a Cu-specific physiology, with a much narrower range of genes encoding putative Cu-specific transport functions differentially expressed. An oxidative stress response was no longer apparent in the Cu-adapted population. Continuous Cu exposure also resulted in a general down-regulation of porins belonging to the OprD family. Iron homeostasis appeared to be particularly fine-tuned in Cu-adapted cultures. Genes encoding biosynthesis and transport of the iron-scavenging siderophore pyochelin were down-regulated in Cu-adapted populations, while genes encoding production of another siderophore, pyoverdine, were up-regulated. Strains bearing mutations in key copper-responsive genes identified in

our study were then analyzed for Cu and Zn sensitivity. Our results suggest that two gene clusters homologous to RND family members, a P-type ATPase, a two component regulatory system, and a periplasmic system involved in proper disulfide bond formation all play important roles in tolerating elevated levels of Cu.

MATERIALS AND METHODS

Bacterial strains, strain construction, and media. A wild-type laboratory strain of *P. aeruginosa* (PAO1) was used in this study. PAO1 was grown in LBM, which consisted of Luria-Bertani (LB) broth (Fisher Scientific, Hampton, NH) amended with 100 mM MOPS [3(4-morpholino)propanesulfonic acid]. Other growth media used in this study were minimal salts vitamin medium with glucose (MSVG), MOPSO-buffered saline solution, Jensens medium (32), and a MOPS minimal medium (53, 65, 72). The form of copper used in this study was CuSO₄, silver was AgNO₃, and zinc was ZnSO₄ (Fisher Scientific, Hampton, NH, and Sigma-Aldrich Co., St. Louis, MO). Antibiotic concentrations used for *P. aeruginosa* in this study were 60 µg/ml for tetracycline and 300 µg/ml for carbenicillin. Ampicillin was used for selection in *E. coli* at 100 µg/ml.

Standard methods were used to manipulate plasmids and DNA fragments. Restriction endonucleases and DNA modification enzymes were purchased from New England Biolabs (Beverly, MA). Chromosomal DNA was isolated using DNeasy Tissue kits (QIAGEN, Valencia, CA), and plasmid isolations were performed using QIAprep spin miniprep kits (QIAGEN). DNA fragments were purified using QIAquick mini-elute PCR purification kits (QIAGEN), and PCR was performed using the Expand Long Template PCR System (Roche, Indianapolis, IN). DNA sequencing was performed by automated sequencing technology either using the University of Oklahoma Health Science Center sequencing core facility or the University of Iowa DNA sequencing core.

A selected group of transposon mutants in PAO1 were obtained from the University of Washington Genome Center and screened for Cu sensitivity (31). The following strains were obtained from the Washington collection: PTL361, PTL396, PTL1518, PTL3663, PTL4456, PTL4590, PTL4823, PTL5121, PTL5506, PTL5729, PTL7641, PTL7760, PTL7968, PTL8385, PTL8750, PTL9314, PTL9648, PTL13061, PTL14395, PTL16055, PTL16683, PTL17602, PTL17732, PTL18229, PTL19549, PTL20046, PTL21569, PTL22097, PTL22200, PTL30399, PTL35346, PTL36065, PTL36283, PTL47603, PTL12403, PTL44858, PTL48465, PTL20694, PTL9334, PTL14744, PTL6615, PTL8802, PTL17142, PTL31953, PTL7848, PTL42174, PTL8844, and PTL12392. There are several strains that have transposon insertions in the same gene. See Table 4 for data from representative strains that have been confirmed by PCR.

Selected mutants were complemented by introducing the native gene on a broad-host-range plasmid. The PA3920 and PA2809 genes were cloned into the broad-host-range vector, pEX1.8. The PA3920 gene was amplified using PCR and the primers PA3920-for (5'-CGGAATTCATGAACAG CGCCACGCTAA CCGAG-3') and PA3920-rev (5'-CGGGATCCCATATGCGCTGGCGTGCTG GCTCA-3') (restriction sites are underlined). The resulting 2.4-kb PCR product was digested with EcoRI/BamHI and cloned into pEX1.8. The resulting plasmid, pEX3920, contains PA3920 under the control of the *Tac* promoter. The PA2809 gene was amplified with PCR and the primers PA2809-F (5'-GGGAATTCAT GAAACTGCTGATCGTCG-3') and PA2809-rev2 (5'-CGGAAGCTTTTCATTC GTCGCGCTCTTCG-3') (restriction sites are underlined). The resulting PCR product was digested with EcoRI/HindIII and cloned into pEX1.8 to create pEX2809.

For construction of the reporter plasmid pAG100, a 496-bp DNA fragment containing the PA3920 promoter was amplified by PCR with the primers PA3920promoter-for (5'-CGGGATCCCGA TCACCTCTGCTAAGGGCCT G-3') and PA3920promoter-rev (5'-GGGGTACCCC TCCTTGAATGGACA GATGG-3') (restriction sites are underlined). The resulting PCR product was digested with the restriction enzymes BamHI/KpnI and then ligated into the BamHI/KpnI digested *lacZ* reporter plasmid pQF50, creating a transcriptional fusion of the PA3920 promoter with *lacZ*.

PCR validation of mutant strains. Strains harboring transposon mutations were verified using PCR. A DNA segment was amplified from the chromosome of mutant strains using primers internal to the transposon (either Hah-138, 5'-CGGGTGCAGTAATATCGCCCT, or LacZ-148, 5'-GGGTAACGCCAGG GTTTTC) paired with a primer complementary to native chromosomal sequence close to the transposon insertion point. An amplified band of DNA of the correct size indicated the presence of the transposon at the expected location. The following primers were used to confirm mutant strains: for PA2809, 5'-CC TGCTTCGCCAGTTTCTCC; for PA3920, 5'-GGCAGCATCCAGTGCAG; for

PA3521, 5'-CCTGTAGCTGGAACAGGACATG; for PA0397, 5'-TCGACC ACACATGCAGGTCG; for PA1435, 5'-GTTGGCGTGACACCGCACGC; for PA1436, 5'-TGAAGTGCAGGGTCAGGGTGG; for PA1549, 5'-ATGGACCG CATCTCGCGCTGG; for PA2064, 5'-CGATCCAGCCGCTGGCGTCC; for PA2065, 5'-CGCCGTGGCCGAGACCTACG; for PA2476, 5'-GCAACCTGA TCACCTTGGC; for PA2477, 5'-AGGTAGCGCGCCAGGCTGCC; for PA2505, 5'-GTAGCGTCGACCTGCTGCCC; for PA2521, 5'-TGCCTGGTTCG GACAACTGC; for PA2522, 5'-GGCCAGCGCGGGAAACGC; for PA2807, 5'-GAAGTCCAGGTCAGCTCGG; for PA2810, 5'-CGACCATATGGCGATGT CCG; for PA3690, 5'-GATGGACTGCCCGACCGAG. The PCR protocol is available at the University of Washington website (<http://www.genome.washington.edu/UWGC/pseudomonas/>).

To further verify the accuracy of transposon insertion strains, PCR products generated from the reactions described above were subjected to sequencing to verify the location of the transposon insertion. Either the Hah-138 or the LacZ-148 primers were used in sequencing reactions. The following strains were confirmed in this manner: PTL12403, PTL16055, PTL20694, PTL17142, PTL12392, and PTL6615.

Transcriptional profiling experiments. For Cu shock conditions, PAO1 was inoculated at an optical density at 600 nm (OD_{600}) of 0.05 in 50 ml of LBM in 250-ml flasks and cultured at 37°C until early logarithmic phase, corresponding to an OD_{600} of 0.2, at which point 10 mM $CuSO_4$ was added to half of the flasks. The other half of the flasks were treated with a small amount of 1 M HCl to lower the pH to a similar level to that of the Cu-treated flasks (6.95 ± 0.04 for Cu-treated versus 7.01 ± 0.07 for the pH control flasks). After 45 min of treatment, cells were harvested and added to the RNA stabilization solution *RNAlater* (Ambion Inc., Austin, TX). Cu-adapted cells were subjected to 10 mM $CuSO_4$ at inoculation. These cultures were grown at 37°C until early logarithmic phase (OD_{600} of 0.2), and then cells were harvested and added to *RNAlater*. The control flasks were grown at 37°C until early log phase (OD_{600} of 0.2). For each condition, all samples were grown in triplicate, and RNA from the different flasks was combined. In addition, biological replicates of each condition were performed on a separate day and run on a separate microarray chip.

RNA was isolated from cells using RNeasy columns (QIAGEN). An additional, off-column DNase treatment with RQ1 RNase-free DNase (Promega Corp., Madison, WI) was performed and then subsequently purified on the RNeasy column. RNA quality was assessed by agarose gel electrophoresis. Synthesis of cDNA and application to the microarray were performed following the instructions of the manufacturer of the GeneChip *P. aeruginosa* Genome Array (Affymetrix Inc., Santa Clara, CA) with some modifications. In the cDNA synthesis, 12 μ g of RNA and semirandom hexamer primers with an average G+C content of 75% were used instead. S. Lory provided the spike transcript of control transcripts from six *B. subtilis* genes to monitor the labeling, hybridization, and staining efficiency of cDNA.

Analysis of DNA microarray data was first performed using Affymetrix Suite version 5.0, and further analysis was done using the online program CyberT (<http://visitor.ics.uci.edu/genex/cybert/>). CyberT utilizes a Bayesian statistical framework to calculate a more robust estimate of variance for a small number of experiments. The Bayesian prior estimate takes into consideration the background variance of the genes with similar expression levels as the gene of interest within a certain range, based on the theory that genes with similar expression levels should also have similar variance (27, 39). A sliding window size of 100 genes and a Bayesian prior estimate of 10 were used to calculate the P value. The posterior probability of differential expression [PPDE($<P$)] was also calculated. PPDE($<P$) ranges from zero to 1, and a low P value corresponds to a PPDE($<P$) closer to 1. Genes were considered differentially regulated if the P value was <0.001 , PPDE($<P$) was >0.996 , and the absolute change (n -fold) was >3 .

Real-time reverse transcription-PCR (RT-PCR). Cultures of PAO1 were grown in 250-ml baffled flasks containing 20 ml of LBM at 37°C and 150 rpm. Triplicate cultures were grown for the control (no Cu addition), Cu shock (10 mM $CuSO_4$ addition when the OD_{600} reached 0.2), and Cu-adapted (10 mM $CuSO_4$ addition at inoculation) experiments. The pH was consistent among the three experimental conditions. RNA was isolated from the control experiments when the OD_{600} was approximately 0.5. The Cu-shocked cells were treated with copper for 45 min; the OD_{600} was approximately 0.5 at the end of the treatment, and RNA was isolated immediately. The Cu-adapted cells also were grown to an OD_{600} of approximately 0.5, at which time the RNA was isolated immediately. One RNA extraction was performed for each flask, and the RNA was pooled for each condition (control, Cu-shocked, and Cu-adapted cultures). RNA was isolated with an RNeasy kit (QIAGEN), and residual DNA was removed with DNase I treatment (RiboPure™-Bacteria kit, Ambion, Austin, TX).

Duplicate cDNA synthesis reactions were performed with random hexamer primers and avian myeloblastosis virus reverse transcriptase (Roche, Germany).

FIG. 1. Growth curves of cultures used for transcriptional profiling. For Cu-shocked cultures, PAO1 was grown in 250-ml flasks containing 50 ml of LBM at 37°C until early logarithmic phase, corresponding to an OD_{600} of 0.2. At this point, 10 mM $CuSO_4$ was added to half of the flasks, and HCl was added to the other half of the flasks to serve as a control for the pH change caused by $CuSO_4$ addition. The cultures were then incubated for a further 45 min. The control culture is indicated by open circles (○), while the Cu-shocked culture is indicated by filled triangles (▲). The time at which Cu is added to the shocked culture or HCl is added to the pH control culture is indicated by the solid arrow. Cu-adapted cultures are indicated by the open triangles (△). In this case, 10 mM $CuSO_4$ was present at inoculation of the culture. The untreated controls for the Cu-adapted samples is indicated by filled squares (■). The dashed arrows represent when the culture was harvested for transcriptional profiling. Dashed arrow 1 indicates when the acute stresses culture was harvested, while the dashed arrow 2 indicates when the adapted culture was harvested. The data points represent three experiments with the standard deviation bars shown.

Four micrograms of RNA was mixed with 2 μ l of the primers (10 μ M) and nuclease-free water (Ambion) to a final volume of 10 μ l. The RNA mixture was denatured at 70°C for 5 min and placed on ice. Four microliters of 5 $\times$ First-Strand Buffer, 2 μ l of deoxynucleoside triphosphate mix (a 10 mM concentration of each nucleoside; New England Biolabs, Beverly, MA), 2 μ l of water, and 2 μ l of avian myeloblastosis virus reverse transcriptase were added to each reaction on ice. Each tube was incubated at 42°C for 1.5 h in a Primus 25 Personal Cycler with a heated lid (MWG Biotech, High Point, NC). Negative controls for RT-PCR were prepared by omitting the reverse transcriptase in the cDNA synthesis reactions and were used to verify that contaminating genomic DNA was absent from the RNA preparations. Duplicate cDNA reactions were pooled and used in triplicate real-time PCRs. Real-time PCRs were run on an Applied Biosystems 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA) using Power Sybr Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The final concentration of each primer was 0.3 μ M for all reactions. The following thermocycler program was used: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Real-time PCR product dissociation curves were used to verify the specificity of the amplified product. Fivefold serial dilutions of cDNA, prepared from a mixture of equal aliquots of cDNA from each experimental condition, were used to construct a standard curve for each gene tested (PA2477, PA2505, PA2522, PA3521, PA3920, PA4067, and PA4946). The cycle number at which fluorescence crossed a selected threshold value during exponential amplification was correlated to a relative quantity. The relative quantity of a gene transcript was normalized to the relative quantity of the housekeeping gene (PA4946) transcript for the same experimental condition. The relative change in expression (n -fold) was calculated as the relative quantity of the target gene transcript under Cu shock or Cu-adapted conditions (normalized to PA4946), divided by the relative quantity of the target gene transcript under the control condition (normalized to PA4946).

FIG. 2. A schematic depicting genes transcriptionally regulated by copper and some of the predicted/putative functions they encode. Gene names are labeled above the arrows. Arrows are color-coded to reflect relative changes in expression (*n*-fold). Arrows are oriented to represent the orientation of the gene on the chromosome. Genetic loci with arrows touching and oriented in the same direction are predicted to belong to part of an operon. A superscript R indicates a putative regulatory gene. Question marks indicate putative functions.

Pyoverdine and pyochelin measurements. Cultures were grown in a modified Jensens medium at 37°C with glucose as the sole carbon source. Pyoverdine concentrations were calculated from the absorbance at 403 nm of cell-free, diluted culture supernatants as previously described (46). Purified pyoverdine was used to generate a standard curve. A modification of a previous protocol was used to measure pyochelin levels. The dried organic fraction of ethyl acetate-extracted cell-free culture supernatants was resuspended at 10× concentration, and pyochelin levels were determined as previously described (69). Purified pyochelin was used to generate a standard curve.

MIC determination for mutant strains. The MIC of Cu was the lowest concentration at which there was no growth after 16 h of exposure. In a 96-well microtiter plate format, PAO1 and mutant strains were subjected to an array of heavy metal concentrations in LBM. Each well contained 100 µl of LBM plus CuSO₄ concentrations ranging from 1 mM to 16 mM. Each well was inoculated with a 10-µl aliquot of cells from an actively growing culture at an OD₆₀₀ of 0.1. The microtiter plate was incubated at 37°C with gentle shaking for 15 s every 30 min. Growth was monitored by reading the OD₆₀₀ every 30 min using a microtiter plate spectrophotometer (Genios microplate reader [Tecan Research Tri-

angle Park, NC) or Synergy HT [Bio-Tek Instruments, Winooski, VT]), and the MIC was determined after 16 h. Each experiment was performed in triplicate.

Disk sensitivity assay. Tested strains were grown to late logarithmic phase in LB at 37°C, and then 500 µl of cell suspension was added to a cooled, molten soft LB agar (0.5% agar). The agar was then poured into petri plates and allowed to solidify. A sterilized Whatman filter disk (catalog no. 2017013) incubated overnight in either 500 mM CuSO₄ or 500 mM ZnSO₄ was added to the center of the agar plate after it had solidified. The plate was then incubated at 37°C overnight, and the zone of clearance surrounding the disk was measured.

Copper viability analysis. Tested strains were grown in 250-ml baffled flasks containing 50 ml of LBM until mid-logarithmic phase (OD₆₀₀ of 0.5). Cells were harvested, centrifuged, and resuspended and washed in the minimal medium MSVG. Aliquots were subjected to an array of Cu concentrations in MSVG and then placed at 37°C. After 5 h, the samples were sonicated for 30 s (3 s on at an output of 7 and 1 s off for 10 cycles) (Ultrasonic liquid processor XL; Misonix Inc., Farmingdale, NY), and viable plate counts were performed on serial dilutions.

β-Galactosidase assays. β-Galactosidase activity was monitored colorimetrically as described by Zubay et al. (77). To examine the expression of PA3920 in response to increasing copper levels, overnight cultures of *P. aeruginosa* containing pAG100 were diluted in Trypticase soy broth to an OD₆₀₀ of 0.05 and incubated with shaking (250 rpm) at 37°C for 1 h. Increasing concentrations of CuSO₄ (50 µM to 1.5 mM) were then added to individual cultures; cultures were incubated for 45 min, and β-galactosidase activities were determined.

Microarray accession number. Array data were submitted to the Gene Expression Omnibus website (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE4152.

RESULTS AND DISCUSSION

Transcriptional profiles of Cu-adapted and Cu-shocked *P. aeruginosa* populations. In this study, *P. aeruginosa* was subjected to two types of Cu stress. Cu-shocked cultures were defined as cultures actively growing under nonstressed conditions that are subjected to a brief pulse of elevated Cu. Transcriptional profiles of early log cultures were assessed shortly after Cu addition (45 min). Cu-adapted populations were defined as cells actively growing in the presence of elevated Cu (approximately 6 h after inoculation of the culture). Cu was present in the growth medium at inoculation, and early log cultures were harvested for transcriptional profiling. The rationale for testing these two conditions was to compare and contrast transcriptional profiles of a sudden Cu stress response to adapted populations growing in the presence of excess Cu.

A range of CuSO₄ was added to actively growing *P. aeruginosa* cultures in order to define acute stress conditions. A buffered complex medium (LBM) was used to maximize biomass yield. A Cu concentration was chosen (10 mM) that impacted *P. aeruginosa* growth rate but was not lethal, as determined by viable plate counts. Forty-five minutes after the addition of 10 mM Cu, only minimal growth had occurred in the treated culture, while growth had occurred in the control culture (OD₆₀₀ of 0.310 versus 0.396, respectively) (Fig. 1).

If present in the growth medium at inoculation, 10 mM Cu resulted in a greater initial lag in growth and a lower growth rate than the untreated culture (Fig. 1). This was chosen to represent a Cu-adapted population. In all cases (control, shocked, and adapted conditions), approximately the same amount of biomass, as determined by viable plate counts and OD₆₀₀ values, was harvested for transcriptional profiling.

Figure 2, Tables 1 to 3, and Table S1 in the supplemental material feature genes that were up- and down-regulated in shocked and adapted populations. Genes were considered differentially regulated if the relative change (*n*-fold) was >3.0, the *P* value was <0.001, and the PPDE(<*P*) was >0.996. Cu

shock resulted in differential expression of 405 genes. Most of these genes were up-regulated. Although more genes would have qualified as repressed, they did not meet the *P* value and PPDE stringency cutoffs. In the case of Cu-adapted cultures, 331 genes were either up- or down-regulated. To further validate the array data, real-time RT-PCR was used to determine the relative quantities of selected transcripts under control and test culturing conditions. These are indicated in a separate column in Tables 1 and 3.

There were 41 genes that were differentially regulated for both types of Cu stress. These genes represented a common core response of *P. aeruginosa* to Cu, including the up-regulation of transporters (*czcCB*, PA3920, and PA3521 to PA3523), a system containing a potential Cu oxidase (*pcoAB*), genes encoding periplasmic thiol-disulfide interchange proteins (*dsbG* and PA2477 to PA2478), putative lyases (PA3516 and PA3517), and transcriptional regulators (PA2479, PA2809, PA2523, and PA3410). Since both the shocked and adapted stress conditions impact growth rate (Fig. 1), subsequent discussion of transcriptional profiling data places little emphasis on features pertaining to general metabolism.

Active efflux is a key aspect of copper tolerance. RND, CDF, and P-type ATPase efflux transporters have been suggested to constitute an efficient, integrated network controlling heavy metal levels in the cytoplasm and periplasm for some gram-negative bacterial species. Several studies have shown that transporters belonging to these families are induced in the presence of elevated levels of their heavy metal substrates.

Cu stress up-regulated expression of genes encoding predicted active transport functions (Table 1). The *P. aeruginosa* chromosome has seven genes homologous to P-type ATPases (43). One, PA3920, was up-regulated in both shocked and adapted populations. In addition, a few RND family transporters were up-regulated under both conditions. The *P. aeruginosa* chromosome has 12 loci harboring genes homologous to RND family members (35, 43). Three were up-regulated in response to Cu exposure (PA1436, PA2520, and PA3522). The most prominently up-regulated locus is PA2520 to PA2522, which encodes the *czcCBA* transport system, previously shown to be important for Cd, Zn, and Co tolerance (26, 57). An adjacent two-component regulatory system (encoded by PA2523 and PA2524) was also up-regulated in Cu-stressed cultures. The PA3521 to PA3523 and PA1435 to PA1436 genes have no known efflux substrates (62). These loci also have no linked regulatory proteins.

A gene encoding a CDF family member, PA0397, was up-regulated in Cu-adapted populations. A homologous gene in *Ralstonia metallidurans* is thought to confer resistance to Cd, Zn, and Co (2). Nies suggested that the Cu ion may be too small to serve as a substrate for transport for this family (43), although Moore et al., recently reported that a CDF family member of *B. subtilis* conferred protection to Cu (41). The PAO1 chromosome has two other genes belonging to the CDF family, PA1297 and PA3693. PA1297 is 50% similar to the zinc transporter, ZntA, of *Staphylococcus aureus* and was up-regulated under Cu shock conditions.

Copper shock appears to produce an oxidative stress response. Two previously published studies characterized the transcriptional profiles of *P. aeruginosa* exposed to reactive oxygen species (50, 59). Comparing our data with the data of

TABLE 1. Regulated genes involved in transport and membrane permeability and associated regulatory proteins

<i>P. aeruginosa</i> ORF ^a	Gene	Change in expression (<i>n</i> -fold)			Annotation ^d
		Cu-shocked culture ^b	Cu-adapted culture ^b	RT-PCR ^c	
Up-regulated					
PA0184 ↑		4			ATP-binding component of ABC transporter
PA0185 ↓		3			Permease of ABC transporter
PA0186		5			Binding protein component of ABC transporter
PA0203 ↑		19			Binding protein component of ABC transporter
PA0204 ↑		19			Permease of ABC transporter
PA0205 ↑		4			Permease of ABC transporter
PA0206 ↓		4			ATP-binding component of ABC transporter
PA0397			17		CDF family, <i>czcD</i> homolog
PA0450		5			Phosphate transporter
PA0913	<i>mgtE</i>	4			Mg transporter MgtE
PA1286		8			MFS transporter
PA1297		10			CDF family
PA1435 ↓		4	12		MFP
PA1436 ↓		3	23		RND efflux transporter
PA1437 ↓		8			Two-component response regulator
PA1438 ↓		4			Two-component sensor
PA1541		89			SMR family transporter
PA2277 ↓	<i>arsR</i>	6			ArsR transcriptional regulator
PA2278 ↓	<i>arsB</i>	3			ArsB protein
PA2279 ↓	<i>arsC</i>	5			ArsC transport protein
PA2505	<i>opdT</i>		83	NC; 9	Predicted porin, OprD family
PA2520 ↑	<i>czrA</i> ^e	3	62		RND metal cation efflux transporter CzcA
PA2521 ↑	<i>czrB</i> ^e	4	118		MFP, CzcB
PA2522 ↓	<i>czrC</i> ^e	20	390	11; 1,430	OMF precursor CzcC
PA2523 ↓	<i>czrR</i> ^e	5	66		Two-component response regulator
PA2524 ↓	<i>czrS</i> ^e	4	28		Two-component sensor
PA3521 ↑		174	34	283; 124	OMF
PA3522 ↑		66	4		RND efflux transporter
PA3523 ↓		460	14		MFP
PA3573		47			MFS transporter
PA3690		43			Metal-transporting P-type ATPase
PA3709			4		MFS transporter
PA3749		10			MFS transporter
PA3920		149	8	520; 75	Metal transporting P-type ATPase
PA3921		3			Transcriptional regulator
PA3936 ↑		3			Permease of ABC taurine transporter
PA3937 ↑		4			ATP-binding component of ABC taurine transporter
PA3938 ↓		6			Periplasmic taurine-binding protein precursor
PA4158	<i>fepC</i>	13			Ferric enterobactin transport protein FepC
PA4159 ↓	<i>fepB</i>	19			Ferric enterobactin-binding periplasmic FepB
PA4160 ↓	<i>fepD</i>	4			Ferric enterobactin transport protein FepD
PA4192 ↑		4			ATP-binding component of ABC transporter
PA4193 ↑		4			Permease of ABC transporter
PA4194 ↑		7			Permease of ABC transporter
PA4195 ↓		7			Binding protein component of ABC transporter
PA4710	<i>phuR</i>	8	3		Heme uptake outer membrane receptor
PA4825	<i>mgtA</i>	44			Mg ²⁺ transport P-type ATPase
PA4909			13		ATP-binding component of ABC transporter

Continued on following page

TABLE 1—Continued

<i>P. aeruginosa</i> ORF ^a	Gene	Change in expression (<i>n</i> -fold)			Annotation ^d
		Cu-shocked culture ^b	Cu-adapted culture ^b	RT-PCR ^c	
PA5311		5			MFS transporter
Down-regulated					
PA0162	<i>opdC</i>		−4		Predicted porin, OprD family
PA0291	<i>oprE</i>		−6		Outer membrane porin OprE precursor
PA0958	<i>oprD</i>		−10		Outer membrane porin protein OprD precursor
PA2760	<i>oprQ</i>		−16		Predicted porin, OprD family
PA3790	<i>oprC</i>		−4		Outer membrane porin protein OprC
PA4067	<i>oprG</i>		−16	1.4; −4	Outer membrane porin protein OprG precursor
PA4179	<i>opdN</i>		−15		Predicted porin, OprD family
PA4221 ↑	<i>fptA</i>		−13		Fe(III)-pyochelin receptor precursor
PA4222 ↑			−8		ATP-binding component of ABC transporter
PA4223 ↑			−17		ATP-binding component of ABC transporter
PA4501	<i>opdP</i>		−4		Predicted porin, OprD family
PA5230			−4		Permease of ABC transporter
PA5231			−4		ATP-binding/permease fusion ABC transporter

^a Gene name, number, and annotation are from the *Pseudomonas* genome project (<http://www.pseudomonas.com>). Putative operons are indicated in boldface, and genetic organization (i.e., the first versus the last gene in the operon) is indicated by an arrow.

^b Values are levels for cultures with Cu/levels for control (no Cu) cultures. Up-regulated and down-regulated genes had an absolute change (*n*-fold) of >3.0, PPDE(<*P*) of >0.996, and *P* of <0.001. Values are not listed in the table if the change value is <3 and >−3.

^c Relative change (*n*-fold) in levels of transcript for cultures with Cu compared to control cultures without Cu as determined by real-time RT-PCR. Data are displayed as follows: change (*n*-fold) in shocked cultures/control cultures; change (*n*-fold) in adapted cultures/control cultures. NC, no change.

^d MFS, major facilitator superfamily; ABC, ATP-binding cassette; MFP, membrane fusion protein; OMF, outer membrane factor; SMR, small multidrug resistance.

^e Nomenclature consistent with reference 20 and opposed to the *czc* designation suggested by the *Pseudomonas* genome project.

Palma et al. and Salunke et al. suggests that Cu shock results in an oxidative stress response. Several genes identified in these studies were also induced in the Cu-shocked cultures, such as superoxide dismutase (*sodM*), catalase (*katB*), genes

involved in organic hydroperoxide resistance (*ohr* and *ahpF*), and genes involved in the DNA SOS response/DNA damage and repair (*dinP*, *recA*, and *recN*) (Table 2; see Table S1 in the supplemental material). Comparing their data sets with ours

TABLE 2. Genes involved in an oxidative stress response

<i>P. aeruginosa</i> ORF ^a	Gene	Change in expression (<i>n</i> -fold) in Cu-shocked culture ^b	Oxidative stress response reported in ^c :		Annotation ^f
			Palma et al. ^d	Salunke et al. ^e	
PA0140	<i>ahpF</i>	43	Y	Y	Alkyl hydroperoxide reductase
PA0472		25	Y	Y	Sigma 70 factor, ECF subfamily
PA0672	<i>hemO</i>	56	Y	N	Heme oxygenase
PA0848		39	Y	Y	Alkyl hydroperoxide reductase
PA0849	<i>trxB2</i>	25	Y	Y	Thioredoxin reductase 2
PA1300		22	Y	Y	Sigma 70 factor, ECF subfamily
PA2826		8	Y	N	Glutathione peroxidase
PA2850	<i>ohr</i>	50	N	Y	Organic hydroperoxide resistance
PA4468 ↑	<i>sodM</i>	8	Y	Y	Superoxide dismutase
PA4469 ↑		20	Y	N	HP
PA4470 ↑	<i>fumC1</i>	23	Y	Y	Fumarate hydratase
PA4471 ↑		164	Y	Y	HP
PA4613	<i>katB</i>	10	Y	Y	Catalase
PA4655	<i>hemH</i>	9	Y	Y	Ferrochelatase

^a Gene name, number, and description from *Pseudomonas* genome project (<http://www.pseudomonas.com>). A putative operon is indicated in boldface, and genetic organization (i.e., the first versus the last gene in the operon) is indicated by an arrow.

^b Values are the levels for cultures with Cu/levels for control (no Cu) cultures. Up-regulated and down-regulated genes had an absolute change (*n*-fold) of >3, PPDE(<*P*) of >0.996, and *P* of <0.001. Values are not listed in the table if change (*n*-fold) was <3 and >−3. Cu-adapted cultures were not relevant.

^c Y, yes; N, no.

^d (50).

^e (59).

^f HP, hypothetical; ECF, extracytoplasmic function sigma factor.

TABLE 3. Other select genes differentially regulated by Cu

<i>P. aeruginosa</i> ORF and function ^a	Gene	Change in expression (<i>n</i> -fold)			Annotation ^d
		Cu-shocked culture ^b	Cu-adapted culture ^b	RT-PCR ^c	
Up-regulated					
Iron homeostasis					
PA2386	<i>pvdA</i>		9		L-Ornithine N5-oxygenase
PA2394 ↓	<i>pvdN</i>		8		PvdN
PA2395 ↓	<i>pvdO</i>		6		PvdO
PA2396	<i>pvdF</i>		6		Pyoverdine synthetase F
PA2397	<i>pvdE</i>		6		Pyoverdine biosynthesis protein PvdE
PA2399	<i>pvdD</i>		3		Pyoverdine synthetase D
PA2424 ↑	<i>pvdL</i>		15		Pyoverdine biosynthesis protein PvdL
PA2425 ↑	<i>pvdG</i>		4		Pyoverdine biosynthesis protein PvdG
PA2426	<i>pvdS</i>	20	5		Sigma factor PvdS
PA3410 (?) ↑		5	10		
PA3411 (?) ↑			12		HP
PA3412 (?) ↑		11	359		HP
PA3413 (?) ↓		7			CHP
PA3414 (?) ↓		10			HP
Copper binding/oxidase					
PA2064 ↑	<i>pcoB</i>	9	136		Copper resistance protein B precursor
PA2065 ↑	<i>pcoA</i>	27	516		Copper resistance protein A precursor
PA2807		762	2,376		Cu binding protein azurin/plastocyanin family
PA3520		5	3		Cu binding protein
Other or unknown functions					
PA0424	<i>mexR</i>	10			Multidrug resistance repressor
PA1390			14		Glycosyl transferase
PA2476 ↑	<i>dsbG</i>	4	26	5; 49	Thiol-disulfide interchange protein
PA2477 ↑	<i>dsbE</i>	6	26		Thiol-disulfide interchange protein
PA2478 ↑	<i>dsbD</i>	8	16		Thiol- thiol:disulfide interchange protein disulfide interchange protein
PA2479 ↓		4	8		Two-component response regulator
PA2480 ↓		4	8		Two-component sensor
PA2808	<i>ptrA</i>	57	392		Negative regulator of type III secretion
PA2809	<i>copR</i>	5	8		Two-component response regulator
PA2810	<i>copS</i>	3	4		Two-component sensor
PA3515 ↑		47	50		HP
PA3516 ↑		70	38		Probable lyase
PA3517 ↑		231	19		Probable lyase
PA3518 ↑		145	28		HP
PA3519 ↑		113	25		HP
PA4818 ↑			32		CHP
PA4819 ↑			31		Glycosyl transferase
PA4820 ↑		166			HP
Down-regulated, iron homeostasis					
PA4224 ↑	<i>pchG</i>		−5		Pyochelin biosynthetic protein PchG
PA4225 ↑	<i>pchF</i>		−13		Pyochelin synthetase
PA4226 ↑	<i>pchE</i>		−20		Dihydroaeruginosic acid synthetase
PA4228 ↓	<i>pchD</i>		−22		Pyochelin biosynthesis protein PchD
PA4229 ↓	<i>pchC</i>		−8		Pyochelin biosynthetic protein PchC
PA4230 ↓	<i>pchB</i>		−10		Salicylate biosynthesis protein PchB
PA4231 ↓	<i>pchA</i>		−55		Salicylate biosynthesis isochorismate synthase

^a Gene name, number, and description from *Pseudomonas* genome project (<http://www.pseudomonas.com>). Putative operons are indicated in boldface, and genetic organization (i.e., the first versus the last gene in the operon) is indicated by an arrow.

^b Values are the levels for cultures with Cu/levels for control (no Cu) cultures. Up-regulated and down-regulated genes had an absolute change (*n*-fold) of >3.0, PPDE(<*P*) of >0.996, and *P* of <0.001. Values are not listed in the table if the change (*n*-fold) was <3 and >−3.

^c Relative change (*n*-fold) in levels of transcript for cultures with Cu compared to control cultures without Cu as determined by real-time RT-PCR. Data are displayed as follows: change (*n*-fold) in shocked cultures/control cultures; change (*n*-fold) in adapted cultures/control cultures.

^d ECF, extracytoplasmic function sigma factor; HP, hypothetical; CHP, conserved hypothetical.

allowed us to filter the Cu shock data set such that none of the genes presented in Tables 1 and 3 are related to an oxidative stress response. This finding is in agreement with the studies of Kershaw et al. and Egler et al., where the addition of either copper sulfate or copper-glycine to *E. coli* cultures was shown to produce an oxidative stress response (12, 34). In contrast, Cu-adapted populations no longer showed elevated expression of these genes, possibly due to decreased intracellular Cu following adaptation of the organism to the stress.

Dealing with excess copper in the periplasm. Genes encoding periplasmic folding functions were up-regulated in response to Cu stress (Fig. 2 and Table 3). One locus, PA2476 to PA2478, has three genes homologous to *dsbG*, *dsbE*, and *dsbD*, respectively. In *E. coli*, this system is thought to repair non-native disulfide bonds in the periplasm (7). These genes were up-regulated in both shocked and adapted populations. Hiniker et al. demonstrated that a member of the *dsb* system, DsbC, is an important Cu resistance determinant in *E. coli* (28). Since free thiols interact with Cu, this system may ensure proper folding of proteins newly imported into the periplasm.

An operon encoding putative metal-binding proteins and Cu oxidases was also up-regulated in both Cu treatments (Fig. 2 and Table 3). Cu stress induced expression of the *pcoAB* genes (PA2065 and PA2064) which are homologous (81% and 75% similarity, respectively) to the *copAB* genes of *Pseudomonas syringae*. CopA and CopB have been demonstrated to bind Cu in the periplasm (CopA) and outer membrane (CopB) (6). PcoA has a multicopper oxidase domain (SufI domain; COG2132 [http://www.ncbi.nlm.nih.gov/COG]), similar to CueO of *E. coli*. This enzyme might participate in the oxidation of Cu^{+1} to the less toxic form Cu^{+2} or the oxidation of catechol-like siderophores (although *P. aeruginosa* is not known to produce its own catechol siderophore). Like *Pseudomonas putida*, *P. aeruginosa* lacks homologs of the *copCD* genes that are present in *P. syringae* (5).

Another gene, PA3520, was up-regulated by Cu and is associated with a cluster of genes that are also Cu responsive. This gene is predicted to encode a small protein that contains a metal binding motif, MXCXXC. This motif is found in a variety of proteins that bind heavy metals, such as CopZ. CopZ in *Enterococcus hirae* is thought to be a periplasmic Cu chaperone (40). However, the localization of the PA3520 gene product to the periplasm is uncertain. Homologous genes are found on the *P. putida* (PP0588) and *P. syringae* (PSPT00752) chromosomes.

Regulation of outer membrane permeability appears to be a feature of adaptation to copper. Some passive transport functions were down-regulated in Cu-adapted populations. Eight different porins or predicted porins were down-regulated (Table 1). One porin, OprD, binds to basic amino acids or basic dipeptides. Interestingly, reduced OprD expression has been linked to resistance to carbapenem antibiotics, and addition of low micromolar levels of Zn to *P. aeruginosa* led to its reduced expression (24). Four more Cu-repressed porins, OprQ, OpdC, OpdN, and OpdP, belong to the OprD family but have no assigned uptake function (24). OprC is TonB dependent and is thought to be Cu specific. Elevated extracellular Cu was previously reported to repress its expression (76). OprG and OprE were also repressed. OprG has been suggested to be involved

in low-affinity Fe uptake, and OprE is an anaerobically induced porin (18, 75). The porin encoded by PA2505 was the only porin induced by long-term Cu exposure. This gene encodes *opdT*, a porin that also belongs to the OprD family. Perhaps induction of *opdT* compensates for repression of the other OprD family members while not compromising the cell in terms of Cu permeability.

Increased porin expression has been previously reported as a feature of Cu resistance in *E. coli*. Egler et al. reported that OmpC expression was increased in response to Cu and controlled through the extracytoplasmic sigma factor, RpoE (12). Mutations in OmpC rendered *E. coli* sensitive to Cu, although the underlying mechanism was unclear.

Effects of copper stress on iron homeostasis. A distinct group of genes involved in Fe homeostasis were induced in Cu-stressed cultures. This group overlapped with genes identified in *P. aeruginosa* exposed to Fe-limiting conditions (46). Of the 30 genes with the highest relative induction (*n*-fold) in response to Fe limitation identified by Ochsner et al., 20 were also up-regulated in response to Cu stress. Many of these 20 genes have consensus binding sequences in their promoter regions for the Fe-responsive regulators, Fur and PvdS (10, 45). In line with this observation is the up-regulation of *pvdS* (PA2426) under both stress conditions. Another interesting similarity with the Ochsner study is that, of the eight genes homologous to extracytoplasmic function family sigma factors they reported to be induced by Fe limitation (PA2387, PA3899, PA4896, PA2468, PA3410, PA1300, PA0472, and *pvdS*), seven are also up-regulated in Cu-shocked populations (all of the above except for PA2387). The heavy metal induction of Fe siderophores has previously been reported for *E. coli* (12, 34). The siderophore enterobactin not only may play a role in Fe transport but also may protect the cell by sequestering heavy metals in the cytoplasm (23).

Many Fe-regulated genes, including *pvdS*, are under control of the regulatory protein Fur, which complexes Fe and represses expression of target genes under Fe-replete conditions. Heavy metals could potentially interact with Fur, displacing Fe and thus influencing its binding to target DNA sequences (9). Another possibility is that ROS generated by Cu stress could oxidize Fur-bound Fe^{2+} , releasing Fe from Fur and relieving Fur-mediated repression.

The major difference between this study and that of Ochsner et al. is that Cu-adapted populations repressed expression of genes involved in synthesis of the siderophore pyochelin, whereas these genes were induced under Fe-limiting conditions. This is consistent with Visca et al., who previously reported that several transition metals, including Cu, repress pyochelin synthesis (67).

In contrast, several genes involved in the biosynthesis of pyoverdine were up-regulated in Cu-adapted populations (Table 3 and Fig. 2). Pyoverdine has been previously shown to be up-regulated in response to Cd and Zn (11, 29, 67). Pyoverdine might provide the bacterium with an iron-specific acquisition system, whereas pyochelin is down-regulated since its broader specificity for metal cations might allow Cu to enter the cell. The regulation of Fe acquisition appears to be carefully fine-tuned in a Cu-stressed environment.

To functionally verify transcriptional profiling data, *P. aeruginosa* was exposed to different levels of CuSO_4 , and

FIG. 3. Long-term exposure to copper induces pyoverdine production in *P. aeruginosa*. (A) Batch cultures were treated with different amounts of CuSO_4 for different periods of time. Pyoverdine production was measured for cultures in triplicate. Long-term copper exposure was seen to induce production of pyoverdine. A mutant unable to make pyoverdine (PAO1-*pvdA*) served as a negative control. (B) A picture of *P. aeruginosa* cultures grown for 20 h in a 96-well microtiter dish. From left to right, PAO1 grown in the absence of CuSO_4 , PAO1 grown in the presence of 100 μM CuSO_4 , and PAO1-*pvdA* grown in the presence of 100 μM CuSO_4 .

pyoverdine and pyochelin levels were measured. Pyoverdine levels were seen to increase more than 10-fold in cultures treated with CuSO_4 for a long period of time (Fig. 3). As suggested by the array data, pyoverdine levels did not increase after short-term exposure. A mutant strain unable to biosynthesize pyoverdine, PAO1-*pvdA*, did not show induced pyoverdine production in the presence of Cu. The array data also predict that long-term copper exposure would result in reduced pyochelin levels. However, even in untreated cultures, pyochelin levels were below detectable limits (data not shown).

The susceptibility of select mutant strains to copper and zinc supports transcriptional profiling data. Transcriptional profiling suggested that a number of functions are important for Cu tolerance. To both validate the array data and explore the relative contribution of different genes to Cu tolerance, strains harboring transposon insertions in select genes were obtained from the mutant library at the University of Washington at Seattle. When available, multiple different mutant strains were obtained for a single gene. Data presented in Table 4 and Fig. 4 and 5 indicate the specific strain used for that experiment. The location of all transposon insertions was verified by PCR. Select PCR products were sequenced to verify the location of the transposon insertion. Polar effects caused by the transposon insertion or secondary mutations not related to the disrupted gene cannot be ruled out here. Due to the number of strains analyzed, a select few mutants with distinct phenotypes were complemented in *trans*, ruling out secondary mutations.

Mutant strains were evaluated for Cu sensitivity using two separate methods. The first was MIC determination for the

TABLE 4. Sensitivity of mutant strains to Cu and Zn

<i>P. aeruginosa</i> ORF ^a	PTL no. ^b	Cu MIC (mM) ^c	Cu disk assay (mm) ^d	Zn disk assay (mm) ^d	Annotation
Wild type		16	8	8	
PA0397	4823	16	9 [†]	8	CDF family, <i>czcD</i> homolog
PA1435	12403	14*	16 [†]	8	MFP
PA1436	44858	16	8 [†]	8	RND efflux pump
PA1549	48465	16	8	18	P-type ATPase
PA2064	47603	16	8 [†]	8 [†]	<i>pcoB</i>
PA2065	16055	16*	15 [†]	9 [†]	<i>pcoA</i>
PA2476	20694	16*	12	10 [†]	<i>dsbG</i> ; thiol-disulfide interchange protein
PA2477	9334	14*	12	8	<i>dsbE</i> ; thiol-disulfide interchange protein
PA2478	14744	16*	10	8	<i>dsbD</i> ; thiol-disulfide interchange protein
PA2505	22097	16	8	8	Predicted porin, <i>opdT</i>
PA2520	6615	16	8	25	<i>czrA</i> ; RND efflux pump
PA2521	20046	16	10 [†]	9	<i>czrB</i> ; MFP
PA2522	8802	14*	17 [†]	19	<i>czrC</i> ; OMF
PA2807	35346	16	12 [†]	8	Azurin/plastocyanin family
PA2809	17142	2*	18	8	Two-component regulator
PA2809C		16	8		
PA2810	31953	16*	10	8	Two-component sensor
PA3521	4456	16*	8 [†]	8	OMF
PA3522	7848	16	8 [†]	8 [†]	RND efflux pump
PA3523	42174	16	8	9	MFP
PA3690	4985	16	8	10 [†]	P-type ATPase
PA3920	12392	4*	26	8	P-type ATPase
PA3920C		16	8		

^a Gene name, number, and description from *Pseudomonas* genome project (<http://www.pseudomonas.com>). PA2809C and PA3920C mutants were complemented in *trans* by pEX2809 and pEX3920, respectively.

^b Strains were obtained from the University of Washington mutant collection. Transposon insertions were verified by PCR.

^c CuSO_4 was used for MIC analysis. *, lag in growth.

^d Diameter of zone of clearance. †, halo of extremely light growth surrounded the zone of clearance.

FIG. 4. Growth curves of mutant and wild-type strains of *P. aeruginosa* in the presence of different CuSO_4 levels. Optical density of the culture was measured as a function of time. Levels of added Cu are indicated by the symbol as it corresponds to the legend inset on the figure. (A) PAO1, the wild-type strain. (B) PA1435, a strain harboring a mutation in the gene PA1435, a homolog of the RND family. (C) PA2522 (*czrC*), a strain harboring a mutation in the gene PA2522, a homolog of the RND family. (D) PA2477 (*dsbE*), a strain harboring a mutation in a gene homologous to a gene involved in thiol-disulfide periplasmic folding in *E. coli*. The specific strains used in this analysis were PTL12403 (PA1435), PTL8802 (PA2522), and PTL9334 (PA2477). There was no discernible growth at 16 mM CuSO_4 , with the slightly elevated OD_{600} due to precipitation of Cu.

strain in liquid culture. To be consistent with the profiling experiments, strains were grown in buffered LB and a variety of CuSO_4 concentrations, and growth was measured at 16 h. The MIC of CuSO_4 for the wild-type strain was 16 mM. The MICs for most of the mutant strains tested were similar to that for the wild type (Table 4). The few exceptions were strains with mutations in PA3920 (a P-type ATPase), PA2809 (a two-component system response regulator), PA1435 (a membrane fusion protein), PA2477 (a periplasmic thiol-disulfide inter-

change protein), and PA2522 (*czrC*; an outer membrane protein). In particular, PA3920 and PA2809 displayed significant Cu-sensitive phenotypes. The failure of the other strains to exhibit Cu sensitivity in this assay may be due to the ability of the organism to compensate with other Cu tolerance mechanisms.

Several strains for which the Cu MIC was comparable to the wild-type level exhibited a lag in growth in the presence of Cu compared to the wild-type strain (Table 4). Examples of such

FIG. 5. Growth curves of mutant strains of *P. aeruginosa* in the presence of different CuSO_4 levels. These strains displayed the most severe Cu sensitivity phenotype of tested strains. (A) PA3920, a strain harboring a mutation in a soft metal P-type ATPase. (B) PA2809, a strain harboring a mutation in a response regulator belonging to the two component family of transcriptional regulators. (C) The PA3920 mutant strain complemented *in trans* with pEX1.8 bearing a functional copy of the PA3920 gene. (D) The PA2809 mutant strain complemented *in trans* with pEX1.8 bearing a functional copy of the PA2809 gene. Complemented strains were grown in the absence of antibiotic selection. Retention of plasmid was verified by plating on selective medium. The specific strains used in this analysis were PTL17142 (PA2809) and PTL12392 (PA3920).

a lag are depicted in the growth curves presented in Fig. 4. The growth of PAO1 is unaffected by added CuSO_4 , until MIC levels are reached (Fig. 4A). However, the mutant strains show a delay in growth and a reduced growth rate compared to PAO1 at near MIC levels of CuSO_4 (e.g., 8 mM in Fig. 4 B, C, and D). In particular, the PA1435 mutant strain showed a delay in growth at a few CuSO_4 concentrations. Other mutant strains that produced a lag in growth in the presence of Cu were PA2476 and PA2478 (both belong to the same operon as PA2477 and show homology to periplasmic thiol-disulfide folding proteins), PA2810 (a homolog of a two-component sensor), and PA3521 (an outer membrane protein).

The two strains judged to be most sensitive to Cu by MIC analysis, PA2809 and PA3920, were extremely sensitive to CuSO_4 (Fig. 5A and B). To verify that the transposon insertion was responsible for the Cu-sensitive phenotype, these strains were complemented *in trans* by supplying a functional copy of the gene on the broad-host-range vector pEX1.8. The complemented mutant strains displayed almost wild-type levels of Cu-tolerance (Fig. 5C and D and Table 4).

A disk sensitivity assay was used as a complementary means to evaluate Cu sensitivity. This assay uses a Cu-impregnated disk, which is set on the surface of soft LB agar. Zones of clearance in a lawn of bacteria were measured around the disk. The wild-type strain showed a clear demarcation between copious growth and no growth on the agar plate. However, some strains produced a halo of very light growth adjacent to the zone of clearance (Table 4).

The Cu disk assay results correlated with MIC data, with PA2809 and PA3920 displaying the largest zone of clearance (Table 4). In some cases, strains showed Cu sensitivity in the disk assay and not the MIC assay. This usually occurred with mutations in a putative operon already identified as important for Cu tolerance in the MIC analysis (e.g., PA2521). There were three exceptions. A strain with a mutation in *pcoA* (PA2065) showed significant Cu sensitivity in the disk assay but not in the MIC analysis. A strain with a mutation in PA0397 (a CDF family homolog) and PA2807 (a gene encoding a member of the plastocyanin/azurin Cu-binding family) were also sensitive in the disk assay. Why these strains have a sensitive phenotype in one assay and not the other is unclear.

Several genes induced by Cu stress showed homology to genes involved in responses to other cations, such as Zn. In particular, the *czt* system (PA2520 to PA2522) is known to be important for Zn tolerance in *P. aeruginosa* (26). Zinc is also a required metal for growth and in excess can present the same problems as Cu to the cell. Therefore, the mutant strains were also subjected to a zinc disk sensitivity assay. This assay demonstrated the metal specificity of certain systems. A strain with a mutation in PA3920, encoding a P-type ATPase, was very sensitive to Cu but not to Zn (Table 4). On the other hand, strains with mutations in two other P-type ATPases, PA3690 (homologous to *zntA*, a Zn P-type ATPase in *E. coli*) and PA1549, were Zn sensitive yet showed no Cu phenotype. As Hassan et al. previously demonstrated, the *cztABC* operon (with the exception of PA2521) appeared to be important for Zn tolerance (26). Also implicated in Zn tolerance by this assay were the *pcoAB* genes.

Our initial analysis suggested that PA3920 and PA2809 con-

FIG. 6. Cu toxicity assay. The viable plate counts of PAO1 and a strain with a mutation in PA2809 after exposure to Cu in MSVG. Cells were first grown in LBM until mid-logarithmic phase and then spun down and resuspended in MSVG. Planktonic cells were exposed to an array of Cu concentrations for 5 h at 37°C, and then cells were sonicated and serial dilutions and viable counts were performed. ○, PAO1; ●, PA2809.

tributed significantly to Cu tolerance in *P. aeruginosa*. Therefore, these genes were subjected to further analysis.

A two-component regulator is important for growth and survival under Cu stress. The two-component response regulator PA2809 was up-regulated in both Cu-shocked and Cu-adapted populations and may be involved in *pcoAB* regulation. This gene is highly homologous to *copR* in *P. syringae* (82% similar), *P. putida*, and *Pseudomonas fluorescens* and to *pcoR* in *E. coli*. The regulators in these systems control expression of the Cu-binding proteins CopAB (*copR*) and PcoAC (*pcoR*) (5, 15, 44). Due to this homology, we propose naming PA2809 *copR* and its adjacent sensor kinase, PA2810, *copS*. Two neighboring genes, PA2807 and PA2808, were also induced by Cu stress. The hypothetical protein PA2807 harbors a Cu-binding motif of the plastocyanin/azurin family. PA2807 encodes a protein similar to the Cu-binding protein Cot from *P. fluorescens* DF57. Cot was identified as a Cu tolerance protein that has elevated expression in response to Cu (66).

Since a mutation in PA2809 significantly influenced Cu tolerance (Table 4), this strain was examined further. The mutant strain was also tested for Zn and silver (Ag) sensitivity and found to be identical to the wild-type strain (data not shown). The toxicity of Cu (as opposed to inhibition of growth) was then tested on the PA2809 mutant and wild-type strain. Cells were exposed to a 5-h treatment of various Cu concentrations. Since complex medium has many components with a high capacity to complex Cu, this assay was performed in a minimal medium designed to minimize complexation. The PA2809 mutant strain was killed at lower Cu concentrations than wild-type (Fig. 6). Resistance to Cu was regained when the mutant strain was complemented *in trans* with a plasmid bearing the PA2809 gene (Table 4 and data not shown). Since PA2809 is up-regulated during both Cu treatments, it appears to be a key regulator involved in Cu tolerance in *P. aeruginosa*.

FIG. 7. Impact of increasing copper concentrations on PA3920 transcription. PAO1 carrying a PA3920-*lacZ* transcriptional fusion on the plasmid pAG100 was grown in the presence of increasing amounts of Cu. β -Galactosidase activity was determined 45 min after Cu addition. Error bars represent standard deviation. Growth without added Cu yielded <10 Miller units of activity.

A soft metal P-type ATPase is important for copper tolerance. Heavy metal resistance in bacteria is often mediated by energy-dependent protein efflux pumps of the P-type ATPase family (1, 43, 63). *P. aeruginosa* possesses seven ORFs with this family signature, many of which, including PA3920, possess putative metal binding motifs. PA3920 is a predicted cytoplasmic membrane protein with a molecular weight of 83,438 and possesses significant homology to several members of the P-type ATPase family including the copper transporter *copA* (54% similarity) from *E. coli* (54) and the Cu transporter *yvgX* (65% similarity) from *B. subtilis* (17). Three conserved domains are present in PA3920 including two heavy-metal-associated domains (amino acids 10 to 70 and 76 to 138) and an E1-E2 ATPase domain (amino acids 258 to 477). Within these domains PA3920 possesses all the characteristics of a soft metal ion-translocating P1-type ATPase, including two Cys-X₂-Cys motifs in the C terminus (amino acids 18 to 21 and 84 to 87) and a Cys-Pro-Cys motif (amino acids 444 to 446) in a putative transmembrane segment.

The MICs of the heavy metals Cu, Co, Zn, and Hg were assayed for a strain harboring a mutation in PA3920 and compared to PAO1. In complex and defined media, the mutant exhibited increased susceptibility to copper compared to PAO1 while no differences were observed for the other heavy metals (Table 4 and data not shown). Cu susceptibility was tested in a complex and a minimal medium, and although the MICs were significantly affected by the medium composition, the mutant was hypersusceptible to copper in all media tested (data not shown).

PA3920 expression responds to external copper. Genes encoding metal-transporting ATPases are often induced by the presence of their metal substrates (1, 47, 54). Thus, a transcriptional fusion of the PA3920 promoter region was cloned upstream of the promoterless reporter gene *lacZ* in the plasmid pAG101. In complex medium, transcription of PA3920 exhibits a linear response to increasing concentrations of copper up to

1.2 mM (Fig. 7). The transcriptional response is immediate, occurring within 45 min after the addition of copper.

Summary. A comparison of shocked and adapted transcriptional profiles revealed interesting insight into the physiology of Cu-stressed *P. aeruginosa*. The response to Cu shock appears to encompass both Cu-specific and general stress responses. Since general stress responses have global effects on physiology, determining what genes were regulated directly in response to Cu is impossible. Perhaps as different stress regulators eventually are defined at the transcriptome level, separating direct and indirect responses to Cu may be possible. The Cu-adapted profiles might provide more insight into the adaptations necessary for elevated environmental Cu. Most of the signature genes associated with general stress responses were no longer up-regulated.

Transcriptional profiling of Cu-stressed *P. aeruginosa* produced both expected and unexpected results. Not surprisingly, many active transport functions were up-regulated in response to Cu. Previous studies have shown that RND efflux systems, P-type ATPases, and CDF transporters play an important role for metal tolerance in other organisms. In particular, the P-type ATPase encoded by PA3920 appeared to be important for Cu tolerance. The expression of several porins appeared to be down-regulated in adapted populations. Most of these porins belonged to the OprD family, which may be involved in the passive transport of positively charged molecules.

Iron homeostasis functions were also regulated under Cu stress conditions. In general, the cells produced a response indicative of iron-limiting conditions. An interesting observation involved the two iron siderophore systems, pyoverdine and pyochelin. Pyoverdine biosynthetic genes were up-regulated in Cu-adapted populations, while pyochelin genes were down-regulated. The explanation for this observation may lie with the specificity of the siderophores. Pyoverdine is highly iron specific, while pyochelin has been shown to complex a variety of divalent metal cations, including Cu.

Finally, a variety of genes not generally associated with metal tolerance were up-regulated in the Cu-adapted population. The *dsb* periplasmic thiol-disulfide folding system was up-regulated and appeared to be functionally important for growth in the presence of excess Cu. Several operons containing genes of unknown functions were also up-regulated in adapted populations. Particularly interesting were the genes PA3515 to PA3520, which constitute an operon adjacent to an induced RND efflux system (PA3521 to PA3523). A few of these genes have homology to lyases, but the function of this operon is a mystery. Determining how genes such as these contribute to Cu adaptation may produce new insight into metal tolerance of microorganisms.

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REFERENCES

1. Adaikkalam, V., and S. Swarup. 2002. Molecular characterization of an operon, *cueAR*, encoding a putative P1-type ATPase and a MerR-type reg-

- ulatory protein involved in copper homeostasis in *Pseudomonas putida*. Microbiology **148**:2857–2867.
2. Anton, A., C. Grosse, J. Reissmann, T. Pribyl, and D. H. Nies. 1999. CzcD is a heavy metal ion transporter involved in regulation of heavy metal resistance in *Ralstonia* sp. strain CH34. J. Bacteriol. **181**:6876–6881.
 3. Anton, A., A. Weltrowski, C. J. Haney, S. Franke, G. Grass, C. Rensing, and D. H. Nies. 2004. Characteristics of zinc transport by two bacterial cation diffusion facilitators from *Ralstonia metallidurans* CH34 and *Escherichia coli*. J. Bacteriol. **186**:7499–7507.
 4. Avery, S. V., N. G. Howlett, and S. Radice. 1996. Copper toxicity towards *Saccharomyces cerevisiae*: dependence on plasma membrane fatty acid composition. Appl. Environ. Microbiol. **62**:3960–3966.
 5. Cánovas, D., I. Cases, and V. de Lorenzo. 2003. Heavy metal tolerance and metal homeostasis in *Pseudomonas putida* as revealed by complete genome analysis. Environ. Microbiol. **5**:1242–1256.
 6. Cha, J.-S., and D. A. Cooksey. 1991. Copper resistance in *Pseudomonas syringae* mediated by periplasmic and outer membrane proteins. Proc. Natl. Acad. Sci. USA **88**:8915–8919.
 7. Collet, J. F., and J. C. Bardwell. 2002. Oxidative protein folding in bacteria. Mol. Microbiol. **44**:1–8.
 8. Connell, D. W., and G. J. Miller. 1984. Chemistry and ecotoxicology of pollution. John Wiley & Sons, New York, N.Y.
 9. Coy, M., and J. B. Neilands. 1991. Structural dynamics and functional domains of the fur protein. Biochemistry **30**:8201–8210.
 10. Cunliffe, H. E., T. R. Merriman, and I. L. Lamont. 1995. Cloning and characterization of *pvdS*, a gene required for pyoverdine synthesis in *Pseudomonas aeruginosa*: PvdS is probably an alternative sigma factor. J. Bacteriol. **177**:2744–2750.
 11. Dao, K.-H. T., K. E. Hamer, C. L. Clark, and L. G. Harshman. 1999. Pyoverdine production by *Pseudomonas aeruginosa* exposed to metals or an oxidative stress agent. Ecol. Appl. **9**:441–448.
 12. Egler, M., C. Grosse, G. Grass, and D. H. Nies. 2005. Role of the extracytoplasmic function protein family sigma factor RpoE in metal resistance of *Escherichia coli*. J. Bacteriol. **187**:2297–2307.
 13. Fergusson, J. E. 1990. The heavy elements: chemistry, environmental impact and health effects. Pergamon Press, New York, N.Y.
 14. Filiatrault, M. J., V. E. Wagner, D. Bushnell, C. G. Haidaris, B. H. Iglewski, and L. Passador. 2005. Effect of anaerobiosis and nitrate on gene expression in *Pseudomonas aeruginosa*. Infect. Immun. **73**:3764–3772.
 15. Finney, L. A., and T. V. O'Halloran. 2003. Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. Science **300**:931–936.
 16. Franke, S., G. Grass, C. Rensing, and D. H. Nies. 2003. Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. J. Bacteriol. **185**:3804–3812.
 17. Gaballa, A., and J. D. Helmann. 2003. *Bacillus subtilis* CPx-type ATPases: characterization of Cd, Zn, Co and Cu efflux systems. Biomol. **16**:497–505.
 18. Gensberg, K., A. W. Smith, F. S. Brinkman, and R. E. Hancock. 1999. Identification of *oprG*, a gene encoding a major outer membrane protein of *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **43**:607–608.
 19. George, S. G. 1991. Cell biochemistry and transmembrane transport of some metals. In E. Merian (ed.), Metals and their compounds in the environment. VCH Publishers, Inc., New York, N.Y.
 20. Goldberg, M., T. Pribyl, S. Juhnke, and D. H. Nies. 1999. Energetics and topology of CzcA, a cation/proton antiporter of the resistance-nodulation-cell division protein family. J. Biol. Chem. **274**:26065–26070.
 21. Grass, G., and C. Rensing. 2001. CueO is a multi-copper oxidase that confers copper tolerance in *Escherichia coli*. Biochem. Biophys. Res. Commun. **286**:902–908.
 22. Grass, G., and C. Rensing. 2001. Genes involved in copper homeostasis in *Escherichia coli*. J. Bacteriol. **183**:2145–2147.
 23. Grass, G., K. Thakali, P. E. Klebba, D. Thieme, A. Muller, G. F. Wildner, and C. Rensing. 2004. Linkage between catecholate siderophores and the multicopper oxidase CueO in *Escherichia coli*. J. Bacteriol. **186**:5826–5833.
 24. Hancock, R. E., and F. S. Brinkman. 2002. Function of pseudomonas porins in uptake and efflux. Annu. Rev. Microbiol. **56**:17–38. Epub 2002 Jan 30.
 25. Haney, C. J., G. Grass, S. Franke, and C. Rensing. 12 May 2005. New developments in the understanding of the cation diffusion facilitator family. J. Ind. Microbiol. Biotechnol. **32**:215–226. [Epub ahead of print.]
 26. Hassan, M. T., D. van der Lelie, D. Springael, U. Römling, N. Ahmed, and M. Mergeay. 1999. Identification of a gene cluster, *czr*, involved in cadmium and zinc resistance in *Pseudomonas aeruginosa*. Gene **238**:417–425.
 27. Hatfield, G. W., S.-P. Hung, and P. Baldi. 2003. Differential analysis of DNA microarray gene expression data. Mol. Microbiol. **47**:871–877.
 28. Hiniker, A., J. F. Collet, and J. C. Bardwell. 8 August 2005. Copper stress causes an in vivo requirement for the *Escherichia coli* disulfide isomerase DsbC. J. Biol. Chem. **280**:33785–33791. [Epub ahead of print.]
 29. Höfte, M., S. Buysens, N. Koedam, and P. Cornelis. 1993. Zinc affects siderophore-mediated high affinity iron uptake systems in the rhizosphere *Pseudomonas aeruginosa* 7NSK2. BioMetals **6**:85–91.
 30. Huffman, D. L., J. Huyett, F. W. Outten, P. E. Doan, L. A. Finney, B. M. Hoffman, and T. V. O'Halloran. 2002. Spectroscopy of Cu(II)-PcoC and the multicopper oxidase function of PcoA, two essential components of *Escherichia coli* pco copper resistance operon. Biochemistry **41**:10046–10055.
 31. Jacobs, M. A., A. Alwood, I. Thaipisuttikul, D. Spencer, E. Haugen, S. Ernst, O. Will, R. Kaul, C. Raymond, R. Levy, L. Chun-Rong, D. Guenther, D. Bovee, M. V. Olson, and C. Manoil. 2003. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **100**:14339–14344.
 32. Jensen, S. E., I. T. Fecycz, and J. N. Campbell. 1980. Nutritional factors controlling exocellular protease production by *Pseudomonas aeruginosa*. J. Bacteriol. **144**:844–847.
 33. Keith, C. H., and W. A. Telliard. 1979. Priority pollutants I: a perspective view. Environ. Sci. Technol. **13**:416–423.
 34. Kershaw, C. J., N. L. Brown, C. Constantinidou, M. D. Patel, and J. L. Hobman. 2005. The expression profile of *Escherichia coli* K-12 in response to minimal, optimal and excess copper concentrations. Microbiology **151**:1187–1198.
 35. Kumar, A., and H. P. Schweizer. 2005. Bacterial resistance to antibiotics: active efflux and reduced uptake. Adv. Drug Deliv. Rev. **57**:1486–1513.
 36. Lebedev, V. S., A. V. Veselovskii, E. Deinega, and I. Fedorov. 2002. The role of reactive oxygen species in copper-induced permeability of plasma membranes in *Escherichia coli*. Biofizika **47**:295–299.
 37. Lee, L. J., J. A. Barrett, and R. K. Poole. 2005. Genome-wide transcriptional response of chemostat-cultured *Escherichia coli* to zinc. J. Bacteriol. **187**:1124–1134.
 38. Legatzki, A., G. Grass, A. Anton, C. Rensing, and D. H. Nies. 2003. Interplay of the Czc system and two P-type ATPases in conferring metal resistance to *Ralstonia metallidurans*. J. Bacteriol. **185**:4354–4361.
 39. Long, A. D., H. J. Mangalam, B. Y. P. Chan, L. Toller, G. W. Hatfield, and P. Baldi. 2001. Improved statistical inference from DNA microarray data using analysis of variance and a Bayesian statistical framework. J. Biol. Chem. **276**:19937–19944.
 40. Lu, Z. H., and M. Solioz. 2001. Copper-induced proteolysis of the CopZ copper chaperone of *Enterococcus hirae*. J. Biol. Chem. **276**:47822–47827. Epub 2001 Oct 3.
 41. Moore, C. M., A. Gaballa, M. Hui, R. W. Ye, and J. D. Helmann. 2005. Genetic and physiological responses of *Bacillus subtilis* to metal ion stress. Mol. Microbiol. **57**:27–40.
 42. Munson, G. P., D. L. Lam, F. W. Outten, and T. V. O'Halloran. 2000. Identification of a copper-responsive two-component system on the chromosome of *Escherichia coli* K-12. J. Bacteriol. **182**:5864–5871.
 43. Nies, D. H. 2003. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol. Rev. **27**:313–339.
 44. Nies, D. H. 1999. Microbial heavy-metal resistance. Appl. Microbiol. Biotechnol. **51**:730–750.
 45. Ochsner, U. A., A. I. Vasil, and M. L. Vasil. 1995. Role of the ferric uptake regulator of *Pseudomonas aeruginosa* in the regulation of siderophores and exotoxin A expression: purification and activity on iron-regulated promoters. J. Bacteriol. **177**:7194–7201.
 46. Ochsner, U. A., P. J. Wilderman, A. I. Vasil, and M. L. Vasil. 2002. GeneChip expression analysis of the iron starvation response in *Pseudomonas aeruginosa*: identification of novel pyoverdine biosynthesis genes. Mol. Microbiol. **45**:1277–1287.
 47. Odermatt, A., H. Suter, R. Krapf, and M. Solioz. 1993. Primary structure of two P-type ATPases involved in copper homeostasis in *Enterococcus hirae*. J. Biol. Chem. **268**:12775–12779.
 48. Outten, F. W., D. L. Huffman, J. A. Hale, and T. V. O'Halloran. 8 June 2001. The independent cue and cus systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli*. J. Biol. Chem. **276**:30670–30677. [Epub ahead of print.]
 49. Outten, F. W., C. E. Outten, and T. O'Halloran. 2000. Metalloregulatory systems at the interface between bacterial metal homeostasis and resistance, p. 145–157. In G. Storz and R. Hengge-Aronis (ed.), Bacterial stress responses. ASM Press, Washington, D.C.
 50. Palma, M., D. DeLuca, S. Worgall, and L. E. N. Quadri. 2004. Transcriptome analysis of the response of *Pseudomonas aeruginosa* to hydrogen peroxide. J. Bacteriol. **186**:248–252.
 51. Paulsen, I. T., and M. H. Saier, Jr. 1997. A novel family of ubiquitous heavy metal ion transport proteins. J. Membr. Biol. **156**:99–103.
 52. Pinto, E., T. C. S. Sigaud-Kutner, M. A. S. Leitão, O. K. Okamoto, D. Morse, and P. Colepicolo. 2003. Heavy metal-induced oxidative stress in algae. J. Phycol. **39**:1008–1018.
 53. Ramsey, M. M., and M. Whiteley. 2004. *Pseudomonas aeruginosa* attachment and biofilm development in dynamic environments. Mol. Microbiol. **53**:1075–1087.
 54. Rensing, C., B. Fan, R. Sharma, B. Mitra, and B. P. Rosen. 2000. CopA: an *Escherichia coli* Cu(I)-translocating P-type ATPase. Proc. Natl. Acad. Sci. USA **97**:652–656.
 55. Rensing, C., M. Ghosh, and B. P. Rosen. 1999. Families of soft-metal-ion-transporting ATPases. J. Bacteriol. **181**:5891–5897.
 56. Rensing, C., and G. Grass. 2003. *Escherichia coli* mechanisms of copper homeostasis in a changing environment. FEMS Microbiol. Rev. **27**:197–213.
 57. Rensing, C., T. Pribyl, and D. H. Nies. 1997. New functions for the three

- subunits of the CzcCBA cation-proton antiporter. *J. Bacteriol.* **179**:6871–6879.
58. Roane, T. M., and I. L. Pepper. 2000. Microbial responses to environmentally toxic cadmium. *Microb. Ecol.* **38**:358–364.
 59. Salunkhe, P., T. Topfer, J. Buer, and B. Tummeler. 2005. Genome-wide transcriptional profiling of the steady-state response of *Pseudomonas aeruginosa* to hydrogen peroxide. *J. Bacteriol.* **187**:2565–2572.
 60. Schuster, M., A. C. Hawkins, C. S. Harwood, and E. P. Greenberg. 2004. The *Pseudomonas aeruginosa* RpoS regulon and its relationship to quorum sensing. *Mol. Microbiol.* **51**:973–985.
 61. Schützendübel, A., and A. Polle. 2002. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *J. Exp. Bot.* **53**:1351–1365.
 62. Schweizer, H. P. 2003. Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions. *Genet. Mol. Res.* **2**:48–62.
 63. Solioz, M., A. Odermatt, and R. Krapf. 1994. Copper pumping ATPases: common concepts in bacteria and man. *FEBS Lett.* **346**:44–47.
 64. Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrenner, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrook-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hancock, S. Lory, and M. V. Olson. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* **406**:959–964.
 65. Teitzel, G. M., and M. R. Parsek. 2003. Heavy-metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* **69**:2313–2320.
 66. Tom-Petersen, A., C. Hosbond, and O. Nybroe. 2001. Identification of copper-induced genes in *Pseudomonas fluorescens* and use of a reporter strain to monitor bioavailable copper in soil. *FEMS Microbiol. Ecol.* **38**:59–67.
 67. Visca, P., G. Colotti, L. Serino, D. Verzili, N. Orsi, and E. Chiancone. 1992. Metal regulation of siderophore synthesis in *Pseudomonas aeruginosa* and functional effects of siderophore-metal complexes. *Appl. Environ. Microbiol.* **58**:2886–2893.
 68. von Canstein, H., Y. Li, J. Leonhäuser, E. Haase, A. Felske, W.-D. Deckwer, and I. Wagner-Döbler. 2002. Spatially oscillating activity and microbial succession of mercury-reducing biofilms in a technical-scale bioremediation system. *Appl. Environ. Microbiol.* **68**:1938–1946.
 69. Wagner, T., G. Soong, S. Sokol, L. Saiman, and A. Prince. 2005. Effects of azithromycin on clinical isolates of *Pseudomonas aeruginosa* from cystic fibrosis patients. *Chest* **128**:912–919.
 70. Wagner, V. E., R. J. Gillis, and B. H. Iglewski. 2004. Transcriptome analysis of quorum-sensing regulation and virulence factor expression in *Pseudomonas aeruginosa*. *Vaccine* **22**:S15–20.
 71. Wang, C. L., P. C. Michels, S. C. Dawson, S. Kitisakkul, J. A. Baross, J. D. Keasling, and D. S. Clark. 1997. Cadmium removal by a new strain of *Pseudomonas aeruginosa* in aerobic culture. *Appl. Environ. Microbiol.* **63**:4075–4078.
 72. Whiteley, M., M. G. Banger, R. E. Bumgarner, M. R. Parsek, G. M. Teitzel, S. Lory, and E. P. Greenberg. 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* **413**:860–864.
 73. Wood, J. M., and H.-K. Wang. 1983. Microbial resistance to heavy metals. *Environ. Sci. Technol.* **17**:582A–590A.
 74. Yamamoto, K., and A. Ishihama. 2005. Transcriptional response of *Escherichia coli* to external copper. *Mol. Microbiol.* **56**:215–227.
 75. Yamano, Y., T. Nishikawa, and Y. Komatsu. 1998. Involvement of the RpoN protein in the transcription of the *oprE* gene in *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* **162**:31–37.
 76. Yoneyama, H., and T. Nakae. 1996. Protein C (OprC) of the outer membrane of *Pseudomonas aeruginosa* is a copper-regulated channel protein. *Microbiology* **142**:2137–2144.
 77. Zubay, G., D. E. Morse, W. J. Schrenk, and J. H. Miller. 1972. Detection and isolation of the repressor protein for the tryptophan operon of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **69**:1100–1103.