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ORIGINAL PAPER

Novel organization of catechol *meta* pathway genes in the nitrobenzene degrader *Comamonas* sp. JS765 and its evolutionary implication

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Abstract The catechol *meta* cleavage pathway is one of the central metabolic pathways for the degradation of aromatic compounds. A novel organization of the pathway genes, different from that of classical soil microorganisms, has been observed in *Sphingomonas* sp HV3 and *Pseudomonas* sp. DJ77. In a *Comamonas* sp. JS765, *cdoE* encoding catechol 2,3-dioxygenase shares a common ancestry only with *tdnC* of a *Pseudomonas putida* strain, while *codG* encoding 2-hydroxymuconic semialdehyde dehydrogenase shows a higher degree of similarity to those genes in classical bacteria. Located between *cdoE* and *cdoG* are several putative genes, whose functions are unknown. These genes are not found in *meta* pathway operons of other microorganisms with the exception of *cdoX2*, which is similar to *cmpX* in strain HV3. Therefore, the gene cluster in JS765 reveals a third type of gene organization of the *meta* pathway.

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Abbreviations

C23O	Catechol 2,3-dioxygenase
HMSD-2	Hydroxymuconic semialdehyde dehydrogenase
HMSH-2	Hydroxymuconic semialdehyde hydrolase

Introduction

The pathways for aerobic dissimilation of aromatic compounds in microorganisms generally consist of two parts: an upper sequence of reactions that prepare the molecule for ring fission and a lower pathway consisting of ring fission and subsequent reactions leading to central metabolites. The catechol *meta* cleavage pathway (Fig. 1) is the archetypal extradiol ring cleavage pathway and it is involved in the degradation of a variety of aromatic compounds including benzene, toluene, naphthalene, biphenyl, nitroaromatic and chloroaromatic compounds [5, 11, 21, 25, 26, 28, 29]. Williams and Sayers [28] proposed that two different classes of evidence can be used to piece together the evolutionary history of degradation pathways: (a) comparison of the nucleotide and amino acid sequences of the genes, and, (b) the study of the organization of the genes of a pathway. They further proposed that the *meta* pathway operons share a common ancestry due to the fact that operons in the four *Pseudomonas* species they reviewed are highly homologous even though the upper pathways are different. Yrjala [29], however, examined the relative positions of the genes encoding the first three enzymes and reported a novel organization of catechol *meta* pathway

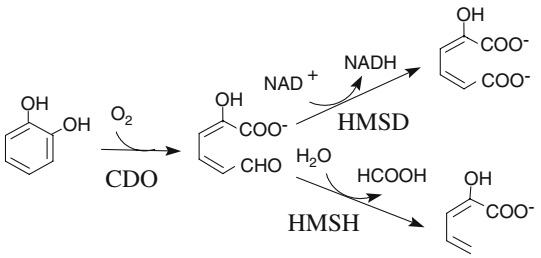

Fig. 1 Initial reactions of the catechol *meta* cleavage pathway. Catechol 2,3-diooxygenase (C23O) catalyzes the ring cleavage reaction. 2-Hydroxymuconinc semialdehyde is further metabolized either through a dehydrogenative route (dehydrogenase, HMSD) or through a hydrolytic route (hydrolase, HMSH)

genes in the *Sphingomonas* sp HV3 pSKY4 plasmid. In addition to the difference in the gene order from that of classical *Pseudomonas* species, a gene with unknown function, *cmpX*, is located between the pathway-relevant genes. A similar gene order was also reported for *Pseudomonas* sp. DJ77 [16]. The sequence of the function-unknown ORF *phnF* is identical to that of *cmpX*. These observations raised the question of whether the *meta* pathway genes evolved from a common ancestor and whether they evolved as a unit as proposed previously [5, 28].

Comamonas sp. JS765, isolated from a nitrobenzene-contaminated site, grows on nitrobenzene as the sole source of carbon, nitrogen and energy [18, 21]. The lower pathway for the degradation of nitrobenzene is the catechol *meta* pathway [9, 21]. A catechol 2,3-dioxygenase gene has been cloned and sequenced from a *Comamonas* sp. JS765 [23]. We report here the nucleotide sequences and analysis of the region downstream of the dioxygenase. Analysis of those genes reveals a third type of gene organization for the catechol *meta* cleavage pathway.

Materials and methods

Bacterial strains and plasmids

Comamonas sp. JS765 was grown in modified LB medium containing no NaCl. *Escherichia coli* strains JM109 and DH5α were grown at 37°C in LB medium with ampicillin (150 μg ml^{-1}) or kanamycin (150 μg ml^{-1}) added as appropriate for plasmid maintenance. When appropriate, isopropyl-β-D-thiogalactopyranoside (1 mM) was added in cultures during the mid growth phase. pDTG912 and pDTG901 were deletion derivatives of the cosmid pDTG900 [23]. pDTG912/A and pDTG912/B were the subclones of pDTG912 in pK18 [24].

DNA manipulation and sequencing

Isolation of plasmid DNA, transformation, restriction endonuclease digestion, ligation, and other recombinant DNA techniques were performed as described previously [3]. DNA sequencing was done by the University of Florida DNA sequencing Core Facility (Gainesville, FL, USA). The sequence was deposited in GenBank and assigned accession number AF190463.

Enzyme assays

Detection of activities of catechol 2,3-dioxygenase (C23O), 2-hydroxymuconic semialdehyde hydrolase (HMSH) and 2-hydroxymuconic semialdehyde dehydrogenase (HMSD) were carried out as described previously [9, 23]. The preparation of 2-hydroxymuconic semialdehyde was described previously [8] and its ethanol solution was kept at −70°C until use. Protein concentrations were determined by the Coomassie plus protein assay reagent from Pierce (Rockford, IL) using bovine serum albumin as a standard.

Results and discussion

C23O, HMSD, and HMSH activities

The portion of pDTG901 encoding the C23O gene (*cdoE*) was sequenced previously [23]. Preliminary data indicated that the other end of the plasmid encoded part of the HMSD gene (Fig. 2). Therefore, the cell-free extracts of the four recombinant strains were tested for the activities of C23O, HMSD and relevant HMSH. C23O activity was detected in both pDTG912 and pDTG901. No HMSH activity was detected in strains carrying any of the four plasmids. HMSD activity was expressed in pDTG912, pDTG912/A, and pDTG912/B. pDTG901/B was 300 bp longer than pDTG912/A. pDTG901 and pDTG912/B had higher activities for C23O or HMSD apparently due to the fact that the two genes were under the control of the *E. coli lac* promoter in the plasmids. Although the gene was in the wrong orientation to be driven by the *E. coli lac* promoter, the low expression of HMSD in pDTG912/A indicated the possible presence of a weak promoter upstream of the HMSD gene (either on the plasmid or insert).

CdoG encoding 2-hydroxymuconic semialdehyde dehydrogenase

Analysis of the nucleotide sequence within the cloned insert of pDTG912/A confirmed the location of the

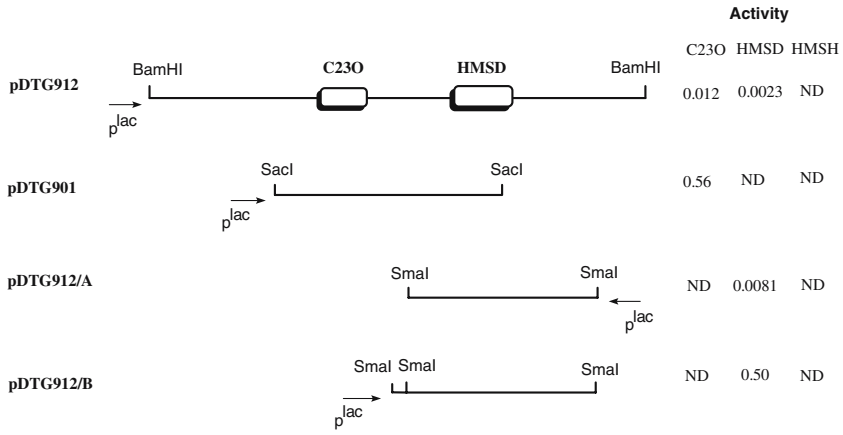

Fig. 2 Plasmids and the activity of relevant enzymes. Enzyme activity is expressed in μmol min^{-1} mg^{-1} of protein in crude extracts. *ND* indicates that no activity was detected. *pLac* indicates the relative position of the *lac* promoter in the plasmids

HMSD gene *cdoG*. A 1,461-bp open reading frame was found that encoded a 52.3-kDa protein with a calculated isoelectric point of 5.61. The deduced amino acid sequence of *CdoG* (Fig. 3) shares significant identity with those of HMSD enzymes in the *meta* pathway of other microorganisms. For example, the identity is 74% for Nah I from *P. stutzeri* AN10 [4], 73% for DmpC from *P. putida* CF600 [22], and 69% for XylG from *P. putida* pWW0 [14], followed by those dehydrogenases with the novel *meta* pathway gene organization: 67% for XylG from *Sphingomonas aromaticivorans* F199 [25] and CmpC from *Sphingomonas* sp. HV3 [29], and 61% for PhnG from *Pseudomonas* sp. DJ77 [16]. Several functionally important amino acid residues for aldehyde dehydrogenases, like the NAD^+-binding sites, FTGXTXXG and GIGXXG, and catalytic glutamate and cysteine residues [1, 13, 27] are conserved in *CdoG* (Fig. 3). It is worth pointing out that no HMSD activity was observed in the crude extracts of *E. coli* carrying pDTG901. Subcloning with the restriction enzyme *Sac*I truncated the gene, resulting in a protein missing the last seven amino acids and adding ten additional amino acids at the C-terminal end (Fig. 3). This observation indicates the importance of the C-terminal part of *CdoG* for enzyme activity. Glu 478 was proposed as a functionally important residue [14, 29]. It is not clear whether the abolishment of the activity is due to the disruption of the neighborhood of glu 478, the deletion of other critical residues (for example, cys 483 which is conserved in all sequences mentioned above), or the change of the tertiary and/or quaternary structures of the enzyme. Further investigation of the reasons for the loss of the activity may shed light on the catalytic mechanism of the aldehyde dehydrogenases.

The HMSD in crude extracts prepared from pDTG912/B showed higher activity in potassium phosphate buffer than in Tris–HCl buffer (50 mM). The optimum pH was 8.5. The results are consistent with the observations with the enzyme from nitrobenzene-grown strain JS765 [9]. When the substrate analog 2-aminomuconic semialdehyde was tested, 12% of the activity, compared to the activity on 2-hydroxymuconic semialdehyde, was observed at pH 8.0. The relative activity of HMSD from strain JS765 on 2-aminomuconic semialdehyde was 18% [9]. Considering the

Fig 3 The deduced amino acid sequence of *cdoG*. Conserved amino acids of functional importance in aldehyde dehydrogenases are marked above the sequence. The sign of ((indicates the location of the corresponding *Sac*I restriction site in pDTG901, leading to the abolishment of the HMDH activity. *Question marks* (?) indicate the potential critical amino acids

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                                                                    70
MLAKQILNFINGEFVATGKWFDNRTPISNAVIGQVAEAGQAEVDAAVAAARAALKGPWGSMTLAQRVELL
                                                                   140
EALVAEITRRFDEFLEAECADTGKPRSLASHIDIPRGAANFKVFADVVKNVPTEFFEMATPDGRGAINYG
                                                                   210
YRKPVGVVGVICPWNLPLLLMTWKVGPALACGNTVVVKPSEETPRTAALLGEVMNHVGIPKGVYNVVHGF
                    FTGXTXXG              E                        280
GPGSAGEFVTSHPGIDAITFTGETRTGEVIMKAAAKGARPVSLEMGGKNAAIVFADCDFDAAIEGTLRSA
          C                                                         350
FVNCGQVCLGTERVYVERPIFDKFVAALKQQVEGWKIGVPEGADTQLGPLISKEHQAKVLSYYQLAVQEG
                                                                   420
ATVVTGGGVPDMPGELKDGCWVQPTIWTGLPETARVIKEEIFGPCCHIAPFDTEDEVLAKANDNEYGLAT
                                          GIGXXG        ?    ?  486
AIWTRDLSRAHRVAQRMEVGISWVNSWFLRDLRTPFGGSKQSGIGREGGVHSLEFYTELKNVCIKL

                                                    ELEFTGRRFTTS
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extreme instability of the substrate and thus the variability of true concentrations of the substrate during the assay [10, 12], we believe that the data are consistent. A previous report provided evidence that the *cdoE* gene encodes the C23O required for nitrobenzene degradation [23]. The similarity of the HMSD in strain JS765 and that encoded on pDTG912/B suggests that *cdoG* encodes the dehydrogenase required for nitrobenzene degradation.

A third type of gene organization of the meta pathway

The distance between *cdoE* and *cdoG* is about 3,000 bp, much longer than that in other gene clusters of the *meta* pathway. Analysis of the sequence between *cdoE* and *cdoG* revealed the presence of five unusual ORFs (Fig. 4). The classical gene order of the *meta* pathway is identical in four soil microorganisms reviewed by Williams and Sayers [28] and the genes are in the same order as the biochemical pathway. The order is also observed in other organisms, as reported recently for *P. stutzeri* AN10 [4]. Alternatively, the order of the HMSH and HMSD genes can be reversed, as in *P. pickettii* PKO1 [17]. In *Sphingomonas* sp. HV3 [29] and *Pseudomonas* sp. DJ77 [16], however, the HMSH gene preceded the C23O gene, and an ORF of unknown function is located between the C23O and HMSD genes. A remarkable conservation in both sequence and gene order is reported between the two organisms and the deep subsurface isolate *S. aromaticivorans* F199 [25]. It was suggested that the deep subsurface *Sphingomonas* strains may be ancestors of the terrestrial surface counterparts [15]. Therefore, those genes and the gene organization in F199 may represent ancestral *meta* pathway models. The gene order in *Comamonas* sp. JS765, which was isolated from a nitrobenzene-contaminated aquifer, is clearly different from those reported, and represents a third type of *meta* pathway gene organization.

Pinpointing the evolutionary kin gene of *cdoE*

The deduced amino acid sequence of *cdoE* has high homology with many catechol 2,3-dioxygenases [23]. In particular, it shared 84–68% identity with 6 other C23O genes with no gaps. A BLAST search with the nucleotide sequence beginning after the stop codon of *cdoE* resulted in only one match with 95% identity (133 identical over 140, 4 bp different and a gap of 3 bp). The sequence was that following the stop codon of the *Pseudomonas putida tdnC* gene for 3-methylcatechol 2,3-dioxygenase (X59790). The sequences of the first 78 nucleotides of the two genes and the sequences in front of the start codons, however, did not show similarity, which suggested that only the nucleotide sequence fragment encoding the dioxygenase, not the entire *meta* pathway, shares a common ancestor in the two organisms.

Sequence analysis of the genes between *cdoE* and *cdoG*

The first ORF *cdoX1* showed significant similarity to several function-unknown sequences in the database

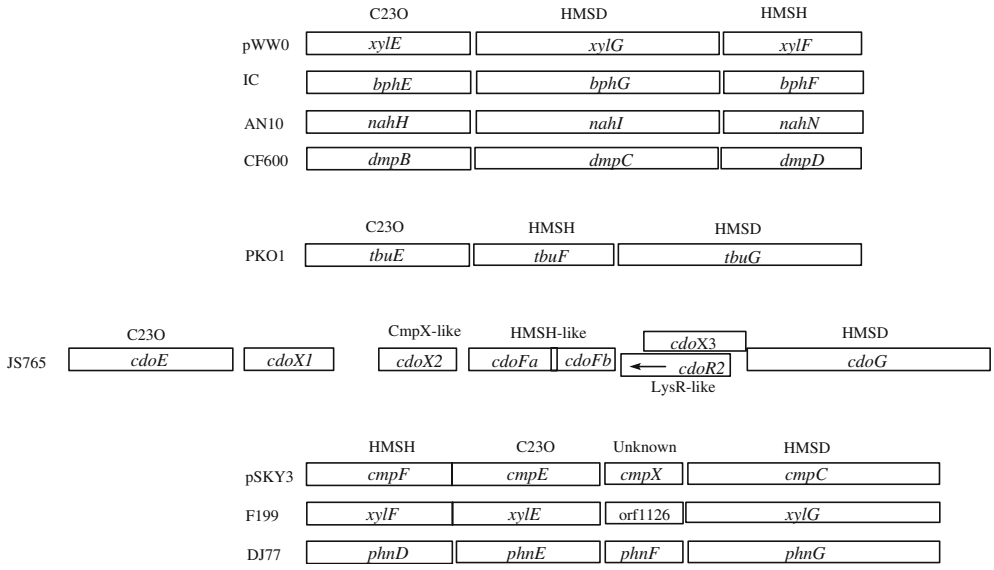

Fig. 4 Comparison of the organization of the *cdo* genes with two other types of *meta* pathway genes organizations: from *P. putida* pWW0 [14], *Pseudomonas* sp. IC [5], *P. stutzeri* AN10 [4], *P. putida* CF600 [22], *R. pickettii* PKO1 [17], *Sphingomonas* sp. HV3 pSKY4 [29], *Sphingomonas aromaticivorans* F199 [25], and *Pseudomonas* sp. DJ77 [16]

(AY940090 and D85415) and *orfX* in *Comamonas testosteroni* TA441 [2]. The second ORF *cdoX2*, which encoded a putative protein of 158 amino acids was similar to some function-unknown ORFs in *meta* cleavage gene clusters: 78% over 137 aa to OrfY of *C. testosteroni* TA441 [2], 69% over 142 aa to CbzX of *P. putida* GJ31 [19], 60% over 113 aa to NahX of a *P. putida* [7], 51% over 113 aa to ORF126 of *S. aromaticivorans* F199 [25], and 50% over 113 aa to CmpX of *Sphingomonas* sp. HV3 [29] and PhnF of *Pseudomonas* DJ77 [16]. Except for *nahX*, the other four genes are all located between C23O and HMSD genes.

Downstream of *cdoX2* are two genes, *cdoFa* and *cdoFb*, which individually are homologous to the N-terminal and C-terminal parts of amino acid sequences deduced from many HMSH genes. The *cdoFab* genes are most similar to the one from *P. putida* CF600 [22] (71% identity over 147 aa for CdoFa and 74% identity over 125 aa to CdoFb). The presence of two genes, rather than one gene as in strain CF600, seems to be caused by a 1-base deletion between positions 1916 and 1917. The absence of HMSH activity in strains carrying either plasmid pDTG912 or pDTG901 confirmed that a mutation had occurred. Genetic and biochemical evidence indicates that the hydrolytic branch of the *meta* pathway does not function in the gene cluster. In wild-type JS765, HMSH activity was observed [9, 21]. Therefore, there must be an HMSH gene, or possibly another *meta* pathway operon elsewhere in the genome of JS765.

ORF *cdoX3*, which is transcribed in the same direction, fills the gap between *cdoFb* and *cdoG* (Fig. 4). The deduced amino acid sequence of CdoX3 did not show significant similarity to any sequences in the database. On the other hand, a *lysR*-like ORF, *cdoR2*, was located in the same region on the opposite strand. A putative *lysR*-like regulatory gene, *cdoR*, is located upstream of the *cdoE* gene [23]. The amino acid sequence of the two *cdoR*s are 67% identical and are also similar to those of activators involved in *ortho* cleavage pathways [6, 20]. No such activator genes have been reported in any other *meta* pathway gene clusters except function-unknown *aphT* in *C. testosteroni* TA441 [2]. To determine whether the region encodes *codX3* or *cdoR2* or any of their biological functions will require further investigation.

Summary

The gene order of the *meta* pathway in pDTG912 of *Comamonas* sp. JS765 is different from both the order in classical soil microorganisms and that in ancestor-relevant *Sphingomonas* strains. Sequence analysis of the genes also indicated that they did not evolve from a single organism. The observations raise questions regarding the relationship of the *meta* pathway gene clusters in strain JS765 to *meta* pathway clusters in other microorganisms. The gene cluster in JS765 could be a result of horizontal gene transfer. The hypothesis, however, raises another puzzle: why the pathway-relevant genes were adapted divergently rather than convergently as a unit which exists already in other microorganisms? On the other hand, the existence of the *cmpX*-like *cdoX* and the relative location of HMSH-like *cdoFa* and *cdoFb* indicate that the gene order in strain JS765 may be a transition cluster between those in the ancestor microorganisms and those in more recent soil microorganisms. The third hypothesis is that considering the presence of more genes of unknown function in the gene cluster of JS765 it may be reasonable to guess that the gene cluster is still in an early stage in the process of evolving a modern and compact gene cluster for the *meta* pathway, hence it may be a representative of even more primitive gene clusters than those in *Sphingomonas* sp HV3 and F199. Only after more information of *meta* pathway genes from more microorganisms has been known, will it be possible to draw a definite conclusion.

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