Puriﬁcation and gene cloning of the oxygenase component of the terephthalate 1,2-dioxygenase system from Delftia tsuruhatensis strain T7

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Received 15 October 2002; received in revised form 3 February 2003; accepted 6 February 2003
First published online 6 March 2003

Abstract

The terephthalate 1,2-dioxygenase system (TERDOS) was found in cell extracts of Delftia tsuruhatensis strain T7 (=IFO16741) grown in terephthalate-salt medium. The cell extract was separated by anion exchange chromatography to yield two fractions (R and Z) that were necessary for oxygenation of terephthalate with NADH and Fe2+. The oxygenase component of TERDOS (TerZ) was puriﬁed from fraction Z by gel ﬁltration chromatography to near homogeneity. An α3β3 subunit structure was deduced from the molecular masses of 235, 46 and 17 kDa of the native complex and the α- and β-subunits, respectively. The N-terminal amino acid sequences of the two subunits of TerZ allowed polymerase chain reaction primers to be deduced and the DNA sequence of the α-subunit was determined. The amino acid sequence of the α-subunit (TerZα) showed signiﬁcant similarities to the large subunits of multicomponent ring-hydroxylation oxygenases. Two motifs in the deduced amino acid sequence, a Rieske [2Fe-2S] center and a mononuclear Fe(II) binding site, were observed. Phylogenetic analyses indicated that TerZα and the large oxygenase component subunits ortho-halo-benzoate 1,2-dioxygenase and salicylate-5-hydroxylase form a cluster that is distant from the rest of the large oxygenase subunits of multicomponent ring-hydroxylation oxygenases.

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Keywords: Terephthalate; Delftia tsuruhatensis; Biodegradation; Enzyme puriﬁcation; Gene cloning

1. Introduction

The biological degradation of terephthalate (1,4-dicarboxybenzene) has been of interest in recent years. Several microorganisms have demonstrated the capability to degrade this compound, including organisms among the Gram-positive bacteria and the Proteobacteria [1–5]. Most of these reportedly produce protocatechuate as an intermediate metabolite. The enzymatic system responsible for the degradation of terephthalate in Comamonas testosteroni strain T-2 has been thoroughly studied. In that strain, terephthalate is degraded to protocatechuate by the terephthalate 1,2-dioxygenase system (TERDOS) and protocatechuate is then metabolized via the 4,5-extradiol (meta) ring fission pathway [5]. However, DNA sequence data of the genes encoding TERDOS have not been reported.

Recently, we isolated a novel terephthalate-assimilating bacterium, Delftia tsuruhatensis strain T7, from activated sludge of a domestic wastewater treatment plant [6]. This isolate was thought to have an enzymatic system similar to TERDOS of C. testosteroni strain T-2, although no other members of the genus Delftia have demonstrated the ability to degrade terephthalate. In this research, we puriﬁed the oxygenase component of TERDOS [5] from strain T7...
and analyzed the base sequence of the gene encoding the α-subunit of the oxygenase component (TerZα). To our knowledge this is the first reported characterization of a TERDOS-encoded gene.

2. Materials and methods

2.1. Organism, growth conditions and preparation of cell extract

D. tsuruhatensis strain T7 (IFO16741) was grown at 30°C in terephthalate-salt medium (pH 7.6) consisting of the following components (per liter): terephthalic acid (disodium salt), 1 g; K2HPO4, 3.48 g; KH2PO4, 0.435 g; (NH4)2SO4, 1 g; MgSO4·7H2O, 0.2 g; FeCl3·6H2O, 0.02 g; NaCl, 0.1 g; and CaCl2·2H2O, 0.1 g. The cells were harvested at an optical density of 1.0 at 546 nm (OD546) and washed in 50 mM Tris sulfate buffer (pH 7.5) containing 1 mM dithiothreitol. Cells, suspended in Tris sulfate buffer, were disrupted with an ultrasonic generator (US-150, Nihonseiki, Tokyo, Japan) for 25 min. The suspension was then centrifuged at 30,000 × g for 50 min and the supernatant collected for use as crude extract. TERDOS activity was determined by oxygen uptake at 30°C using a Clarke-type oxygen electrode with a 1.1-ml reaction vessel (Oxygraph 9, Central Kagaku, Tokyo, Japan) following the method of Locher et al. [7].

2.2. Purification of TERDOS

TERDOS was purified from the crude extract following the method of Schlüff et al. [5], which is as follows.

Step 1: Nucleic acids were removed from the crude extract by precipitation with protamine sulfate using the method of Locher et al. [7].

Step 2: An anion exchange column (MonoQ HR 10/10, Amersham Bioscience) was equilibrated (4 ml min⁻¹) with buffer A (0.5 ml min⁻¹) consisting of the following components: (NH4)2SO4, 1 g; MgSO4·7H2O, 0.2 g; FeCl3·6H2O, 0.02 g; NaCl, 0.1 g; and CaCl2·2H2O, 0.1 g. The cells were harvested at an optical density of 1.0 at 546 nm (OD546) and washed in 50 mM Tris sulfate buffer (pH 7.5) containing 1 mM dithiothreitol. Cells, suspended in Tris sulfate buffer, were disrupted with an ultrasonic generator (US-150, Nihonseiki, Tokyo, Japan) for 25 min. The suspension was then centrifuged at 30,000 × g for 50 min and the supernatant collected for use as crude extract. TERDOS activity was determined by oxygen uptake at 30°C using a Clarke-type oxygen electrode with a 1.1-ml reaction vessel (Oxygraph 9, Central Kagaku, Tokyo, Japan) following the method of Locher et al. [7].

2.3. Amplification, nucleotide sequencing and sequence analysis of the terZα gene

Total DNA from strain T7 was extracted with a Qiagen Genomic DNA Buffer Set using a Qiagen Genomic-Tip 20/G (Qiagen). Degenerate primer pairs for polymerase chain reaction (PCR) amplification were deduced from the N-terminal amino acid sequences of TerZα and TerZβ. The successful pair was identified as taf, 5’-ATGCAGGACWSSATCATCCAGTGG-3’, based on the N-terminal sequence of oxygenase Zα from TERDOS of C. testosteroni strain T-2, and tb2r, 5’-ATYGDA-TYTCTTIDATCAT-3’, based on the N-terminal sequence of TerZβ from strain T7. PCR was performed with total DNA from strain T7 using the reagents supplied with the AmpliTaq Gold kit (Applied Biosystems) with 200 ng of DNA and 40 pmol of each primer. The reactions were carried out using a GeneAmp PCR System 2400 (Applied Biosystems) with preincubation at 95°C for 3 min and 35 cycles at 95°C for 1 min, 50°C for 1 min and 72°C for 2 min. The amplified DNA fragment was then ligated into pT7-Blue T-Vector (Novagen) and sequenced using an ABI 373S-18 DNA sequencer (Applied Biosystems) with a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Bioscience).

DNA and deduced amino acid sequences were analyzed with the GENETYX-WIN (version 5.1) software package (Software Development, Tokyo, Japan). Searching for homologous proteins was done with the BLAST program [9]. Multiple alignments were run using the Clustal X program, version 1.8 [10]. Phylogenetic analyses were conducted with MEGA version 2.1 [11]. The reference sequences used in the comparison were retrieved from the DDBJ, EMBL and GenBank nucleotide sequence databases. The DNA sequence of terZα from strain T7 was deposited in the DDBJ, EMBL and GenBank databases with accession number AB081091.
3. Results and discussion

3.1. Purification of TERDOS of strain T7

Crude extracts from cells of strain T7 grown in terephthalate-salt medium catalyzed a TER-dependent uptake of oxygen in the presence of NADH and FeSO₄. We attribute this to the presence of a multicomponent oxygenase system that is similar to the TERDOS system of C. testosteroni strain T-2 [5]. TERDOS activity in crude extract showed a linear response to protein concentrations (data not shown).

When proteins in crude extract were separated by anion exchange chromatography, no single fraction was active. However, TERDOS activity was obtained when the red-brown fraction Z was combined with fraction R. The proteins in fraction R demonstrated the capability to oxidize NADH and reduce cytochrome c (data not shown), thus this fraction was considered to contain the reductase function of TERDOS. The oxygenase component of TERDOS (TerZ) was purified to near homogeneity by anion exchange chromatography and gel filtration chromatography (Fig. 1). Even though most contaminants were removed during the gel filtration step, this did not result in an increase in specific activity (Table 1), which is most likely due to the occurrence of protein denaturation during the gel filtration procedure.

Typical results for purification of TerZ are shown in Table 1. Approximately 0.8 mg of TerZ was obtained from 400 mg of soluble protein (about 6 g wet cells). TerZ was determined to have an $M_r$ value of about 235000 under native conditions (gel filtration chromatography; data not shown) and to consist of subunits with $M_r$ values of 46000 and 17000 (Fig. 1). Determinations of the N-terminal amino acids in these subunits (see below) yielded unique sequences, thus each subunit was considered to be homogeneous. The subunits were labeled α (46000) and β (17000) and the $M_r$ of TerZ from strain T7 was higher than that of oxygenase Z from strain T-2, which was reported to be 126000. The oligomeric structure of TerZ from strain T7 appeared to be different (possibly α₂β₂) from that of strain T-2 (α₂β₂ [5]). The isoelectric points (pI) of the two subunits were 5.3 (TerZα) and 4.9 (TerZβ) as determined by two-dimensional electrophoresis.

The N-terminal amino acids of the two subunits were determined to be TerZα (Met-Gln-Glu-Ser-Ile-Ile-Gln) and TerZβ (Met-Ile-Asn-Glu-Ile-Ala-Ala-Phe-Asn-Ala-Ala-Tyr-Ala-Lys-Thr-Ile-Asp-Ser-Asp-Ala-Met-Glu-Gln), which are identical to the TERDOS oxygenases Zα and Zβ from C. testosteroni strain T-2, respectively. These results suggest that TERDOS of strain T7 has similar properties to that of C. testosteroni strain T-2, in spite of the phylogenetic distance between the two strains [6].

3.2. The terZα gene sequence from strain T7

The forward primer taf, which had a codon (TGG) for the eighth Trp residue of oxygenase Zα from strain T-2, was able to amplify a 1.3-kb fragment of the total DNA from strain T7 when the reverse primer tb2r was used. The DNA sequence of the amplified fragment was determined and the deduced 10-amino acid sequence contiguous with the primer taf was determined to be identical to the known sequence from strain T-2 (Fig. 2a). These results suggest that the amplified fragment contains the gene encoding TerZα. The termination codon (TAG) of terZα was found on the complementary sequence of the primer tb2r, sug-

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total activity (nkat)</th>
<th>Amount of total protein (mg)</th>
<th>Specific activity (mKat kg⁻¹)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
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<tr>
<td>Crude extract</td>
<td>14.5</td>
<td>996.9</td>
<td>424.56</td>
<td>2.35</td>
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<td>58.9</td>
<td>17.2</td>
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<td>Gel filtration</td>
<td>3.0</td>
<td>39.5</td>
<td>0.85</td>
<td>46.6</td>
<td>4.0</td>
<td>19.8</td>
</tr>
</tbody>
</table>

Note: *Activity was measured by TER-dependent oxygen uptake. One kat was defined as 1 mol oxygen uptake in 1 s.*
suggesting that the 3'-end of terZa overlaps with the 5'-end of terZβ encoding TerZβ (Fig. 2a). A 413-amino acid sequence of TerZα was thus deduced and the calculated molecular mass (46.4 kDa) of this sequence corresponded well with the observed value (Fig. 1).

The deduced amino acid sequence of TerZα showed significant similarities to large (α) subunits of terminal oxygenases (iron-sulfur proteins, ISP) for multicomponent ring-hydroxylating oxygenases. Two motifs in the amino acid sequence, a Rieske [2Fe-2S] center and a mononuclear Fe(II) binding site [12], were observed (Fig. 2b). This agrees with the results of others [5] who demonstrated a Rieske [2Fe-2S] center for TERDOS oxygenase Z from C. testosteroni strain T-2 by absorption and electron paramagnetic resonance spectrum analyses. In addition, TerZα had overall identity levels of 40.1 and 40.1% to the α-ISP of ortho-halobenzoate 1,2-dioxygenase (OhbB) from Pseudomonas putida strain 142 (EMBL/GenBank accession number AF121970 [13]) and NagG of Ralstonia sp. strain U2 (AF036940 [14]) are aligned. The consensus residues for the Rieske [2Fe–2S] center and the Fe²⁺ binding site [12] are marked with asterisks. The gaps introduced into the alignment are indicated with hyphens.

The phylogenetic analysis revealed that the large subunits of the oxygenase components for ring-hydroxylating oxygenases could be divided into five major clusters (Fig. 3). Four of the five clusters corresponded to the four groups of the classification system by Nam et al. [15], which was based on the relationships of the large subunits. TerZα, along with OhbB and NagG, was assigned into a noble cluster distant from other four groups. Nam et al.
Fig. 3. Phylogenetic relationships of the deduced amino acid sequences with large (α) subunits of oxygenase components from ring-hydroxylating oxygenases. The tree was constructed from phylogenetic distances obtained by the neighbor-joining method [17]. The bar represents 20 amino acid substitutions per 100 amino acids. Each cluster bears the appropriate nomenclature from Nam et al. [15]. AbsA O1-1, 2-amino benzenesulfonate dioxygenase of *Alcaligenes* sp. strain O-1 (AF109074 [18]); AntA ADP1, antranilate dioxygenase of *Acinetobacter* sp. strain ADP1 (AF071556 [19]); BnaA BE-81, benzene 1,2-dioxygenase of *Pseudomonas putida* strain BE-81 (M17904 [20]); BphA1 KF707, biphenyl dioxygenase of *Pseudomonas* pseudocaldichromatensis strain KF707 (M83673 [21]); CarAa CB3, carbazole dioxygenase of *Sphingomonas* sp. CB3 (AF060489 [22]); CdbA 2CBS, 2-halobenzoate 1,2-dioxygenase of *Burkholderia cepacia* strain 2CBS (X97067 [23]); NagA U2, salicylate-5-hydroxylase of *Ralstonia* sp. strain U2 (AF036940 [14]); NdcA JS42, 2-nitrotoluene dioxygenase of *Pseudomonas* sp. strain JS42 (U49504 [24]); OhbB 142, o-halobenzoate 1,2-dioxygenase of *P. aeruginosa* strain 142 (AF121970 [15]); OhbB JB2, o-halobenzoate 1,2-dioxygenase of *P. aeruginosa* strain JB2 (AF087482 [25]); OphA2a DBO1, phthalate dioxygenase of *B. cepacia* strain DBO1 (AF093748 [26]; Orf2 DNT, 2,4-dinitrotoluene dioxygenase of *Burkholderia* sp. strain DNT (U62430 [27]); PahAc OUS82, polycyclic aromatic hydrocarbon dioxygenase of *P. putida* strain OUS82 (AB004059 [28]); PhnAc RP007, polycyclic aromatic hydrocarbon dioxygenase of *Burkholderia* sp. strain RP007 (AF061751 [29]); PobA POB310, phenoxoaza benzoate dioxygenase of *P. pseudocaldichromatensis* strain POB310 (Q52185 [30]); VanA BF13, vanillate-O-demethylase of *Pseudomonas* sp. strain BF13 (AJ245887 [31]). All reference sequences were taken from the DDBJ/EMBL/GenBank database.

classified OhbB into group II. However, their analysis [15] as well as our analysis (Fig. 3) showed that OhbB is phylogenetically distant from other members of group II. In conclusion, TerZα, along with OhbB and NagG, would most likely belong in a new fifth phylogenetic group among the large subunits of oxygenase components for ring-hydroxylating oxygenases.

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