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Cloning, Sequencing and Comparison of Genes for early Enzymes of the Protocatechuate (*ortho*-Cleavage) Pathway in *Pseudomonas putida*

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Abstract : The major portions of two DNA fragments, one from degradative plasmid, pRA4000 from *Pseudomonas putida* NCIMB 9866, and the other from degradative plasmid, pRA500 from *P. putida* NCIMB 9869, which harbor the structural genes for the flavoprotein (*pchF*) and cytochrome (*pchC*) subunits of *p*-cresol methylhydroxylase (PCMH), have been sequenced. The DNA and deduced amino acid sequences for *pchC* and *pchF* have been published.[15] In these fragments, a coding region (*dhal*) for an aldehyde dehydrogenase has been identified. It is proposed that this gene encodes for the aldehyde dehydrogenase which converts *p*-hydroxybenzyaldehyde to *p*-hydroxybenzoate. *p*-Hydroxybezealdehyde is the product of oxidation of *p*-cresol by PCMH. The fragment from *P. putida* 9869 also harbors the genes for the α (*pcaG*) and β (*pcaH*) subunits of protocatechuate 3,4-dioxigenase. The fragment from 9866 does not have any portion of these genes in the corresponding region A possible open reading frame (ORF) between *pchC* and *pchF* is seen for both clones, and a second putative open reading frame (ORF') also exists in the 9866 clone. The gene organizations are *dhal-pchC-ORF-pchF-pcaGH* for the DNA fragment from 9869, and *ORF'-dhal-pchC-ORF-pchF* for the DNA fragment from 9866.(Received June 19, 1996; accepted September 23, 1996)

Introduction

A fair amount is known concerning the biochemistry and molecular biology of the enzymes in the bacterial pathway for p-hydroxybenzoate degradation. Seven enzymes are required for its conversion to succinyl-CoA, acetyl-CoA, and compounds that feed into the citric acid cycle. The seven enzymes are p-hydroxybenzonate hydroxylase (PHBH), protocatechuate 3,4-dioxygenase (PcaG and PcaH subunits), β-carboxymuconate lactonizing enzyme (PcaB), γ-carboxymuconolactone decarboxylase (PcaC), β-ketoadipate enol-lactone hydroxylase (PcaD), β-ketoadipate succinyl-CoA transferase (PcaE) and β-ketoadipyl-CoA thiolase (PcaF). In *Acetobacter calcoacticus* the structural (*pca*) genes coding for the last six of these enzymes are organized in a cluster (*pcaEFDBCHG*) on the chromosome,[3,21] where *pcaG* codes for the α subunit and *pcaH* for the β subunit of protocatechuate 3,4-dioxygenase. This organization varies between species. For example, in *P. putida, pcaE, pcaF* and *pcaBCD* are separated from each other on the chromosome and are transcribed separately, although they are under positive control of the transcriptional activator, *pcaR*.[7,21] The organization of pcaBDC is different from that of A. calcoacticus. Additionally, genes coding for enzymes with the same activity as those produced by *pcaEFG* in *A. calcoaceticus*, are duplicated in the cat gene cluster (*catEFD*) which encodes for enzymes required for catechol catabolism in the organism.[19] The *catEFD* cluster does not exist in *P. putida*, so for the catabolism of catechol, this organism makes use of *pcaE* and *pcaD*. So far, various other aspects of the regulation of these genes have been known.[7,19,21]

A number of pca genes have been cloned and sequenced.[19,21] The *pdbA* gene for PHBH from *A. calcoaceticus*,[2] and from two species of *Pseudomonas* have also been cloned and sequenced.[4,18] *PobA* is about 5 Kbp downstream from the *pcaG* locus of the *pcaEFDBCHG* cluster in *A. calcoaceticus*, and together likely constitute members of a supraoperonic cluster.[8]

In contrast, less is known about these aspects of enzymes which produce *p*-hydroxybenzonate from more reduced compounds. The most reduced analog of *p*-hydroxybenzonate is toluene, however, this compound is usually metabolized via an extradiol dioxygenase pathway, for which *p*-hydroxybenzonate is not an intermediate (*meta*-cleavage pathway involving the en-

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zymes are encoded by *xyl* genes).[19] The next most reduced species are benzyl alcohol, *o*-cresol, *m*-cresol, and *p*-cresol (2-, 3- and 4-methylphenol, respectively). Benzyl alcohol is oxidized to benzonate, which is converted to catechol. *o*-Cresol and *m*-cresol are converted to 3-methylcatechol by toluene monooxigenase, and 3-methyl catechol feeds into the meta cleavage pathway.[6] On the other hand, *p*-cresol is oxidized to *p*-hydroxybenzonate by the combined action of *p*-cresol methylhydroxylase (PCMH) and a specific NAD^+-linked aldehyde dehydrogenase.[7,14] A bezaldehyde dehydrogenase of this type has never been isolated. Interestingly, *P. mendocina* KR1 is capable of converting toluene to *p*-cresol by toluene-4-monooxigenase.[20]

PCMH oxidizes *p*-cresol to *p*-hydroxybenzyl alcohol, and then to *p*-hydroxybenzaldehyde.[10] Although we have extensively studied the physical and biochemical properties of PCMH from various strains of *P. putida*, until recently, nothing was known of its molecular biology. We have cloned and sequenced the structural genes of the α flavoprotein (PchF) and the β cytochrome c (PchC) subunit of this enzyme from *P. putida* NCIMB 9869 and NCIMB 9866. Several open reading frames, in the cloned DNA fragments harboring, *pchF* and *pchC* have been identified.[15] This report focuses on the identity of these coding regions.

Materials and Methods

Materials

Ampicilin, bromophenol blue, CsCl, Tris, EDTA, SDS Acrylamide and ammonium persulfate were purchased from Sigma Chemical Co., St. Louis, MO. Agarose and restriction enzymes were obtained from Gibco/BRL., Life technologies, Gaitherburg, MD.

Cloned DNA

The details for cloning, identifying cloned fragments of DNA from plasmid pRA4000 from *P. putida* 9866 and plasmid pRA500 from *P. putida* 9869, construction of gene library, etc., are described previous paper.[15] For sequencing genes from *P. putid* 9866, clone 12[15] which consist of 6.2 kb *SstI* fragment from plasmid pRA4000[13] was digested with *SstI* and *SmaI*. The resulting 2.9 kb *SstI-SmaI* fragment and the 3.3 kb *SmaI-SstI* fragment were sequentially subcloned into the pUC18. A 3.0 kb *SmaI* fragment from *P. putida* 9869, which containing *pchC, pchF* and the 3' end of the putative aldehyde dehydrogenase (*dhal*) gene was sequenced in both direction (Fig. 1). Only the first 620 bases of the sense strand of 1.6 kb *SmaI* subfragment, harboring *pcaH* and *pcaG*, could be sequenced due to instability of the nested deletion insert for the following region. The antisense strand of the 1.6 kb *SmaI* subfragment was also sequenced until the unstable region was reached.

DNA sequencing

Plasmids with progressive unidirectional deletions were constructed by exonucleaseIII digestion using Erase-a-base system from Promega Corp (Madison, WI). Double-stranded DNA sequencing was performed by dideoxynucleotide chain termination method[16] using Sequenase, Version 2.0 from United Biochemical, Inc. (Cleveland, OH). The sequence data were analyzed with the PCGENE group program (Intelli- Genetics, Inc., Mountain View, CA).

Results and Discussion

The entire 6.2 kb SstI fragment from plasmid pRA 4000[13] of *P. putida* harboring *pchC* and *pchF* was sequenced. The entire 3.0 kb *SmaI* subfragment, and 620 bases of the coding strand and 320 bases of the noncoding strand of a *SmaI* subfragment of a *SstI-HindIII* fragment from plasmid pRA500[13] were sequenced (Fig. 1). The gene organization of the 9866 and 9869 region are identical, except that the far end of the 9866 fragment lacks a coding region found at the far end of 9869 fragment.

In both the *P. putida* 9866 and 9869 clones, between *pchC* and *pchF*, resides a putative open reading frame (ORF) that is not homologus with any protein in several databases. This region was sequenced several times in both directions. The nucleotide and the presumed, translated amino acid sequences for ORF for both clones is provided in Fig. 2.

Near the far end of the 9869 clone, the beginning of an ORF was easily identified as coding for *pcaH*. Its DNA and translated amino acid sequences are displayed in Fig. 2. *PcaH* follows *pchG* by about 200 bp. A putative ribosomal binding site (RBS) was found at nucleotides 4229 to 4234 (Fig. 2). A signal sequence was

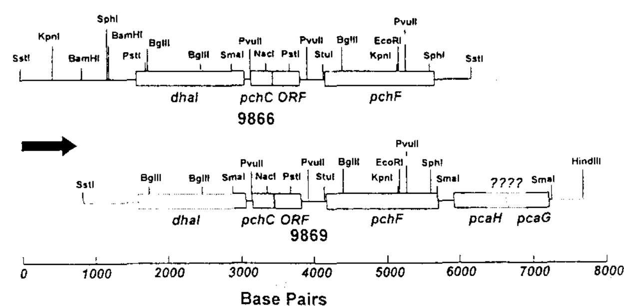

Fig. 1. The organization of the genes neighboring *pchC* and *pchF*. 9866 and 9869 denote fragments cloned from the degradetive plasmids, pRA4000 from *P. putida* NCIMB 9866, and pRA500 from *P. putida* NCIMB 9869. The region marked by ???? indicates the locus that contains the unstable DNA. Fragments isolated from this region were unstable in the sequencing plasmid. The arrow indicates the direction of transcription.

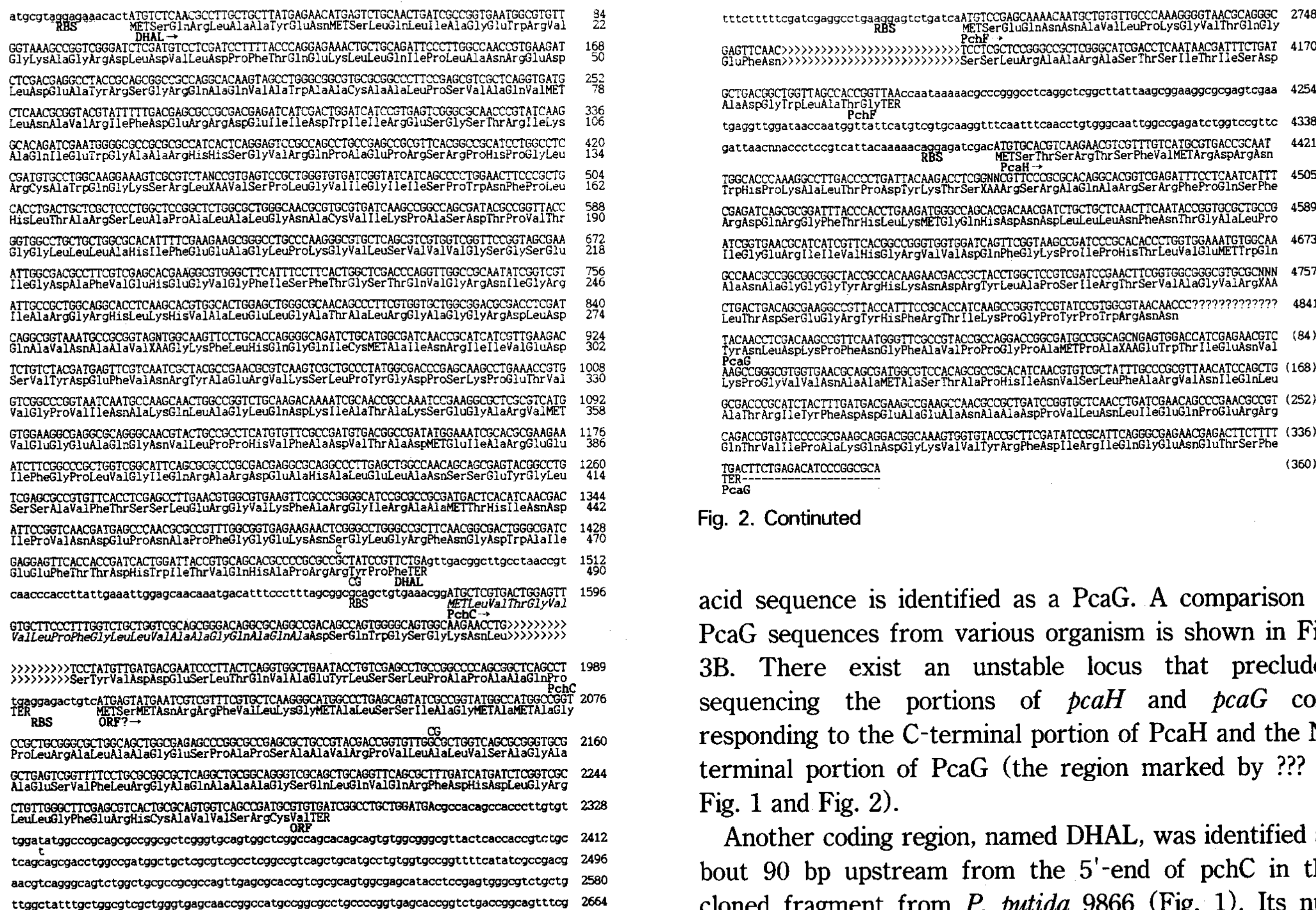

Fig. 2. Continued

Fig. 2. The nucleotide and translated amino acid sequence for reading frames depicted in Fig. 1.The first reading frame is that of the putative bezaldehyde dehydrogenase (*dhal*) determined for the clones from *P. putida* 9866 and 9869. The main nucleotide and translated amino acid sequences are those for the genes from the *P. putida* 9866 clone. Following the *dhal* sequence, the *pchC* and *pchF* are not displayed completely. Putative open reading frame (ORF) between *pchC* and *pchF*, for the cloned 9866 and 9869, vary by four nucleotides. Following these regions are the sequences for the *pchF* and *PchF* are denoted by the >>>>>symbols. Next is the reading frame for the 5'-end of *pcaH*, and the corresponding N-terminal portion of the deduced amino acid sequence for PcaH, only found in the *P. putida* 9869 clone. The following region, denoted by ????, contains the unstable portion of DNA described in the text. The last reading frame in this figure is for the 3' end of *pcaG*, shown with the corresponding C-terminal amino acid sequence for PcaG. The sites labeled RBS are putative ribosomal binding sites. TER indicates termination codons. Putative leader sequences of cytochrome are italicized.

not identified for PcaH, thus it is a cytoplasmic protein. A comparison of its partial, N-terminal amino acid sequences with that of PcaH from other species indicates that all are very similar (Fig. 3A). By comparison, tyrosyl groups that are iron ligands in this protein were located at position 105 and 144 in the protein sequence for the β subunit of the enzyme from *P. putida* 9869.

At the far 3' end of the 9869 clone, a region corresponding to the C- terminus of the deduced amino acid sequence is identified as a PcaG. A comparison of PcaG sequences from various organism is shown in Fig. 3B. There exist an unstable locus that precluded sequencing the portions of *pcaH* and *pcaG* corresponding to the C-terminal portion of PcaH and the N-terminal portion of PcaG (the region marked by ??? in Fig. 1 and Fig. 2).

Another coding region, named DHAL, was identified about 90 bp upstream from the 5'-end of pchC in the cloned fragment from *P. putida* 9866 (Fig. 1). Its nucleotide and deduced amino acid sequences are provided in Fig. 2. Using the method of Stormo *et al.*[17] in the PCGENE program SIGNAL, the RBS for this gene gave a score higher than any other RBS in the clone. A search of Protein Identification Resource (PIR) database demonstrated that the protein encoded by this gene is very similar to numerous NAD^+-dependent aldehyde dehydrogenases. Fig. 3C presents a comparison of selected aldehyde dehydrogenase and DHAL. All have characteristic "active site" glutanyl and cysteinyl residues[9] (position 248 and 281 in DHAL), and a putative NAD^+ binding domain located at DHAL residue 224~229 (Fig. 3C). The N-terminal sequences of three benzaldehyde dehydrogenases from the benzyl alcohol and mandelate pathway of *A. calcoaceticus*, and toluene pathway of *P. putida* have been published (1), however, there is no similarity between any of these sequences and the N-terminal sequence of DHAL.

Although we have no biochemical evidence, we speculate this DHAL is the specific aldehyde dehydrogenase needed to convert *p*-hydroxybezaldehyde to *p*-hydroxybenzonate and experiment is going on to prove this. If there is operonic control of the enzymes required for the catabolism of *p*-cresol, *via* the *ortho* cleavage pathway, to citric acid cycle intermediates, they should be clustered on the *P. putida* plasmids. Three enzymes of in this pathway are clustered on pRA

(A)

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PcaG-Psp-1  ??????????????????????????????????????????????????????????????????????
PcaG-Psp-2  MP---IELLPETPSQTAGPYVHIGLALEAAGNPTRDQEIWNRLAKPDAPGEHILLLGQVYDGNGHLVRDSFL   69
PcaG-PSEAE  -P---IELLPETPSQTAGPYVHIGLALEAAGNPTRDQEIWNRLAKPDAPGEHILLLGQVYDGDGHLVRDSFL   68
PcaG-ACICA  MNCWNFQELKETPSQTGGPYVHIGLLPKQANIEVFEHNLGNNLVQDNTQGQRIRLEGQVFDGLSLPLRDVLI   72
PcaG-PSECE  MTT-----LKQTPSQTVGPYFAYGLCPQQYGYDLKSL-FTPTIAAPHADGEHVLLVGQVFDGDGNVVSDAML   66

PcaG-Psp-1  ???????????????????YNLDKPFNGFAVPPGPAMPAXEW-TIENVKPGVVNAAMAST-APHINVSLFAR   51
PcaG-Psp-2  EVWQADANGEYQ-----DAYNLENAFNSFG-RTATTFDAGEW-TLHTVKPGVVNNAAGVPMAPHINISLFAR  134
PcaG-PSEAE  EVWQADANGEYQ-----DAYNLENAFNSFG-RTATTFDAGEW-TLHTVKPGVVNNAAGVPMAPHINISLFAR  133
PcaG-ACICA  EIWQADTNGVYPSQADTQGKQVDPNFLGWG-RTGADFGTCFW-SFNTIKPGAVPGRKGSTQAPHISLIIFAR  142
PcaG-PSECE  EFTQVDGAGRFPASRDDVA---KSGFTGFA-RVGTGTDAQHRFVVETVKPGRIAAD----EAPHINVTVMMR  130

PcaG-Psp-1  -VNIQLATRIYFDDEAEANAADPVLNLIEQPERRQTVIPAK--QDGK-VVYRFDIRIQGENETSF---  112
PcaG-Psp-2  GINIHLHTRLYFDDEAQANAKCPVLNLIEQPQRRETLIAKRCEVDGK-TAYRFDIRIQGEGETVFFDF  201
PcaG-PSEAE  GINIHLHTRLYFDDEAQANAKCPVLNLIEQPQRRETLIAKRCEVDGK-TAYRFDIRIQGEGETVFFDF  202
PcaG-ACICA  GINIGLHTRVYFDDEAEANAKDPVLNSIWATERRQTLVAKREERDGE-VVYRFDIRIQGENETVFFDI  209
PcaG-PSECE  GILTHAFTRVYFDDEAAANAADPVLNLVP-AERRATLVAKRDAQPGRPVVYRFDVRMQGPDETVFFDV  197
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(B)

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PcaH-PSP-1  MST-SRTSFVMRDRNWHPKALTPDYKTSK-RSPRQALSVIPQSFRDQRG--FT-HLKMGQHDNDLLLNFNTG   67
PcaH-PSP-2  MPAQDNSRFVIRDRNWHPKALTPDYKTSIARSPRQALVSIPQSISETTGPNF-SHLGFGAHDHDLLLNFNNG   71
PcaH-PSEAE  MPAQDNSRFVIRDRNWHPKALTPDYKTSIARSPRQALVSIPQSISETTGPNF-SHLGFGAHDHDLLLNFNDG   71
PcaH-ACICA  MSQIIWGAYAQSNTEDHPPAYRPGYKTSVLRSPKNRLISIAETLSEVTATHF-SADKFGPKDNDLILNYAKD   71
PcaH-PSECE  MDSP--TILTPRDWPSHPAYVHPDYRSSVKRGPTRPMIPLKERLRDQYAPVY-GADELGPLDHDLTKNAVKN   69

PcaH-PSP-1  ALPIGERIIVHGRVVDQFGKPIPHTLVEMWQANAGGGYRHKNDRYLAPSIRTSVAGVRXLTDSEGRYHFRTI  139
PcaH-PSP-2  GLPIGERIIVAGRVVDQYGKPVPNTLVEMWQANAGGRYRHKNDRYLAPLDPNFGGVGRCLTDSDGYYSFRTI  143
PcaH-PSEAE  -LPIGERIIVAGRVVDQYGKPVPNTLVEMWQANAGGRYRHKNDRYLAPLDPNFGGVGRCLTDSDGYYSFRTI  142
PcaH-ACICA  GLPIGERVIVHGYVRDQFGRPVKNALVEVWQANASGRYRHPNDQYIGAMDPNFGGCGRMLTDDNGYYVFRTI  143
PcaH-PSECE  GEPLGERIVVTGRVLDEGGKPVRNTLVEVWQANAAGRYVHKVDQHDAPLDPNFLGAGRCMTDAEGRYRFLTI  141

PcaH-PSP-1  KPGPYPWRNN????????????????????????????????????????????????????????????
PcaH-PSP-2  KPGPYPWRNGPNDWRPAHIHFGISGPSIATKLITQLYFEGDPLIPMCPIVKSIANPEVAQQLIAKLDMNNAN  215
PcaH-PSEAE  KPGPYPWRNGPNDWRPAHIHFGISGPSIATKLITQLYFEGDPLIPMCPIVKSIANPEVAQQLIAKLDMNNAN  214
PcaH-ACICA  KPGPYPWRNRINEWRPAHIHFSLIADGWAQRLISQFYFEGDTLIDSCPILKTIPSEQQRRALIALEDKSNFI  215
PcaH-PSECE  KPGAYPWGNHPNAWRPNHIHFSLFGDYFGSRLVTQMYFPGDPLLAYDPIFQGTPEA-ARDRLISRFSLDTTE  212

PcaH-PSP-1  ????????????????????????
PcaH-PSP-2  PMDCLAYRFDIVLRGQRKTHFENC   239
PcaH-PSEAE  PMDCLAYRFDIVLRGQRKTHFENC   237
PcaH-ACICA  EADSRCYRFDITLRG--RADLLRK   237
PcaH-PSECE  EGHALGYEFDIVLRGRDATPMER-   235
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(C)

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DHAL-PSE66  M-------------------------------SLQLIAGEWRVGKAGRDLDVLDPFTQEKLLQIPLAN   37
DHAL-ASPNI  M-----------SDLFTTIETPVIK--YEQPLGL----FINNEFVKGVEGKTFQVINPSNEKVITSVHEAT   54
DHAL-HUMAN  MLRAAARFGPRLGRRLLSAAATQAVPAPNQQPEVFCNQIFINNEWHDAVSRKTFPTVNPSTGEVICQVAEGD   72
ALD-ECOLI   -------------------------SVPVQHP------MYIDGQFVTWRGDAWIDVVNPATEAVISRIPDGQ   41

DHAL-PSE66  REDLDEAVRSGRQA---QVAWAACGPSERAQVMLNAVRIFDERRDEIIDWIIRESGSTRIKA-QIEWGAARH  105
DHAL-ASPNI  EKDVDVAVAAARAAFE--GPWRQVTPSERGILINKLADLMERDIDTLAAIESLDNGKAFTMA-KVDLANSIG  123
DHAL-HUMAN  KEDVDKAVKAARAAFQLGSPWRRMDASHRGRLLNRLADLIERDRTYLAALETLDNGKPYVISYLVDLDMVLK  144
ALD-ECOLI   AEDARKAIDAAERA---QPEWEALPAIERASWLRKISAGIRERASEISALIVEEGGKIQQLA-EVEVAFTAD  109

DHAL-PSE66  HSGVRQPAEPRSRPHPGLRCAWQGKSRLXVS---PLGVIGIISPWNFPLHLTARSLAPALALGNACVIKPAS  174
DHAL-ASPNI  CLRYYAGWADKIHGQT-IDTN---PETLTYTRHEPVGVCGQIIPWNFPLLMWSWKIGPAVAAGNTVVLKTAQ  191
DHAL-HUMAN  CLRYYAGWADKYHGKT-IPID---GDFFSYTRHEPVGVCGQIIPWNFPLLMQAWKLGPALATGNVVVMKVAE  212
ALD-ECOLI   YIDYMAEWARRYEGEI-IQSDRPGENILLFKR--ALGVTTGILPWNFPFFLIARKMAPALLTGNTIVIKPSE  178

DHAL-PSE66  DTPVTGGLLLAHIFEEAGLPKGVLSVVVGSGSEIGDAFVEHEGVGFISFTGSTQVGRNIGRIARGRHLKHVA  246
DHAL-ASPNI  QTPLSA-LYAAKLIKEAPFPAGVINVISGFGRTAGAAISSHMDIDKVAFTGSTLVGPTILQAAAKSNLKKVT  262
DHAL-HUMAN  QTPLTA-LYVANLIKEAGFPPGVVNIVPGFGPTAGAAIASHEDVDKVAFTGSTEIGRVIQVAAGSSNLKRVT  283
ALD-ECOLI   FTPNNA-IAFAKIVDEIGLPRGVFNLVLGRGETVGQELAGNPKVAMVSMTGSVSAGEKIMATAAK-NITKVC  248

DHAL-PSE66  LELGATALRGA-GGRDLDQAVNAAVXGKFLHQGQICMAINRIIVEDSVYDEFVNRYAERVKSLPYGDPSKPE  317
DHAL-ASPNI  LELGGKSPNIVFDDADIDNAISWANFGIFFNHGQCCCAGSRILVQEGIYDKFVARFKERAQKNKVGNPFEQD  334
DHAL-HUMAN  LELGGKSPNIIMSDADMDWAVEQAHFALFFNQGQCCCAGSRIFVQEDIVDEFVERSVARAKSRVVGNPFDSK  355
ALD-ECOLI   LELGGKAPAIVMDDADLELAVKAIVDSRVINSGQVCNCAERVYVQKGIYDQFVNRLGEAMQAVQFGNPAERN  320

DHAL-PSE66  TV-VGPVINAKQLAGLQDKIATAKSEGARVMVEG---EAQGNVLPPHVFADVTADMEIAREEIFGPLVGIQR  385
DHAL-ASPNI  TFQ-GPQVSQLQFDRIMEYINHGKKAGATVATGGDRHGNEGYFIQPTVFTDVTSDMKIAQEEIFGPVVTIQK  405
DHAL-HUMAN  TEQ-GPQVDETQFKKILGYINTGKQEGAKLLCGGGIAADRGYFIQPTVFGDVQDGMTIAKEEIFGPVMQILK  426
ALD-ECOLI   DIAMGPLINAAALERVEQKVARAVEEGARVAFGGKAVEGKCYYYPPTLLLDVRQEMSIMHEETFGPVLPVVA  392

DHAL-PSE66  ARDEAHALELANSSEYGLSSAVFTSSLERGVKFARGIRAAMTHINDIPVNDEPNAPFGGEKNSGLGRFNGDW  457
DHAL-ASPNI  FKDVAEAIKIGNSTDYGLAAAVHTKNVNTAIRVSNALKAGTVWINNYNMISY-QAPFGGFKQSGLGRELGSY  476
DHAL-HUMAN  FKTIEEVVGRANNSTYGLAAAVFTKDLDKANYLSQALQAGTVWVNCYDVFGA-QSPFGGYKMSGSGRELGEY  497
ALD-ECOLI   FDTLEDAISMANDSDYGLTSSIYTQNLNVAMKAIKGLKFGETYINRENFEAM-QGFHAGWRKSGIGCADGKH  463

DHAL-PSE66  AIEEFTTDHWITVQHAPRRYPFX   480
DHAL-ASPNI  ALENYTQIKTVHYRLGDAL--FA   497
DHAL-HUMAN  GLQAYTEVKTVTVKVPQKN---S   517
ALD-ECOLI   GLHEYLQTQVVYLQ--------S   478
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Fig. 3. Amino acid sequences for the α subunits (*Panel A*) and subunits (*Panel B*) of protocatechuate 3,4-dioxygenase from *P. putida* NCIMB 9869 (PcaG-PSP-1 and PcaH-PSP-1), *P. putida* ATCC 23975 (PcaG-PSP-2 and PcaH-PSP-2) (5), *P. ceppacia* (PcaG-PSECE and PcaH-PSECE), *P. aeruginosa* (PcaG-PSEAE and PcaH-PSEAE) and *A. calcoaceticus* (PcaG-ACICA and PcaH-ACICA). Only about 60~65% of the *P. putida* 9869 sequences are known. The ▼ symbol indicate the ligands for essential iron bound in the β subunits. The *P. cepacia, P. aeruginosa,* and *A. calcoaceticus* sequences were obtained from the EMBL database. Panel C. A comparison of the amino acid sequence of the putative bezaldehyde dehydrogenase derived from the cloned DNA of *P. putida* 9866 (DHAL-PSE66), with sequences for other aldehyde dehydrogenases: DHAL-ASPNI, aldehyde dehydrogenase from *Aspergillus nidulans*; DHAM-HUMAN, human mitochondrial aldehyde dehydrogenase precursor (class2); ALD-ECOLI, lactaldehyde dehydrogenase from *E. coli.* The ▼ symbols mark the position of the active site glutamyl and cysteinyl residues common for all NAD-linked aldehyde dehydrogenases. The ↓ symbols indicate a putative NAD^+ binding domain. The dehydrogenase sequences are from the Swiss Protein databank. The *** denote identical residue, and the period denotes residues with similar properties.

500 in *P. putida* 9869: PCMH, the putative *p*-hydroxybezaldehyde dehydrogenase and protocatechuate 3, 4-dioxygenase. The first is a periplasmic protein[11] and the latter two cytoplasmic. It has been suggested[7] that the unusual periplasmic location of an enzyme involved in the catabolism of aromatic compounds, i.e., PCMH, is to prevent the formation of "toxic aldehydes" in the cytoplasm. However, one must realize that the four electrons extracted from *p*-cresol to produce *p*-hydroxybezaldehyde, are passed into the cytoplasmic electron transport chain for ATP production. Thus, PCMH must be in the periplasm.

The results indicate yet another variant of the organization of genes for *ortho*-cleavage-pathway enzymes. For *A. calcoaceticus,* the chromosomal organization is *pcaEFDBCHG* and *pobA* at two loci, for *P. putida,* it is *pcaE, pcaF* and *pcaBDC* at three separate loci, and for P. putida 9869, it is *dhal-pchC-ORF-pchF-pcaHG* at a single loci on plasmid pRA500. It is not unusual for degradative plasmids to have altered gene organization of *ortho*-cleavage enzymes, in particular, the gene clusters coding for enzymes required for the catabolism of chlorinated aromatics.[19] For *P. putida* 9869, PCMH that is encoded on plasmid pRA500, is made constitutively. The plasmid readily loses the *pch* genes and constitutive genes required for the catabolism of 3,5-xylenol when grown on nonselective media.[12] Perhaps, other enzymes of the protocatechuate pathway, encoded on pRA500, are constitutively produced. When *P. putida* is cured of this plasmid, the organism is still able to grow of *p*-cresol, due to a second, chromosome-encoded (B form) of PCMH.[11] It will be interesting to see how the chromosome-encoded *ortho*-cleavage enzymes are organized.

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*Pseudomonas putida*의 Protocatechuate 경로에 관여하는 초기 효소들의 유전자의 클로닝 및 염기서열 분석비교

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Abstract : *P.putida* NCIMB 9869와 *P. putida* NCIMB 9866의 분해 plasmid인 pRA 4000과 pRA500으로부터 *p*-cresol methylhydroxylase(PCMH)의 flavoprotein(*pchF*) 및 cytochrome(*pCHC*) subunit의 구조유전자를 sequencing하였다. 이 두개의 유전자의 DNA 및 아미노산의 염기 서열은 이미 발표를 하였다.[14] 이 두 개의 유전자 이외에도 aldehyde dehydrogenase 유전자가 확인되었다. 이 aldehyde dehydrogenase는 *p*-hydroxybezaldehyde를 *p*-hydroxybenzonate로 전환시키는데 *p*-hydroxybezaldehyde는 *p*-cresol의 PCMH에 의한 분해 산물이다. 그외에도 *P. putida* 9869의 protocatechuate 3,4-dioxigenase의 alpha(*pcaG*) 및 beta(*pcaH*) subunit 가 확인되었다. 반면에 *P. putida* 9866는 상응하는 영역에 이 유전자들을 가지고 있지 않았다(protocatechuate는 *p*-hydroxybenzonate hydroxylase에 의해 *p*-hydroxybenzonate로부터 생성된다). *pchC*와 *pchF*사이에 open reading frame이 존재하며 9866로 부터는 추가로 다른 하나의 open reading frame (ORF')가 존재한다. 9869과 9866의 유전자 구조는 각각 *dhal-pchC-ORF-pchF-pcaGH*과 *ORF'-dhal-pchC-ORF-pchF*다.

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*연락저자