RESEARCH LETTER

Mutational, proteomic and metabolomic analysis of a plant growth promoting copper-resistant *Pseudomonas* spp.

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Abstract

Pseudomonas sp. TLC6-6.5-4 is a multiple metal resistant plant growth-promoting bacteria isolated from copper-contaminated lake sediments. In this study, a comprehensive analysis of genes involved in copper resistance was performed by generating a library of transposon (Tn5) mutants. Two copper-sensitive mutants with significant reduction in copper resistance were identified: CSM1, a mutant disrupted in *trpA* gene (tryptophan synthase alpha subunit), and CSM2, a mutant disrupted in *clpA* gene (ATP-dependent Clp protease). Proteomic and metabolomic analyses were performed to identify biochemical and molecular mechanisms involved in copper resistance using CSM2 due to its lower minimum inhibitory concentration compared with CSM1 and the wild type. Proteomic analysis revealed that disruption of Clp protease gene up-regulated molecular chaperones and down-regulated the expression of enzymes related to tRNA modification, whereas metabolomic analysis showed that amino acid and oligosaccharide transporters that are part of ATP-binding cassette (ABC) transporters pathways were down-regulated. Further, copper stress altered metabolic pathways including the tricarboxylic acid cycle, protein absorption and glyoxylate metabolism.

Introduction

Copper is an essential micronutrient for bacterial growth because it is the cofactor for many key enzymes such as cytochrome c oxidases or monooxygenases (Frangipani *et al.*, 2008). However, high levels of copper are toxic to bacteria and lead to cell damage by generating reactive oxygen species, affecting the function of proteins or inactivating the enzymes (Teitzel *et al.*, 2006). Bacteria have developed different mechanisms to confer resistance to copper, which vary significantly among the species. In *Pseudomonas* species, the well characterized copper resistance system is the plasmid-encoded *cop* system in *Pseudomonas syringae* pv. tomato (Cha & Cooksey, 1991; Cooksey, 1993). In this organism, a 35-kb plasmid pPT23D carries the *cop* operon, which consists of four structural genes (*copABCD*) and two regulatory genes (*copRS*).

Recent proteomic analysis of *Pseudomonas putida* KT2440 in response to copper and cadmium identified that the bacterial isolate is able to survive under copper stress by up-regulation of the expression of copper-binding proteins (CopA and CopR), oxidative stress protective proteins and several enzymes involved in the Krebs cycle (Miller *et al.*, 2009). Besides genetic and proteomic studies, the metabolomic approach provides additional information on how the bacteria adapt to various environments (Frimmersdorf *et al.*, 2010). Changes in tricarboxylic acid cycle (TCA) cycle, glycolysis, pyruvate and nicotinate metabolism of *Pseudomonas fluorescens* planktonic culture in response to copper stress were found using a combined gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) approach (Booth *et al.*, 2011).

Pseudomonas sp. TLC6-6.5-4 isolated from Torch Lake sediment contaminated by copper mine tailings shows high resistance with the minimum inhibitory concentration of 5 mM in basic salt medium (BSM) and 6 mM in Luria broth (LB) medium (Li & Ramakrishna, 2011). The bacteria produce indole-3-acetic acid and siderophores and solubilize phosphate, which promotes plant growth. The objective of this study was to investigate how this bacterium adapts to the toxic levels of copper. We created

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a transposon insertion library, screened for copper-sensitive mutants and found that the disruption of ATP-dependent *clp* protease (*clpA*) gene caused a significant reduction in copper resistance of *Pseudomonas* sp. TLC6-6.5-4. Further, we performed proteomic and metabolomic analyses to compare the copper-sensitive mutant with the wild type.

Materials and methods

Morphological characterization

Bacterial strain *Pseudomonas* sp. TLC6-6.5-4 was grown in Luria broth (LB) with 4 mM Cu^{2+} at 30 °C and shaken at 140 r.p.m. until the $OD_{600\,mm}$ reached 0.4 (exponential phase). This concentration challenged the bacteria but did not inhibit growth. Bacteria grown in LB medium without copper were used as control. Bacterial cells were stained using a gram staining kit (BD) and observed under an Olympus BX51 microscope (Leeds Precision). In addition, the morphology of the bacterial isolate was examined using a scanning electron microscope (SEM) (JSM-6400, JEOL). Sample preparation was carried out as described by Shi & Xia (2003). The bacterial length was measured using IMAGE J software (http://rsb.info.nih.gov/ij). The average length was calculated using 20 individual cells per treatment. The statistical difference in bacterial length between the two groups was analyzed by *t*-test.

Transposon insertion mutagenesis and screen of copper-sensitive mutants

Transposon insertion mutants were created using an EZ-Tn5™ <KAN-2> Tnp Transposome™ kit (Epicenter). Copper-sensitive mutants were screened by replica plating kanamycin-resistant colonies on BSM with 3 mM Cu^{2+} (BSM + 3 mM Cu; sodium glycerophosphate was used instead of sodium phosphate to reduce copper–phosphate precipitate). Mutants that were not able to grow on BSM + 3 mM Cu but which grew on BSM without copper after incubation for 3 days at 30 °C were regarded as copper-sensitive mutants.

Identification of the disrupted genes

Genomic DNA of the copper-sensitive mutants was isolated using a ZR fungal/bacterial DNA miniprep kit (Zymo Research). The genomic regions harboring the insertion of transposon in the copper-sensitive strains were rescued by self-ligation of EcoRI-digested genomic DNA and electroporation into *Escherichia coli* TransforMax™ EC100D™ (*pir*+) electrocompetent cells (Epicenter). Plasmid DNA was extracted using a Zyppy plasmid miniprep kit (Zymo Research) from the *E. coli* transformants selected on LB agar with kanamycin (50 μg mL^{-1}). The sequence flanking the transposon element was sequenced using primers KAN-2 FP-1 and R6KAN-2 RP-1 provided with an EZ-Tn5™<KAN-2> Tnp Transposome™ kit.

Real-time PCR analysis of *clpA* and *trpA* gene expression in response to copper stress

TLC6-6.5-4 was grown in 1 mL LB broth with or without 4 mM Cu^{2+} at 30 °C until $OD_{600\,mm}$ reached 0.4. CSM1 and CSM2 grown without copper were used as controls. Three replicates were used in each group. Total RNA was extracted with an RNeasy Mini kit (Qiagen) and cDNA was synthesized using a QuantiTect Reverse Transcription kit (Qiagen). Real-time PCR was performed on the StepOnePlus™ system (Applied Biosystems) using Fast SYBR Green Master Mix. Primers for *clpA*, *trpA* and *gyrB* (reference gene) are listed in Supporting Information, Table S1.

Proteomic analysis of *Pseudomonas* sp. TLC6-6.5-4 in response to copper stress

TLC6-6.5-4 was grown in LB with 4 mM Cu^{2+} at 30 °C until $OD_{600\,mm}$ reached 0.4. TLC6-6.5-4 and copper-sensitive mutant CSM2 grown in LB broth were used as controls. Three replicates were used in each group. Total protein was isolated from the bacterial pellets using the ZOOM® 2D-protein solubilizer kit (Invitrogen). The protein concentration was measured by the Bradford method (Bio-Rad protein assay kit). The protein extract was separated by two-dimensional gel electrophoresis (Noel-Georis *et al.*, 2004). The protein spots were visualized by silver staining and scanned with a GS 800 scanner (Bio-Rad) and analyzed using IMAGEMASTER 2D-Platinum v7.0 for spot detection, background subtraction and protein spot intensity quantification. Significant changes in protein expression levels were set to at least a twofold change compared with the control group (Miller *et al.*, 2009). Spots of interest were excised from gels and subjected to in-gel trypsin digestion (Shevchenko *et al.*, 2006). The digested peptides were analyzed using electrospray ionization mass spectrometry (ESI-MS) (Thermo). The MS data from the protein samples were searched against NCBI_nr database using MASCOT (www.matrixscience.com). Molecular weight and pI were calculated based on amino acid sequence and compared with gel location. Functional annotation of the identified protein was carried out using the gene ontology (GO) database (http://geneontology.org) and UniProtKB (http://www.uniprot.org).

Metabolomic analysis of *Pseudomonas* sp. TLC6-6.5-4 in response to copper stress

Bacterial sample preparation was same as described for proteomic analysis. Extraction of intracellular metabolites was performed as previously described with slight modifications (Frimmersdorf *et al.*, 2010). Four replicates were used in each group. The compounds were derivatized with methoxyamine hydrochloride and *N*-methyl-*N*-trimethylsilyltrifluoracetamide. A set of alkane standards were added to calculate retention indices. The derivatized extracts were analyzed with a GC-MS-QP-5050A (Shimadzu). Spectral deconvolution, calibration and identification of metabolites were performed using AMDIS software from NIST (Natural Institute of Standards and Technology). Prior to statistical analysis, each compound was normalized by the peak area of the standard (ribitol) and the optical density of each bacterial culture (Strelkov *et al.*, 2004). These relative ratios can be compared directly among different groups without knowledge of the absolute compound concentrations. Hierarchical cluster analysis with Pearson correlation as the distance measure and a one-way ANOVA test was performed with TIGR MEV 4.7.4. Significant differences in the metabolite level were determined by comparing the *P* values ($P < 0.05$). The metabolites with significant changes were submitted to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/pathway.html) to obtain the compound IDs and then submitted to Metabolite Pathway Enrichment Analysis (MPEA) Analysis (http://ekhidna.biocenter.helsinki.fi/poxo/mpea/mpea) to determine which metabolic pathways are most likely to be involved with these compounds. *P*-values were calculated by Monte Carlo simulation.

Results

Effect of copper on bacterial cell morphology

Pseudomonas sp. TLC6-6.5-4 is a rod-shaped bacterial strain with an average length of 6.52 ± 1.60 μm when grown in LB broth without copper (Fig. 1b, d and e). However, when the bacterial isolate was exposed to 4 mM copper, about 70% reduction in bacterial cell length was observed. The mean cell length was 1.92 ± 0.38 μm, which is significantly ($P < 0.05$) shorter than cells grown in LB broth without copper (Fig. 1a, c and e).

Characterization of copper-sensitive mutants generated by random transposon mutagenesis

A comprehensive genome-wide analysis of *Pseudomonas* sp. TLC6-6.5-4 was performed to identify the genes involved in copper resistance using random transposon mutagenesis. A total of 5023 colonies with transposon insertions were screened for copper-sensitive mutants, which resulted in the identification of three mutants with a decrease in resistance to copper. These mutants were designated CSM1 (Copper Sensitive Mutant), CSM2 and

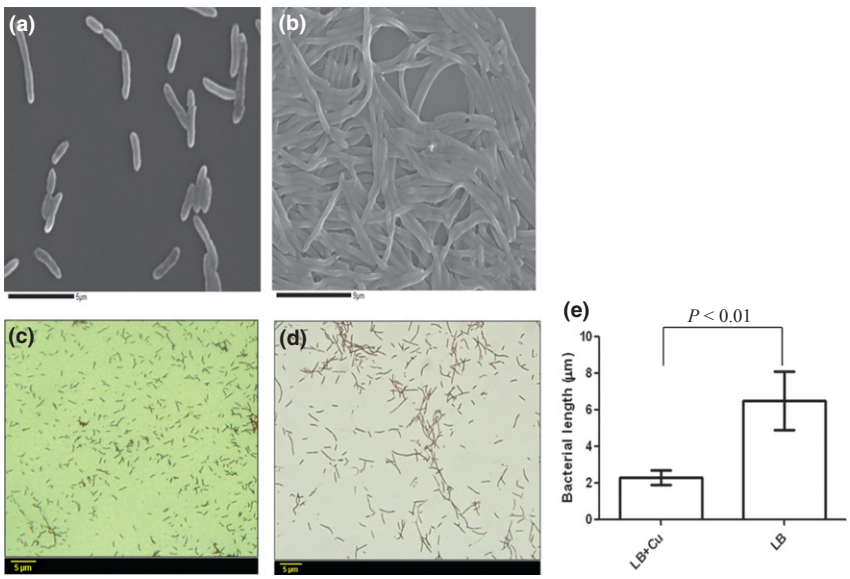

Fig. 1. Photomicrographs of *Pseudomonas* sp. TLC6-6.5-4 with and without exposure to copper (4 mM). (a,c) Bacterial isolate grown in LB with copper. (b,d) Bacterial isolate grown in LB without copper. (a,b) SEM images (magnification × 6000). (c,d) Digital microscopic images (magnification × 700). **(**e) Length of *Pseudomonas* sp. TLC6-6.5-4 cells grown with and without copper stress. Data are mean ± SD ($n = 20$). The difference was significant between the two groups according to Student's *t*-test ($P < 0.01$).

CSM3. Growth of these mutants in the medium without copper was not affected (Fig. 2). Sequencing of the transposon-flanking region and BLAST analysis showed 83% homology of the disrupted gene in CSM1 and CSM2 with *trpA* (accession no. AM181176, locus-tag PFLU_0035) and *clpA* (locus-tag PFLU_3805) genes of *P. fluorescens* encoding tryptophan synthase alpha-subunit and ATP dependent Clp protease, respectively. CSM3 had transposon insertion site identical to that of CSM2. The difference in the copper tolerance between the wild-type strain and the mutants (CSM1 and CSM2) was investigated by growth inhibition experiments in LB broth with increasing concentrations of copper (Fig. 2). The growth of the mutants was comparable to the wild-type strain grown in the presence of 2 mM copper and no copper, suggesting that the mutations did not limit the bacterial fitness in 2 mM copper. The growth of the two mutants was significantly inhibited in 4 mM copper compared with the wild-type control ($P < 0.05$). CSM2 and CSM1 did not grow in 4.5 and 5 mM copper, respectively. Quantitative RT-PCR analysis showed that the relative expression of *clpA* and *trpA* genes in wild type under copper stress (4 mM) was 13- and 3.2-fold, respectively, compared with wild type grown without copper (Fig. S1). No *clpA* and *trpA* expression was detected in the mutants.

Proteomic analysis of the copper-sensitive mutant CSM2

Proteomic analysis of the wild type and the copper-sensitive mutant CSM2 grown without copper identified 21 protein spots with a greater than twofold change, of which the relative intensity of 13 protein spots decreased by 2- to 4.3-fold and eight spots increased two to eight-fold. Five protein spots were selected for mass spectrometry analysis based on more than threefold changes in protein expression and the possibility of clean excision. Expression of proteins involved in carbohydrate metabolism, energy production and tRNA processing was down-regulated in CSM2 compared with the wild type (Table 1). However, the expression of DnaJ-class molecular chaperone and HpcH/HpaI aldolase was up-regulated compared with the wild-type strain. Interestingly, the protein expression of all the five identified spots was up-regulated in wild-type strain grown in 4 mM copper compared with the wild-type strain and CSM2 grown without copper.

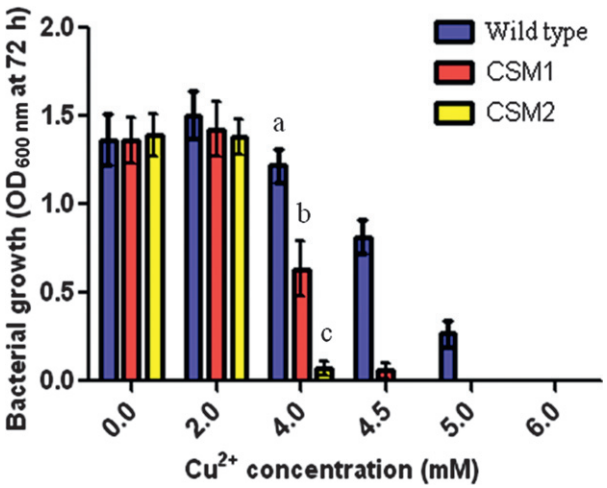

Fig. 2. Effect of varying copper concentrations on the growth of the copper-sensitive mutants CSM1 and 2. *Pseudomonas* sp. TLC6-6.5-4 (wild type) and its mutants CSM1 and CSM2 were grown in LB broth supplemented with 0–6 mM copper. Data are mean ± SD. Different letters above the column at 4 mM indicate a significant difference among the three groups according to one-way ANOVA followed by Tukey's test.

Our next step was to investigate proteins whose expression was altered in wild type exposed to copper and which, at the same time, showed no change in CSM2 compared with the wild type. This experiment identified eight proteins that have a role in efflux of macromolecules, small molecules and ions, and act as transporters of amino acids (Table 1). Proteins related to amino acid metabolism and histidine kinase, which is part of the bacterial two-component sensor system involved in environmental sensing (Swartz *et al.*, 2007), were up-regulated compared with the wild-type strain grown without copper. However, the expression levels of a transcriptional regulatory protein (MalR) and a hypothetical protein (GSU1247) in wild-type strain grown in 4 mM copper were about two- and fourfold lower than wild type grown without copper, respectively.

Metabolomic analysis of the copper-sensitive mutant CSM2

The intracellular metabolites produced by *Pseudomonas* sp. TLC6-6.5-4 and the mutant strain CSM2 grown with or without copper was analyzed by GC-MS. A total of 44 compounds – organic acids, sugars, amino acids, nucleosides and lipids – were identified. To examine the overall metabolic changes, the relative metabolite concentrations were analyzed in an unsupervised hierarchical cluster analysis (HCA) using Pearson correlation as the distance metric (Fig. S2). A more robust statistical method, one-way ANOVA, was applied to examine the changes in relative metabolic levels, which identified significant changes of 15 compounds (Fig. 3). Several sugars and amino acids such as glycerol-3-phosphate, alpha-D-glucopyranoside, L-proline and L-isoleucine decreased significantly in the CSM2 mutant compared with wild type grown without copper. However, these compounds significantly increased in wild type grown with 4 mM copper. In addition, the

Table 1. Identification of differentially expressed proteins between wild type, CSM2 mutant and wild type grown in 4 mM copper

Spot ID	Known essential function	Protein name	Accession no.	MW kDa pI^{-1}	Fold changes: CSM2 vs. wild type	Fold changes: Wild type (+ Cu) vs. wild type
53	Heat shock protein	DnaJ-class molecular chaperone	gi|341615709	22.2/7.78	↑8	↑20
39	Carbon metabolism	HpcH/HpaI aldolase	gi|110635597	32.4/5.30	↑8	↑20
15	tRNA processing	tRNA (guanine-N(7)-)-methyltransferase	gi|27375899	26.8/8.91	↓3.5	↑10
19	Carbohydrate metabolism	Glycosyl transferase	gi|322380228	28.1/9.59	↓4	↑3.1
81	Energy production and conversion	Ubiquinone biosynthesis protein	gi|323527440	23.4/5.90	↓4.3	↑4.1
41	Transporter	ABC transporter-like protein	gi|284032206	32.8/5.26	—	↑2.3
38	Regulator of citrate/malate metabolism	Transcriptional regulatory protein MalR	gi|91781471	33.6/4.78	—	↓2.1
32	Amino acid metabolism	Tryptophan synthase β subunit	gi|119775268	42.6/5.91	—	↑3.39
13	Amino acid metabolism/TCA cycle	Ketol-acid reductoisomerase	gi|32266703	37.1/5.75	—	↑2.5
115	Efflux	Putative lipoprotein	gi|124003301	24.4/8.73	—	↑4.7
36	Two-component sensor	Histidine kinase	gi|325528613	24.2/6.44	—	↑4
182	Unkonwn	Conserved hypothetical protein	gi|170696365	22.3/9.76	—	↑3
164	Unkonwn	Hypothetical protein GSU1247	gi|39996349	21.6/9.88	—	↓4.3

↑ indicates upregulated proteins and ↓ indicates downregulated proteins. — indicates no significant changes in spot abundance ($P > 0.05$) between the two groups.

concentration of several organic acids including phosphoric acid, butanedioic acid and hexadecanoic acid were significantly reduced in wild-type strain with copper exposure, whereas the concentration of these compounds was not altered in the CSM2 mutant compared with wild-type strain grown without copper. Transposon insertion in CSM2 mutant resulted in the down-regulation of the ABC transporter pathway compared with its up-regulation in wild-type strain in the presence of copper (Table 2). Besides ABC transporters, TCA cycle, protein digestion, and absorption and glyoxylate metabolism were affected by exposure to high levels of copper.

Discussion

In this study, the response of *Pseudomonas* sp. TLC6-6.5-4 to elevated copper ion concentrations was evaluated using morphological, transposon insertion, proteomic, and metabolomic analyses. Alternation in cell morphology is a visible indicator of bacterial adaptation to environmental stress (Justice *et al.*, 2008). A significant reduction of bacterial cell size observed in the wild type in the presence of copper was similar to that of a lead-resistant *Pseudomonas aeruginosa* strain exposed to 0.8 M lead nitrate (Naik & Dubey, 2011). *Pseudomonas* outer-membrane has two major groups of lipoproteins with peptidoglycan binding lipoproteins and efflux porins (Remans *et al.*, 2010). Bacterial shape is controlled by peptidoglycan and its associated lipoproteins (Pierce *et al.*, 2011). It is likely that a peptidoglycan-binding lipoprotein or the efflux lipoprotein identified in this study may have a role in cell size regulation.

The copper sensitive mutant CSM1 identified in this study has transposon insertion in *trpA* gene, which is part of the *trp* operon that includes genes encoding enzymes required for the synthesis of tryptophan. The up-regulation of tryptophan synthase in *Pseudomonas* sp. TLC6-6.5-4 in the presence of copper was consistent with our transposon mutational analysis (CSM1 *trpA*) (Table 1). The overexpression of *trpA* gene induced by copper treatment was reported in *Helicobacter pylori* (Waidner *et al.*, 2002). Besides tryptophan, our metabolomic analysis showed that the levels of several other amino acids such as L-proline and L-isoleucine were significantly increased when *Pseudomonas* sp. TLC6-6.5-4 was grown in the presence of 4 mM copper (Fig. 4), which correlates with the up-regulation of ke-

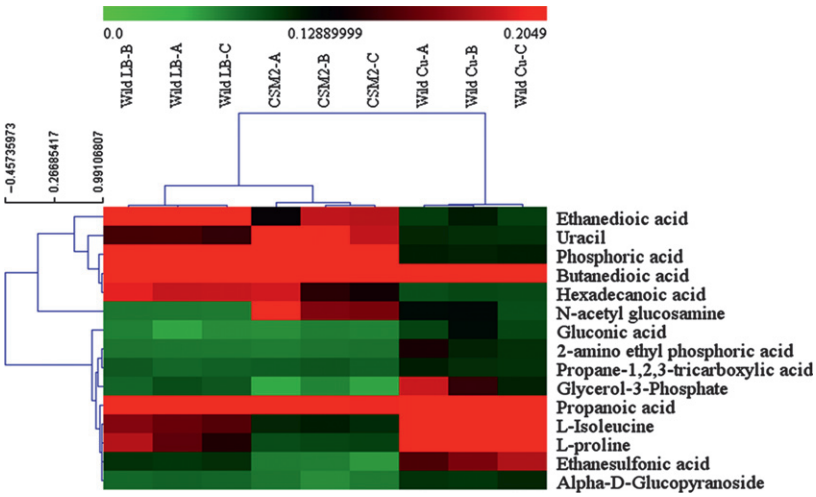

Fig. 3. Significant changes in relative intracellular metabolite concentrations of *Pseudomonas* sp. TLC6-6.5-4 (wild type) and CSM2 (mutant) grown in LB broth with or without copper, illustrated by one-way ANOVA clustering. Three replicates (A,B,C) in each group. Clustering was performed using Pearson correlation as the distance metric. The color bar on top represents the relative metabolite level.

Table 2. Metabolic pathways associated with the compounds with relative concentrations significantly different in wild type strain in presence and absence of copper and CSM2 mutant.

Wild + Cu vs. wild		CSM2 mutant vs. wild	
Metabolic pathway	P-value	Metabolic pathway	P-value
ABC transporters	0.000023	ABC transporters (amino acid; organic ion and oligosacchride)	0.0000011
TCA cycle	0.000049		
Protein digestion and absorption	0.00011		
Glyoxylate and dicarboxylate metabolism	0.00078		

The identified compounds with significant changes were submitted to KEGG to acchieve KEGG compound IDs. The most likey matabolic pathways involved with the compounds were determined by MPEA. *P*-values were calculated by Monte Carlo simulation.

tol-acid reductoisomerase, an enzyme involved in the biosynthesis of leucine and isoleucine (Table 1). An increase in amino acid synthesis was also identified in the multiple metal-resistant bacteria *P. fluorescens* in both biofilm and planktonic culture, which could be a protective mechanism against enzyme inhibition or replacement of damaged proteins caused by the presence of copper (Booth *et al.*, 2011). Furthermore, the accumulation of L-proline itself is the protective mechanism that bacteria (and plants and yeast) use to cope with the oxidative stress caused by heavy metals (Nandakumar *et al.*, 2011).

The Clp proteases play an important role in regulating cellular functions by refolding or degrading damaged proteins and also regulate the expression of genes involved in oxidative stress and DNA repair (Hengge & Bukau, 2003; Michel *et al.*, 2006). However, very little is known about the role of Clp proteases in *Pseudomonas* species except for the basic function of proteolysis. Disruption of ClpA in *P. putida* CA-3 decreased polyhydroxyalkanoates, the intracellular granules, in response to inorganic nutrient limitation (Goff *et al.*, 2009). In the present study, we demonstrated that the transposon insertion mutant, CSM2, disrupted in Clp protease subunit ClpA showed a significant reduction in copper resistance compared with the wild-type strain. A recent study on *Staphylococcus aureus* also showed that the expression of ClpA was up-regulated in response to copper (Baker *et al.*, 2010). The disruption of ClpA caused the down-regulation of glycosyl transferase and tRNA (guanine-N (7)-)-methyltransferase (Table 1). Glycosyl transferase is essential for bacterial biofilm formation and resistance to oxidative stress (Erb *et al.*, 2009; Tao *et al.*, 2010). The higher levels of tRNA methyltransferase under cellular stress response are likely to reduce the degradation of tRNAs by ribonucleases activated under stress conditions (Thompson & Parker, 2009; Chan *et al.*, 2010). DnaJ-class molecular chaperone (Table 1), whose expression was up-regulated in wild-type strain grown with copper compared with wild type without copper, binds unfolded polypeptide chains, preventing their irreversible aggregation (Düppre *et al.*, 2011). Clp proteases including ClpA act as molecular chaperones with a similar function as DnaK/DnaJ (Wickner *et al.*, 1994). It is likely that the up-regulation of DnaJ-class molecular chaperone in CSM2 mutant is due to the substitution of the partial Clp protease function by DnaJ when Clp protease is dys-

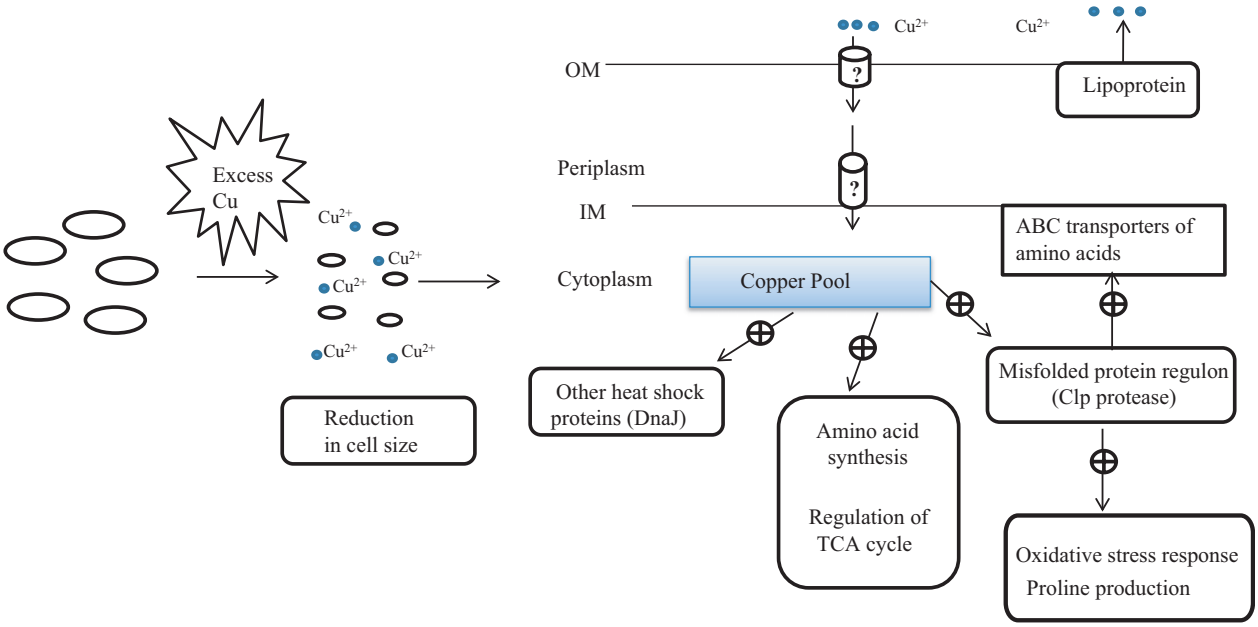

Fig. 4. A simple model for the mechanism of copper resistance in *Pseudomonas* sp. TLC6-6.5-4. ⊕ indicates up-regulation. OM, outer membrane; IM, inner membrane. ? represents unknown proteins.

functional. Further, our metabolomic analysis of the Clp protease mutant identified down-regulation of amino acid and oligosaccharide transporters that are part of ABC transporter pathways (Table 2). The mechanism is likely to be similar to that observed in *Mycobacterium tuberculosis* under hypoxia, where Clp proteases degrade the factors that inhibit DNA replication and transcription to initiate the synthesis of amino acids during stressful conditions (Sherrid *et al.*, 2010).

Changes in the levels of TCA cycle enzymes in response to copper identified by the metabolomic analysis (Table 2) were confirmed by the proteomic analysis with up-regulation of ketol-acid reductoisomerase, which participates in the production of CoA (Table 1). In addition, down-regulation of MalR, a transcriptional regulatory protein for malate and citrate metabolism (Table 1) under copper stress would result in the accumulation of malate and citrate, the intermediate products in TCA cycle (Papa *et al.*, 2009). Higher levels of malate allow the organism to cope with oxidative stress caused by copper toxicity, by producing more NADPH, an important antioxidant (Singh *et al.*, 2007). Citrate is a metabolite involved in the sequestration of aluminum and the increase of citrate accumulation was previously shown in *P. fluorescens* grown under aluminum stress (Mailloux *et al.*, 2008). Our results suggest that citrate is involved in the sequestration of copper.

Based on our results, we propose a model for the response to toxic levels of copper in *Pseudomonas* sp. TLC6-6.5-4 (Fig. 4). High copper concentrations reduce its cell size, which decreases the amount of copper bound on the cell surface. In addition, smaller cells need less energy for maintenance under copper stress. CopA and lipoprotein mediate sequestration and efflux of copper outside the cytoplasm. Heat shock proteins including Clp protease and DnaJ-class molecular chaperone either degrade the damaged proteins or prevent their irreversible aggregation under copper stress. Furthermore, Clp protease is directly involved in copper resistance by up-regulation of amino acid transporters, proteins related to oxidative stress and proline accumulation. This organism maintains a fine metabolic balance to enable the cells to survive in environment with high copper concentration by increasing amino acid production and regulating TCA cycle.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Real-time RT-PCR analysis of *clpA* and *trpA* genes of wild type grown with or without copper and mutants grown without copper.

Fig. S2. Hierarchical clustering analysis of the relative intracellular metabolite concentrations of *Pseudomonas* sp. TLC 6-6.5-4 (wild type) and CSM2 (mutant) growing in LB broth with or without copper.

Table S1. Primers used in this study.

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