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ORIGINAL PAPER

Proteomic and molecular investigation on the physiological adaptation of *Comamonas* sp. strain CNB-1 growing on 4-chloronitrobenzene

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Abstract *Comamonas* sp. strain CNB-1 can utilize 4-chloronitrobenzene (4CNB) as sole carbon and nitrogen source for growth. Previous studies were focused on 4CNB degradative pathway and have showed that CNB-1 contained a plasmid pCNB1 harboring the genes (*cnbABCaCbDEFGH*, *cnbZ*) for the enzymes involving in 4CNB degradation, but only three gene products (CnbCa, CnbCb, and CnbZ) were identified in CNB-1 cells. *Comamonas* strain CNB-2 that lost pCNB1 was not able to grow on 4CNB. In this study, physiological adaptation to 4CNB by CNB-1 was investigated with proteomic and molecular tools. Comparative proteomes of strains CNB-1 and CNB-2 grown on 4CNB and/or succinate revealed that adaptation to 4CNB by CNB-1 included specific degradative pathway and general physiological responses: (1) Seven gene products (CnbA, CnbCa, CnbCb, CnbD, CnbE, CnbF, and CnbZ) for 4CNB degradation were identified in 4CNB-grown cells, and they were constitutively synthesized in CNB-1. Two genes *cnbE* and *cnbF* were cloned and simultaneously expressed in *E. coli*. The CnbE and CnbF together catalyzed the conversion of 2-oxohex-4-ene-5-chloro-1,6-dioate into 2-oxo-4-hydroxy-5-chloro-valeric acid; (2) Enzymes involving in glycolysis, tricarboxylic acid cycle, and synthesis of glutamate increased their abundances in 4CNB-grown cells.

Keywords 4-Chloronitrobenzene degradation · *Comamonas* sp. strain CNB-1 · Proteome · Pathway

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Abbreviations

CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate
CHCA	α-Cyano-4-hydroxycinnamic acid
4CNB	4-Chloronitrobenzene
DTT	Dithiothreitol
HED	Hydroxyethyldisulfide
IEF	First-dimensional isoelectric focusing
IPG	Immobilized pH gradient
IPTG	Isopropylthiogalactopyranoside
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TFA	Trifluoroacetic acid

Introduction

Many chlorinated and nitroaromatic compounds were assumed to be resistant to microbial degradation, but later it became evident that microbes are quickly adapt to polluted environments and are able to degrade these compounds (van der Meer 1994; Symons and Bruce 2006). An example is the recent work on microbial degradation of 4-chloronitrobenzene (4CNB). 4CNB is produced in large quantities and widely used as an intermediate for chemical syntheses of drugs, herbicides, dyes, etc (as cited by Katsivela et al. 1999). Although this compound has been introduced into the environment for a relatively short period, its occurrence in the environment has selected for microorganisms that are able to utilize 4CNB as carbon and/or nitrogen sources over the last few years (Katsivela et al. 1999; Park et al. 1999; Wu et al. 2005; Zhen et al. 2006). *Comamonas* sp. strain CNB-1, a recently isolated 4CNB degrader, was obtained from the wastewater treatment facility of a 4CNB production factory (Wu et al. 2005). Previous studies showed that CNB-1 degraded 4CNB *via* a partial-reductive pathway. Two enzymes [2-amino-5-chlorophenol 1,6-dioxygenase (CnbCaCb) and 2-amino-5-chloromuconic acid deaminase (CnbZ)] and their encoding genes were identified at genetic and proteomic levels, and three other genes encoding for 4CNB nitroreductase (CnbA), hydroxylaminobenzene mutase (CnbB), 2-amino-5-chloromuconic semialdehyde dehydrogenase (CnbD)] were identified at genetic but not proteomic level (Wu et al. 2005, 2006; Liu et al. 2007). These enzymes sequentially convert 4CNB to 2-oxohex-4-ene-5-chloro-1,6-dioate (Wu et al. 2006). Some other enzymes (CnbG, CnbE and CnbF) were suggested to be involved in the partial-reductive pathway, however, solid evidence is still missing (Wu et al. 2006).

Microorganisms can use aromatic compounds as carbon and energy sources for growth. Microbial cells cultivated on aromatic compounds usually exhibit an induced catabolic pathway. In addition, a number of other cellular processes such as gluconeugenesis and stress responses are also affected (Kurbatov et al. 2006; Tomas-Gallardo et al. 2006; Qi et al. 2007). Due to the robust ability to resolve hundreds of proteins simultaneously, proteomic tools such as 2-dimensional electrophoresis (2-DE) have been exploited to understand the physiological adaptation to the environment in the past few years (Segura et al. 2005). By using 2-DE approach, enzymes of degradative pathways for phenol, benzoate, 3-hydroxybenzoate, aniline and gentisate were identified in *Pseudomonas putida* (Kim et al. 2004b, 2006), *Pseudomonas alcaligenes* (Zhao et al. 2004, 2005), *Rhodoccocus* (Tomas-Gallardo et al. 2006), *Acinetobacter lwoffii* (Kahng et al. 2002; Kim et al. 2002, 2004a) and *Acinetobacter radioresistens* (Giuffrida et al. 2001). Moreover, novel proteins involving in aromatic degradation have been recently discovered in *Corynebacterium glutamicum* (Qi et al. 2007).

In this study, comparative proteomic studies were conducted on *Comamonas* sp. strains CNB-1 and CNB-2 that were cultivated with 4CNB or succinate. The goals of this study were (1) to identify enzymes that involved in 4CNB degradation pathway; (2) to reveal that how other cellular processes are affected in 4CNB grown cells.

Materials and methods

Bacterial strains and culture conditions

Comamonas sp. strain CNB-1 was pre-cultivated aerobically at 30°C on a rotary shaker in 10 ml Luria–Bertanni (LB) broth. The overnight precultures were subsequently inoculated in 200 ml mineral salt basic (MSB) medium (Dorn et al. 1974) supplemented with 10 mM succinate and 10 mM $(NH_4)_2SO_4$ or 1.27 mM 4CNB as the sole carbon and nitrogen source. The pH of the medium was adjusted to 7.0. For the proteomic comparison, strain CNB-2, a plasmid-free mutant of CNB-1 (Wu et al. 2006) was cultivated in parallel. Cell growth was determined by measuring the optical density at 600 nm (OD_{600}). Samples for 2-DE were harvested at the late exponential phase of growth (Fig. 1). For each experiment, three cultures were performed and sampled in parallels.

All *Escherichia coli* strains were cultivated aerobically at 37°C in LB medium. When required, amplicillin was added to the medium at a final concentration of 100 μg ml^{-1}.

Sample preparation

Bacterial cells were harvested by centrifugation (7,000 × *g*, 10 min, 4°C). The cell pellets were

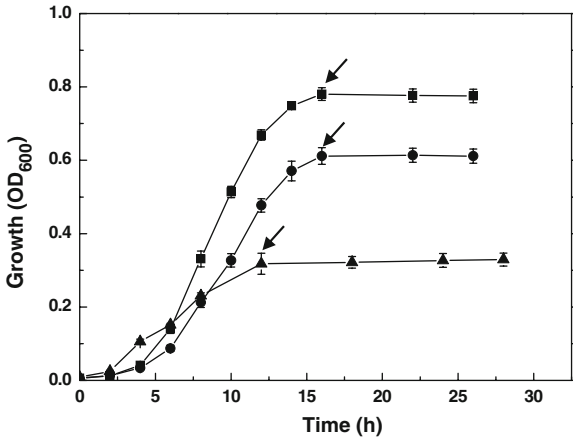

Fig. 1 Growth of CNB-1 and CNB-2 in mineral salt broth (MSB) medium with 4CNB and succinate as the carbon source, respectively. The *black arrows* indicate the harvesting time for 2-DE. For cultivation with 4CNB or succinate, mean values of three parallel cultures under same conditions are given and the deviations are shown. ■, CNB-1/Succinate; ●, CNB-2/Succinate; ▲, CNB-1/4CNB

washed twice with 50 mM Tris–HCl (pH 7.4) and resuspended in 1.2 ml lysis buffer (8 M urea, 4% CHAPS, 2% Bio-Lyte). Cells were broken by sonication on ice, and the resulting cellular lysate was centrifuged at 14,000 × *g* and 4°C for 10 min. The supernatant was collected and stored at −20°C for proteomic analysis. Protein concentration was determined by the Bradford method using BSA as a standard (Bradford 1976).

Samples of supernatants were treated with DNaseI and RNaseA at final concentrations of 1 and 0.25 mg ml^{-1}, respectively, and at 4°C for 30 min. An aliquot of the supernatant containing 300 μg of proteins was analyzed by 2-DE.

Operation of IFE and SDS-PAGE

IEF was performed with two types of IPG strips on an Ettan IPGphor II instrument (GE Healthcare, Little Chalfont, United Kingdom). The IPG strip of pH 4–7 (size 13 cm) was rehydrated and loaded according to the instruction from the supplier (Cat. No. 80-6429-60Ac_2D, GE Healthcare, Little Chalfont, United Kingdom). IEF was performed as previously described (Qi et al. 2007). The IPG strip of pH 6–11 (size 11 cm) was rehydrated and loaded differently. To reduce cathodic drift and reverse-endosmotic flow during IEF (Molloy et al. 2002) at alkaline condition, the IPG strip (pH 6–11) was rehydrated overnight in a modified buffer (8 M urea, 4% CHAPS, 10% isopropanol, 5% glycerol, 1.5% HED and 1% IPG buffer pH 6–11). IEF was carried out by the stepwise program at 150 V for 0.5 h, 300 V for 3 h, 600 V for 2 h, and 3,500 V for 35,000 Vh.

After the IEF was completed, the IPG strips were treated according to instruction by the supplier (Cat. No. 80-6429-60Ac_2D, GE Healthcare, Little Chalfont, United Kingdom). Proteins on the IPG strip were further separated according to their molecular size by SDS-PAGE, which was performed on a 13% polyacrylamide gel using the SE600 Ruby^{TM} instrument (GE Healthcare, Little Chalfont, United Kingdom). The SDS-PAGE was run firstly at 20 mA/gel for 30 min, and subsequently the current was increased to 30 mA/gel. At the end of each run, the 2-D gels were fixed for 30 min in solution containing 40% ethanol and 10% acetic acid, and visualized with Coomassie blue R-250 (Bio-Rad, Hercules, CA, USA).

Image analysis and statistics

2-DE gels were scanned using UMAX Powerlook 2100XL (Novax Technologies Inc.) at a resolution of 400 dpi, and the images were analyzed with the software ImageMaster^{TM} 2D Platinum 6.0 (GE Healthcare, Little Chalfont, United Kingdom). Three parallel gels were run for each protein sample and were analyzed. The normalized volume (%) for each protein spot was defined as the fraction of that spot volume to the total spot volume of the gel. A two-tailed Student's *t*-test was adopted to evaluate the significantly different spots between the control and experimental gels ($P < 0.05$). Significant change of protein abundance was defined as the normalized volume over 2-folds in the two sets of 2-DE spots.

Tryptic in-gel digestion and protein identification by mass spectroscopy

Tryptic in-gel digestion and protein identification by mass spectroscopy were carried out as previously described (Zhang et al. 2007).

Cloning and expression of *cnbEF* genes in *E. coli*

The primers used for cloning *cnbEF* are listed in Table 1. The entire gene(s) were amplified by PCR from the strain CNB-1 genome. Purified PCR products were treated with *Nde*I and *EcoR*I and were ligated to the similarly treated pET-21a (+) vector. The resulting plasmid was used to transform *E. coli* cells for the expression of the *cnbEF* genes. The expression of the CnbEF was induced with 1 mM IPTG when the culture reached an optical density at 600 nm of 0.6.

Preparation of cell lysate, substrates, enzymatic assays and identification of catalyzed product

Cell lysate of recombinant *E. coli* was prepared by sonification of cell suspensions in 10 mM phosphate buffer (pH 8.0). Cell debris was removed by centrifugation at 12,000 × *g* for 10 min and the supernatant was used for enzymatic assays. The substrate, 2-oxohex-4-ene-5-chloro-1,6-dioate, was prepared by enzymatic conversion of 2-amino-5-chloromuconic acid with 2-hydroxy-5-chloromuconic acid tautomerase (Wu et al. 2006). The reaction buffer (3 ml) consisted of 10 mM phosphate buffer (pH 8.0), 0.2 mM 2-oxohex-4-ene-5-chloro-1,6-dioate and 1 µl of the cell lysate. The reaction was performed in 10 min at 30°C. The reaction mixture was acidified to pH 2.0 with HCl and centrifugated at 12,000 × *g* for 10 min. The supernatant was extracted with an equal volume of ethyl acetate, dried under a constant stream of N_2, and dissolved in 50 µl methanol. The product was identified by liquid chromatography-mass spectrometry (LC-MS) (Wu et al. 2006).

Results

Growth and proteomes of CNB-1 and CNB-2 on 4CNB and/or succinate

CNB-1 is able to grow on 4CNB or succinate as source for carbon and energy. In contrast, strain CNB-2, which lost the 4CNB-degrading plasmid pCNB1, grows on succinate but not on 4CNB. Due to the low solubility (200 mg l^{-1}) and high toxicity of 4CNB, the final cell yield of CNB-1 was lower on 4CNB than on succinate (Fig. 1). Interestingly, the cell yield of CNB-1 was higher than that of strain CNB-2 when both were cultivated on succinate (Fig. 1). This result indicated that plasmid pCNB1 was also involved in the assimilation of other carbon sources than 4CNB.

Cells of CNB-1 and CNB-2 grown on 4CNB and/or succinate were harvested at the late exponential phase (as indicated in Fig. 1) and were subjected to proteomic analysis. When the protein fractions were separated on a broad pH range IPG strip (pH 3–10), only few protein spots were located on the acidic sides (pH < 4), but a large number of protein spots were located in the area of pH 5–10. We therefore employed two different IPG strips covering pH 4–7 and pH 6–11, respectively, for better separation of the CNB-1 and CNB-2 proteomes.

When strain CNB-1 was cultivated with 4CNB, 553 ± 6 ($n = 3$) spots in the pH range 4–7 and 201 ± 4 ($n = 3$) spots in the pH range 6–11 were resolved (Fig. 2). When succinate was used as carbon source, 573 ± 8 ($n = 3$) spots in the pH range 4–7 and 212 ± 9 ($n = 3$) spots in the pH range 6–11 were separated. Cells of CNB-2 grown on succinate

Table 1 Bacterial strains and plasmids used in this study

Strains or plasmids	Characteristics	Sources
Comamonas sp. CNB-1	Wild-type strain isolated from activated sludge, assimilating 4CNB	Wu et al. (2005)
Comamonas sp. CNB-2	Mutant derived from strain CNB-1, devoid of pCNB1	Wu et al. (2006)
E. coli BL21(DE3)	Expression host	Novagen
pET-21a(+)	Expression vector	Novagen
pET*cnbEF*	pET-21a(+) derivative for expression of CnbEF	This work
Primers		
cnbEFPf	5′-GTAAATACATATGAACCGAACACAAGCCAAAG-3′ (*Nde*I)	This work
cnbEFPr	5′-CGGAATTCTCAGACGAATCGCATGCCAAC-3′ (*EcoR*I)	This work

The restriction enzyme sites are underlined

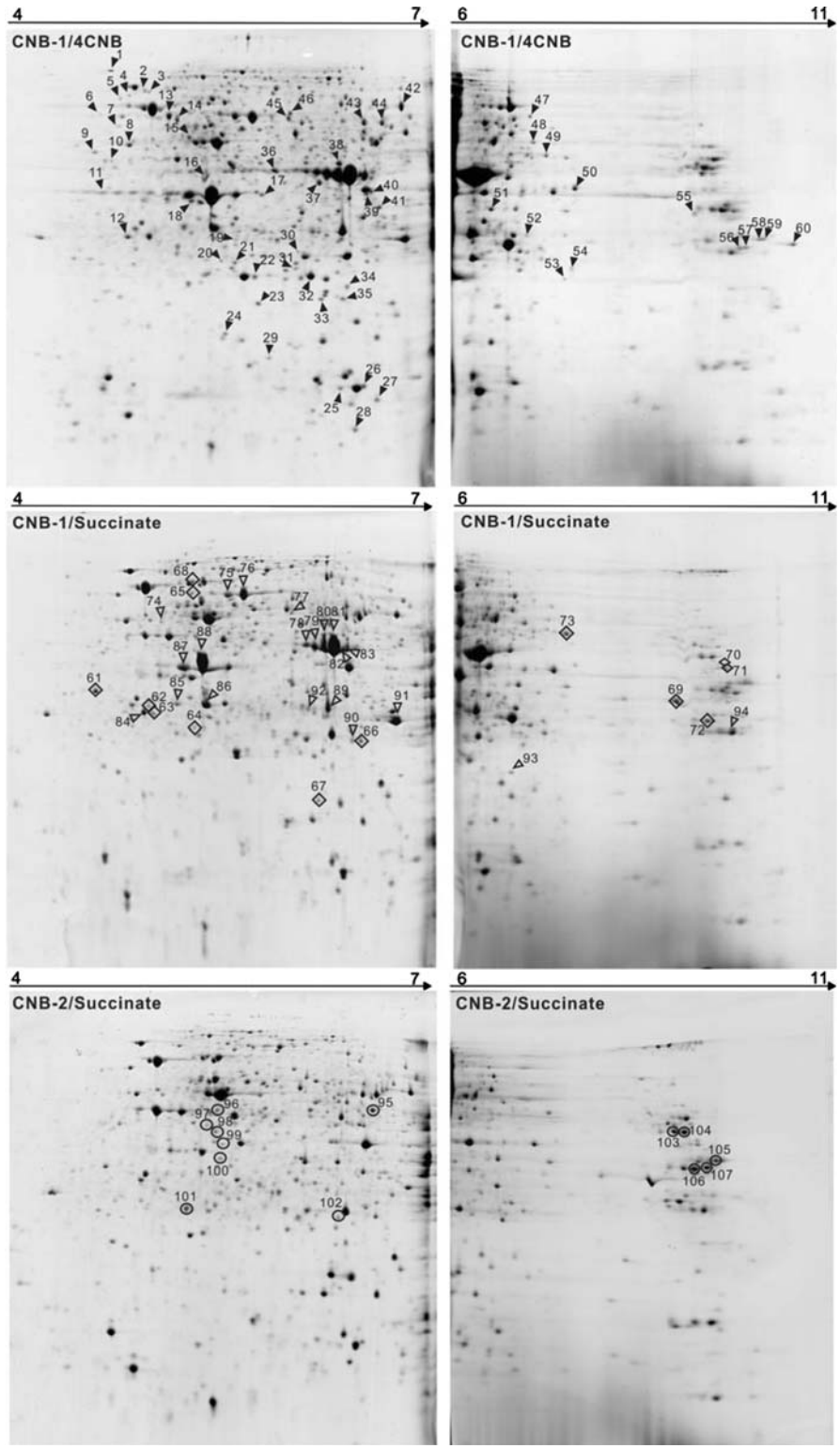

Fig. 2 2-DE of the total soluble protein extracts of CNB-1 cultured in 4CNB or succinate and CNB-2 cultured in succinate. 2-DE were performed using different IPG strips (pH 4–7 and pH 6–11), following by 13% SDS-polyacrylamide gels. Description of proteins: (i) comparison of panel CNB-1/4CNB with panel CNB-1/succinate: ▼ proteins present exclusively or in higher abundance in panel CNB-1/4CNB, ◇ proteins present exclusively or in higher abundance in panel CNB-1/succinate. (ii) comparison of panel CNB-1/succinate with panel CNB-2/succinate: ▽ proteins present exclusively or in higher abundance in panel CNB-1/succinate, ○ proteins present exclusively or in higher abundance in panel CNB-2/succinate. Each protein sample was run in 3 parallels for 2-DE, and the 3 DE gels were analyzed. Only a representative gel of each protein sample is shown

Table 2 Proteins specific or more abundant in 4CNB-grown cells identified by MALDI-TOF mass spectroscopy

Spot no.	Function	Score	Coverage (%)	Match	pI/MW^T	pI/MW^E	*P*-value	Fold
Central carbon metabolism								
36	Fructose-bisphosphate aldolase, class II, Calvin cycle subtype	227	50	23	5.51/38	5.98/36	0.004	2.01
38	Glyceraldehyde-3-phosphate dehydrogenase, type I	261	68	24	5.92/36	6.40/37	0.015	2.83
8	Enolase	180	36	15	4.88/46	5.00/41	0.022	3.50
17	Malate dehydrogenase	145	48	15	6.00/34	5.90/32	+	+
40	Malate dehydrogenase	112	43	14	6.00/34	6.62/33	+	+
19	Succinyl-CoA synthetase, beta subunit	100	45	17	5.08/41	5.76/26	+	+
Nitrogen metabolism								
1	Urocanate hydratase	153	35	19	6.33/61	4.95/60	0	10.82
42	Urocanate hydratase	196	37	24	6.33/61	6.77/51	0.036	10.27
47	Glutamate synthases, NADH/NADPH, small subunit	115	33	15	6.54/53	7.04/56	0.001	5.19
Nucleotides metabolism								
39	Ribose-phosphate pyrophosphokinase	185	50	17	6.00/33	6.58/32	+	+
23	Phosphoribosyltransferase	67	47	8	5.50/19	5.91/20	0.000	3.13
46	Phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	81	31	17	5.45/62	6.07/49	0.005	2.23
Stress proteins								
2	Chaperone protein DnaK	131	32	16	4.90/68	5.17/54	+	+
3	Chaperone protein DnaK	149	33	17	4.90/68	5.15/54	+	+
4	Chaperone protein DnaK	80	19	10	4.90/68	5.06/53	+	+
5	Chaperone protein DnaK	99	21	13	4.90/68	4.99/52	+	+
6	Chaperone protein DnaK	99	21	10	4.90/68	4.87/48	+	+
7	Chaperonin GroEL	129	33	13	5.01/57	4.99/45	+	+
9	Chaperonin GroEL	190	35	19	5.01/57	4.84/39	0.037	4.47
10	Chaperonin GroEL	123	23	11	5.01/57	4.97/36	0.025	5.88
13	Chaperonin GroEL	257	48	21	5.01/57	5.31/47	+	+
14	Chaperonin GroEL	148	38	18	5.01/57	5.37/47	+	+
15	Chaperonin GroEL	171	36	22	5.02/57	5.45/43	0.041	2.24
25	Chaperonin Cpn10	97	58	5	6.10/11	6.45/14	0.002	2.61
27	Heat shock protein HSP20	115	56	9	6.13/13	6.68/13	+	+
29	Chaperone protein DnaK	99	21	13	4.90/68	6.01/16	+	+
30	Chaperonin GroEL	97	15	9	5.01/57	6.18/24	+	+
50	Heat shock protein DnaJ-like	95	42	17	7.08/36	7.56/36	0.000	4.55
26	UspA	207	68	13	6.28/15	6.59/14	0.001	3.02
18	Thioredoxin reductase	87	47	11	5.21/34	5.47/31	0.000	2.30
20	NAD(P)H:quinone oxidoreductase	90	45	7	5.37/21	5.67/23	+	+
32	Manganese and iron superoxide dismutase	89	24	8	6.11/24	6.25/22	0.031	5.74
22	ATP-dependent Clp protease, proteolytic subunit ClpP	60	35	6	5.31/22	5.88/22	0.004	3.81
Transcription and translation								
35	Putative transcriptional regulator	81	44	8	5.97/18	6.44/20	+	+
16	Translation elongation factor Tu	126	35	13	5.28/43	5.55/34	+	+

Table 2 continued

Spot no.	Function	Score	Coverage (%)	Match	pI/MW^T	pI/MW^E	*P*-value	Fold
31	Ribosome recycling factor	52	15	5	7.88/21	6.13/23	+	+
34	Single-strand binding protein	163	67	17	5.90/20	6.46/21	0.001	2.16
57	Ribosomal 5S rRNA E-loop binding protein Ctc/L25/TL5	88	34	10	9.34/22	9.57/25	0.000	2.49
58	Ribosomal protein L1	133	48	14	9.75/24	9.72/26	0.002	2.89
59	Ribosomal protein L1	186	61	15	9.75/24	9.77/26	0.000	2.39
60	Ribosomal protein L3	129	55	10	10.08/24	10.1/25	0.001	3.35

In the CNB-1 protein database, scores higher than 49 are significant ($P < 0.05$); pI/MW^T are the theoretical values from the Mascot research result; pI/MW^E are the experimental values; *P*-value indicates that the change of protein is significant (<0.05); Fold indicates the change of protein in quantity; "+" indicates that the spot is found only on gels for CNB-1 grown on 4CNB

revealed 587 ± 3 ($n = 3$) spots in the pH range of 4–7 and 220 ± 4 ($n = 3$) protein spots in the pH range of 6–11.

Comparative proteomics of CNB-1 and CNB-2 cells grown on 4CNB and/or succinate

Comparison of the CNB-1 proteomes from 4CNB and succinate grown cells revealed that 52 protein spots were specifically detected and 42 protein spots significantly increased their abundances. Among these spots (52 + 42), 73 were successfully identified. Except of the 2-hydroxypent-2,4-dienoate hydratase, 4-oxalocrotonate decarboxylase, acetaldehyde dehydrogenase (acylating), and 2-hydroxymuconic semialdehyde dehydrogenase, the majority of the specific or more abundant proteins were enzymes involved in the central carbon metabolism, nitrogen metabolism,

Table 3 The proteins involved in 4CNB degradation pathway identified by MALDI-TOF and MALDI-TOF/TOF mass spectroscopy

Spot no.	Function	Score	Coverage (%)	Match	pI/MW^T	pI/MW^E
92	Chloronitrobenzene nitroreductase (CnbA)	110 (PMF)	67	16	5.74/26	6.19/27
87	2-Aminophenol 1,6-dioxygenase alpha subunit (CnbCa)	68 (MS/MS)	7	2	5.30/29	5.34/34
88	2-Aminophenol 1,6-dioxygenase alpha subunit (CnbCa)	141 (PMF)	65	15	5.30/29	5.47/35
78	2-Aminophenol 1,6-dioxygenase beta subunit (CnbCb)	76 (PMF)	20	8	6.14/35	6.20/36
79	2-Aaminophenol 1,6-dioxygenase beta subunit (CnbCb)	147 (MS/MS)	7	2	6.14/35	6.24/38
80	2-Aminophenol 1,6-dioxygenase beta subunit (CnbCb)	90 (MS/MS)	7	2	6.14/35	6.30/38
81	2-Aminophenol 1,6-dioxygenase beta subunit (CnbCb)	222 (MS/MS)	14	3	6.14/35	6.37/39
75	2-Aminomuconic semialdehyde dehydrogenase (CnbD)	129 (PMF)	44	16	5.49/53	5.65/59
76	2-Aminomuconic semialdehyde dehydrogenase (CnbD)	249 (PMF)	67	32	5.49/53	5.77/60
84	2-Keto-4-pentenoate hydratase (CnbE)	189 (MS/MS)	13	3	5.34/30	6.19/24
85	2-Keto-4-pentenoate hydratase (CnbE)	155 (MS/MS)	10	2	5.34/30	5.30/28
86	2-Keto-4-pentenoate hydratase (CnbE)	248 (MS/MS)	14	2	5.34/30	5.50/27
89	4-Oxalocrotonate decarboxylase (CnbF)	127 (PMF)	82	18	6.51/24	6.33/27
90	2-Amino-5-chloromuconic deaminase (CnbZ)	182 (PMF)	62	16	6.34/27	6.33/26
91	2-Amino-5-chloromuconic deaminase (CnbZ)	147 (PMF)	79	17	6.34/27	6.79/25
12	2-Hydroxypent-2,4-dienoate hydratase	124 (PMF)	33	11	4.90/27	5.06/26
52	4-Oxalocrotonate decarboxylase	59 (PMF)	10	2	6.49/27	7.00/27
37	Acetaldehyde dehydrogenase (acylating)	107 (PMF)	41	16	6.09/32	6.28/34
45	2-Hydroxymuconic semialdehyde dehydrogenase	68 (PMF)	28	13	5.65/52	6.07/48

PMF indicates that the protein is identified by MALDI-TOF mass spectroscopy; MS/MS indicates that the protein is identified by MALDI-TOF/TOF mass spectroscopy

nucleotides metabolism, stress responses, and transcription/translation (Table 2).

Comparative proteomics between the 4CNB- and succinate-grown cells of CNB-1 did not disclose much information on the 4CNB-degrading enzymes/proteins, in particular on those proteins involved in the upper pathway of 4CNB degradation. However, further comparison of the proteomes of CNB-1 and CNB-2 on succinate revealed that 21 spots were specifically detected or observed in higher abundance in CNB-1 cells. Among these spots, seven spots were identified as enzymes involved in the upper pathway of 4CNB degradation, and other eight spots were identified as enzymes involved in the lower 4CNB degradation pathway (Table 3). These results indicated that the proteins involved in the upper pathway of 4CNB degradation were constitutively synthesized by CNB-1, regardless of whether 4CNB or succinate was used as carbon and energy source.

Pathway for 4CNB degradation as revealed by proteomic analysis of 4CNB-grown cells and functional identification of CnbE and CnbF

Previous studies showed that nine genes (*cnbA*, *cnbB*, *cnbCa*, *cnbCb*, *cnbD*, *cnbE*, *cnbF*, *cnbG*, and *cnbZ*) encoding the enzymes responsible for 4CNB degradation were dispersedly located on the plasmid pCNB1 (Ma et al. 2007). Except for 2-amino-5-chlorophenol 1,6-dioxygenase (CnbCaCb) and deaminase (CnbZ) that had been purified from 4CNB grown cells, none of the gene products was identified in CNB-1 cells. In this study, seven (CnbA, CnbCa, CnbCb, CnbD, CnbE, CnbF, and CnbZ) of the nine gene products were successfully identified (Fig. 3; Table 3). The CnbA and CnbF were identified as single spots on the gel. However, others proteins were represented by more than one spot: two spots represented CnbCa, four spots CnbCb, two spots CnbD, two spots CnbZ and three sports for CnbE (Fig. 3).

According to the protein homology searches and amino acid sequence alignments, CnbE and CnbF had high sequence identities to the 2-keto-4-pentenoate hydratase and 4-oxalocrotonate decarboxylase, respectively (Wu et al. 2006). Both CnbE and CnbF proteins were identified in CNB-1 cells grown on 4CNB. In order to identify the two enzymes at biochemical level, the genes *cnbE* and *cnbF* were cloned and expressed in *E. coli*. Results indicated that individual synthesis of either CnbE or CnbF did not show any enzymatic activity. When the two genes were simultaneously expressed, cellular lysate of recombinant *E. coli* showed weak but detectable hydratase and decarboxylase activity. This cellular lysate of the recombinant *E. coli* converted 2-oxo-hex-4-ene-5-chloro-1,6-dioate into a product that was identified to be 2-oxo-4-hydroxy-5-chloro-valeric acid (Fig. 4).

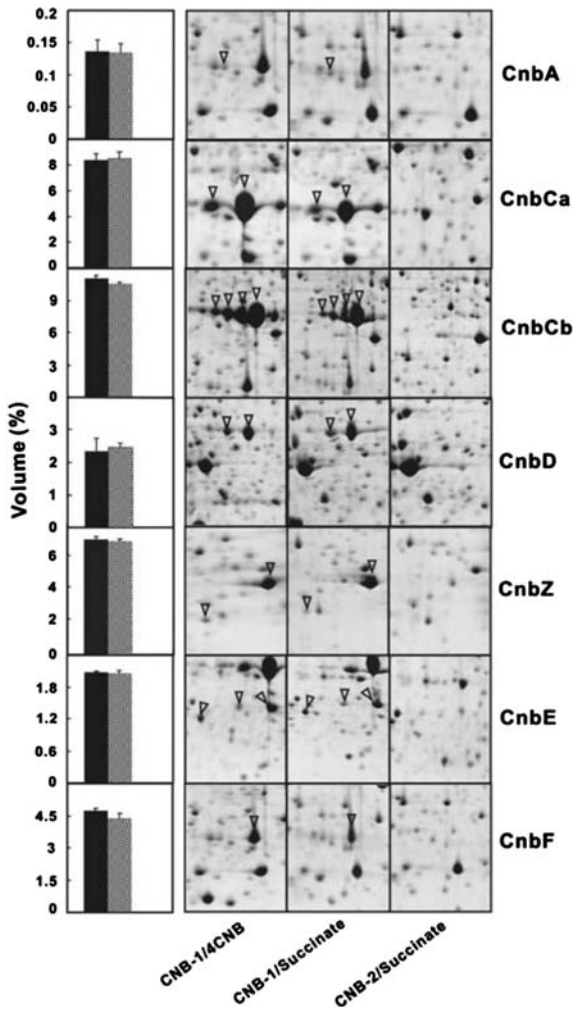

Fig. 3 Sections of 2-DE gels showing the proteins involved in the 4CNB degradation pathway of the CNB-1 cellular proteome. The changes of proteins normalized volume (%) were shown on the left of the 2-DE gels (black bar, CNB-1 grown on 4CNB; grid bar, CNB-1 grown on succinate)

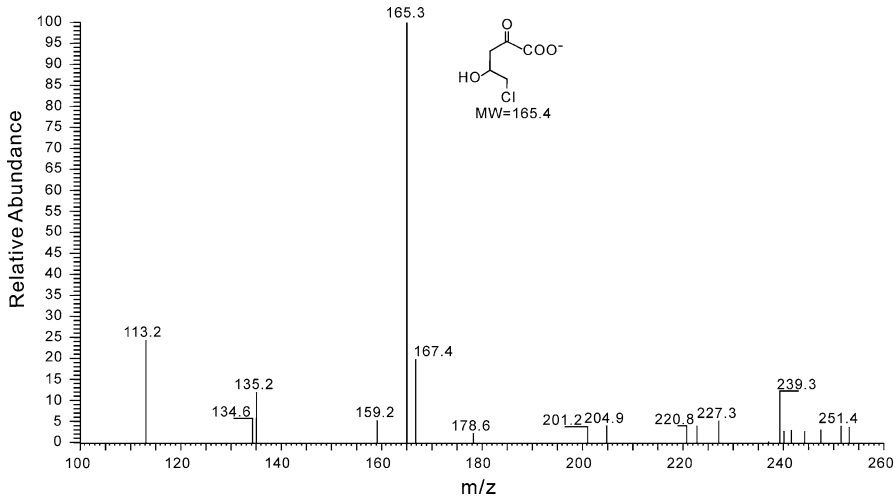

Fig. 4 Mass spectrometry of products of hydratase and decarboxylase using 2-oxohex-4-ene-5-chloro-1,6-dioate as substrate

General changes of proteins involving in other cellular activities and metabolisms of CNB-1 cells-grown on 4CNB

Besides the specific proteins responsible for the degradation of 4CNB, other proteins involving in various cellular activities and metabolisms altered their abundances in 4CNB-grown cells (Table 2). Comparison of the proteomes of CNB-1 cells grown on succinate and on 4CNB revealed that three enzymes (fructose-bisphosphate aldolase, glyceraldehydes-3-phosphate dehydrogenase, and enolase) involved in glycolysis were more abundant in the 4CNB-grown cells. In addition, two enzymes (malate dehydrogenase and succinyl-CoA synthetase) involved in tricarboxylic acid cycle were induced in 4CNB-grown cells. In addition, two enzymes (glutamate synthase, urocanate hydratase) related to glutamate synthesis and three enzymes (phosphoribosylpyrophosphate synthetase, phosphoribosyltransferase and phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase) related to nucleotide metabolism were significantly up-regulated or induced in 4CNB-grown cells.

Discussion

In this study, we used the proteomic approach to get a comprehensive understanding of the biodegradative pathway for 4CNB. The results demonstrated that seven proteins involved in 4CNB degradation pathway (CnbA, CnbCa, CnbCb, CnbD, CnbZ, CnbE and CnbF) were detected both in 4CNB-grown and succinate-grown CNB-1 cells. This indicated that these proteins were constitutively expressed and were not under the control of a regulatory system responding to 4CNB. Among these proteins, five proteins were represented by more than one spot (Fig. 3). Multiple spots of the same protein occurred in most cases with different p*I* values and similar molecular masses, which may reflect posttranslational modifications such as phosphorylation, glycosylation and acylation or may be the result of systematic process-induced modifications including urea-mediated carbamylation or deamidation (Lutter et al. 2001; Berven et al. 2003). Posttranslational modification of 4CNB degradative enzymes has yet not been reported, and it is an interesting topic to be followed in the future. Three proteins (CnbB, CnbH and CnbG) could not be detected on 2-DE gels. Failure to identify CnbB does not imply its absence in 4CNB-grown cells. It may be the result of the low abundance or overlap with other proteins. Moreover, CnbG is unlikely to be detected using the current 2-DE protocols due to its low molecular mass (7,160 Da). CnbH shared sequence similarity with the subunit A of glutamyl-tRNA^{Gln} amidotransferases. The expression in *E. coli* showed CnbH might function as a deaminase (Wu et al. 2006). However, the product of *cnbH* (6.5/45 kDa) could not be detected on the 2-DE gels in this study, and transcription of cnbH was not detectable in previous study (Liu et al. 2007). The alternative deaminase, CnbZ (6.4/28 kDa), was

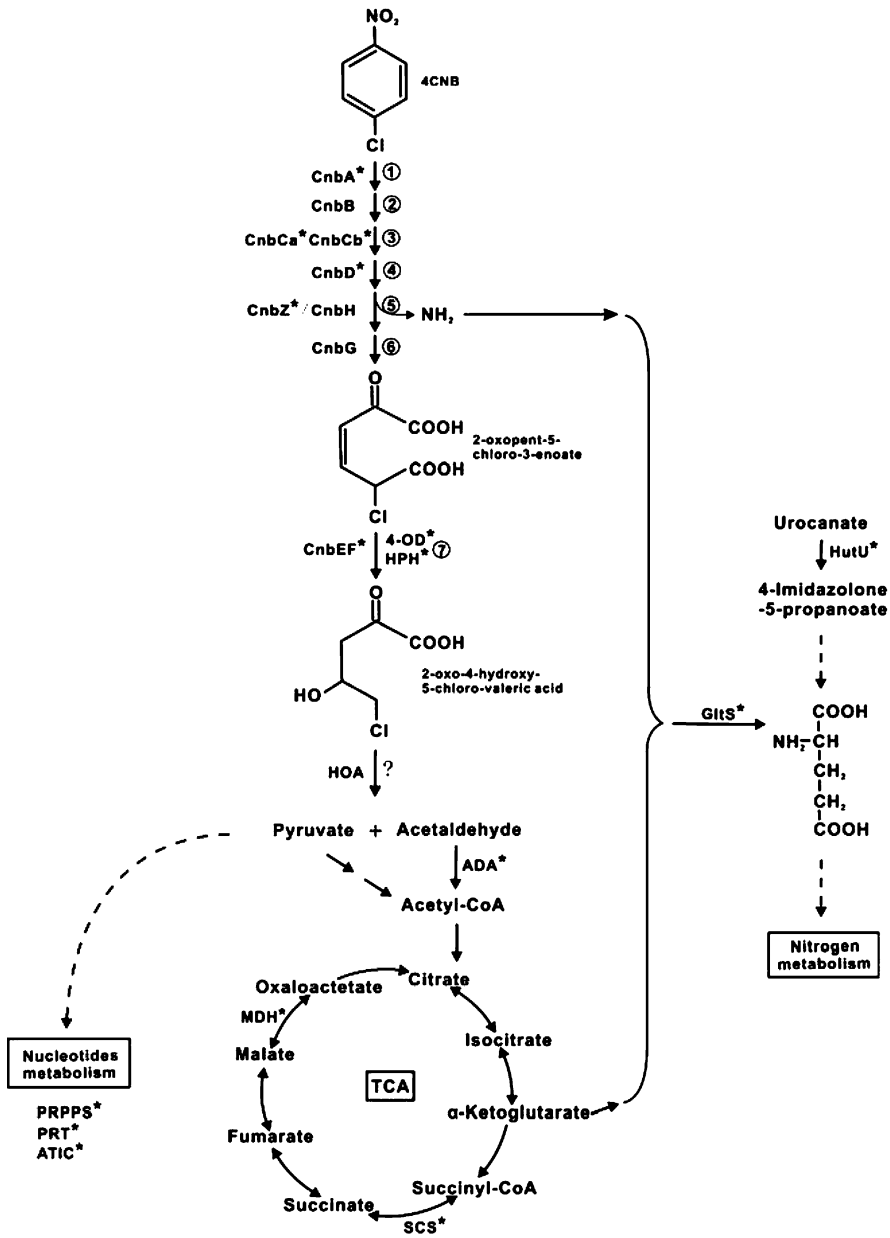

Fig. 5 Expression of proteins involved in 4CNB degradation and central metabolism of CNB-1 grown on 4CNB based on 2-DE results. The proteins marked with "*" were detected in the 4CNB-grown cells. Abbreviations: CnbA, 4CNB nitroreductase; CnbB, 1-hydroxylaminobenzene mutase; CnbCa, 2-amino-5-chlorophenol 1,6-dioxygenase alpha subunit; CnbCb, 2-aminophenol 1,6-dioxygenase beta subunit; CnbD, 2-amino-5-chloromuconic semialdehyde dehydrogenase; CnbZ, 2-amino-5-chloromuconic acid deaminase; CnbH, 2-amino-5-chloromuconic acid deaminase; CnbG, 2-hydroxy-5-chloromuconic acid tautomerase; CnbE, 2-keto-4-pentenoate hydratase; CnbF, 4-oxalocrotonate decarboxylase; GltS, glutamate synthase; HPH, 2-hydroxypent-2,4-dienoate hydratase; 4-OD, 4-oxalocrotonate decarboxylase; HOA, 4-hydroxy-2-oxovalerate adolase; ADA, acetaldehyde dehydrogenase; MDH, malate dehydrogenase; SCS, succinyl-CoA synthetase; PRPPS, ribose-phosphate pyrophosphokinase; PRT, phosphoribosyltransferase; ATIC, phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase; TCA cycle, tricarboxylic acid (Krebs) cycle. For the details of reaction steps **1–6**, see Wu et al. (2006)

clearly observed on the 2-DE gels. The current study provided additional evidence that only CnbZ was involved in the deamination of 2-amino-5-chloromuconate (Liu et al. 2007).

CnbE and CnbF were encoded on plasmid pCNB1 and identified in this study from strain CNB-1 cells grown on 4CNB and succinate. Based on amino acid sequence analysis, CnbE and CnbF have 65% and 69% identities to the putative 2-keto-4-pentenoate hydratase (Protein ID BAB62054) of *P. putida* and the putative 4-oxalacrotonate decarboxylase (Protein ID AAG17138) of *Comamonas* sp. JS765. In this study, we further evidence that CnbE and CnbF were involved in conversion of 2-oxohex-4-ene-5-chloro-1,6-dioate to 2-oxo-4-hydroxy-5-chloro-valeric acid (Fig. 5, step 7). Based on these facts and analysis, we proposed that CnbE and CnbF be involved in 4CNB degradation. Interestingly, two other proteins (represented by spots 12 and 52) that function putatively similarly to CnbE and CnbF, were also observed in 4CNB-grown cells. These two proteins shared high sequence identities with 2-hydroxypent-2,4-dienoate hydratase and 4-oxalocrotonate decarboxylase which involved in the lower pathway of phenol degradation in *Comamonas testosteroni* TA441 (Arai et al. 2000). It was deduced that these two proteins which encoded by genes on the chromosome might also be involved in the conversion of 2-oxohex-4-ene-5-chloro-1,6-dioate to 2-oxo-4-hydroxy-5-chloro-valeric acid (Fig. 5).

Previous studies did not provide any clues as to how 2-oxo-4-hydroxy-5-chloro-valeric acid was further converted to intermediates that enter the TCA cycle or other metabolic processes. Sequence analysis of pCNB1 did not show any candidate enzymes possibly involving in the conversion of 2-oxo-4-hydroxy-5-chloro-valeric acid (Ma et al. 2007). In this study, it was observed that acetaldehyde dehydrogenase increased its abundances in 4CNB-grown cells. Considering that this enzyme together with 4-hydroxy-2-oxovalerate aldolase are involved in the last steps of the *meta*-cleavage pathway, their involvements in the cleavage of 2-oxo-4-hydroxy-5-chloro-valeric acid and production acetyl-CoA and pyruvate were highly possible (Fig. 5).

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