Proteomic analysis of the benzoate degradation pathway in Acinetobacter sp. KS-1

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Abstract

The purpose of this study was to perform proteome analysis of Acinetobacter sp. KS-1, a bacterium capable of degrading benzoate as a sole carbon source. In order to understand the benzoate degradation pathway used by strain KS-1, proteomes of benzoate-cultured and succinate-cultured KS-1 were comparatively analyzed by two dimensional gel electrophoresis (2-DE). Eighteen protein spots proteins were exclusively induced from the benzoate-cultured strain KS-1. Of these 18 spots, two benzoate-degrading enzymes (catechol 1,2-dioxygenase and β-ketoadipate succinyl-CoA transferase) were identified by MS/MS analysis by MALDI-TOF/TOF mass spectrometry, which suggests that strain KS-1 degrades benzoate by the β-ketoadipate pathway. DEAE-chromatography suggested that strain KS-1 induced only one type of catechol 1,2-dioxygenase during benzoate degradation. The catechol 1,2-dioxygenase was purified using three steps of ammonium sulfate precipitation, DEAE-sepharose, and Mono-Q chromatography. The purified catechol 1,2-dioxygenase of strain KS-1 had strong dioxygenase activity for 4-methylcatechol as well as catechol. Sequencing analysis using N-terminal and internal amino acid sequences showed that this catechol 1,2-dioxygenase is highly homologous with catechol 1,2-dioxygenase of Acinetobacter radioresistens. These results suggest that comparative proteomic analysis of biodegrading bacteria cultured under different conditions may be a useful initial step toward the elucidation of the aromatic compound degradation pathway.

Keywords: Acinetobacter sp. KS-1; Benzoate degradation pathway; Proteome analysis; Catechol 1,2-dioxygenase

1. Introduction

Many environmental aromatic pollutants have been reported to be biodegraded by a variety of microorganisms, which contain various dioxygenases capable of cleaving aromatic compounds [12]. Such dioxygenases play a major role in the biodegradation of aromatic compounds. Aromatic compounds are invariably converged by dioxygenases into more reactive dihydroxylated intermediates such as catechol, protocatechuate, and gentisate, and these intermediates are subjected to either intradiol (ortho) cleavage, extradiol (meta) cleavage, or gentisate cleavage in accord with the characteristics of the bacterial dioxygenase concerned [13]. Because of the importance of dioxygenases, many (catechol oxygenases, protocatecuate oxygenases, and gentisate oxygenases in Gram-negative and Gram-positive bacteria have been purified and extensively studied [8,10,22,26]. In particular, two Acinetobacter calcoaceticus strains (NCIB8250 and ADP1) have been well studied in terms of the biodegradation of benzoate and phenol, and the related gene clusters, including the dioxygenases of these bacteria, have been reported [9,13].

Proteome analysis has become a powerful tool for investigating global changes in prokaryotic gene expression [15,16,27]. Because 2-DE displays all bacterial soluble proteins expressed at specific culture conditions on gel, high throughput screening of these induced proteins is possible. We have applied this approach to biodegradation in order to characterize the proteins induced by aromatic compounds. To date, proteome analysis has been performed upon several bacteria including Acinetobacter radioresistens [11], Acinetobacter lwolfii K24 [17,20], and Pseudomonas sp. K82 [19]. Aromatic compounds (e.g., benzoate, aniline, phenol) have
been found to induce not only the expected biodegradation-related enzymes, including various dioxygenases, but also other metabolic enzymes such as the stimulus.

Strain KS-1 was screened at the Soonchunhyang University Hospital located in Cheonan, Korea, and was identified as Acinetobacter by using the BIOLOG analysis system. Moreover, Acinetobacter sp. KS-1 is known to biodegrade benzoate as the sole carbon source [28]. In this study, we performed proteome analysis upon benzoate-induced Acinetobacter sp. KS-1 in an effort to understand the metabolic pathway of benzoate degradation and to identify the proteins induced. We found that Acinetobacter sp. KS-1 utilizes the β-keto-5,6-dioxygenate pathway for benzoate degradation, and thus we purified the catechol 1,2-dioxygenase involved and investigated its characteristics.

2. Material and methods

2.1. Bacterial strain and cultivation condition

Acinetobacter sp. KS-1 was pre-cultured in potassium phosphate buffer (pH 6.25) containing 3.4 mM MgSO4, 0.3 mM FeSO4, 0.2 mM CaCO3, 10 mM NH4Cl and 10 mM sodium succinate as previously described and cultured in a benzoate (5 mM) and the succinate medium 10 mM sodium succinate as previously described and cultured in a benzoate (5 mM) and the succinate medium [28]. For isoelectric focusing (IEF), dried crude cell extracts (about 250 µg) were dissolved in 50 µl buffer I (SDS 0.3%, DTT 0.2 M, Tris–HCl 50 mM) and heated at 95 °C for 5 min. After incubation with 5 µl buffer II (MgCl2 50 mM, DNAse I 10 unit, RNAsase 3.75 unit, Tris–HCl 50 mM) on ice for 10 min, the samples were dissolved in 350 µl of IPG buffer (urea 8 M, CHAPS 2% (w/v), 0.5% IPG buffer). The protein sample solutions so obtained were applied to immobilized pH 3–10 nonlinear gradient strips using IPGphor (Pharmacia). Focusing was performed in 3 steps (500 V for 1 h, 1000 V for 1 h and 8000 V for 8 h). The second dimension was run on a 12% polyacrylamide SDS gel using a PROTEAN II xi electrophoresis kit (BIO-RAD, Hercules, CA, USA). Silver staining was carried using the method of Heukeshoven and Dernick [14] using a silver staining kit purchased from Pharmacia Biotech.

2.4. 2-D PAGE

For isoelectric focusing (IEF), dried crude cell extracts were suspended in 20 mM Tris–HCl buffer (pH 8.0) and disrupted using a French pressure cell (SLM AMINCO, Urbana, IL, USA) at 20000 lb/in2. The supernatant (crude cell extract), obtained by centrifugation at 15 000 g for 45 min was subjected to 30–55% ammonium sulfate precipitation and then dialyzed against Tris–HCl buffer (50 mM, pH 7.0). The resulting enzyme solution was applied to a Fast-Flow DEAE-sephase column and eluted with a 100–500 mM NaCl gradient at a flow rate of 2 ml/min for 40 min. Active fractions were pooled, dialyzed against 20 mM Tris–HCl buffer (pH 8.0) and used for Mono-Q of Pharmacia using FPLC (Pharmacia, Uppsala, Sweden). Proteins were eluted with a 100–500 mM NaCl gradient at a flow rate 0.5 ml/min for 30 min, and fractions were collected and used for enzyme assay.

Protein contents were determined by the method of Bradford [4] and SDS-PAGE was performed as described by Laemmli [23].

2.5. In-gel digestion

The stained protein spots were excised from the gel and digested with trypsin. After washing with 10 mM ammonium bicarbonate and 50% acetonitrile, gel pieces were swollen in digestion buffer containing 50 mM ammonium bicarbonate, 5 mM CaCl2 and 12.5 ng/µl trypsin, and incubated at 37 °C for 12–16 h. The peptides were recovered by a two-stage extraction using 50 mM ammonium bicarbonate and 100% acetonitrile. The resulting peptide extracts were pooled and lyophilized in a vacuum centrifuge and stored at −20 °C.

2.6. Peptide sequencing by MALDI-TOF

The resulting trypsinized peptides were dissolved using a 0.5% TFA solution. To reduce chemical background noise for MALDI-MS, sample peptides were purified using Zip-tip C18 (Millipore, Bedford, MA, USA). Peptides were eluted onto the MALDI target plate using a matrix solution containing 10 mg of α-cyano-4-hydroxycinnamic acid dissolved in 1 ml of a solution containing 50% acetonitrile and 0.5% TFA. Sample peptides were analyzed by TOF-MS and TOF-MS/MS mode (4700 Proteomics analyzer, Applied Biosystems, Framingham); UV light (355 nm) of an Nd: YAG laser with a 200 Hz laser repetition rate. For peptide sequencing by MS/MS analysis, collision-induced dissociation was performed using air as the collision gas. The collision energy was set to 1 kV. MS/MS spectra were analyzed using the Mascot program compared with the NCBI nr database.
2.7. N-terminal protein sequencing

Protein spots separated on the 2-D gel were transferred onto a PVDF membrane using a semi-dry blotting apparatus (BIO-RAD Trans-Blot SD) at 2 mA/cm² for 50 min. The PVDF membrane was stained with Coomassie Brilliant Blue R250 and washed with 50% methanol. Coomassie-stained protein spots were excised from the PVDF membrane and installed in the blot cartridge of a model 491A protein sequencer (Perkin–Elmer, Foster City, CA, USA) for sequencing analysis. The obtained N-terminal sequence was used for protein identification by BLAST search of NCBI.

2.8. PCR amplification of the catechol 1,2-dioxygenase

Degenerate primers for catA were designed using N-terminal and internal amino acid sequences (Table 1). Two sets of oligonucleotides (forward and reverse primers) were synthesized as follows: primer 164 [ATGAA(C/T)TCA-(A/G)CA(G/AT)(C/G/T)GA]; primer 165 [AA(A/G)CA-(A/G)ATGGT(G/AT)(C/G/TA(T)AC]; primer 166 [AG-(A/G)TC(A/G)AA(A/G)TC(G/AT)(C/G/TGA]; primer 167 [AC(A/G)AA(A/G)TA(G/AT)(C/G/T)GA]. PCR reactions were performed for 25 cycles of 1 min at 95 °C, 1 min at 50 °C and 1 min at 72 °C. PCR products were purified on the 1% agarose gel for sequencing.

2.9. DNA sequencing and analysis

The DNA sequences of about 630-bp PCR product containing catA gene was determined by the dideoxy-chain termination methods using a BigDye terminator cycle sequencing kit (Perkin–Elmer). Sequencing reactions were prepared according to the supplier’s instructions and analyzed using a Perkin–Elmer Model 3700 DNA sequencer. Homology search and protein identification were performed in NCBI program using the BLAST [1].

2.10. Southern hybridization

Chromosomal DNAs of Acinetobacter sp. KS-1 were prepared according to previously described methods [28]. Chromosomal DNAs were digested with EcoR1 and Hind III (NEB), run on a 1% agarose gel, and transferred to Zeta-probe blotting membranes. Hybridization with 32P-labeled 750-bp PCR product of catA was performed at 65 °C and membranes were developed using a phosphoimager (Amersham Pharmacia).

3. Results and discussion

3.1. Growth of strain KS-1 in benzoate media

Acinetobacter sp. KS-1 can utilize benzoate (5 mM) as a sole carbon source. In order to determine the range

### Table 1
Proteome analysis of benzoate-induced protein spots of Acinetobacter sp. KS-1

<table>
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<tr>
<th>Spot no.</th>
<th>Mw, pI [M + H]⁺</th>
<th>MW of peptides</th>
<th>Sequence</th>
<th>Accession no.</th>
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<tr>
<td>2</td>
<td>38.0, 4.2</td>
<td>1523.71</td>
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<td>Unidentified</td>
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<tr>
<td>3</td>
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<td>AGITGTPR</td>
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<tr>
<td></td>
<td></td>
<td>841.53</td>
<td>IQQVVVR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>966.46</td>
<td>SQSFENLR</td>
<td></td>
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<td></td>
<td></td>
<td>1933.98</td>
<td>TIEGILVYGAPESVGFAR</td>
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<td></td>
<td></td>
<td>2081.00</td>
<td>HGNRPSHIVYFVSAPGYR</td>
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<td></td>
<td></td>
<td>2082.00</td>
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<td></td>
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<tr>
<td>4</td>
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<td>GMFALHYR</td>
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<tr>
<td></td>
<td></td>
<td>1125.49</td>
<td>DISKTHIVR</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>1315.66</td>
<td>CVVEIKPKGTNKK</td>
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<td>DISKTHIVR</td>
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<tr>
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<td>1504.69</td>
<td>QSDSYVFDEL</td>
<td>β-ketoadipate succinyl-CoA transferase subunit A (catI) Q01103</td>
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of benzoate concentrations required for survival, different concentrations of benzoate (e.g., 10, 100, 500 mM, and 1 M) were used. Even though survival rates at 100 and 500 mM benzoate were reduced after 6 h, *Acinetobacter* sp. KS-1 was able to use benzoate as carbon source. *Acinetobacter* sp. KS-1 survival in 1 M benzoate was poor (Fig. 1).

### 3.2. Proteomic analysis

In order to identify the proteins of *Acinetobacter* sp. KS-1 induced under benzoate-cultured conditions, 2-DE and comparative analyses were performed using benzoate-grown and succinate-grown bacteria, respectively. The crude extract of *Acinetobacter* sp. KS-1 was prepared from succinate and benzoate media according to the method described [22]. Approximately 350 protein spots were detected on the 2-D gel when a pH 3–10 and 12% polyacrylamide SDS gel was used and more than 200 protein spots were located in the acidic region, meaning that the majority of proteins of *Acinetobacter* sp. KS-1 were acidic. 18 protein spots appeared with significantly different intensities on the 2-D gel of benzoate grown bacteria and respectively numbered 1–18 (Fig. 2). Even though numerous spots were detected on the gel, the protein amount of only 18 spots was enough to analyze using MALDI-TOF mass spectrometry. These proteins were selected and in-gel digested with trypsin for MS/MS analysis. 10 of 18 proteins were successfully analyzed and 8 proteins were identified by sequence homology using the elucidated amino acid sequences (Table 1). Interestingly, spot 3 was identified as catechol 1,2-dioxygenase, suggesting that *Acinetobacter* sp. KS-1 uses the β-ketoacid pathway for benzoate degradation. This suggestion was confirmed by the identifications of spots 9, 10, 18, which were identified as β-ketoacid succinyl-CoA transferase (catI). Surprisingly, spots 4, 5, 6, 8 were identified as trehalose phosphatase (otsB). Trehalose phosphatase is known to be involved in the synthesis of trehalose and trehalose is a nonreducing disaccharide (α-D-glucopyranosyl-1,1-α-D-glucopyranoside), which is present in many prokaryotic and eukaryotic organisms [18,24] and plays a major role in cell protection against harsh environmental conditions such as heat shock [26], cold shock, and osmotic stress [7]. Further study is needed to determine the relation between benzoate stress and trehalose induction. Proteome analysis of *A. lwoffii* K24 and *A. radiorresistens* found that these bacteria induce enzymes of the β-ketoacid pathway when cultured in aniline and benzoate condition but enzymes related with trehalose synthesis were not detected under these conditions [11,20].

![Fig. 1. Survival of *Acinetobacter* sp. KS-1 after benzoate shock. Strain KS-1 cells were maintained at benzoate concentration of 0 mM (■), 10 mM (○), 100 mM (▲), 500 mM (◇), 1 M (◆) benzoate, respectively.](image)

![Fig. 2. 2-DE of the water soluble protein extracts of succinate-cultured (A) and benzoate-cultured (B) *Acinetobacter* sp. KS-1. The protein extract were separated on the 2-D gels by electrofocusing using an IPG strip (pH 3–10 NL) and 12% SDS-PAGE. The 2-D gels were silver-stained [13]. Numbered protein spots were analyzed by MS/MS.](image)
analysis using 2-DE revealed that several major proteins, including β-ketoacidipate succinyl-CoA transferase and trehalose phosphatase, exist as isoforms with different pIs. Further study will be needed to explain the different pIs. On the other hand, we could not detect other related enzymes such as muconate cycloisomerase (catB) or benzoate 1,2-dioxygenase. In our next study, fractionation of crude protein mixtures using column or narrow range pH strip gel will be performed for the screening undetected enzymes.

3.3. Purification of catechol 1,2-dioxygenase

Because the proteome analysis of benzoate-induced Acinetobacter sp. KS-1 revealed the induction of catechol 1,2-dioxygenase, the catechol 1,2-dioxygenase activity of Acinetobacter sp. KS-1 was assayed. Enzymatic activity increased during exponential growth and decreased gradually during the stationary phase (Fig. 3). The purification of catechol 1,2-dioxygenase was performed in three stages, i.e., ammonium sulfate precipitation, and DEAE-Sepharose, followed by Mono-Q chromatography. This procedure increased its activity 12.22-fold (Table 2 and Fig. 4). Several bacteria have been reported to possess more than two catechol 1,2-dioxygenases induced by benzoate, aniline or phenol [5,7,25,26]. However DEAE-Sepharose chromatography showed that Acinetobacter sp. KS-1 expressed only catechol 1,2-dioxygenase in benzoate culture (data not shown), which was consistent with the result of 2-DE. The molecular weight of catechol 1,2-dioxygenase was determined at about 36 kDa by SDS-PAGE (Fig. 4). The N-terminal amino acid sequence of the catechol 1,2-dioxygenase of Acinetobacter sp. KS-1 was MNYYQIDALVKQMNVDTAKG, and was highly related to the catechol 1,2-dioxygenase (IsoB) of A. radioresistens, which was reported to express two catechol 1,2-dioxygenases (IsoA and IsoB), as major cleavage enzymes, in phenol and in benzoate cultures. IsoB of A. radioresistens was found to be strongly induced in benzoate culture, and to be expressed at lower levels in phenol-containing culture [7].

3.4. Characterization of catechol 1,2-dioxygenase

The catechol 1,2-dioxygenase of Acinetobacter sp. KS-1 showed considerable cleavage activity for 4-methylcatechol (58.6% of catechol) but had a cleavage activity of <10% for other catechol analogues such as 3-methyl, 4-chloro, and 3-methoxycatechol (data not shown). These results suggest that the catechol 1,2-dioxygenase of Acinetobacter sp. KS-1 has cleavage characteristics similar to those of Pseudomonas sp. [25], Acinetobacter lwofii [22], and Frateuria sp. [3], and that it is a type I catechol 1,2-dioxygenase [12]. The optimal temperature and pH of catechol 1,2-dioxygenase were 35°C and 7.5, respectively. Metal ion effects on enzyme activity were assayed using the described methods [3,6]. In particular, Ag⁺ and Hg⁺ (0.1 mM) inhibited its activity by more than a 90% inhibitory effect, which suggests that the sulfhydryl group of cysteine(s) is involved

### Table 2

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (Unit)</th>
<th>Specific activity (Unit/mg)</th>
<th>Total protein (mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
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<td>Crude extract</td>
<td>110.9</td>
<td>1.03</td>
<td>107.5</td>
<td>100</td>
<td>1</td>
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<tr>
<td>Ammonium precipitation 74.0</td>
<td>1.32</td>
<td>64.1</td>
<td>66.7</td>
<td>1.28</td>
<td>1</td>
</tr>
<tr>
<td>(30–55%)</td>
<td></td>
<td></td>
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<tr>
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in the enzyme activity of catechol 1,2-dioxygenase [3]. PCR was performed with degenerate primers, which were designed using the internal amino acid sequences of catechol 1,2-dioxygenase, and obtained PCR products (Fig. 5A). Homology analysis of the amino acid sequence deduced from the DNA sequence of its PCR product showed that the catechol 1,2-dioxygenase of Acinetobacter sp. KS-1 was highly homologous with enzymes of A. radioresistens S13 (93%) and A. calcoaceticus Z36909 (86%) with respect to the 174 amino acid sequence of internal region (Figs. 5B and 5C). Two conserved tyrosines were identified, which are probably involved in the iron ligand function. A conserved cysteine was located at the same site as the 202 cysteine in the catechol 1,2-dioxygenase of A. lwoffii K24. This 202 cysteine is known not to be directly involved in enzyme activity, but to be the sole inhibition site by Ag⁺ and Hg⁺ in A. lwoffii [21]. Thus, this result strongly suggests that the conserved cysteine of Acinetobacter sp. KS-1 is also inhibited by Ag⁺ and Hg⁺.

Acinetobacter sp. KS-1 can utilize benzoate as the sole carbon source by the β-ketoadipate pathway over a broad range of concentrations (5–500 mM). Enzymes related to the β-ketoadipate pathway, i.e., catechol 1,2-dioxygenase and β-ketoadipate succinyl-CoA transferase, were identified by proteome analysis using 2-DE. In addition, trehalose phosphatase was found to be induced in benzoate. These results suggest that comparative proteome analysis is a useful screening tool for the elucidation of bacterial metabolisms like biodegradation. However, other related enzymes such as muconate cycloisomerase (catB) or benzoate 1,2-dioxygenase were not detected in this work. This study shows that Acinetobacter sp. KS-1 possesses a single major catechol 1,2-dioxygenase, and describes its purification and characterization. It was found that this catechol 1,2-dioxygenase is a type I catechol 1,2-dioxygenase and that it shares a high level of homology with the IsoB of A. radioresistens.

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References


