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The catechol 2,3-dioxygenase gene and toluene monooxygenase genes from *Burkholderia* sp. AA1, an isolate capable of degrading aliphatic hydrocarbons and toluene

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***Burkholderia* sp. AA1 isolated from a diesel fuel-contaminated site degraded toluene, as well as a wide range of alkanes from decane (C_8) to pentacosane (C_{25}) as sole carbon and energy sources. This strain also utilized *m*-toluate, *p*-toluate, *o*-toluate, and *m*-cresol as sole carbon and energy sources. Toluene- and toluate-grown cells showed catechol 2,3-dioxygenase activity and indole oxidation activity that is exhibited by some toluene oxygenation enzymes. The catechol 2,3-dioxygenase gene (*catB*) was cloned and sequenced. Its deduced amino acid sequence is analogous to the extradiol dioxygenases cloned from a variety of microorganisms. A DNA fragment containing the genes for the indole oxidation activity was cloned and sequenced. A seven-gene cluster designated as *tbhABCDEFG* was identified. Significant similarities were found with multicomponent monooxygenase systems for toluene, benzene and phenol from different bacterial strains.** *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 127–131.

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Introduction

Although many microorganisms utilize aliphatic or aromatic hydrocarbons as sole carbon and energy sources, it has rarely been reported that a single bacterium could metabolize both types of hydrocarbons. In addition, degradation of the three isomers of toluate, *o*-toluate, *p*-toluate, and *m*-toluate, by a single microorganism has only been reported for *Burkholderia cepacia* MB2 [12]. Preliminary studies suggest that *Burkholderia* sp. AA1 can utilize a wide range of alkanes, toluene, benzoate, *m*-, *p*- and *o*-toluate as sole carbon and energy sources. Cells grown on the aromatic compounds exhibited catechol 2,3-dioxygenase activity indicating the presence of a *meta* cleavage pathway in strain AA1.

Toluene degradation has been reported for a number of microorganisms, from which at least five different pathways have been characterized. Dioxygenation of the aromatic ring by the toluene dioxygenase of *Pseudomonas putida* F1 results in the formation of *cis*-2,3-toluene dihydrodiol, which is dehydrogenated to 3-methylcatechol by a diol dehydrogenase [10]. The *o*-hydroxylation of the aromatic ring by the toluene-2-monooxygenase of *B. cepacia* G4 [27] yields *o*-cresol. *m*-Hydroxylation of the aromatic ring by toluene-3-monooxygenase (T3MO) of *B. pickettii* PK01 produces *m*-cresol [5]. Both *m*-cresol and *o*-cresol are oxidized by respective enzymes to form 3-methylcatechol. The monooxygenation of the methyl group of toluene by *P. putida* (*arvilla*) strain mt-2 containing the TOL plasmid [14] results in the formation of benzoic acid, which is further oxidized to form catechol. Catechol or 3-methylcatechol undergoes *meta* ring cleavage to produce intermediates of the tricarboxylic acid cycle. The *p*-hydroxylation of the aromatic ring by toluene-4-monooxygenase of *P. mendocina* KR1 results in *p*-cresol formation [32]. *p*-Cresol is further oxidized to protocatechuate, which undergoes *ortho* ring cleavage to form intermediates of the tricarboxylic acid cycle [32].

Indigo formation from indole oxidation is a characteristic of the dioxygenase enzyme systems and some monooxygenase systems. Among the five enzymes which catalyze the first step in toluene degradation pathways, toluene 2,3-dioxygenase from *P. putida* F1, xylene/toluene monooxygenase from *P. putida* mt-2, and toluene-4-monooxygenase from *P. mendocina* KR1 also oxidize indole to indigo. The other two enzymes, toluene-2-monooxygenase from *B. cepacia* G4 and *Pseudomonas* sp. strain JS150 and T3MO from *B. pickettii* PK01, do not oxidize indole to indigo.

In this paper, we report the cloning and sequencing of a C230-encoding structural gene, *catB*, and indole-oxidizing toluene monooxygenase gene cluster, *tbhABCDEF*, from strain AA1.

Materials and methods

Bacterial strains and plasmids

Isolates were obtained from a diesel fuel-contaminated site. The one used in the current study was identified using 16*S* rRNA analysis as a *Burkholderia* species and was most closely related to *B. phenazinium*. Gene cloning was carried out using pTZ19R (Ap^r, T_7 promoter, *lac* promoter, *lacZ′*) as a vector (United States Biochemical Corp., Cleveland, OH, USA) and *Escherichia coli* SURE strain (*el4*$^-$(*mcrA*), Δ(*mcrCB-hsdSMR-mrr*)171, *sbcC*, *recB*, *recJ*, *umuC*::Tn5(Kan^r),*uvrC*, *supE44*, *lac*, *gyrA96*, *relA1*, *thi-1*, *endA1*, [F′, *proAB*, *Lac1*^{q}ZM15, Tn10(Tet^r)]) (Stratagene, La Jolla, CA, USA) as the recipient strain. *E. coli* does not contain genes for catechol *meta* cleavage or toluene oxygenation.

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Media and growth conditions

Burkholderia sp. AA1 was incubated in a basal salt medium supplemented with a single carbon source at 30°C [12]. The *E. coli* SURE strain was incubated in 2× YT medium [26] at 37°C.

Utilization of alkanes and aromatics

The isolate was inoculated into basal salt medium supplemented with a variety of alkanes and aromatic hydrocarbons as carbon sources. Alkanes such as hexane, octane, decane, dodecane, tetradecane, pentadecane, hexadecane, heptadecane, octadecane, eicosane, pentacosane, and triacontane were added in microtiter plates or tubes at concentrations from 0.1% to 1.0%. Volatile hexane and octane were also provided as vapor. Among the aromatic hydrocarbons, xylene, benzene, toluene, *o*-, *p*-, or *m*-chlorotoluene were provided as liquid or as vapor; phenanthrene and 2,3-dimethylnaphthalene were added into microtiter plates and tubes. *m*-Toluate, *p*-toluate, *o*-toluate, benzoate and 2,4,5-trichlorophenol were added to basal salt medium at 5 mM. *m*-Cresol, *o*-cresol and *p*-cresol were added to basal salt medium at 1 mM. Toluene was mixed with hexadecane prior to its addition into the side arm of a biometer flask. This isolate was stored at 4°C on basal salt medium plates supplemented with 5 mM *m*-toluate.

The tubes or the flasks were incubated with or without shaking at 30°C. The microtiter plates were incubated in sealed plastic bags at 30°C. Increased turbidity was used as an indication of growth on the substrates while no growth was observed in the absence of these substrates.

Enzymes and chemicals

Ampicillin, catechol and other chemicals were obtained commercially at the highest purity available (Sigma, St. Louis, MO, USA). Restriction endonucleases and T4 DNA ligase were used as suggested by the suppliers (Promega, Madison, WI, USA).

DNA manipulation

Chromosomal and plasmid DNA extraction, *E. coli* transformation, DNA ligation, gene subcloning and agarose electrophoresis were carried out as described in Sambrook *et al.* [26].

Recombinant plasmid construction

Chromosomal DNA from *Burkholderia* sp. AA1 was digested with *Hin*dIII restriction endonuclease. The completely digested DNA was ligated into the linearized vector, pTZ19R, which was cut by the same enzyme. Ligation was carried out with 600 ng of insert DNA, 100 ng of vector DNA and 1 U of T_4 DNA ligase. After transformation of competent *E. coli* SURE in the presence of ampicillin, colonies exhibiting catechol 2,3-dioxygenase activity were screened by spraying the plate with a 0.1 M catechol solution. A positive one from 5000 colonies turned yellow due to the formation of 2-hydroxymuconic semialdehyde from *meta* cleavage of catechol [20]. A similar procedure was used to clone T3MO with *Kpn*I restriction endonuclease-digested *Burkholderia* sp. AA1 DNA. The plates were incubated at 37°C overnight and then incubated at room temperature for another 24 h. A positive one among 10,000 colonies turned blue due to the formation of indigo from indole oxidation by the cloned oxygenase.

Nucleotide sequencing and sequence analysis

Sequencing was performed on both DNA strands using Ready Reaction PCR Sequenase Dye Determinator Reaction Kit (24 cycles) (Perkin Elmer, Branchburg, NJ, USA) with M13 forward and reverse primers used for the first round of sequencing. The subsequent primers used were designed on the basis of the previously determined sequences. Sequence analysis was performed using the GCG Program (Madison, WI, USA). Similarity searches were carried out with the GenBank database. The identity and similarity were determined using GCG Gap program with gap weight 12 and length weight 4.0.

Nucleotide sequence accession numbers

The nucleotide sequences for the gene encoding the catechol 2,3-dioxygenase from *Burkholderia* sp. AA1 have been deposited in the GenBank database under accession no. U47111 (1.5 kb). The accession number for the T3MO genes is AF001356 (6.5 kb).

Enzyme assays

E. coli recombinants containing the cloned gene were inoculated into 2× YT broth medium and incubated overnight at 37°C. Cells were harvested by centrifugation and broken by sonication. Catechol 2,3-dioxygenase activity was measured spectrophotometrically by monitoring the increase in absorbance at 375 nm with catechol as the substrate, at 382 nm with 4-methylcatechol as the substrate, and at 388 nm with 3-methylcatechol as the substrate [15]. The amounts of 2-hydroxymuconic semialdehyde produced from catechol, 4-methyl-2-hydroxymuconic semialdehyde from 4-methylcatechol and 2-hydroxy-6-oxohepta-2,4-dienoic acid formed from 3-methylcatechol were estimated according to Rast *et al.* [25]. One unit (U) of activity was defined as the amount of enzyme which cleaves 1 μmol substrate per minute.

Results

Characterization of degradative capabilities of Burkholderia sp. AA1

Individual hydrocarbon degradation tests showed that strain AA1 can utilize decane, dodecane, tetradecane, pentadecane, hexadecane, heptadecane, octadecane, eicosane and pentacosane. There was no growth on pentane, hexane, octane, *m*-xylene, naphthalene, phenanthrene, triacontane (C_{30}), 2,3-dimethyl-naphthalene, 2-methylphenanthrene, 2-, 3- or 4-chlorotoluene individually. Strain AA1 was also able to use toluene, benzoate, *m*, *p*, *o*-toluate and *m*-cresol but not the chlorobenzoates, *o*-cresol or *p*-cresol as sole carbon sources. Strain AA1 formed yellow colonies on basal salts plates supplemented with benzoate, any of the toluates, or toluene, when sprayed with 0.1 M catechol solution.

Cloning of the catechol 2,3-dioxygenase gene (catB)

A genomic library of *Burkholderia* sp. AA1 was constructed with *Hin*dIII-digested total genomic DNA and the plasmid vector, pTZ19R, in *E. coli* SURE. Colonies were screened for catechol 2,3-dioxygenase activity (C230) based on the ability to form the yellow compound, 2-hydroxymuconic semialdehyde, from *meta* cleavage of catechol. A positive colony was obtained from some 5000 colonies and the recombinant plasmid was designated as pTZ19R102 (Figure 1A). Restriction endonuclease site analysis of

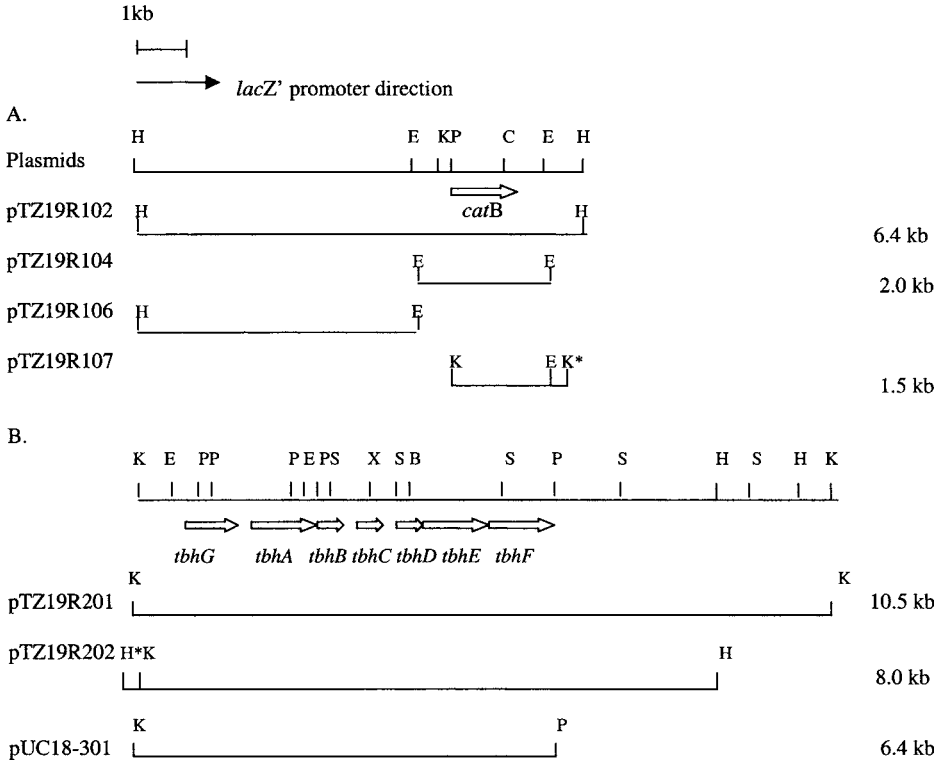

Figure 1 (A) Restriction endonuclease site map of the *Hin*dIII fragment containing the catechol 2,3-dioxygenase gene and the recombinant plasmids constructed from it. (B) Restriction endonuclease map of the *Kpn*I fragment containing the T3MO activity and the recombinant plasmids constructed from it. Abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sma*I and X, *Xba*I. *Indicates that this enzyme site is in the vector.

pTZ19R102 indicated that it contained a 6.4-kb inserted DNA fragment. The *catB* gene was located on a 2.0-kb *Eco*RI fragment through the first subcloning (pTZ19R104). *Kpn*I digestion of pTZ19R104 produced a 1.5-kb DNA fragment whose one *Kpn*I site originated from the vector. Religation of such a fragment into a *Kpn*I cut vector produced pTZ19R107.

Sequence analysis of the C230 gene, catB

A total of 1436 bp, corresponding to the region between the *Kpn*I and *Eco*RI restriction sites (Figure 1A), was sequenced. The nucleotide sequence was determined in both directions and all the restriction nuclease sites used for cloning and mapping were verified. A 942-bp open reading frame (ORF) initiating at ATG (bp 106) and terminating at TAA (bp 1047) was identified within this sequence. The ORF predicts a 314-amino acid residue protein with a molecular weight of 34 kDa. This value is similar to the molecular weight of catechol 2,3-dioxygenase from other microorganisms [11]. Six base pairs upstream from the initiation codon is a putative ribosome-binding sequence, 5′-AGGAG-3′ [28].

The percent G+C content of the *catB* coding region was 54%. The value is significantly lower than the G+C contents reported for closely related *Burkholderia* species [22,31], indicating the possibility of *catB* gene recruitment by horizonal gene transfer. Codon usage in the *catB* gene showed a high (68.8%) usage of G or C as the wobble base.

The deduced amino acid sequences of *catB* have a high homology with several catechol 2,3-dioxygenase genes. The most similar ones are CbzE (71.7% identity) from *P. putida* GJ31 (accession no. AF109307 [18]), CdoE (71.0% identity) from the nitrobenzene-degrading *Comamonas* sp. JS765 (accession no. U93090 [23]) and AphB (70.7% identity) from the phenol-utilizing *Comamonas testosteroni* TA441 (accession no. AB006479 [1]). The others include PhnE2 (69.4% identity) from the phenanthrene-degrading *Burkholderia* RP007 (accession no. AF112137 [17]) and the two proteins of the I.2.C subfamily of extradiol dioxygenases [6]: the TdnC from *P. putida* UCC22 at 68.5% identity (accession no. X59790 [19]) and the C230II from *P. putida* MT15 at 65.3% (accession no. U01826 [13]).

The substrate range of catechol 2,3-dioxygenase, CatB

The enzymatic activity of catechol 2,3-dioxygenase was determined using the crude extracts of *E. coli* cells containing pTZ19R107 grown in 2× YT broth with catechol, 3-methylcatechol and 4-methylcatechol as substrates (0.1 M). No activity was detected with the extracts of *E. coli* containing the cloning vector, pTZ19R. The catechol 2,3-dioxygenase oxidized 3-methylcatechol at 37% (265 U) and 4-methylcatechol at 35% (252 U) of the rate for catechol oxidation (725 U), respectively.

Cloning of the T3MO genes (tbhABCDEF)

The cloning strategy was based on the wide substrate specificity of the T3MO, which oxidizes indole to indigo. The recombinant positive colonies were blue on 2× YT plates containing 1 mM indole.

Total DNA was isolated from strain AA1 and digested with the restriction enzyme, *Kpn*I. A library of the digested DNA was constructed using pTZ19R in *E. coli* SURE. Recombinant *E. coli* colonies were incubated at 37°C overnight and then stored at room

temperature overnight in the presence of antibiotics. A blue colony from some 10,000 colonies was obtained and plasmid DNA was isolated from this recombinant and designated pTZ19R201. Restriction endonuclease site analysis indicated a 10.5-kb *Kpn*I fragment insert (Figure 1B). Digestion of this recombinant plasmid by *Hin*dIII produced an 8.0-kb fragment, whose one *Hin*dIII site was determined to be from the vector, pTZ19R. Religation of this fragment into the *Hin*dIII-digested pTZ19R produced two types of recombinant colonies. A blue colony showing T3MO activity was designated pTZ19R202. It was shown that the inserted DNA in pTZ19R202 was in the same orientation as in pTZ19R201. Clones containing the insert in an opposite orientation (data not shown) did not show T3MO activity. This indicated that the cloned T3MO genes probably did not contain their own promoter region, and are transcribed using the vector's promoter (Figure 1B). Finally, a 6.5-kb *Kpn*I–*Pst*I fragment was cloned into vector pUC18. The resulting pUC18–301 contained the T3MO genes.

Nucleotide sequences of the T3MO genes

Recombinant plasmid pUC18–301 was used as a template to determine the entire sequences of the T3MO genes. A total of 6658 bp of sequence was determined. Seven ORFs were identified in the fragment cloned in pUC18–301 (Figure 1B). These genes were tentatively designated as *tbhG*, *tbhA*, *tbhB*, *tbhC*, *tbhD*, *tbhE*, and *tbhF* (in expected order of transcription). A putative ribosomal binding site [28] was identified upstream from the initiation codon of each ORF. The G+C content of the whole sequence was 54.3%. This level is lower than the reported G+C contents for closely related *Burkholderia* species [22,31]. The G+C content and G or C usage at the wobble position for each gene are: 55.6% and 60.1% for *tbhG*; 54.3% and 65.1% for *tbhA*; 54.3% and 49.4% for *tbhB*; 51.2% and 57.6% for *tbhC*; 51.6% and 56.7% for *tbhD*; 53.8 and 66.9% for *tbhE* and 52.8 and 58.3% for *tbhF*, respectively. Considering the G+C content for each gene, there is a codon usage preference for G or C at the wobble position of each codon in the *tbh* gene cluster except *tbhB*. TbhG showed a significant similarity to the 4-oxalocrotonate decarboxylases DmpH from *P. putida* CF600 (accession no. P49156 [29]) at an identity of 45%.

Sequence analysis of toluene monooxygenase encoded by tbhABCDEF

Searching the homologous sequences to *tbhABCDEF* in GenBank showed that it has a high degree of similarity to the subunits of toluene and benzene monooxygenase from *P. aeruginosa* JI104 (accession no. D83068 [16]), *B. pickettii* PK01 (accession no. U04052 [5]), *P. mendocina* KR1 (accession no. M65106 [33]) and *P. stutzeri* OX1 (accession no. AJ005663 [4]) and phenol hydroxylase from *Ralstonia eutropha* JMP 134 (accession no. AF065891 [3]). Lower but significant similarities to polypeptides from other enzyme systems were also found. TbhA (501aa), the largest polypeptide, and TbhE, the smaller oxygenase subunit, are similar to their counterparts in other systems. A motif characterized by a pair of conserved domains with the amino acid sequence Asp–Glu–X-Arg–His was found in some enzymes which catalyze reactions involving activated oxygen [8]. This motif was identified in TbhA by alignment with TbuA1, TmoA, BmoA and TouA (alignment not shown) and is similar to a dinuclear iron binding ligand. TbhB has a lower similarity to the small polypeptides, while TbhC is highly homologous to the ferredoxin parts of the T3MOs and toluene-4-monooxygenases. TbhF showed a significant similarity to the oxidoreductase components from other di- and monooxxygenase systems. It was putatively considered to have the oxidoreductase function in the *tbh* monooxygenase system. The identification of two putative redox proteins, TbhC and TbhF, in the *tbh* system is consistent with what has been observed in other mono- or dioxygenase systems [6,33]. TbhD also shared similarity to MmoB from *Methylocystis* sp. strain M at an identity of 29.4% [30], and DmpM from *Pseudomonas* sp. CF 600 at 24.4% [21]. TmoB, TmoD and DmpM were reported to be necessary for the whole enzyme activity, although their exact functions are not clear [33]. Froland *et al.* [9] suggested that MmoB may function as a regulatory protein which affects the overall reaction rate and quantities of different products from a given substrate. Sequence similarity with MmoB suggests that TbhD may play a similar role.

Discussion

Many microorganisms have been reported to utilize aliphatic or aromatic hydrocarbons with few capable of using both types of hydrocarbons. It has also been noted that there are many differences in the range of hydrocarbons utilized by different microorganisms. This is a reflection of underlying genotypes: different genes are involved in the metabolism of low-molecular-weight alkanes and high-molecular-weight alkanes [30]. Most alkane-utilizing microorganisms metabolize only a narrow range of alkanes [2]. *B. cepacia* MB2 was the only organism reported to utilize *o*-toluate, *m*-toluate or *p*-toluate as a sole carbon and energy source [12] until now. *Burkholderia* sp. AA1 utilized a wide range of alkanes (C_8–C_{25}), toluene, *o*-toluate, *m*-toluate and *p*-toluate as sole carbon and energy sources. These combined degradative capacities of strain AA1 may be of great interest for bioremediation and in studying the molecular genetics of hydrocarbon utilizing microorganisms.

The monocyclic *meta* cleavage enzymes possess different substrate specificities. C230I enzymes, which are represented by Xy1E from *P. putida* mt-2, oxidize 3-methylcatechol at about 60% of the rate for catechol oxidation [13], while C230II and TdnC oxidize 3-methylcatechol at about twice the rate for catechol oxidation [13,19]. The new catechol 2,3-dioxygenase enzyme oxidizes 3-methylcatechol at about 40% of the rate for catechol oxidation, which is similar to the rate seen with C230I, although CatB has high homology to C230II and TdnC at the nucleotide and amino acid sequence levels. It is interesting that the relative activity of CatB is similar to that of C230I enzymes, C230II or TdnC when 4-methylcatechol is provided as the substrate [13,19]. It has been reported that the substrate specificity of catechol 2,3-dioxygenase can be dramatically changed by single amino acid substitutions [24]. This may explain the observation that CatB has an amino acid sequence which is similar to C230II, but has a substrate specificity more closely related to the C230I enzyme.

E. coli cells containing the cloned T3MO genes from *Burkholderia* sp. AA1 do not form blue colonies when grown on indole-supplemented 2× YT plates at 37°C. However, when such cells are incubated at room temperature or 30°C, the blue colonies form through production of indigo. A possible explanation is that the T3MO from *Burkholderia* sp. AA1 is active at 37°C. The other cloned enzymes which oxidize indole to indigo such as toluene/xylene monooxygenase [14], toluene-4-monooxygenase [33],

naphthalene dioxygenase [7], and toluene dioxygenase [10] were not reported to have such a temperature sensitivity.

Toluene-4-monooxygenase from *P. mendocina* KR1 [33] oxidizes indole to indigo, while T3MO from *B. pickettii* PKO1 does not [5]. Although the *tbh* enzyme also oxidizes indole to indigo, it shares a higher similarity with *tbu* T3MO than with *tmo* toluene-4-monooxygenase; therefore, the new *tbh* enzyme may be designated as T3MO. According to the pathways proposed by Byrne *et al.* [5], *m*-cresol is the intermediate of the toluene degradation pathway oxidized by T3MO; *p*-cresol is the intermediate of the pathway which involves toluene-4-monooxygenase. *Burkholderia* sp. AA1 utilizes *m*-cresol but not *o*-cresol or *p*-cresol as a sole carbon and energy sources. This further supports the conclusion that the cloned toluene monooxygenase encoded by *tbhABCDEF* gene cluster is T3MO.

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