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Nucleotide Sequence of the *Pseudomonas* sp. DJ77 *phnG* Gene Encoding 2-Hydroxymuconic Semialdehyde Dehydrogenase

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The nucleotide sequence of a 1520 bp region, spanning the coding region for the *meta*-cleavage pathway enzyme, 2-hydroxymuconic semialdehyde dehydrogenase, was determined. This enzyme, encoded by the *phnG*, is the first of three sequential enzymes required for conversion of 2-hydroxymuconic semialdehyde, which is produced from catechol by the PhnE catechol 2,3-dioxygenase, to 2-hydroxypent-2,4-dienoate in the dehydrogenative branch of the pathway. The deduced protein sequence is 484 amino acid residues long with a M_r of 51504. The *phnG* has a high degree of homology with genes encoding isofunctional proteins from other *Pseudomonas* strains. We now show that the relative position of the *phnG* dehydrogenase gene in the *phn* operon is unique compared to the other *meta*-cleavage operons which have a dehydrogenative branch of the pathway.

The *meta*-cleavage pathway is versatile, being used in the dissimilation of a range of substituted catechols. Part of this metabolic versatility is due to divergence of the *meta*-pathway after ring cleavage of catechol to form *meta*-cleavage products (MCPs) such as 2-hydroxymuconic semialdehyde (HMS). This MCPs are processed through either a hydrolytic (H) or a dehydrogenative (D) route. Both branches converge with the formation of 2-hydroxypent-2,4-dienoate (HPD). The H branch converts the MCPs directly to HPD through the activity of HMS hydrolase (HMSH). The D path converts the MCPs to enol form of 4-oxalocrotonate by NAD^+-dependent HMS dehydrogenase (HMSD). The enol form of 4-oxalocrotonate is then converted to HPD through the successive activities of an isomerase and a decarboxylase [1].

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The metabolic roles of these two branches of the *meta*-cleavage pathway have been elucidated. The MCP of catechol and 4-methylcatechol are metabolized primarily by the 4-oxalocrotonate (D) branch because the affinity of the dehydrogenase (HMSD) towards these compounds, containing an oxidizable aldehyde group, is much higher than that of the hydrolase (HMSH) [2,3]. The H pathway is devoted to the catalysis of the MCP of 3-methylcatechol (a ketone). The rate of this reaction is significantly greater than that occurring with the alternative aldehyde substrates [3,4].

Up to now, several HMSDs were discovered from various strains including *Pseudomonas putida* CF600 [5], *P. putida* mt-2 [6], *P. putida* NCIB9816 [7,8], and *Pseudomonas* sp. IC [9], and the genes encoding these isofunctional enzymes were designated as *dmpC*, *xylG*, *nahI*, and *bphG*, respectively. It is interesting that both the biochemical route and the gene organization of these *meta*-cleavage pathway operons are identical with each other and all of genes are encoded by the large plasmids which were found in each strain as the name of pVI150, pWW0, NAH7, and pWW110, respectively. In addition, these operons are able to encode both D and H enzymes.

We have undertaken a detailed study of the *meta*-cleavage pathway of phenanthrene-utilizing *Pseudomonas* sp. strain DJ77 [10] which is able to use both H and D routes, too. The genes responsible for the *meta*-cleavage pathway has been cloned [11], this cloned 6.8-kb *Xho*I fragment of chromosomal DNA for HSMH (*phnD*), catechol 2,3-dioxygenase (C23O; *phnE*), unknown polypeptide (*phnF*), and HPDH (*phnH*) have been mapped, and their nucleotide sequences have been determined [12,13,14,15]. In this paper we define the precise location and hitherto unknown nucleotide sequence of the *phnG* gene encoding an HMSD. We have also compared the sequences and the gene order between those operons which are able to use both H and D branches of the pathway and analyzed evolutionary

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relationships among dehydrogenases which are involved in the *meta*-cleavage pathway.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *Pseudomonas* sp. strain DJ77 [10] is a natural isolate capable of degrading phenanthrene, biphenyl and 4-chlorobiphenyl. The recombinant plasmid pHENX7 [11] contains the *phnDEFGH* genes, which are involved in the phenanthrene degradation pathway from *Pseudomonas* sp. strain DJ77. Isolation of plasmid DNA, transformation, restriction endonuclease digestion, ligation, agarose gel electrophoresis and other standard recombinant DNA techniques were performed as described by Sambrook et al. [16].

Nucleotide sequencing, sequence analyses, and phylogenetic analysis. Nucleotide sequences were determined directly from plasmids by using the Applied Biosystems automated DNA sequencer (Version 2.1.0). Suitable subcloning of various fragments constructed from pHENX7 into the polycloning site of the pBluescript SK (+) sequencing vector allowed sequencing of both strands. Plasmid DNAs were purified by standard procedures using Qiagen Plasmid Kit (Qiagen Co., Cat. No. 12125). The sequence of the *phnG* gene was deposited in the GenBank database under the accession number AF023839. Sequence analyses and phylogenetic analysis were carried out as described previously [15]. The EMBL and GenBank database accession numbers for the sequences used as reference sequences for analyses were as follows: *P. putida* CF600 (*dmpC*), X52805; *P. putida* mt-2 TOL plasmid pWW0 (*xylG*), M64747; *Cycloclasticus oligotrophus* (*xylG*), U51165; *E. coli* ATCC11105 (*hpaE*), Z37980; *E. coli* C (*hpcC*), X81322; *Pseudomonas* sp. IC (*bphG;* 5′ partial sequence), U01825.

RESULTS AND DISCUSSION

The *phnG* gene of the *Pseudomonas* sp. DJ77 *meta* operon have been previously localized by partial sequencing [11,15]. In the present study, we generated subclones containing defined fragments within this region and determined the sequence of 1520 bp of DNA extending from downstream of *phnF* [14] to immediate upstream of *phnH* gene [15].

The complete nucleotide sequence of the *phnG* gene is shown in Fig. 1 along with translation of open reading frame (ORF) that lie in an operon structure together with the HPDH gene. The deduced amino acid sequence indicates that the protein contains 484 amino acids with M_r 51504. The putative ribosome binding site, 5′-GGA-3′, is followed by an open reading frame extending from the methionine codon at bp 50 to a TAA translational stop codon at bp 1504 which is 5 bp upstream of the initiation codon of *phnH*. Interestingly, when the locations of stop condon of *phnG* gene is compared with downstream RBS of *phnH,* overlap is observed (Fig. 1). Considering that the *phnG* gene is solely transcribed from the *meta*-operon promoter, which is located at immediate upstream of *phnD* [12,15], the translational stability of a message may be partly accomplished by the coincidence of translational stop codon and contiguous downstream RNA. This arrangement would tend to reduce dissociation of ribosomes from the transcript before downstream genes could be translated.

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5'CGTAGGTAATATGCACCAATGACTTCCGTCTCCTCCTCTCCAATCACGGATGACTATCCTGAACTTCATCGACGGGTCCT   80
                                        phnG:M  T  I  L  N  F  I  D  G  S
ATCGCGAAGGCAGCGAGGGCAAGTCGTTTTCCAACGTCAATCCGGCCACCGGGGGCCGAGATCGGGGTTGTGCACGAAGCA  160
 Y  R  E  G  S  E  G  K  S  F  S  N  V  N  P  A  T  G  A  E  I  G  V  V  H  E  A
AGCCAGGCAGAGGTCGAAGACGCCGTGGAGGCCGCCAAGGCCGCGCTTACCGGGCCGTGGGGCAAGATGACCACGGCCCG  240
 S  Q  A  E  V  E  D  A  V  E  A  A  K  A  A  L  T  G  P  W  G  K  M  T  T  A  R
AACGGGTCAAGCTGATCACCGCGTGGCGACCGAGATCGAACGCCGAGCGGATGATTTCCTGGCTGCCGAAGTGGCCGACA  320
   T  G  Q  A  D  H  R  V  A  T  E  I  E  R  R  A  D  D  F  L  A  A  E  V  A  D
CCGTGAAGCCGCGTCATGTTGTGTCGCATATCGATATTCCGCGCGGAGCCGCTAACTTCCGCATGTTCGCCGATGTCGTC  400
 T  V  K  P  R  H  V  V  S  H  I  D  I  P  R  G  A  A  N  F  R  M  F  A  D  V  V
TCGACGATGCCGGGAGAAAGCTTCAACACGCCAACCCCCGATGGCGGCCAGGCGTTCATCTATACCGTGAGCAAGCCCAA  480
 S  T  M  P  G  E  S  F  N  T  P  T  P  D  G  G  Q  A  F  I  Y  T  V  S  K  P  K
GGGTGTGGACGGCCGCCGTCTGTCCGCGGAACTTCCCGCTGCTGCTGATGATCTGGAAGGTTGGACCTGAGCTTGCTTGCG  560
   G  V  D  A  A  V  C  P  R  N  F  P  L  L  L  M  I  W  K  V  G  P  E  L  A  C
GTAATACCGCGGTGGTCAAACCGTCCGAGGAAATCGCTCGAACCGCTGCCCTACTGGGCGATGTGATAGACGCGGGTGTC  640
 G  N  T  A  V  V  K  P  S  E  E  I  A  R  T  A  A  L  L  G  D  V  I  D  A  G  V
AATCACCATGGTGTCTTCAACGTCGCCCAACGATTCGGTCCGGCTTCGGCGGGAGAATTCCTCACGTCCAACCCCGATGT  720
 N  H  H  G  V  F  N  V  A  Q  R  F  G  P  A  S  A  G  E  F  L  T  S  N  P  D  V
CGATGCCATCACCTTCACCGGCGAAACCGGCACCGGACAGGCCATCATGCAGAAGGCCGCGACCGGCGTTCGCGACATCT  800
   D  A  I  T  F  T  G  E  T  G  T  G  Q  A  I  M  Q  K  A  A  T  G  V  R  D  I
CGTTCGAACTCGGTGGCAAGAACCCGGCGATCGTGTTCGCCGATGCCGACCTCGACAAAGCGGTCGAGGGTCTGTCGCGC  880
 S  F  E  L  G  G  K  N  P  A  I  V  F  A  D  A  D  L  D  K  A  V  E  G  L  S  R
TCGGTCTTCCTGAACACCGGGCAGGTCTGCCTCGGAACCGAGCGGGTCTATGTCGAACGGCCGATCTTCGATGCCTTCGT  960
 S  V  F  L  N  T  G  Q  V  C  L  G  T  E  R  V  Y  V  E  R  P  I  F  D  A  F  V
GGCGCGGATGGCGGCGGCGGCGCAGGACTTCAAGCCGGGCGTGACCGGTGATCGCGCCTATCTCGGCCCTCTGATCAGCG 1040
   A  R  M  A  A  A  A  Q  D  F  K  P  G  V  T  G  D  R  A  Y  L  G  P  L  I  S
CCGAGCACCGCGAGAAAGTCCTGGCCTACTATCCCCGTGCGGTCGAGGACGGGCCCACCGTGTTCACCGGCGGCGGCGTT 1120
 A  E  H  R  E  K  V  L  A  Y  Y  P  R  A  V  E  D  G  P  T  V  F  T  G  G  G  V
CCTGAAATCTCGGGCGCGGAAACCGGCGGCTTCTTCGTGGAACCGACGTTGTGGATCGACGTCGCCCACGGCGACACCGT 1200
 P  E  I  S  G  A  E  T  G  G  F  F  V  E  P  T  L  W  I  D  V  A  H  G  D  T  V
GATGCGCGAGGAAATCTTCGGGCCGTGCTGCGACATCTTACCGTTCGACAGCGAGGACGAGGTGATCGCGCTGGCAAACG 1280
   M  R  E  E  I  F  G  P  C  C  D  I  L  P  F  D  S  E  D  E  V  I  A  L  A  N
ATACGGTATACGGCCTGTGCGCCTCAGTCTGGACCGAAAACCTGTCCCGCGACACCGCGTGGCGGCGGCGATGGAGGTG 1360
 D  T  V  Y  G  L  C  A  S  V  W  T  E  N  L  S  R  G  H  R  V  A  A  A  M  E  V
GGGGTGTGCTGGGTCAATTCCTGGTTCCTGCGCGATCTGCGCACGGCTTTCGGCGGGTCCGGCCATTCCGGCATAGGCCG 1440
 G  V  C  W  V  N  S  W  F  L  R  D  L  R  T  A  F  G  G  S  G  H  S  G  I  G  R
GGAAGGCGGGGTGCACAGCCTCGAATTCTACACCGAGATCACCAACATCTGCGTAAAGCTTTAAGACGATGACTATCGAC 3' 1520
 E  G  G  V  H  S  L  E  F  Y  T  E  I  T  N  I  C  V  K  L  *  phnH:M  T  I  D
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FIG. 1. Nucleotide sequence of the *phnG* gene encoding HMSD (GenBank accession number AF023839). Amino acid sequences are given in their one letter code with asterisk indicating stop codon. Underlined sequences indicate putative ribosome binding sites. The end of the following *phnH* (GenBank accession number U97697) gene of the pathway, encoding HPDH, is also shown.

The G + C content of the newly identified *phnG* gene (63.3%) is slightly higher than those of the *phnD* (60.5%), *phnE* (57.4%), and *phnH* (62.4%) gene encoding an HMSH, C23O, and HPDH, respectively, with the exception of *phnF* (67.1%) encoding unknown polypeptide.

A Computer-assisted search using Blast E-mail server [17] was conducted to find sequence similarities between HMSD and other proteins in the database. This enzyme was found to have more than 35% identity to a number of MCPs dehydrogenases from a variety of sources. This confirms that the PhnG protein is an MCP dehydrogenase. This enzyme is more closely related to HMSD of the phenol-degrading pathway in *P. putida* CF600 (DmpC, [5]), and isofunctional protein in the TOL pathway of *P. putida* mt-2 (XylG, [6]), which are also able to utilize both D and H branches of the pathway, and a 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate dehydrogenase (HMODD) of xylene-degrading pathway in *Cycloclasticus oligotrophus* RB1 (XylG, [18]), being 61.9%, 58.8%, or 58.6% identical, respectively. In this respect, this high level of sequence identity reflects that the PhnG dehydrogenase is active on the MCPs (HMS, and HMOD, respectively) from cate-

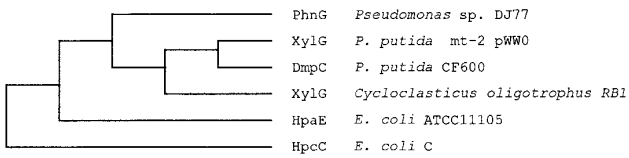

FIG. 2. Phylogram of the best tree obtained by PAUP analyses of alignment of 6 dehydrogenase sequences. HpcC and HpaE were used as an outgroup. The bootstrap analyses show that these clades are very stable (bootstrap values over 93%, results not shown).

chol and 4-methylcatechol formed by the PhnE, C23O [13], and *Pseudomonas* sp. DJ77 might utilize the PhnG dehydrogenase in the metabolic pathways of *p*-xylene and 4-methylbiphenyl or *p*-toluate.

The PhnG dehydrogenase is more distantly related to the sequenced 5-carboxy-2-hydroxymuconic semialdehyde dehydrogenase (CHMSD), and 5-carboxymethyl-2-hydroxymuconic semialdehyde dehydrogenase (CMHMSD) from *E. coli* ATCC11105 (HpaE, [19]), and *E. coli* C (HpcC, [20], being 37.4%, and 36.7% identical, respectively. These enzymes are known to be involved in the degradation of 4-hydroxyphenylacetate or homoprotocatechuate.

These differences are clearly shown in the phylogram obtained by PAUP analysis (Fig. 2), the *Pseudomonas* sp. DJ77 (PhnG), *P. putida* CF600 (DmpC), *P. putida* mt-2 (XylG) and *Cycloclasticus oligotrophus* RB1 (XylG) are actually in evolutionary terms closely related.

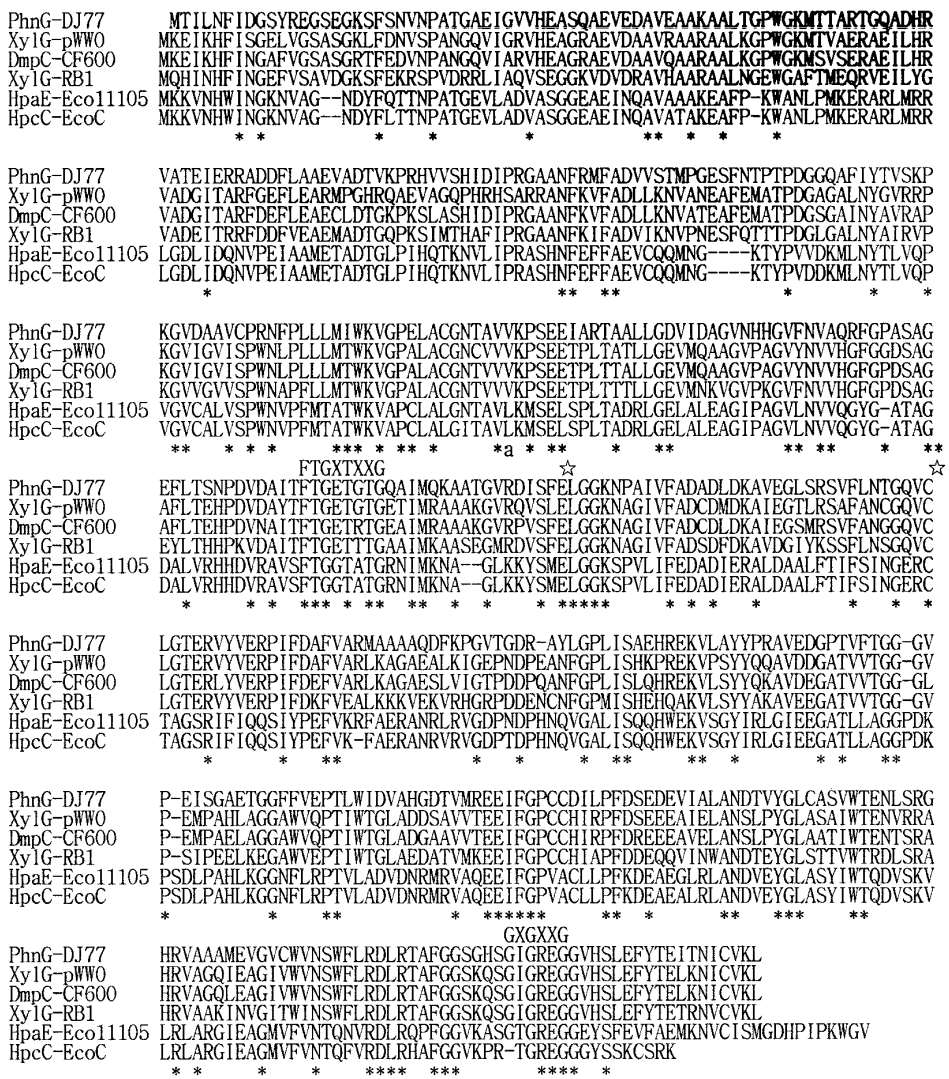

FIG. 3. Amino acid sequence comparison of PhnG with other dehydrogenases from various sources. The amino acids identical in all proteins are indicated by asterisks. References for individual sequences are given in the materials and methods. FTG(S)TXXG and GXGXXG mark the segment expected in the NAD^+ binding site [5,22,24] and a star indicates the location of conserved cysteine and glutamate residues that is believed to be important for the catalytic activity of aldehyde dehydrogenases [6,21,22,23]. The full sequences of the NahI and BphG dehydrogenases have not yet been reported.

Alignment of the MCPs dehydrogenases primary sequences of known prokaryotic organisms (Fig. 3) demonstrates significant shared homology, indicating that the common activities of these enzymes results from shared structural features. Amongst all 6 enzymes shown in the alignment, 124 amino acids were found to be completely conserved. Among these conserved amino acids the highly conserved residues of the aldehyde dehydrogenase family, Cys-302 and Glu-268 (the numbering system refers to the human cytosolic ADH), which have been implicated as essential residues for actual catalytic nucleophile or a charge relay network [6,21,22,23], are identical in all six sequences compared. These two active sites are derived from a sequence alignment of various classes of ADH, mainly from mammalian ADH sequences although Glu is little bit shaky because some of the ADH sequences do not have Glu in the sequences [22]. Two segments, (F/Y)TG(S)(T)XX(G), and GXGXXG, which are considered as putative NAD^+ binding region are also shown [5,22,24].

In other studies, the gene order of the catabolic operon of NAH7 for salicylate oxidation was determined to be: promoter-*nahG* (the structural gene for salicylate hydroxylase)-*nahH* (C23O)-*nahI* (HMSD)-*nahN* (HMSH)-*nahL* (HPDH) [8]. Interestingly this order is identical to that of the isofunctional genes of TOL plasmid pWW0 [25], phenol plasmid pVI150 [5,26,27], and biphenyl plasmid pWW110 [9]. These results were regarded as strong evidence that they have a shared ancestry and constitute a tightly clustered evolutionary unit and therefore evolved in a modular fashion [9,28].

However, in the strain DJ77, the relative position of the dehydrogenase gene, *phnG*, in the *phn* operon, is unique compared to those of the other four *meta*-cleavage operons, *dmp* [5], *xyl* [6], *nah* [7,8], and *bph* [9] (Fig. 4). The PhnG dehydrogenase gene is located immediately 5 bp upstream of the PhnH hydratase gene and 66 bp downstream of PhnF polypeptide (function unknown), while the equivalent dehydrogenase gene of the *xyl/dmp/nah/bph* operons is preceded by the C23O gene and followed by the hydrolase gene (in order of C23O-HMSD-HMSH-HPDH).

There is also *meta* pathway where the gene order is different. The order of the *tbu meta* pathway sequence for toluene/benzene/phenol catabolism in *P. pickettii* PKO1 differs: promoter-*tbuE* (C23O)-*tbuF* (HMSH)-*tbuG* (HMSD)-*tbuK* (4-hydroxy-2-oxovalerate aldolase)-*tbuI* (4-oxalocrotonate decarboxylase)-*tbuH* (4-oxalocrotonate isomerase)-*tbuJ* (HPDH) [29]. And it is noteworthy that the gene arrangement of the *cmp* operon (HMSH-C23O-ORF-HMSD) from *Pseudomonas* sp. HV3 plasmid pSKY4 [30] which appears to be involved in naphthalene, salicylate and *m*-toluate catabolism is distinct from the operons of *xyl/dmp/nah/bph/tbu* [9]. Rather it is identical to that of *phn* operon. Therefore although the order for the other *meta* pathway genes of *cmp* was not determined, this is an another example showing the MCPH-EDO-ORF-HMSD gene order. In this respect, these seven *meta* pathways may therefore represent either very distant divergences or possibly examples of convergent evolution.

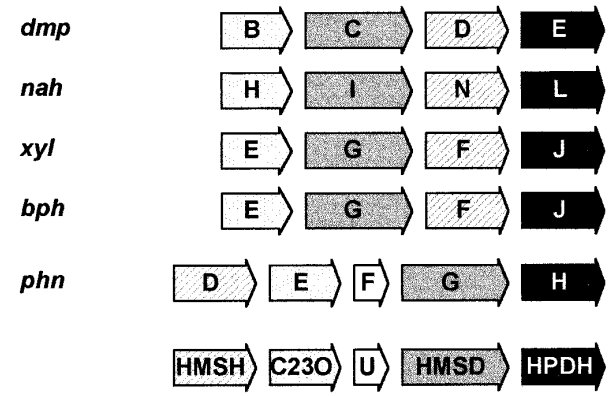

FIG. 4. Differences in the genetic organization of the *phn* operon from the other four operons, which can utilize both hydrolytic (H) and dehydrogenative (D) routes of the *meta*-cleavage pathway, such as *dmp, nah, xyl,* and *bph*. Only the genes analyzed in this study are shown. Arrows indicate the direction of transcription. Abbreviations are as follows: *dmp* is from *P. putida* CF600 plasmid pVI150 [5]; *nah* from *P. putida* NCIB9816 [7,8]; *xyl* from *P. putida* TOL plasmid pWW0 [6]; *bph* from *Pseudomonas* sp. IC [9]; HMSH, 2-hydroxymuconic semialdehyde hydrolase; C23O, catechol 2,3-dioxygenase; U, function unknown polypeptide; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase; HPDH, 2-hydroxypent-2,4-dienoate hydratase.

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