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EXPERIMENTAL ARTICLES

Cloning and Characterization of a Chromosome-Encoded Catechol 2,3-Dioxygenase Gene from *Pseudomonas aeruginosa* ZD 4-3[1]

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Abstract—Catechol 2,3-dioxygenase (C23O), an extradiol-type dioxygenase cleaving the aromatic C–C bond at the *meta*-position of dihydroxylated aromatic substrates, catalyzes the conversion of catechol to 2-hydroxymuconic semialdehyde. Based on a curing experiment, PCR identification, and Southern hybridization, the gene responsible for C23O was localized on a 3.5-kb *Eco*RI/*Bam*HI fragment and cloned from *Pseudomonas aeruginosa* ZD 4-3, which was able to degrade both single and bicyclic compounds via a *meta*-cleavage pathway. A complete nucleotide sequence analysis of the C23O revealed that it has one ORF, which showed a strong overall amino acid similarity to the known gram-negative bacterial mesophilic C23Os. The alignment analysis indicated a distinct difference between the C23O in this study and the 2,3-dihydroxybiphenyl dioxygenases that cleave bicyclic aromatic compounds. The heterogeneous expression of the *phe*B gene in *E. coli* BL21(DE3) demonstrated that this C23O possesses a *meta*-cleavage activity.

Key words: catechol 2,3-dioxygenase, cloning, expression, *meta*-cleavage, *phe*B gene.

Aromatic compounds, which are widely used and released into the natural environment in industrial and agricultural activities, have become increasingly serious pollutants in China. Because some of them are quite recalcitrant or toxic when released into the environment, these hazardous aromatic compounds have been receiving ever greater attention and various ways of eliminating or reducing their environmental presence have been pursued, including bioremediation using soil microorganisms [1, 2].

The biodegradation of aromatic compounds has been intensively studied, and many bacteria strains have been isolated for their ability to degrade and use these toxic compounds as source of carbon and energy. The catabolic pathway in some gram-negative soil bacteria has been fully elucidated at the biochemical and even at the molecular genetic level [3]. Generally, most aromatic compounds are aerobically degraded through a common intermediate, catechol or protocatechuate, depending on the chemical structure of the starting compound. The catechol is further degraded either by cleavage between the two hydroxyl groups or by cleavage adjacent to the hydroxyl groups by catechol 2,3-dioxygenase via a *meta*-pathway [4, 5]. Catechol dioxygenases are key enzymes in many bacterial pathways for the degradation of aromatic compounds, and the reactions catalyzed by these enzymes are the rate-limiting steps for the decomposition of some aromatic compounds, such as *p*-xylene/*p*-toluate and 3-chlorotoluene/*s*-chlorobenzoate [6].

Previously, we isolated two aromatic degrading bacteria that were designated as strains *Pseudomonas aeruginosa* ZD 4-3 and *Comamonas testosteroni* ZD 4-1. It was found that strains ZD 4-3 and ZD 4-1 possessed *meta*- and *ortho*-cleavage pathways, respectively. As for biodegradation properties, although strain ZD 4-1 was superior in terms of the scope of pollutant degradation and adaptation to pH fluctuation compared with strain ZD 4-3, the *meta*-pathway in ZD 4-3 was obviously of higher efficiency than the *ortho*-pathway in strain ZD 4-1, partly due to the higher activity of C23O than C12O. As part of our ongoing effort to characterize the biochemical and genetic properties of extradiol-type dioxygenases at the molecular level, the C23O gene in *P. aeruginosa* ZD 4-3 was cloned and expressed in *Escherichia coli*. The sequence of the C23O gene and its deduced amino acid sequence were also characterized in this study.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation conditions. *Pseudomonas aeruginosa* ZD 4-3 isolated in

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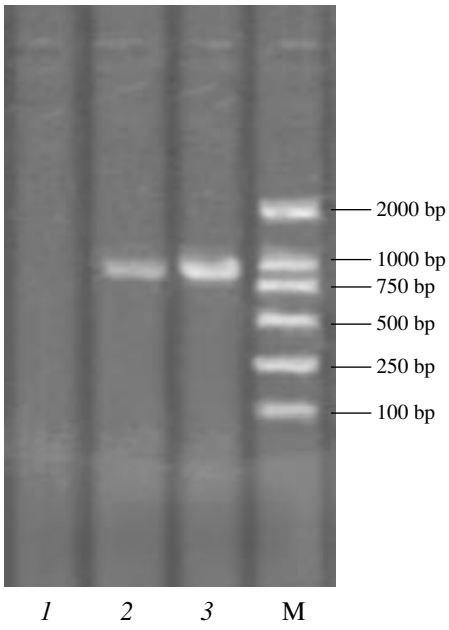


Fig. 1. Agarose gel electrophoresis of PCR amplification products: (*1*) PCR result of reextracted plasmid DNA from *E. coli*; (*2*) PCR product of crude plasmid DNA from ZD 4-3; (*3*) PCR product of chromosome DNA from ZD 4-3.

our laboratory was cultured at 30°C in mineral medium containing 250 mg/l phenol as the sole carbon source as described previously [7]. *E. coli* TG1 and BL21(DE3) were routinely grown at 37°C on LB medium. The cloning vector used was pGEM-T Easy (Promega, United States) and the expression vector was pET-22b(+) (Novagen, United States). Ampicillin at a final concentration of 100 mg/ml was used for the selection of plasmids.

DNA manipulations. Isolation of plasmid, restriction enzyme digestion, DNA ligation, and gel electrophoresis were performed using standard procedures [8]. The *P. aeruginosa* ZD 4-3 chromosome DNA used for Southern hybridization was isolated by using a Total DNA Isolation Kit (Promega) following the manufacturer's instructions. The Southern hybridization followed the instructions of the DIG System (Roche Molecular Biochemicals, Germany) User's Guide. A PCR fragment labeled with digoxigenin was used as a probe for hybridization with template DNA.

Curing experiment. The method of the curing experiment was described elsewhere [9].

Cloning of the *phe*B gene from *P. aeruginosa* ZD 4-3. Standard molecular cloning procedures were employed [8, 10]. A pair of PCR primers was designed based on the conserved end sequence of the known C23O-encoding genes from *Pseudomonas* sp. (forward primer: 5'-GGCCA TGGTC ATGAA CAAAG GTGTA ATGCG-3'; reverse primer: 5'-GGGAA TTCTC AGGTC AGCAC GGTCA TGAA-3'). In the above primers, the underlined sequences indicate the endonuclease sites *Nco*I and *Eco*RI, respectively. For *phe*B gene amplification, the total DNA of strain ZD 4-3 was used as a template and the PCR reaction (50 μl final volume) contained 10× PCR buffer, 5 μl; 25 mM $MgCl_2$, 4 μl; 5 mM dNTP, 2 μl; 20 μM primer, 1 μl each; template DNA, 100 ng; *Taq* polymerase (5000 U/ml), 0.5 μl; and sterile distilled water, 35.5 μl. The cycling parameters were 2 min at 94°C followed by 30 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 2 min, with the 72°C segment of the final cycle extended to 10 min before cooling to 4°C. The resulting PCR product was purified and ligated with the pGEM-T Easy vector. Subsequently, the target segment was digested with *Nco*I and *Eco*RI and then ligated with the pET-22b(+) vector restricted by the same two endonucleases.

Expression of the *phe*B gene in *E. coli*. The host bacterium used to express the *phe*B gene was *E. coli* BL21(DE3). After the recombinant vector (designated pET-LH12) was constructed, it was transformed into *E. coli* BL21(DE3) and induced with IPTG (1 mmol/l) for 4 h after being cultured for 2 h for *phe*B gene expression [11].

Preparation of clear supernatant and crude lysate. Cells of strain ZD 4-3 and *E. coli* containing plasmid pET-LH12 were cultivated overnight at 30 and 37°C, respectively, in LB medium. Cells were harvested by centrifugation at 12000 rpm for 5 min, and the clear supernatant was collected for enzyme assay. Consequently, the pellet was washed with phosphate buffer solution, disrupted by sonication with 99 3-s bursts on ice, and centrifuged at 12000 rpm for 10 min. The clear supernatant obtained was used as a crude lysate for enzyme assay.

C23O enzyme assay. The in vitro C23O enzyme assay method was described previously elsewhere [7].

Sequence analysis. For analyzing the relationship of the sequence and function of the *phe*B gene and also the evolutionary relationship among the extradiol dioxygenases, the deduced amino acid sequence of *phe*B cloned in this paper was aligned with other extradiol-type dioxygenases. These extradiol-type dioxygenases were found in GenBank and were divided into three groups: mesophilic C23O for the biodegradation of single-ring aromatic compounds (AY112717, AF320981, AB035539, D85415, AB004065, U93090, U01825); thermophilic C23O for single-ring fission (AF140605); and bicyclic fission dioxygenases—2,3-bihydroxy-dioxygenases (U22355, X97984, X66122, D44550). The bioinformatics software BioEdit was used for alignment analysis, with all parameters set at their default values. DNAstar was used to construct the phylogenetic tree, and the parameters "protein weight matrix" and "DNA weight matrix" were set as the identity matrix and CLUSTALW, respectively.

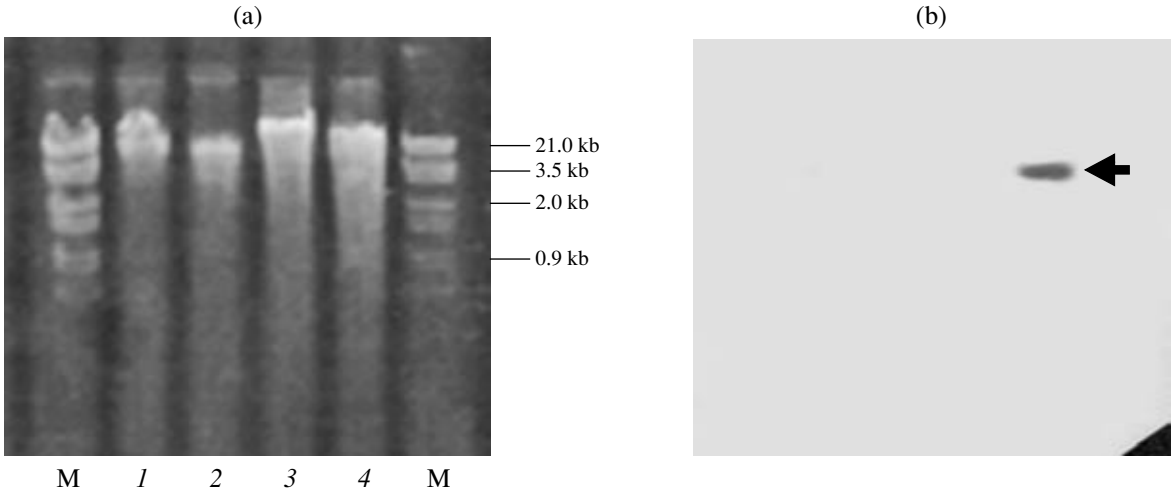


Fig. 2. Southern hybridization of chromosome and reextracted plasmid DNA. (a) Agarose gel electrophoresis of chromosome and reextracted plasmid DNA fragments digested by *Eco*RI or *Eco*RI/*Bam*HI: (*1*) reextracted plasmid DNA (*Eco*RI/*Bam*HI-digested); (*2*) reextracted plasmid DNA (*Eco*RI-digested); (*3*) chromosome DNA (*Eco*RI/*Bam*HI-digested); (*4*) chromosome DNA (*Eco*RI-digested). (b) Signal of endonuclease-digested fragments hybridized with the *phe*B gene probe. The arrowhead indicates the position of the DNA fragments hybridized to the probe.

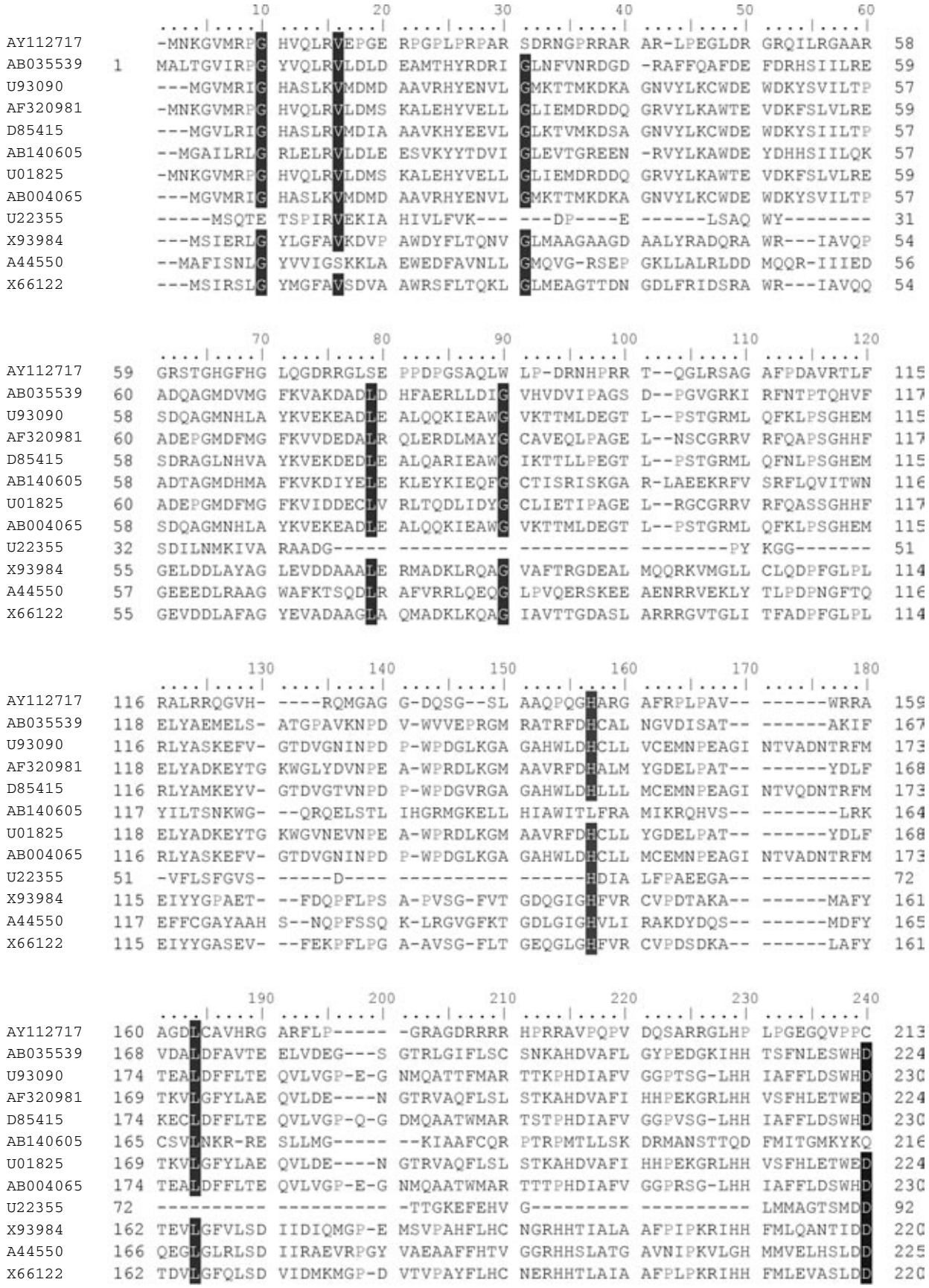
RESULTS AND DISCUSSION

Evidence for the existence of the C23O gene on the chromosome. In the curing experiment, 95% of the colonies of strain ZD 4-3 treated with acridine orange could still grow with phenol. This indicated that the acridine orange had not impaired the phenol utilization ability of strain ZD 4-3 and that the C23O gene may be a chromosome-encoded gene. The chromosome and plasmid DNA of strain ZD 4-3 were extracted and used respectively as PCR templates to further clarify the position of the C23O gene. The plasmid DNA was first extracted from ZD 4-3 as crude plasmid DNA, transformed into *E. coli* TG1, and then reextracted from the *E. coli* so the chromosome DNA mixed in with the crude plasmid DNA was excluded. As shown in Fig. 1, the *phe*B products were amplified with both the chromosome and the crude plasmid DNA as templates but could not be amplified by using the reextracted plasmid DNA as the template. This result not only indicated that the C23O gene was likely to be chromosome-encoded but also verified that the crude plasmid DNA perhaps contained some chromosome DNA on which the C23O gene was located. The reason was that the alkaline lysis method used for plasmid DNA extraction may have resulted in the mixture of linear chromosome DNA with the crude plasmid DNA, so the PCR amplification probably provided a false-positive result. To avoid the mixture of linear chromosome DNA with plasmid DNA, we transformed the circular plasmid DNA into *E. coli* and subsequently reextracted the plasmid DNA from the bacteria. As linear chromosome DNA cannot multiply in *E. coli*, it was removed from the reextracted plasmid DNA.

The chromosome and reextracted plasmid DNA were used to detect the C23O gene by Southern hybridization detection with a PCR product from the total DNA of strain ZD 4-3 as a probe. Figure 2a shows the electrophoresis results of chromosome and reextracted DNA digested by *Eco*RI and *Eco*RI/*Bam*HI, respectively. As seen in lanes *1* and *2*, many DNA fragments were more than 20 kb even after digestion by endonucleases, suggesting that the plasmid DNAs were from strain ZD 4-3 because normal plasmid DNAs (e.g., a plasmid vector) used in standard cloning manipulation were frequently several kilobases long. In Fig. 2b, a strong hybridization signal was observed only for the 3.5-kb DNA fragment of chromosome DNA, while no hybridization signal appeared for the plasmid DNA fragments.

Recapitulating the above results, we explicitly demonstrated that the C23O gene lies on the chromosome DNA. To our knowledge, this may be somewhat unexpected. To date, many papers have indicated that most catabolic genes for aromatic hydrocarbon degradation are located on the plasmid and many C23O genes were confirmed on the plasmid [12, 13].

Cloning of the C23O gene. A PCR using the forward and reverse primers amplified a DNA fragment of the expected size (about 0.9 kb). This fragment was directly cloned into the pGEM-T Easy vector and then transformed into *E. coli* TG1. Consequently, a positive recombinant vector (designated pGEMT-LH5) was identified through PCR and endonuclease digestion with *Nco*I and *Eco*RI. To show whether the 0.9-kb insert of pGEMT-LH5 contained and expressed a functional C23O enzyme, the *Nco*I/*Eco*RI-digested DNA segment was ligated onto the expression vector pET-22b(+) digested with the same endonucleases. A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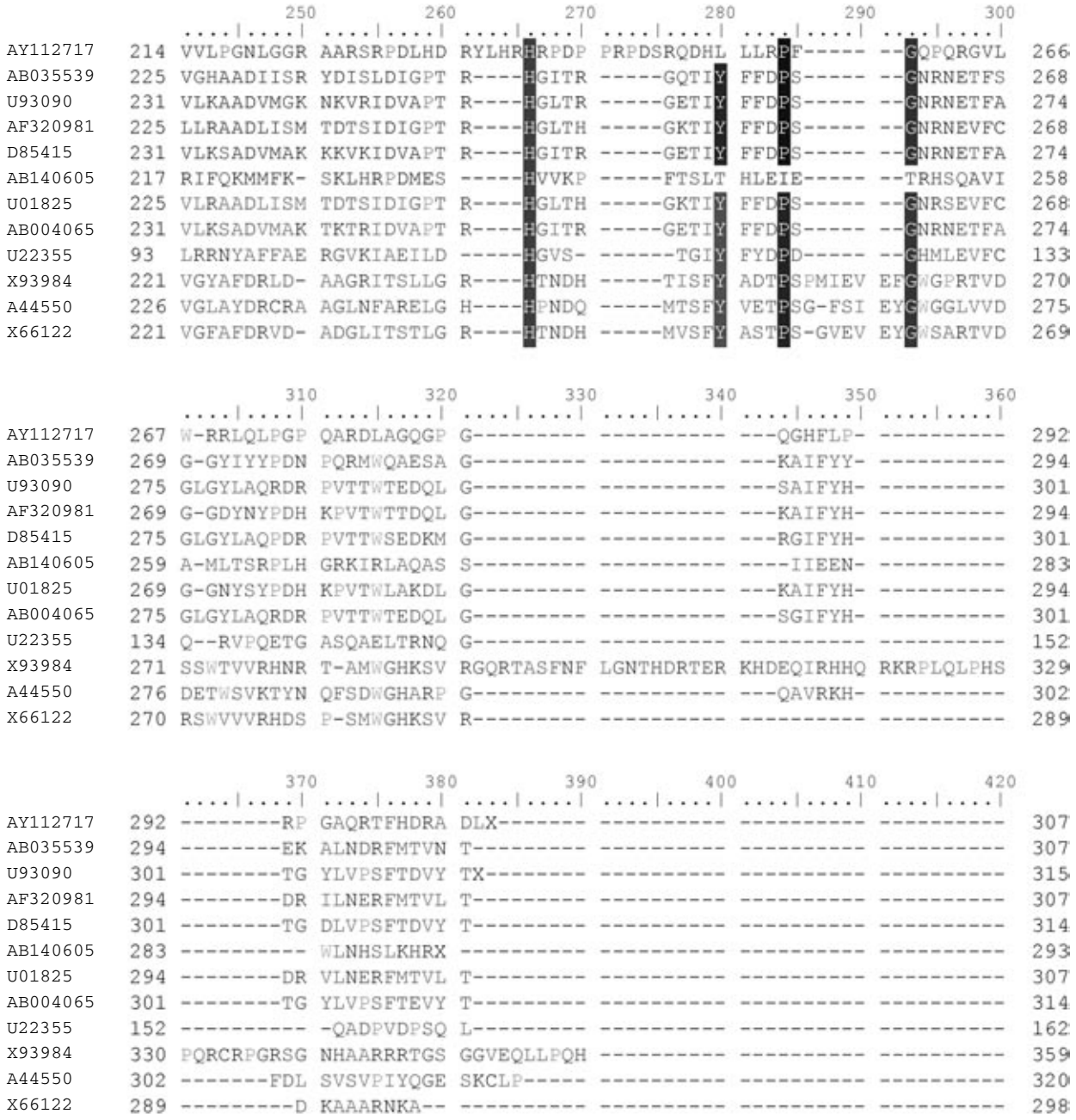

Fig. 3. (Contd.)

recombinant expression vector (designated pET-LH12) was identified by using *Nco*I and *Eco*RI as endonucleases to show whether the DNA fragment was on the expression vector. After the recombinant plasmid was isolated, it was transformed into the expression host *E. coli* BL21(DE3) for expression.

Nucleotide sequence analysis. To verify and analyze the *phe*B gene, the 0.9-kb DNA insert of pGEMT-LH5 was completely sequenced. The complete nucleotide sequence and deduced amino acid sequence have been deposited in GenBank under accession number AY112717. A complete open reading frame, encompassing 924 bp and encoding a protein of 307 amino acids, was identified. The base composition of the cloned 0.9-kb fragment is 61.0% G+C. This value falls within the wide range of G+C content of mesophilic

Fig. 3. Alignment of deduced amino acids of 12 extradiol dioxygenases. Gaps were inserted for optimal alignment, and the highly homologous amino acid residues were shaded.

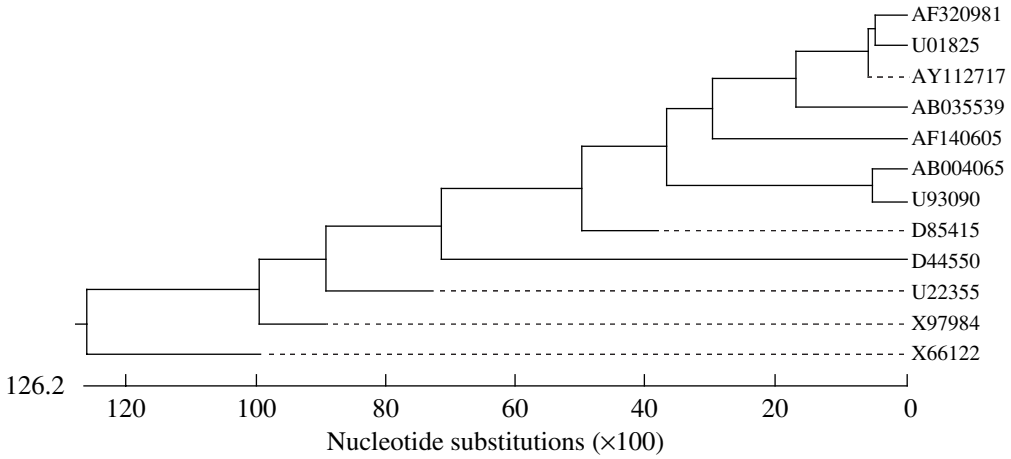

Fig. 4. The phylogenetic tree of 12 extradiol dioxygenases.

C23O in *Pseudomonas* bacteria but is very different from the C23O in thermophilic bacilli, which usually have a relatively low G+C value [14]. The deduced amino acid sequence of the *phe*B gene has a theoretically calculated molecular mass of 37 kDa.

In order to investigate the relationship between the structure and function of C23O encoded by the *phe*B gene, alignment was performed by comparing the homology between the *phe*B deduced amino acid sequence and the other extradiol dioxygenases using the BioEdit software (Fig. 3). The alignment analysis showed that 12 areas of homology exist in this group of sequences, and these areas of homology are mainly distributed near the C end and the N end. Eltis and Bolin reported that the middle strictly conserved residues of extradiol dioxygenases probably constitute the active site residues and the other strictly conserved residues remote from the active site are likely to play structural or folding roles [15]. Based on the three-dimensional crystal structure of BphC-LB400 and BphC-kks102, we speculated that the strongly conserved residues Gly-31, His-157, and Leu-184 were likely to be the active sites of the extradiol dioxygenases in this study. In addition, pairwise alignment scores between *phe*B and the other extradiol dioxygenases were as follows: XylE (AF320981), 0.807; CatA (AB035539), 0.447; TdnC (D85415), 0.283; AlnE (AB004065), 0.285; CdoE (U93090), 0.294; pheB (AF140605), 0.298; bphE (U01825), 0.848; bphC (U22355), 0.117; bphC (X97984), 0.162; bphC (X66122), 0.190; and pcbC (D44550), 0.152. The homology values revealed that the C23O shared low homology (<0.2) with the bicyclic cleaving extradiol dioxygenases (U22355, X97984, X66122, D44550).

Activity of C23O in *E. coli* BL21 and wild-type strain ZD 4-3

Strains	Activity of C23O, μmol/(min mg)	
	culture supernatant	crude lysate
E. coli BL21 (containing ET-LH12)	0.0237	3.5617
strain ZD 4-3	0.0831	0.4520
E. coli BL21	n.d.	n.d.

Note: n.d., not detected.

A phylogenetic tree was constructed using DNAstar to show the evolutionary relationship among the extradiol dioxygenases (Fig. 4). As shown in Fig. 4, the extradiol dioxygenases cleaving bicyclic substrates (U22355, X97984, X66122, D44550) exhibited a remote genetic distance from the C23Os preferentially cleaving monocyclic substrates. The evolutionary analysis was consistent with their homology values data, suggesting that a distinct difference existed between the C23O in this study and the extradiol dioxygenases cleaving bicyclic substrates. This result was in agreement with the proposal of Harayama [16] that the extradiol dioxygenases could be divided into two families: those showing a preference for bicyclic substrates and those showing a preference for monocyclic substrates. Also, we found that no significant differences existed between the amino acids of the C23Os and the thermophilic C23O (pheB, AF140605).

C23O gene expression and activity in *E. coli* strains. The crude lysate supernatant and culture supernatant of *E. coli* containing pET-LH12 were used for the C23O enzyme assay, and the C23O activity was also compared with that in wild-type strain ZD 4-3 (table). It can be seen from the table that, in the crude lysate extract, the C23O activity of *E. coli* was much higher than that in the wild-type strain ZD 4-3. Moreover, it can be inferred that, in both wild-type strain ZD 4-3 and *E. coli*, the C23O activity in the crude lysate supernatant was higher than that in the bacterial culture supernatant.

In brief, it can be demonstrated that the cloned C23O gene from the *P. aeruginosa* strain ZD 4-3 is a functional

gene and has a high activity in the *E. coli* expression system. The high C23O activity in *E. coli* facilitated the forward transgenetic and also the enzyme purification operation for further analysis of C23O features.

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