The Journal of Microbiology, December 2006, p. 632-640 Vol. 44, No. 6
Copyright © 2006, The Microbiological Society of Korea

Characterization of β-Ketoadipate Pathway from Multi-Drug Resistance Bacterium, *Acinetobacter baumannii* DU202 by Proteomic Approach

Soon-Ho Park, Jae-Woo Kim[1], Sung-Ho Yun, Sun Hee Leem[2], Hyung-Yeel Kahng[3], and Seung Il Kim*

**Proteomics Team, Korea Basic Science Institute, Daejeon 305-333, Republic of Korea*
[1]Department of Laboratory Medicine, Dong-A University Medical Center, Busan 602-715, Republic of Korea
[2]Department of Biology, Dong-A University, Busan 604-714, Republic of Korea
[3]Department of Environmental Education, Sunchon National University, Sunchon 540-742, Republic of Korea

(Received October 20, 2006 / Accepted Decembdr 27, 2006)

In this study, the biodegradative activities of monocyclic aromatic compounds were determined from the multi-drug resistant (MDR) *Acinetobacter baumannii*, which were studied in the form of clinical isolates from a hospital in Korea. These bacteria were capable of biodegrading monocyclic aromatic compounds, such as benzoate and *p*-hydroxybenzoate. In order to determine which pathways are available for biodegradation in these stains, we conducted proteome analyses of benzoate and *p*-hydroxybenzoate-cultured *A. baumannii* DU202, using 2-DE/MS analysis. As genome DB of *A. baumannii* was not yet available, MS/MS analysis or *de novo* sequencing methods were employed in the identification of induced proteins. Benzoate branch enzymes [catechol 1,2-dioxygenase (CatA) and benzoate dioxygenase α subunit (BenA)] of the β-ketoadipate pathway were identified under benzoate culture condition and *p*-hydroxybenzoate branch enzymes [protocatechuate 3,4-dioxygenaseα subunit (PcaG) and 3-carboxy-*cis,cis*-muconate cycloisomerase (PcaB)] of the β-ketoadipate pathway were identified under *p*-hydroxybenzoate culture condition, respectively, thereby suggesting that strain DU202 utilized the β-ketoadipate pathway for the biodegradation of monocyclic aromatic compounds. The sequence analysis of two purified dioxygenases (CatA and PcaGH) indicated that CatA is closely associated with the CatA of *Acinetobacter radiresistance*, but PcaGH is only moderately associated with the PcaGH of *Acinetobacter sp.* ADP1. Interestingly, the fused form of PcaD and PcaC, carboxymuconolactone decarboxylase (PcaCD), was detected on benzoate-cultured *A. baumannii* DU202. These results indicate that *A. baumannii* DU202 exploits a different β-ketoadipate pathway from other *Acinetobacter* species.

Keywords: β-Ketoadipate Pathway, Aromatic compound biodegradation, MDR *Acinetobacter baumannii*

Acinetobacter baumannii is a nonfermentative gram-negative bacterium which is commonly located in water and soil, and was known to be susceptible to the majority of antibiotics in the 1970s (Fournier *et al.*, 2006). However, these bacteria now constitute 73% of all *Acinetobacter* clinical isolates, which are most commonly involved in clinical infections, and have become resistant to almost all currently available antibacterial agents (Van Looveren and Goossens, 2004).

Nosocomial infections induced by mutidrug-resistant (MDR) *A. baumannii* have recently been reported worldwide (Bergogone-Berezin and Towner, 1996; Kuo *et al.*, 2004). These opportunistic bacteria have been implicated in epidemic pneumonia, urinary tract infections, septicemia, and meningitis (Magnet *et al.*, 2001). *A. baumannii* is capable of survival under extreme culture conditions (temperatures, pH, and dry condition) and is known to exploit a variety of carbon and energy sources (Wendt *et al.*, 1997; Abbo *et al.*, 2005). These properties explain the ability of *A. baumannii* to spread both in hospital and natural environments (Guardabassi *et al.*, 1998). *A. baumannii* has now become a major representative of environmental antibiotic-resistant bacteria (Navon-Venezia *et al.*, 2005). Therefore, the elucidation of adaptation mechanisms under different environmental conditions,

* To whom correspondence should be addressed.
(Tel) 82-42-865-3451; (Fax) 82-42-865-3419
(E-mail) ksi@kbsi.re.kr

as well as the elucidation of antibiotic resistance mechanisms, is important for us to understand these pathogenic bacteria, and in the development of new antibiotics. However, the metabolic potentials of these strains have yet to be thoroughly elucidated. In this regard, the degradability of monocyclic aromatic compounds was assayed in four selected *A. baumannii*, which were detected in patients undergoing long-term treatment and antibiotics, and evidence strong multi-drug resistance (MDR). Monocyclic aromatic compounds are one of the principal carbon sources in the soil environment. Additionally, biodegradation pathways for monocyclic aromatic compounds and related enzymes have been analyzed in one *A. baumannii* strain (DU202) via proteomic techniques. Unfortunately, as genome sequence information of *A. baumannii* was not available for protein identification, bacterial DB including *Acinetobacter sp.* ADP1 was utilized as a reference DB (Barbe *et al.*, 2004). In this study, the enzymes of the β-ketoadipate pathway were identified, and their sequences were characterized. The β-ketoadipate pathway is widely distributed among bacteria and plays a central role in the degradation of naturally occurring aromatic compounds and environmental pollutants (Harwood and Parales, 1996). To our knowledge, this is the first report regarding the characterization of β-ketoadipate pathway enzymes from MDR *A. baumannii*.

Materials and Methods

Bacteria identification and antibiotic resistance assay

Acinetobacter baumannii (DU201 - DU204) were identified at the Dong-A Medical Center (Korea). These strains were detected in the sputum of hospital patients aged 48-69, who underwent long-term treatment with a variety of antibiotics. These strains were identified using the Vitek Auto Microbic System (bioMérieux Vitek Systems Inc., USA) (Murray *et al.*, 2003).

Antibiotic resistance tests were conducted using the AST-N041 card and the AST-N022 card from Vitek Systems. The MIC (minimal inhibitory concentration assay) of several antibiotics was conducted via standard microdilution (Llanes *et al.*, 2004). Bacteria were pre-cultured in Luria broth (LB) until optical density at 600 nm reached 0.8 - 1.0, after which diluted bacterial inocula of approximately 1.0×10^5 CFU per ml were transferred into LB plates containing different antibiotic concentrations. The antibiotic resistances of these bacteria were checked following 24 h of cultivation.

Biodegradation activity and aromatic ring cleavage dioxygenase activity assay

For the biodegradation assay, bacteria were pre-cultured overnight at 30°C with vigorous shaking in culture media [50 mM potassium phosphate buffer (pH 6.25), 3.4 mM $MgSO_4$, 0.3 mM $FeSO_4$, 0.2 mM $CaCO_3$, 10 mM NH_4Cl and 10 mM sodium succinate]. They were then transferred to culture media plates containing succinate, benzoate, *p*-hydroxybenzoate, *o*-hydroxybenzoate (salicylate), chlorobenzoate, vaniline, or aniline as a sole carbon source. Vaniline was dissolved in ethanol (7.6 g/ 50 mL) due to its low solubility. The biodegradability of these bacteria was determined after 48 h of cultivation. For the enzyme assay and purification, the bacteria were cultured in identical minimal media with each monocyclic aromatic compound (5 mM). The bacterial culture was harvested during the early stationary phase (about OD 1.0), after which the harvested cells were suspended in 20 mM Tris- HCl buffer (pH 8.0) and disrupted twice with a French pressure cell (SLM AMINCO, Urbana, IL, USA) at 20,000 lb/in^2. The supernatants (crude cell extracts) were collected via 45 min of centrifugation at 15,000 x g and used to determine the activity of a variety of dioxygenases. The enzyme activities of catechol 1,2-dioxygenase (CD1,2), and protocatechuate 3,4-dioxygenase (PCD3,4) were spectrophotometrically measured using a Beckman DU7500 UV spectrometer at 25°C, as reported previously (Bull and Ballou, 1981; Aoki *et al.*, 1984). One unit of enzyme activity was defined as the amount of enzyme required to generate 1 μmol of product per minute, as usual. The protein concentrations of the crude extracts from different substrate-cultured bacteria were determined via the Bradford method, using bovine serum albumin as a standard (Bradford, 1976).

2-D gel electrophoresis

2-DE was conducted in accordance with the previously reported procedure (Kim *et al.*, 2006). For the isoelectric focusing (IEF) step, 250 μg of dried proteins were resolved in 50 μl of Buffer I (SDS 0.3%, DTT 0.2 M, Tris-HCl 50 mM), and were heated for denaturation for 5 min at 95°C. After 10 min of incubation with 5 μl of Buffer II ($MgCl_2$ 50 mM, DNAse I 10 unit, RNAse 3.75 unit, Tris-HCl 50 mM) on ice, the samples were resolved in 350 μl of IPG buffer (urea 8 M, CHAPS 2% (w/v), IPG buffer 0.5%).

The sample solutions were then applied to immobilized pH 4-7 linear strips (18 cm) for isoelectric focusing, using IPGphor (Amersham Pharmacia Biotech, Sweden).

After isoelectric focusing, the IPG strips were incubated with equilibration solution (Tris-HCl 50 mM, urea 6 M, glycerol 30%, SDS 2%, DTT 75 mM, iodoacetamide 135 mM, BPB) for 15 min, after which SDS-polyacrylamide electrophoresis was conducted using a Hoeffer Dalt system (Pharmacia Biotech). Silver staining was conducted using a Pharmacia Biotech staining kit. Image acquisition was conducted on a Pharmacia Biotech image scanner. Each 2-DE

was conducted 3 times to verify the induced proteins.

PMF, MS/MS analysis, de novo sequencing, and N-terminal Edman sequencing

In-gel digestion and PMF (Peptide Mass Fingerprinting) were conducted in accordance with previously described methods (Kim *et al.*, 2004). Protein bands were excised from the SDS-polyacrylamide gels. After the reduction and alkylation of cysteines, the proteins were digested using 7-8 μl trypsin (0.1 μg/μl) for 12 to 16 h at 37°C. The digested peptides were then recovered via two extraction steps, using a solution containing 50 mM ammonium bicarbonate, 50% acetonitrile and 5% trifluoroacetic acid (TFA). The digested peptide extracts were then employed for PMF analysis using a 4700 Proteomic Analyzer (Applied Biosystems, Framingham, MA). The proteins were identified using MASCOT software (Matrixscience, London). For the MS/MS search and *de novo* sequencing, two MS analyzers (ESI-Q-TOF MS and MALDI-TOF MS) were utilized. For protein identification, the MS/MS spectra were searched using MASCOT software (Matrix science, www.matrixscience.com, UK) and *de novo* sequencing was manually conducted by Masslynx software 3.5 (Micromass, UK) in ESI-Q-TOF MS. Proteins harboring at least one significant peptide (≥ individual score) were selected from the database search results. For N-terminal amino acid sequencing via the Edman method, the purified enzymes were electro-transferred onto PVDF membranes, using a previously described method (Yun *et al.*, 2004) and were analyzed with a Perkin-Elmer protein sequencer (Model 491A). A sequence homology search was conducted using the BLAST program in the NCBI database (www.ncbi.nlm.nih.gov).

Purification of catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase

For the fractionation of CD1,2 and PCD3,4 from the bacteria cultured with *p*-hydroxybenzoate and benzoate, the crude extracts were applied to a Hi-trap Q (Amersham Pharmacia, Sweden) column FF (5 ml), and eluted with a 100 to 500 mM NaCl gradient at a flow rate of 2 ml/min for 40 min. The active fractions were concentrated and loaded onto Superdex (Amersham Pharmacia, Uppsala, Sweden) 200 GPC column (16 x 60 cm) for the second purification step.

Results

Antibiotic Resistance of Acinetobacter baumannii MIC assay

Strains were selected from different patients. All of the strains evidenced substantial resistance to antibiotics such as ampicillin, kanamycin, tetracycline, and chloramphenicol (Table 1). It is notable that three strains (DU202, DU203, DU204) of *Acinetobacter baumannii* evidence resistance even to high concentrations of antibiotics (over 8192 μg/ml ampicillin and kanamycin and 1024 μg/ml of tetracycline). These strains were also resistant to other antibiotics, including gentamicin, imipenem, piperacillin, and cefepime (data not shown). The results from the antibiotic tests conducted in this study indicated that the four strains utilized in this experiment are multiple drug-resistant (MDR) bacteria.

Biodegradation activity of Acinetobacter baumannii

A biodegradability assay for four strains using minimal media containing monocyclic aromatic compounds as a sole carbon source indicated that all strains of *A. baumannii* are capable of utilizing *p*-hydroxybenzoate and benzoate up to concentrations of 50 mM and 25 mM, respectively, but no other monocyclic aromatic compounds were used. *A. baumannii* DU202 was selected for the elucidation of the biodegradation pathways of benzoate and *p*-hydroxybenzoate (Table 2).

Proteomic analysis of A. baumannii DU 202 by 2-DE/MS approaches (biodegradation pathway analysis)

In order to identify enzymes associated with the biodegradation of benzoate and *p*-hydroxybenzoate in *A. baumannii* DU202, we conducted a comparative analysis

Table 1. Resistance of *A. baumannii* to various antibiotics

Antibiotics MIC (μg/ml)	DU201	DU202	DU203	DU204
Ampicilline	2048	8192>	8192>	8192>
Kanamycin	8192>	8192>	8192>	8192>
Tetracycline	1024	2048	2048	2048
Prepemen	2	32	32	32
Chloramphenicol	256	512	512	512

Table 2. Degradation activity of *A. baumannii* for monocyclic aromatic compounds

Substrates (mM)	DU201	DU202	DU203	DU204
Benzoate	25	25	25	25
o-Hydroxybenzoate	N.D.	N.D.	N.D.	N.D.
Chrolobenzoate	N.D.	N.D.	N.D.	N.D.
p-Hydroxybenzoate	50>	50>	50>	50>
Vaniline	N.D.	N.D.	N.D.	N.D.
Aniline	N.D.	N.D.	N.D.	N.D.

ND; Not detected for biodegradation activity

Table 3. Proteins induced or increased on the 2D gel of *A. baumannii* DU202 cultured in benzoate and *p*-hydroxybenzoate media

Spot (no)	Protein name	Matching peptide (no)	Mowse score	Sequences from MS/MS analysis	*de novo* Sequence analysis	gi. no of related protein & bacterial origin	Mr (kDa) & pI
B1	Not identified						
B2	dihydrodipicolinate synthase	2	91	LPLTPLAEQYR, AIAEAVDIPQILYNVPGR		gi 49532511, *Acinetobacter* sp. ADP1	34.7 & 5.57
B3	malate dehydrogenase	6	266	NFTAMLR, AGVAVADIEK, DADYALLVGSRP, GLSSAASAANAAIDHMR, VLVVGNPANTNAYIAMK, LTVWGNHSPTMYADYR		gi 49532132, *Acinetobacter* sp. ADP1	35.4 & 5.14
B4	malate dehydrogenase	6	205	AGVAVADIEK, DVFLPTVGK, DADYALLVGSRPR, GLSSAASAANAAIDHMR, VLVVGNPANTNAYIAMK, GVMMELDDCAFPLLAGMIGTDDPK		gi 49532132, *Acinetobacter* sp. ADP1	35.4 & 5.14
B5	malate dehydrogenase	9	403	NFTAMLR, AGVAVADIEK, DVFLPTVGK, DADYALLVGSRPR, GLSSAASAANAAIDHMR, VAVTGAAGQIGYSLLFR, VLVVGNPANTNAYIAMK, LTVWGNHSPTMYADYR, GVMMELDDCAFPLLAGMIGTDDPK		gi 49532132, *Acinetobacter* sp. ADP1	35.4 & 5.14
B6	malate dehydrogenase	5	155	AGVAVADIEK, DADYALLVGSRPR, GLSSAASAANAAIDHMR VAVTGAAGQIGYSLLFR, LTVWGNHSPTMYADYR		gi 49532132, *Acinetobacter* sp. ADP1	35.4 & 5.14
B7	Not identified						
B8	catechol 1,2-dioxygenase (CatA)	2	74	TIEGPLYVAGAPESVGFAR HGNRPSHVHYFVSAPGYR		gi 535286, *A. calcoaceticus*	33.6 & 5.01
B9	catechol 1,2-dioxygenase (CatA)	2	62	TIEGPLYVAGAPESVGFAR, HGNRPSHVHYFVSAPGYR		gi 535286, *A. calcoaceticus*	33.6 & 5.01
B10	catechol 1,2-dioxygenase (CatA)	2	71	TIEGPLYVAGAPESVGFAR, HGNRPSHVHYFVSAPGYR		gi 535286, *A. calcoaceticus*	33.6 & 5.01
B11	benzoate 1,2 dioxygenase alpha subunit (BenA)				PDDLEEFR	gi 31407697, *A. calcoaceticus*	52.3 & 5.51
B12	ribose 5-phosphate isomerase	4	95	FVCIVDDSK, LVSLGGDPVYR, KLVSLGGDPVYR, DFPLPVEVIPMAR		gi 49530513 *Acinetobacter* sp. ADP1	23.7 & 5.16
B13	Not identified						
B14	carboxymuconolactone decarboxylase (PcaCD)	1	50	FPKIVLANTAAK		gi 72122184 *Ralstonia eutropha* JMP134	42.9 & 5.95
B15	succinylglutamate desuccinylase				YEELQLTQR	gi 88818195 *Pseudoalteromonas tunicata* D2	38.8, 5.27
B16	Not identified						
B17	Not identified						
B18	Not identified						
B19	Not identified						
B20	Not identified						
B21	putative oxydoreductase related to nitroreductase				LVFGSVE	gi 50085029, *Acinetobacter* sp. ADP1	22.6 & 5.42
B22	acetolactate synthase III, large subunit	3	72	FAYEYPEK, HASEIPAIIK, MELLSGGEMLVR		gi 49532084 *Acinetobacter* sp. ADP1	62.8 & 6.13
B23	Not identified						
B24	Not identified						
B25	Not identified						
P1	putative alcohol dehydrogenase	3	140	GIGLVTAGGGHIR, CTPLIAIDDPK, FCIITNTDTHVK		gi 49531126, *Acinetobacter* sp. ADP1	40.9 & 5.41
P2	mandelate racemase/muconate lactonizing enzyme				DNDLMLLK	gi 78063902 *Burkholderia* sp. 383	44.4 & 5.68
P3	Not identified				FNVLT		
P4	protocatechuate 3,4-dioxygenase alpha chain (PcaG))	2	55	GINIGLHTR, LEGQVFDGLGLPLR		gi 49530842 *Acinetobacter* sp. ADP1	23.5 & 5.01
P5	putative GTP-binding protein	3	62	STLFNALTK, ETDAIAHVVR, GEGLGNQFLANIR		gi 49531170 *Acinetobacter* sp. ADP1	39.7 & 4.82
P6	Not identified				ELTY		
P7	3-carboxy-cis,cis-muconate cycloisomerase (PcaB)	1	64	NPVAAASVLAAANR		gi 141778, *Acinetobacter* sp. ADP1	49.5 & 5.74
P8	3-carboxy-cis,cis-muconate cycloisomerase (PcaB)	2	125	NPVAAASVLAAANR, VPALMSSIYQSMVQEHER		gi 141778 *Acinetobacter* sp. ADP1	49.5 & 5.74
P9	hydrolase (haloacid dehalogenase family)	1	49	VDLAIATNK		gi 52629615 *Legionella pneumophila* subsp. pneumophila str. Philadelph	24.1 & 4.81

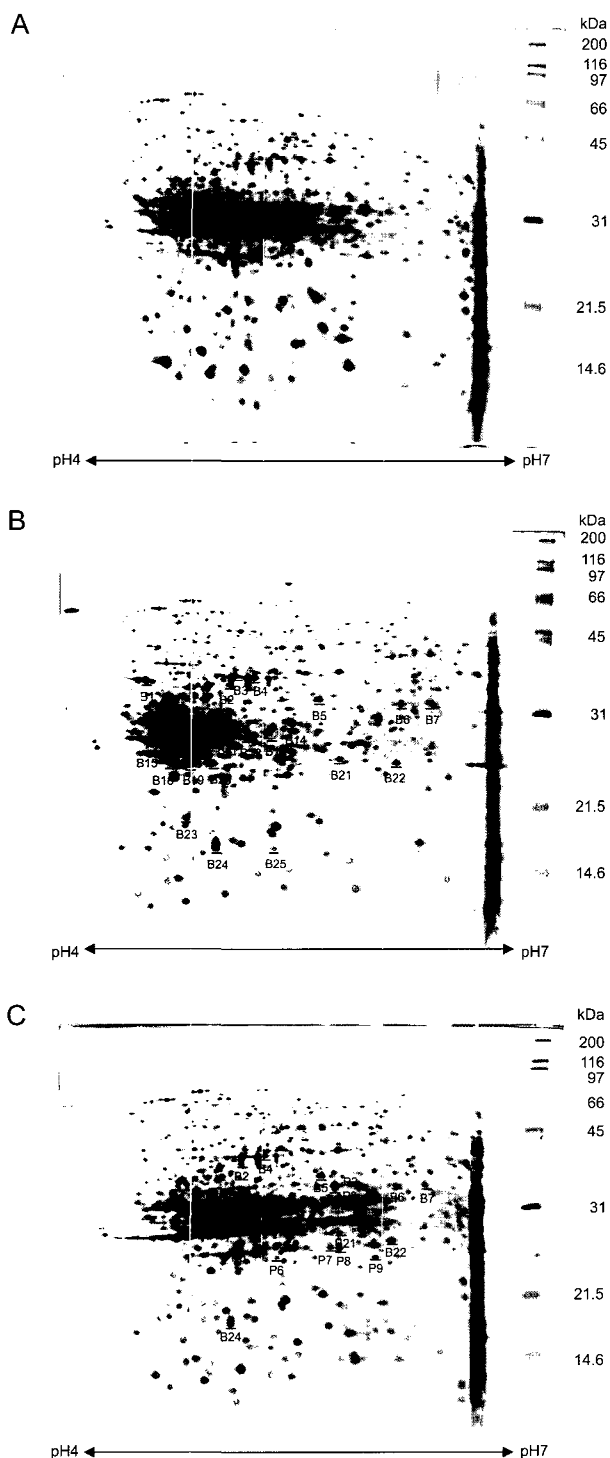

Fig. 1. 2-DE of the soluble fraction from *A. baumannii* DU202 induced by benzoate and *p*-hydroxybenzoate. (A) succinate-cultured; (B) benzoate-cultured; (C) *p*-hydroxybenzoate-cultured. Numbered proteins were identified via MS/MS spectra analysis or *de novo* sequencing.

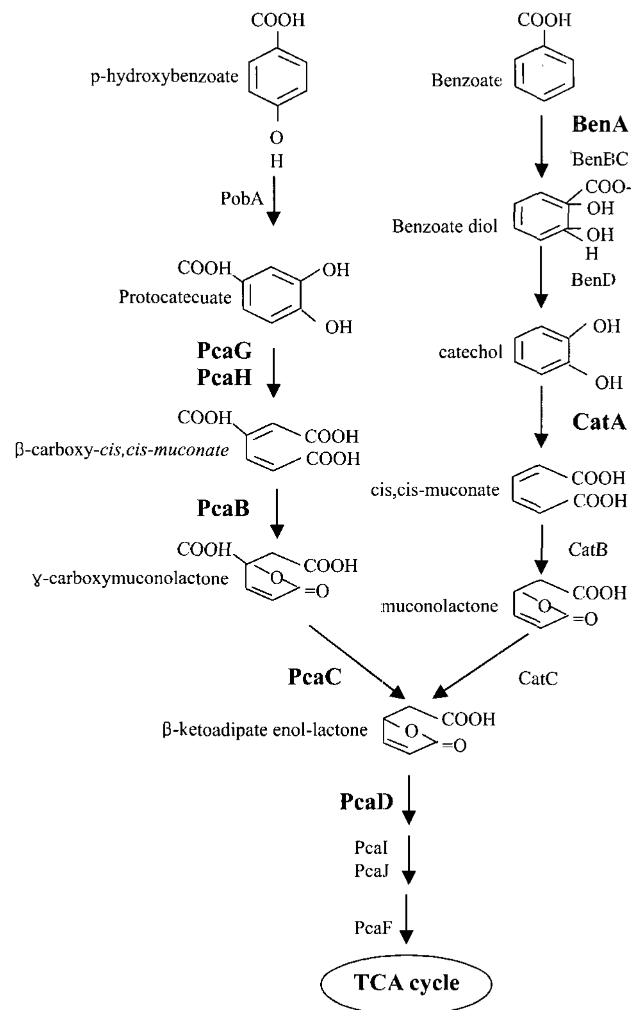

Fig. 2. Proposed β-ketoadipate pathways for mono monocyclic aromatic compounds in *A. baumannii* DU202. Identified enzymes in this study were indicated with bold letter.

of 2-DE/MS using succinate, benzoate, and *p*-hydroxybenzoate-cultured bacteria. Nearly 25 proteins were determined to have been upregulated in the benzoate-cultured bacteria (Fig. 1). 14 proteins were identified successfully via MS/MS analysis or *de novo* sequencing with ESI-Q TOF MS (Table 3). Among them, three proteins (catechol 1,2-dioxygenase, benzoate 1,2-dioxygenase, and carboxymuconolactone decarboxylase) were directly associated with the degradation of benzoate. All of them are members of the catechol branch of the β-ketoadipate pathway. Interestingly, the carboxymuconolactone decarboxylase identified herein was determined to be a merged enzyme, consisting of PcaC and PcaD. Additionally, malate dehydrogenase was strongly upregulated on benzoate media. Approximately 17 proteins were upregulated in *p*-hydroxybenzoate-cultured bacteria. 8 proteins were upregulated simultaneously on benzoate media. Three upregulated proteins [two subunits of protocatechuate 3,4-dioxygenase (α and β), 3-catboxy-*cis,cis*-mucoante cycloisomerase] ere included in the protocatechuate branch of β-ketoadipate pathway. Proteomic results indicate that *A.*

baumannii DU202 selectively induced enzymes of two branches of the β-ketoadipate pathway, according to exposure to different aromatic compounds, and the β-ketoadipate pathway is a principal pathway for the biodegradation of aromatic compounds (Fig. 2).

Purification of induced dioxygenase in benzoate and p-hydroxybenzoate culture condition

In order to verify the results of proteomic analysis, we conducted an enzyme activity assay and purified the primary dioxygenases, which were induced in the presence of aromatic compounds. *A. baumannii* DU202 evidenced catechol 1,2-dioxygenase (CD1,2) activity (1.41 U/mg) and protocatechuate 3,4-dioxygenase (PCD3,4) activity (0.77 U/mg) under benzoate and *p*-hydroxybenzoate cultivation conditions, respectively. No extradiol cleavage activity (CD2,3 or PCD4,5) was detected under our culture conditions. The purification of CD1,2 induced in *A. baumannii* DU202 by benzoate was conducted via successive chromatography using a Hi-trap Q FF anion exchange column and a Superdex 200 gel filtration column, and the enzyme activities were assessed. The purification fold of CD1,2 was 10.59 (Table 4A). PCD3,4 induced in *A. baumannii* DU202 by *p*-hydroxybenzoate was also purified via the procedure mentioned above, and the purification fold of PCD3,4 was 12.76 (Table 4B). Two enzymes were identified on the SDS-polyacrylamide gels (data not shown), and the N-terminal amino acid sequences of two enzymes were sequenced via the Edman method (Table 5).

Sequence analysis of catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase

In order to determine to which bacteria groups the dioxygenases belonged, we conducted PMF (Peptide

Table 4. Purification steps of catechol 1,2-dioxygenases (A) and protocatechuate 3,4-dioxygenase (B) from *A. baumannii* DU202 cultured in benzoate and *p*-hydroxybenzoate media

A

Purification step	Volume (ml)	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Fold
Crude extract	15.00	40.50	57.00	1.41	1.00
Hi-trap Q FF	1.20	2.40	16.26	6.78	4.81
Superdex 200	1.50	0.32	4.70	14.90	10.59

B

Purification step	Volume (ml)	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Fold
Crude extract	20.00	33.50	25.80	0.77	1.00
Hi-trap Q FF	12.00	5.76	9.36	1.63	2.11
Superdex 200	4.00	0.11	2.24	20.74	12.76

Table 5. Sequence analysis of catechol 1,2-dioxygenases and protocatechuate 3,4-dioxygenase of *A. baumannii* DU202

Protein Name	Amino acid sequence	Analysis Methods
Catechol 1,2-dioxygenase (CatA)	MNRQQIDALV KLTTQFNIEGDEYLWDD LTTQFNIEGDEYLWDDF YVALTTMPVGYGCPPEG LLGDLFQAIEDLDIQPS HGNRPSHVHYFVSAPGY VEVWHANSLGNYSFFDK TIEGPLYVAGAPESVGF SQSDFNLRR QQIDADVK	Edman sequencing PMF
Protocatechuate 3,4-dioxygenase (PcaG)	MNNWNFQELK LEGQVFDGLGLPLR	Edman sequencing MS/MS analysis
Protocatechuate 3,4-dioxygenase (PcaH)	SQHSWGAYAQ TLTDENGFYIFR TIKPGPYPW	Edman sequencing MS/MS analysis MS/MS analysis

Mass Fingerprinting) using purified CD1,2 and PCD3,4. The results of PMF analyses indicated that CD1,2 was matched with the CD1,2 of *A. radioresistance* (gi:10440998). The PMF sequence coverage of CD1,2 was determined to exceed 45% (Fig. 3, Table 5). The N-terminal amino acid sequence of 10 residues also matched completely with the N-terminal sequence of CD1,2 from *A. radioresistance*. However, PMF was not an efficient identification method for the two subunits of PCD3,4 of *A. baumannii* DU 202. Therefore, as an alternative method, we utilized MS/MS analyses of tryptic peptides. Our analyses indicated

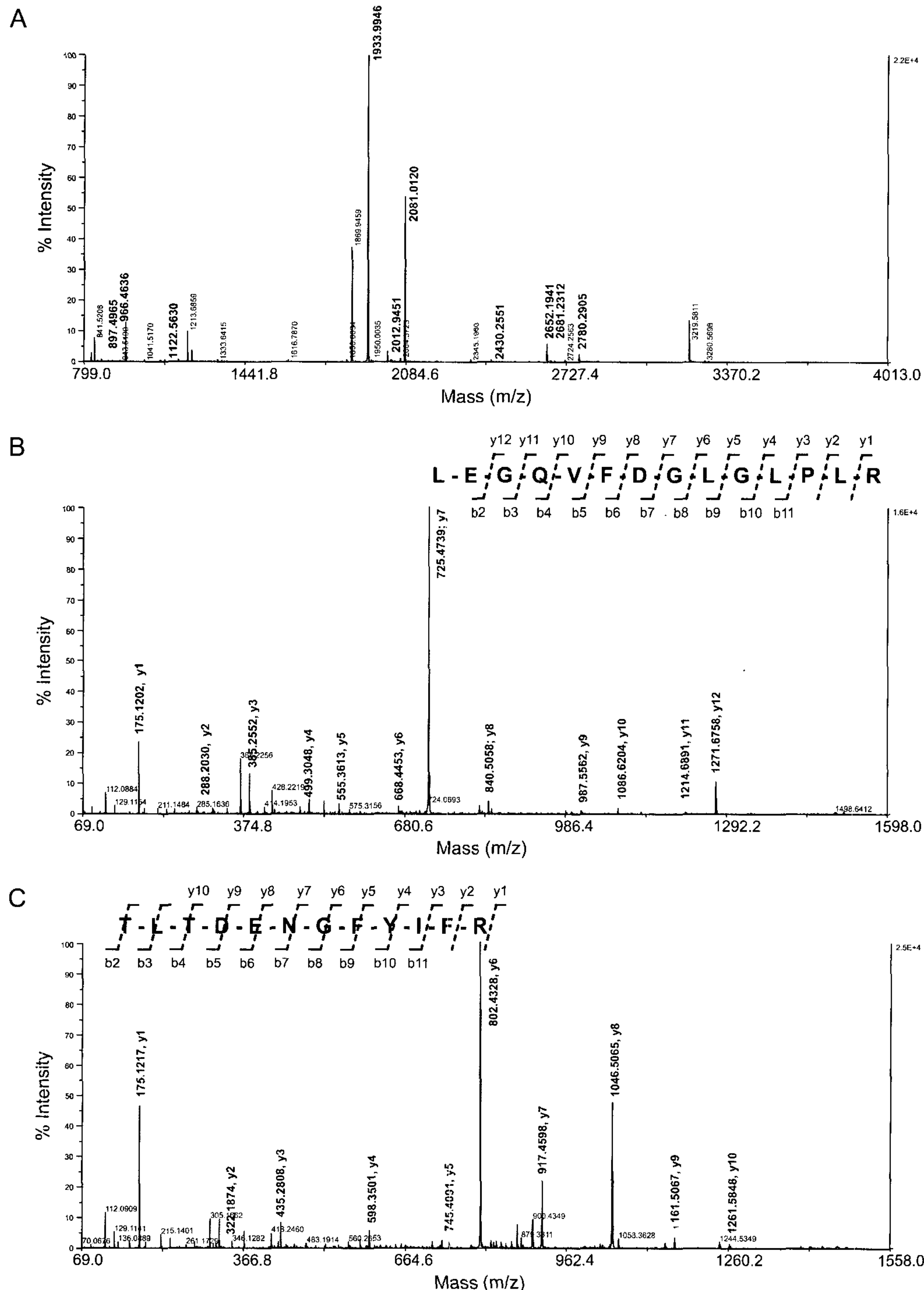

Fig. 3. Peptide mass fingerprinting (PMF) of CD1,2 from *A. baumannii* DU202 using Matrix-assisted laser desorption (MALDI) ionization spectrum (A) and MS/MS spectra analysis of PCD3,4 from *A. baumannii* DU202. α subunit (B); β subunit (C).

that the subunits of PCD3,4 harbor one or two internal sequence matches with the subunits of PCD3,4 of *Acinetobacter* sp. ADP1 or *Marine bacterium* SE197 (Fig. 3, Table 5).

Discussion

In this study, multi-drug resistant *Acinetobacter baumannii*, which was detected and isolated from patients undergoing long-term antibiotic treatment, were assessed with regard to biodegradation activities for monocyclic aromatic compounds. *A. baumannii* was reported in a previous study to be one of the bacterial species most frequently involved in clinical infections (Kuo *et al.*, 2004). Therefore, attention should be directed toward the pathogenic MDR *A. baumannii*, which is capable of adapting to or resisting hostile conditions, such as an aromatic hydrocarbon-contaminated environment. This ability allows such species to exhibit enhanced capabilities to disseminate throughout the environment, or to more easily infect other hosts. All tested strains of *A. baumannii* proved capable of utilizing benzoate and p-hydroxybenzoate, but not other aromatic compounds (Table 2). In order to determine which biodegradation pathways were generally utilized in *A. baumannii*, proteome analysis, major dioxygenase activity assays, and enzyme purification were conducted. In recent studies, proteome analysis using 2-DE has been shown to be a useful tool (Giuffrida *et al*, 2001; Kim *et al.*, 2004; Kim *et al.*, 2006). Due to the unavailability of the genome sequence of *A. baumannii*, PMF was not considered to be a favorable method for protein identification. Our results also indicated that the genome sequence of this bacterium does not match completely with the genomic sequence of any other bacteria obtainable from a public database, such as the NCBI bacterial genome DB (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). Via either de novo sequencing or MS/MS analysis, 21 of 34 protein spots (61.8%) were identified successfully (Table 3). A great many proteins have been identified through the genome DB of *Acinetobacter* sp. ADP1 as a reference, thereby suggesting that the two bacteria share a significant relationship. The results of 2-DE provided several interesting results, as follows: 1) two branches of the β-ketoadipate pathway were induced according to the degree to which they were exposed to benzoate and p-hydroxybenzoate, respectively. 2) Several *Acinetobacter* sp. have been reported to harbor multiple catechol branches of the β-ketoadipate pathway (Pessione *et al.*, 2001; Caposio *et al.*, 2002). However, *A. baumannii* DU202 harbors only one copy of the catechol branch. 3) Carboxymuconolactone decarboxylase (PcaC; protein spot B14) was detected in excess of 42 kDa under benzoate culture conditions (Table 3). Carboxymuconolactone decarboxylase (PcaC) is generally detected at less than 16 kDa (Barbe *et al.*, 2004; Kim *et al.*, 2004). More than 42 kDa of carboxymuconolactone decarboxylase was initially reported in *Rhodococcus opacus* (Eulberg *et al.*, 1998) and was detected as a merged enzyme with 4-carboxymuconolactone decarboxylase (PcaC) and 3-oxoadiopate enollactone hydrolase (PcaD). 4) Malate dehydrogenase (protein spot B3 - B6) was upregulated under benzoate and p-hydroxybenzoate culture conditions. Malate dehydrogenase converts malate into oxaloacetate, which is an essential metabolite for the running of the Krebs cycle with acetyl CoA made from benzoate or p-hydroxybenzoate. The PMF of purified CD1,2 from *A. baumannii* DU202 indicated that this enzyme was completely matched with the CD1,2 of *A. radioresistens* (Table 5). The CD1,2 of *A. radioresistens* evidences 85% identity with the CD1,2 of *Acinetobacter* sp. ADP1. However, the results of PMF and N-terminal sequencing of purified PCD3,4 showed that the PCD3,4 of *A. baumannii* DU202 was not matched with any reported PCD3,4. In conclusion, the results of proteome analysis and sequence analysis suggest that the β-ketoadipate pathway of *A. baumannii* DU202 differed from that of other *Acinetobacter* sp. To the best of our knowledge, this is the first report of CD1,2 and PCD3,4 from *A. baumannii*.

Acknowledgement

This work was supported by Basic Research Program (N26047) funded by the Korean Basic Science Institute.

References

Abbo, A., S. Navon-Venezia, O. Hammer-Muntz, T. Krichali, Y. Siegman-Igra, and Y. Carmeli. 2005. Multidrug-resistant *Acinetobacter baumannii*. *Emerg. Infect. Dis.* 11, 22-29.

Aoki, K., K. Konohana, R. Shinke, and H. Nishira. 1984. Purification and characterization of catechol 1,2-dioxygenase from aniline-assimilating *Rhodococcus erythropolis* AN-13. *Agric. Biol. Chem.* 48, 2087-2095.

Barbe, V., D. Vallenet, N. Fonknechten, A. Kreimeyer, S. Oztas, L. Labarre, S. Cruveiller, C. Robert, S. Duprat, P. Wincker, N.L. Ornston, J. Weissenbach, P. Marliere, G.N. Cohen, and C. Medigue. 2004. Unique features revealed by the genome sequence of *Acinetobacter sp.* ADP1, a versatile and naturally transformation competent bacterium. *Nucleic Acids Res.* 19, 5766-5779.

Bergogone-Berezin, E. and K.J. Towner. 1996. *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. *Clin. Microbiol. Rev.* 9, 148-165.

Bradford, M.M. 1976. Rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.

Bull, C. and D. Ballou. 1981. Purification and properties of

protocatechuate 3,4-dioxygenase from *Pseudomonas putida*. *J. Biol. Chem.* 256, 12673-12680.

Caposio, P., E. Pessione, G, Giuffrida, A, Conti, S. Landolfo, C. Giunta, and G. Gribaudo. 2002. Cloning and characterization of two catechol 1,2-dioxygenase genes from *Acinetobacter radioresistens* S13. *Res. Microbiol.* 153, 69-74.

Eulberg, D., S. Lakner, L. Golovlerva, and M. Schlömann. 1998. Characterization of a protocatechuate catabolic gene cluster from *Rhodococcus opacus* 1CP: Evidence for a merged enzyme with 4-caboxymuconolactone-dcarboxylating and 3-oxoadipate enol-lactone-hydroyzing activity. *J. Bacteriol.* 180, 1072-1081.

Fournier, P.E., D. Vallenet, V. Barbe, S. Audic, H. Ogato, L. Poirel, H. Richet, C. Rabert, S. Mangenot, C. Abergel, P. Nordmann, J. Weissenbach, D. Raoult, and J.M. Claverie. 2006. Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. *Plos. Genetics* 2, 62-72.

Giuffrida, M.G., E. Pessione, R. Mazzoli, G. Dellavalle, C. Barello, A. Conti, C. Giunta, 2001. Media containing aromatic compounds induce peculiar proteins in *Acinetobacter radioresistens*, as revealed by proteome analysis. *Electrophoresis* 22, 1705-1711.

Guardabassi, L., A. Petersen, J.E. Olsen, and A. Dalsgaard. 1998. Antibiotic resistance in *Acinetobacter* spp. isolated from sewers receiving waste effluent from a hospital and a pharmaceutical plant. *Appl. Environ. Microbiol.* 64, 3499-3502.

Harwood, C.S. and R.E. Parales. 1996. The β-ketoadipate pathway and the biology of self-identity. *Annu. Rev. Microbiol.* 50, 553-590.

Kim, S.I., J.Y. Kim, S.H. Yun, J.H. Kim, S.H. Leem, and C.H. Lee. 2004. Proteome analysis of *Pseudomonas* sp. K82 biodegradation pathways. *Proteomics* 4, 3610-3621.

Kim, Y.H., K. Cho, S.H. Yun, J.Y. Kim, K.H. Kwon, J.S. Yoo, and S.I. Kim. 2006. Analysis of aromatic catabolic pathways in *Pseudomonas putida* KT 2440 by combined proteomic approach: 2-DE/MS and cleavable ICAT analysis. *Proteomics* 6, 1301-1318.

Kuo, L.C., L.T. Teng, C.J. Yu, S.W. Ho, and P.R. Hsueh. 2004. Dissemination of a clone of unusual phenotype of pan-drug-esistant *Acinetobacter baumannii* at a university hospital in Taiwan. *J. Clin. Microbiol.* 42, 1759-1763.

Llanes, C., D. Hocquet, C. Vogne, D. Benali-Baitich, C. Neuwirth, and P. Plésiat. 2004. Clinical strains of *Pseudomonas aeruginosa* overproducing MexAB-OprM and MexXY efflux pumps simultaneously. *Antimicrob. Agents Chemother.* 48, 1787-1802.

Magnet, S., P. Courvalin, and T. Lambert. 2001. Resistance-odulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. *Antimicrob. Agents Chemother.* 45, 3375-3380.

Murray, P.R., E.J. Baron, J.H. Jorgensen, M.A. Pfaller, and R.H. Yolken. 2003. Manual of clinical microbiology, 8th ed. American Society for Microbiology, Washington DC, USA

Navon-Venezia, S., R. Ben-Ami, and Y. Carmeil. 2005. Updata on *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections in the healthcare setting. *Curr. Opin. Infect. Dis.* 18, 306-313.

Pessione, E., M.G. Giuffrida, R. Mazzoli, P. Caposio, S. Landolfo, A. Conti, C. Giunta, and G. Gribaudo. 2001. The catechol 1,2 dioxygenase system of *Acinetobacter radioresistens*: isoenzymes, inductors and gene localisation. *Biol. Chem.* 382, 1253-1261.

Van Looveren, M. and H. Goossens. 2004. Antimicrobial resistance of *Acinetobacter* spp. in Europe. *Clin. Microbiol. Infect.* 10, 684-704.

Wendt, C., B. Dietze, E. Dietze, and H. Rüden. 1997. Survival of *Acinetobacter baumannii* on dry surfaces. *J. Clin. Microbiol.* 35, 1394-1397.

Yun, S.H., C.Y. Yun, and S.I. Kim. 2004. Characterization of protocatechuate 4,5-dioxygenase induced from p-Hydroxybenzoate-ultured *Pseudomoans* sp. K82. *J. Microbiol.* 42, 152-155.