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A proteome analysis of the tetracyanonickelate (II) responses in *Klebsiella oxytoca*

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Summary

Tetracyanonickelate (II) (TCN) has been proved to be degraded by *Klebsiella oxytoca*. In order to examine the physiological responses of TCN degradation by this bacterium, two-dimensional (2-DE) electrophoresis approach and Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry allow us to identify 91 proteins spots that were significantly altered in the presence of 1 mM TCN in relative to that in 1 mM ammonia when *K. oxytoca* grown at the late-log phase. Among them, 43 proteins were successfully identified. Fractions enriched in hydrophobic proteins were obtained with a specific extraction method based on temperature-dependent phase partitioning with Triton X-114, with the successful identification of 26 proteins out of 41 differential proteins. Some proteins were related with TCN metabolism. OsmC-like protein, molecular chaperone DnaK, glutathione S-transferase, alkyl hydroperoxide reductase, DNA protection during starvation conditions and DNA binding ferritin-like protein can counteract the oxidative stress from TCN biodegradation. The nitrogenase had been suggested to participate in TCN degradation by *K. oxytoca*, and was upregulated in TCN-treated cells as expected. The induction of glutamine synthetase could enhance the assimilation of limited nitrogen source produced from the bioconversion of TCN into ammonia as the alternate nitrogen source for bacteria growth. These findings could provide new insights into the inducible mechanisms underlying the capacity of *K. oxytoca* to tolerate TCN stress.

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Introduction

Cyanide (KCN) is highly toxic to living organisms (Chena and Liu, 1999; Yanase *et al*., 2000) by inactivating the respiration system by tightly binding to terminal oxidases (Porter *et al*., 1983), and is produced by industry with a total amount of 2–3 millions of tons per year (Raybuck, 1992). About 80% of KCN is used in the synthesis of organic compounds such as nitriles, nylon, acrylic plastics, painting, adhesives, cosmetics, dyes, drugs, chelating agents, etc. (Luque-Almagro *et al*., 2007). As cyanide is a very toxic compound and the waste products generally contain other contaminants including heavy metals such as nickel, copper, zinc and iron (Silva-Avalos *et al*., 1990), the metal–cyano complexes are major forms in metal containing waste because of the quick binding of KCN with metals (Chen *et al*., 2009). Such metal–cyano complexes are highly stable and more resistant to biological attack compared with free cyanide (Chen *et al*., 2009). Currently, wastewater containing cyanide is treated by chemical oxidation methods (alkaline chlorination, ozonization, wet-air oxidation) (Watanabe *et al*., 1998). However, these methods are expensive and hazardous chemicals are used as the reagent (chlorine and sodium hypochlorite) (Watanabe *et al*., 1998). Moreover, these techniques can not completely degrade all cyanide complexes in many cases (Dubey and Holmes, 1995).

Based on the above discussions, biological treatment would be a cost-effective and environmentally acceptable method for KCN removal compared with the other techniques currently in use (Raybuck, 1992; Dubey and Holmes, 1995). It has been reported that tetracyanonickelate (II) (TCN) can be utilized as the nitrogen source by some microorganisms such as *Pseudomonas fluorescens* NCIMB 11764 (Rollinson *et al*., 1987), *P. putida* BCN3(Silva-Avalos *et al*., 1990), *Klebsiella* sp. (Silva-Avalos *et al*., 1990), *Fusarium oxysporum* (Barclay *et al*., 1998), *F. Solani* (Barclay *et al*., 1998), *F. oxysporum* N-10 (Yanase *et al*., 2000), *Cryptococcus humicolus* MCN2 (Kwon *et al*., 2002) and *Azotobacter vinelandii* (Kao *et al*., 2005). We previously showed that *Klebsiella oxytoca*, a TCN-resistant strain, can effectively degrade 1 mM TCN

into ammonia as an alternate nitrogen source for the bacterial growth (Kao *et al*., 2004). Nevertheless, little is known about the mechanisms of TCN biodegradation carried out by aforementioned microorganisms, except for Yanase *et al*. (Yanase *et al*., 2000) and our previous study (Kao *et al*., 2004).

The two-dimensional (2-D) electrophoresis approach has been successfully used to understand the molecular basis of the physiological changes that occur in living organisms to adapt to environmental changes (Luque-Almagro *et al*., 2007). To study the physiological response of TCN biodegradation by *K. oxytoca*, we first utilized a proteomic approach based on the 2-D electrophoresis technique and the Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) analysis to check differential protein expression in response to TCN.

Results and discussion

Effect of TCN on the growth of K. oxytoca

The measured optical densities of ammonia- and TCN-treated cells over time were shown in Fig. S1. According to the plots, 1 mM ammonia-treated cells started to grow quickly and finished at OD_{600nm} 0.8. This bacterial density was also observed in 1 mM TCN-treated cells, although this growth rate was much more delayed compared with that of ammonia-treated cells. This delayed growth in TCN-treated cells could be attributed to the toxicity of TCN on growth. The OD_{600nm} 0.8 was chosen for harvesting the cells.

Differential proteins

Protein lysates obtained from these cells treated with 1 mM ammonia and 1 mM TCN, respectively, were subjected to 2-DE. A substantial part of the proteome of this bacterium was visualized by using a isoelectric point (pI) range of the IPG strips from 4 to 7. Among the differential proteins, the upregulation of 67 proteins (at least twofold) ($P < 0.05$) were found but expression of 24 proteins was repressed in the TCN-treated cells relative to ammonia-treated cells. These proteins were indicated on the 2-DE protein map with an identification number (Fig. S2), and then were excised from the gels and analysed by MALDI-TOF-MS. After searching on-line using peptide mass fingerprinting (PMF), 34 upregulated and nine downregulated proteins (Table S1) were successfully identified. The bacterial membrane proteins are encoded by about one quarter to one-third of all bacterial genes and perform essential physiological functions (Poetsch and Wolters, 2008). However, the analysis of this group of proteins has been traditionally difficult (Alvarez-Chaver *et al*., 2007), which could be due to the inability of the detergents employed for separating hydrophobic proteins (HPB) effectively, and the tendency of aggregation of these proteins at their isoelectric point (Molloy, 2000).

In this study, we isolated HPB by using a specific protein extraction method based on temperature-dependent phase portioning with Triton X-114 (Bordier, 1981; Santoni *et al*., 2000). Comparing 2-D maps obtained from HPB proteins of 1 mM TCN-treated and 1 mM ammonia-treated cells, we observed variations in the level of expression of 41 proteins (Fig. S3), 26 of which were identified (Table S2).

Proteins involved in oxidative stress

As shown in Table 1, there were eight proteins involved in oxidative stress. Some differential proteins related with oxidative stress were enlarged in Fig. 1. The role of glutathione S-transferase (GST) in bacterial growth was reported in many studies. For example, the upregulation of OaGST, one type of GSTs, in *Ochrobacturm anthropi* could function as a detoxifying agent within catabolism of phenols and chlorophenols (Tamburro *et al*., 2004); three GSTs of distinct classes in *Escherichia coli* were reported to defence oxidative stress in spite of the structural diversity (Tamburro *et al*., 2004), and the expression of *Proteus mirabilis* glutathione-S-transferase B1-1 (PmGST B1-1) is involved in the detoxification of antimicrobial agents and oxidative stress generated by H_2O_2 (Allocati *et al*., 2003). In our previous study (Tang *et al*., 2008), we indicated that GST in *K. oxytoca* could be viewed as the detoxifying enzyme for counteracting the oxidative stress generated by succinonitrile, a complex of cyanide with organic acid. The induction of alkyl hydroperoxide reductase (AHR) (spot no. 48) in this study is involved in degrading H_2O_2 and organic peroxides, which can be generated at the level of the upper complexes of the respiratory chain (Messner and Imlay, 1999). Heat shock protein (spot no. 39) and chaperone (DnaK) (spot no. 8) could protect the TCN-treated cells against oxidative stress. Heat shock proteins can assist abnormal proteins accumulating under stress conditions to regain their proper folding or assist their proteolyic degradation (Lund, 2001). Most of chaperons and proteases, in addition to their protective functions, may help reorchestrate the cell metabolism to the needs of the oxidative stress response (Godon *et al*., 1998). The induction of DNA starvation/stationary phase protection protein (Dps) (spot no. 52) in TCN-treated cells suggested that Dps can provide protection from oxidative stress, as Dps homologues are widespread conservation among prokaryotes, and may be a general strategy for coping with oxidative stress (Martinez and Kolter, 1997). The induction of osmotically inducible protein C (OsmC) was observed in *K. oxytoca* treated with cyanide (Tang

Table 1. Identification of differential proteins related with TCN metabolism in TCN-treated and non-treated bacterial cells.

Function	Spot No.[a]	Protein name	NCBI Accession number	Exp. pI/Mw[b]	Theo. pI/Mw[c]	Score[d]	Sequence coverage	Peptides[e] match/total	PSORTb predicted location[f]	Spot volume/total volume (%)[g] NH_4Cl	Spot volume/total volume (%)[g] TCN
Stress protein	1	OsmC-like protein	gi:118046300	5.8/18	5.7/16.3	56	24%	5/41	Cytoplasmic	ND	0.023 ± 0.0070
	8	Molecular chaperone DnaK	gi:152968596	4.6/31.5	4.8/69.1	55	21%	11/68	Unknown	ND	0.003 ± 0.0007
	24	Peptidyl-prolyl cis-trans isomerase B (rotamase B)	gi:152969053	5.7/18.7	5.1/18.2	56	39%	6/28	Cytoplasmic	ND	0.004 ± 0.0005
	29	Glutathione S-transferase	gi:157159060	6.4/25	5.9/32.3	76	41%	8/37	Unknown	ND	0.002 ± 0.0005
	39	Proteolytic subunit of clpA-clpP ATP-dependent serine protease, heat shock protein F215	gi:62126693	5.6/23.4	5.6/23.2	57	36%	8/39	Cytoplasmic	ND	0.006 ± 0.0006
	48	Alkyl hydroperoxide reductase	gi:152969221	4.8/20.0	5.0/20.8	102	55%	13/58	Cytoplasmic	0.003 ± 0.0009	0.006 ± 0.0007
	52	DNA protection during starvation conditions	gi:152969398	6.0/18.4	5.6/18.6	113	75%	16/98	Cytoplasmic	ND	0.017 ± 0.0002
	116	DNA binding ferritin-like protein	gi:75178395	6.2/18.7	5.73/19.4	54	43%	6/57	Cytoplasmic	ND	0.279 ± 0.0295
Nitrogen metabolism	10	Glutamine synthetase	gi:3808290	4.5/30.5	5.3/51.8	91	35%	11/57	Cytoplasmic	ND	0.004 ± 0.0002
	47	Nitrogenase	gi:67155565	5.8/17.7	5.1/15.5	57	59%	7/70	Cytoplasmic	0.006 ± 0.0005	0.053 ± 0.0050

a. Spot number as stated in Fig. S2B.
b. pI and Mw values were experimentally determined.
c. Predicted pI and Mw according to protein sequence.
d. Scores in MASCOT greater than 53 were considered significant ($P < 0.05$).
e. Criteria for confident identification were that the protein should match at least four peptides.
f. Output of computer algorithms that predicts subcellular location of protein [PSORTb (Nakai and Horton, 1999)].
g. Spot quantity was expressed as the volume of a spot divide by total volume of all the spots in a gel.
The SD of normalization (each spot volume/total spot volume × 100) means that every experiment repeat three times ($n = 3$).
The cytoplasmic protein sample solution (80 μg) was mixed with a rehydration buffer containing 6 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer, 40 mM dithiothreitol (DTT) and a trace of bromophenol blue (BPB). 2-D electrophoresis was conducted as described by Tang and colleagues (2010). Briefly, the IEF was carried out with commercially available immobilized pH gradients (pH 4–7, 11 cm; GE Healthcare) using the Ettan IPGphor II (GE Healthcare) apparatus. After IEF, the strip was equilibrated with 130 mM DTT and then 250 mM iodoacetamide in 10 ml of equilibration buffer (0.375 M Tris-HCl/pH 8.8, 6 M urea, 20% glycerol, 2% SDS) for 15 min. In the second dimension SDS-PAGE (Bio-Rad), the IPG strips were embedded in 0.5% melted agarose prior to running on the SDS-PAGE slabs. The running conditions were 15 mA per gel and 300 V per total for 5 h until the BPB as a tracking dye reached the end of the gel. After separation in the SDS-PAGE gels, the proteins were visualized with silver-staining according to Blum and colleagues (1987). Computer analysis of the 2-D gels for protein detection, spot matching between gels, and change of protein expression levels were performed with Phoretix 2-D advanced software, version 5.1 (Phoretix, Newcastle upon Tyne, UK). Volume normalization was used to allow accurate comparison of measurements between gels as provided by the Phoretix 2-D analysis package. The threshold of the expression level of differentially expressed proteins was more than or less than twofold for cyanide-treated cells as compared with untreated cells. Protein spots of interest were excised from the gel and digested with trypsin (Tang *et al.*, 2010). This extraction was repeated three times with 50% acetonitrile (ACN) and 5% trifluoroacetic acid (TFA) at room temperature and dried in a vacuum centrifuge. The samples were reconstituted in 10 μl of 0.1% TFA and treated with ZipTips containing C18 resin (Millipore) according to the manufacture's instructions. The washed peptides were eluted with a saturated matrix solution (CHCA in 60% ACN, 0.1% TFA) and were then analysed with a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) in Chang Gung Proteomics Core Laboratory (Taoyan, Taiwan). The protein identities were assigned if at least five peptide masses matched within a maximum error of 50 ppm, and the candidate agreed with the estimated pI and molecular weight from the 2-DE gel.
The isolation of hydrophobic proteins (HPB), 1 mM ammonia- and 1 mM TCN-treated cells were subjected to temperature-dependent phase portioning using the Ready Prep™ Protein Extraction Kit (Membrane I) (Bio-Rad) according to the manufacture's instructions. The final protein concentration was solubilized in a buffer containing 7 M urea, 2 M thiourea and 4% CHAPS (w/v) and was measured using the Bradford assay (Bradford, 1976). Subsequently, HPB were performed for proteomic analysis.
ND, not detectable.

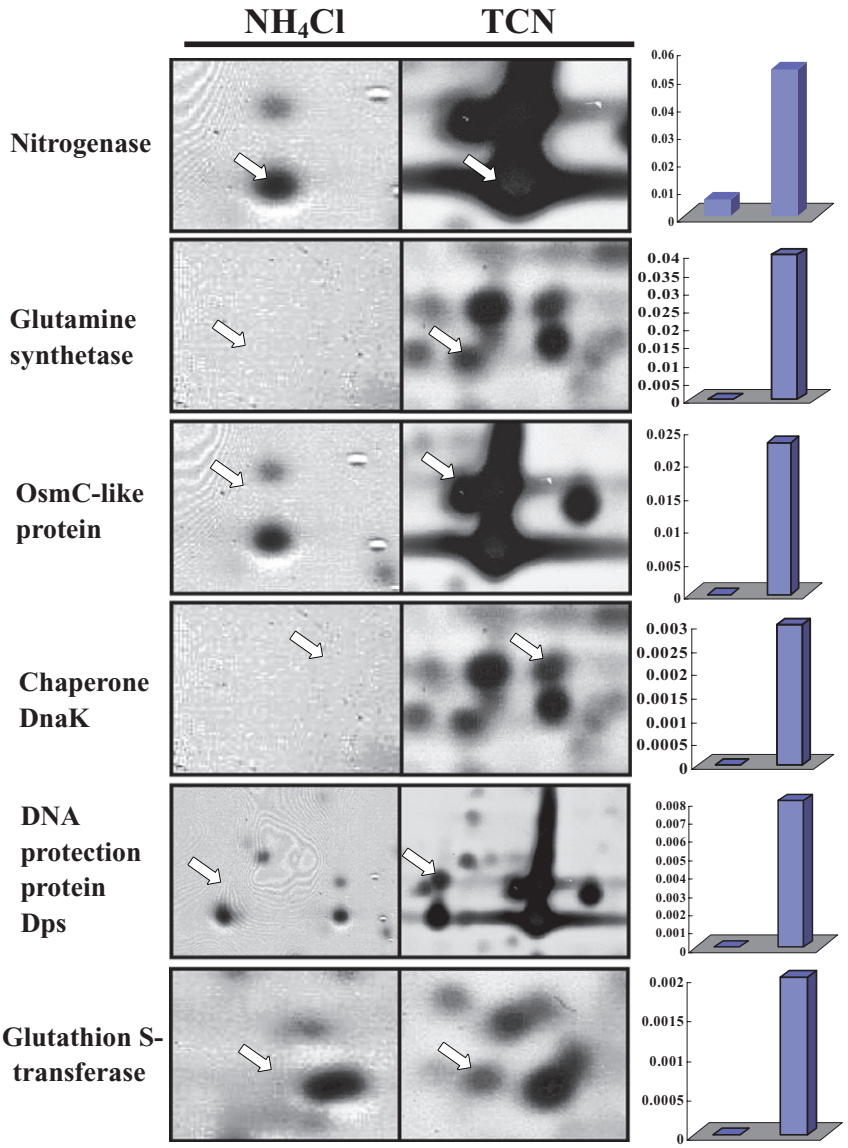

Fig. 1. Enlarged regions of some proteins related with oxidative stress identified from 2-DE gels of the control group versus TCN-treated cells.

et al., 2008). OsmC is one component of peroxiredoxins functional in bacterial antioxidant defence and can be controlled by multiple general stress responsive regulators (Dubbs and Mongkolsuk, 2007). Similarly, the induction of OsmC (spot no. 1) in *K. oxytoca* can enable this bacterium to detoxify oxidative stress generated by TCN.

As the respiratory inhibition of *K. oxytoca* by cyanide accompanied by the production of H_2O_2 (Kao *et al.*, 2007), we proposed that the induction of these above described proteins can protect *K. oxytoca* from oxidative stress in this study.

Proteins involved in the nitrogen metabolism

We previously suggested the utilization of TCN as the alternate nitrogen source by the induction of nitrogenase in *K. oxytoca* (Kao *et al.*, 2004). Although the nitrogenase is an anaerobic enzyme, we have demonstrated that *K. oxytoca* with the self-protective mechanism can protect the nitrogenase from oxygen destruction when this bacterium was grown in nitrogen-free glucose medium with KCN as the sole nitrogen source (Chena and Liu, 1999). As expected, the induction of nitrogenase (spot no. 47) was detected using the analysis of 2-DE map in this study.

Ammonia is the preferred nitrogen source for most microorganisms (Muro-Pastor *et al.*, 2001). In the nitrogen-limited conditions for bacterial growth, the high level of glutamine synthetase could warrant the assimilation of the small amount of free ammonium available (Muro-Pastor *et al.*, 2001). Thus, the glutamine synthetase activity (spot no. 7 and 10) was activated by the induction of glutamine synthetase enhancing the assimi-

lation of limited nitrogen source produced from the bioconversion of TCN to ammonia as the alternate nitrogen source for bacterial growth. This protein was reported to be induced only under nitrogen deprivation conditions and be responsible for the ability of nitrogen-starved cells to survive and thrive rapidly once ammonia is supplied as nitrogen source (Atkinson *et al*., 2002).

Conclusion

Klebsiella oxytoca is able to induce many defence mechanisms upon TCN stress such as the induction of antioxidant enzymes and heat shock proteins. Additionally, the induction of nitrogenase could be related with TCN degradation. Overall, the resulting data identifying a number of differential expressed TCN-associated proteins may provide clues about the understanding of the underlying mechanisms of TCN biodegradation by *K. oxytoca*

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Supporting information

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

Fig. S1. Growth curves of *K. oxytoca* in nitrogen-free glucose (NFG) media containing 1 mM NH_4Cl (◆) or 1 mM TCN (●). Protein samples were taken at late-log phase points indicated by the arrows. *K. oxytoca* was grown on a NFG medium containing Na_2HPO_4 $2H_2O$ (50 mM), KH_2PO_4 (100 mM), $MgSO_4$ (1 mM), $CaCl_2$ (0.1 mM) and glucose (0.8%). The pH value of this medium was adjusted to 7.0. Filter-sterilized TCN or ammonia at indicated doses was added as nitrogen source. *K. oxytoca* grown in NFG medium containing TCN (1 mM) or ammonium (1 mM) was incubated in a Gyrotory shaker at 30°C.

Fig. S2. 2-D electrophoresis of the cytoplasmic proteins in *K. oxytoca* growth in NFG medium supplemented with 1 mM NH_4Cl (A) or with 1 mM TCN (B) at late-log phase. Differential proteins were marked by arrowheads and numbered.

Fig. S3. 2-D electrophoresis of the hydrophobic proteins in *K. oxytoca* growth in NFG medium supplemented with 1 mM NH_4Cl (A) or with 1 mM TCN (B) at late-log phase. Induced proteins or overexpressed proteins of at least twofold were marked by arrowheads and numbered.

Table S1. Identification of cytosolic proteins on 2-DE gels of control versus TCN-treated cells.

Table S2. Isolation of proteins using Triton X-114 and then identification of these proteins on 2-DE gels of control versus TCN-treated cells.

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