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Molecular Cloning and Biochemical Characterization of Protocatechuate 3,4-dioxygenase in *Burkholderia* sp. NCIMB 10467

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Abstract: Strain NCIMB 10467, a lignin degrader, was reclassified as genus *Burkholderia* according to its 16S rDNA sequence. It seems that the metabolism of protocatechuate by this strain is diverse under the induction of various substrates. A 9505-bp DNA fragment extending from a conserved region of the gene, which encodes α subunit of *ortho* cleavage protocatechuate 3,4-dioxygenase (P34D; EC 1.13.11.3), was obtained by genome walking. Sequence analysis revealed two deduced open reading frames, *pcaG* and *pcaH*, encoding the α and β subunits of P34D respectively in this fragment. The P34D activity could be detected when *pcaGH* were expressed in *E. coli* and the disruption of *pcaH* in strain NCIMB 10467 has lead to loss of its ability to catabolize protocatechuate. It was proved that the cloned *pcaGH* were encoding a functional protocatechuate 3,4-dioxygenase which was necessary for the protocatechuate metabolism in this strain.

Keywords: Protocatechuate 3,4-dioxygenase, *Burkholderia* sp. NCIMB 10467, *pcaH*

Burkholderia sp. NCIMB 10467 菌株中原儿茶酸 3, 4-双加氧酶基因的分子克隆和生化特性研究

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摘　要：NCIMB 10467 是一株木质素降解菌，根据其 16S rDNA 序列将其重新分类为 *Burkholderia* 菌属。研究显示，在 NCIMB 10467 菌株中，不同的底物可以诱导该菌株对于原儿茶酸的多种代谢形式。根据克隆到的一段原儿茶酸邻位开环酶，即原儿茶酸 3, 4-双加氧酶(P34D; EC 1.13.11.3)α-亚基的保守序列，通过染色体步移的方法，得到一段 9505 bp 的 DNA 片段。序列分析显示，在这段 9.5 kb 的 DNA 片段中，两个可能的开放阅读框 *pcaG* 和 *pcaH* 分别编码 P34D 的α-亚基和β-亚基。将 *pcaGH* 克隆并在大肠杆菌中进行表达后，可以检测到 P34D 的活性。而 *pcaH* 在 NCIMB 10467 菌株中的敲除则使该菌完全丧失了代谢原儿茶酸的能力。由此证实，克隆到的 pcaGH 基因确实编码原儿茶酸 3, 4-双加氧酶，并且对于 NCIMB 10467 菌株对原儿茶酸的代谢是必需的。

关键词：原儿茶酸 3, 4-双加氧酶, *Burkholderia* sp. NCIMB 10467, *pcaH*

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Aromatic compounds are widespread in the environment and persist over a long period of time. Lignin, a plant biopolymer, is the second most abundant aromatic compounds in nature[1], and its decomposition is essential to the terrestrial carbon cycle[2] However, aromatic compounds are exceptionally stable due to the delocalization of their π orbitals[3]. Sequential metabolic steps were involved in the degradation pathways of lignin, including depolymerization of the phenolic units, simplification of polycyclic aromatic compounds into monocyclic compounds, and their further metabolism into key intermediates, such as catechol, gentisate, protocatechuate and etc[3–5].

The aromatic ring of protocatechuate could be cleaved via three pathways by protocatechuate 2,3-dioxygenase[6], protocatechuate 4,5-dioxygenase[7] and protocatechuate 3,4-dioxygenase (P34D; EC 1.13.11.3)[8] respectively. The former two enzymes, requiring ferrous iron as cofacter, catalyze the *meta* cleavage of aromatic ring adjacent to the hydroxyl substitution[6,9]. In contrast, protocatechuate 3,4-dioxygenase, requiring nonheme ferric iron as sole cofactor, catalyzes the *ortho* cleavage of aromatic ring between the vicinal hydroxyls to form β-carboxy-*cis*, *cis*-muconic acid (Fig. 1)[10].

Strain NCIMB 10467, a versatile aromatic compounds degrader, was isolated from decaying wood in Helsinki in the 1960s, and could catabolize lignin completely into CO_2[11]. Although this strain had been shown to have the constitutive ability to oxidize protocatechuate[11–13], the metabolic pathway of protocatechuate and the enzymes involved is unknown. In this study, we report the reclassification of this strain, molecular and biochemical characterization of a protocatechuate 3,4-dioxygenase.

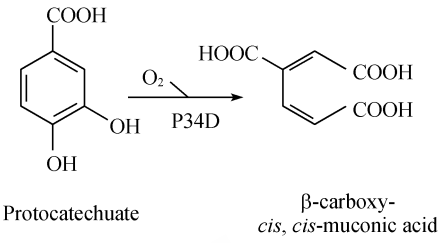

Fig. 1 Protocatechuate 3,4-dioxygenase converts protocatechuate to β-carboxy-*cis, cis*-muconic acid

1 Materials and methods

1.1 Bacterial strains, plasmids, primers, media, and culture conditions

The bacterial strains and plasmids used are listed in Table 1. *E. coli* strains were grown and transformed as

Table 1 Strains and plasmids used in this study

Strains and plasmids	Relevant characteristic(s)	Reference or source
Burkholderia sp.		
NCIMB 10467	Lignin utiliser, wild type	[11]
1441	*pcaH* gene disrupted mutant of 10467	This study
E.coli		
DH5α	*sup E44*, *lac*U169 (Φ80*lac*ZΔM15), *rec*A1, *end*A1, *hsd*R17, *thi*$^{-1}$, *gyr*A96, *rel*A1	Gibco, BRL
Rosetta (DE3)	pLysS [F^- ompT hsd $S_B(r_B^- m_B^-)$ gal dcm *lac*Y1(DE3) pLysSRARE^2 (Cm^r)]	Novagen Co
S17-1λpir	Tp^r Sm^r *recA thi pro hsdR M*+ RP4::2-Tc::Mu::Km Tn7 *pir* lysogen	[16]
Plasmid		
pGEM-T	Cloning vector, Ap^r	Promega Co
pET5a	Expression vector, Ap^r	Novagen Co
pTn*Mod*-OKm	Km^r, source of Km resistant gene	[17]
pEX18Tc	Tc^r, *sacB*$^+$, gene replacement vector with MCS from pUC18	[18]
pZWLS001	Ap^r, pGEM-T derivative carrying 298 bp fragment of *pcaG*	This study
pZWLS005	Ap^r, pET5a derivative carrying *pcaGH* from the start codon through the end codon	This study
pZWLS006	Tc^r, Km^r, pEX18Tc derivate designed for *pcaH* knockout where *pcaH* was disrupted by Km resistant gene	This study

described previously[14]. Strain NCIMB 10467 was grown in Luria-Bertani (LB) medium or minimal medium (MM) [15] with 5 mmol/L substrate as a sole carbon and energy source at 30°C.

1.2 Cloning of the 16S rRNA gene from strain NCIMB 10467

The 27f and 1492r universal primers[19] were used to amplify the 16S rDNA from strain NCIMB 10467 by PCR. The fragment generated was purified by agarose gel electrophoresis and band extraction before it was cloned into the pGEM-T vector for sequencing.

1.3 Whole-cell biotransformations

Cells were harvested by centrifugation, washed in phosphate buffer (50 mmol/L, pH 7.4), and resuspended in a minimal volume of phosphate buffer to give an OD_{600} of 5.0. Protocatechuate was added to the cell suspensions at a final concentration of 0.22 mmol/L and samples were collected at appropriate intervals to monitor the progress of reaction.

1.4 Cloning of protocatechuate degradation genes and sequence analyses

Primers were designed based upon a conserved region of protocatechuate 3,4-dioxygenase α subunit gene from *Burkholderia gladioli* (accession no. AAC99962), and *Burkholderia xenovorans* LB400 (accession no. YP552569). The primers were used to amplify a potential protocatechuate 3,4-dioxygenase α subunit gene fragment from strain NCIMB 10467. Primer sequences were: forward, 5′-CTCAAGCAAA CGCCTTCGCAAACGGTT-3′; reverse, 5′-TGTGC GGATCGGTACCGGTGCCCACG-3′. Genome walking was then conducted to clone the flanking regions of the dioxygenase gene with the methods described previously[20]. The primer sequences for genome walking used are available upon request. Nucleotide sequences were determined by Invitrogen Technologies Co. (Shanghai, China). Analyses of potential open reading frames (ORFs) and comparison of amino acid sequences (or nucleotide sequences) were performed with the ORF finder and Blast programs on the National Center for Biotechnology Information website[21].

1.5 Cloning of *pcaGH* genes and protein expression in *E. coli*

pcaGH were amplified from strain NCIMB 10467 via PCR with DNA polymerase (*Ex Taq*™, Takara). The purified PCR products were inserted into *Nde*I and *Eco*RI sites of pET5a to produce pZWLS005. The sequence was verified by DNA sequencing to ensure that no mutation had been incorporated during the PCR. *E. coli* Rosetta strains carrying this resulting plasmid were grown in LB at 37 °C to an OD_{600} of 0.6 and then induced for 4 h by addition of 0.4 mmol/L IPTG at 30°C. The expressed proteins were identified by SDS-PAGE.

1.6 Enzyme assays

Cell extracts were prepared by sonication as described previously[22]. Protocatechuate 3,4-dioxygenase activities were determined spectrophotometrically by measuring the decrease in absorbance at 290 nm with a molar extinction coefficient of 3890 L/(mmol·cm^2) due to substrate consumption[23]. The reference cuvette contained the same but omitting the substrate and the assay was initiated by the addition of substrates. All assays were conducted with strains carrying vectors only as negative controls. One unit of enzyme activity is defined as the amount required for the disappearance of 1 μmol of substrate per min at 30°C. Specific activities are expressed as units per gram of protein. Protein concentration were determined according to the Bradford method[24], with bovine serum albumin as the standard.

1.7 Gene knockout experiments

Target *pcaH* gene was cloned into the gene replacement vector pEX18Tc[18] with insertion of a kanamycin resistance gene (*nptII*) from plasposon pTn*Mod*-Okm[17] as a selectable marker. The 5′and 3′end of *pcaH* were amplified by primer pairs BHS (5′-GCGCTCGAAAAGCTTTACCCCGAGAATCG-3′)-BPA(5′-TCGGTGCTGCAGACGGGCACATGC-3′)(with *Hin*d III and *Pst* I sites respectively) and BPS (5′-CGACACCCTGCAGGAAGCCTATGCG-3′)-

BXA(5′-CGACCACATCTAGATGCGGCGCTGC-3′) (with *Pst* I and *Xba* I sites respectively), these two PCR fragments were cloned into *Hin*d III and *Xba* I sites of pEX18Tc by three-fragment ligation and then the kanamycin cassette was inserted into the *Pst* I site of this fragment to form pZWLS006. This construct was then transformed into mobilizing strain *E. coli* S17-1[25] before being conjugated into strain NCIMB 10467 by biparental matings as described previously[26]. Finally, double crossover recombinants were screened on sucrose plates[26] and mutated strains were confirmed by their kanamycin resistance and PCR analysis.

1.8 Nucleotide sequence accession numbers

The sequences of the 1451 bp 16S rRNA gene and the 9505 bp protocatechuate degradation gene cluster from strain NCIMB 10467 had been deposited in the GenBank database under accession numbers EU165544 and EU165546 respectively.

2 Results

2.1 Reclassification of strain NCIMB 10467

The almost complete 16S rDNA of strain NCIMB 10467 was cloned and sequenced. The DNA sequence exhibited the highest identity (99%) to the 16S rDNA from three strains of the genus *Burkholderia* (accession nos. AY741358, EF139188, and AJ971351), indicating that it should be reclassified as *Burkholderia* sp. strain NCIMB 10467 rather than as a manuscript name *Lignobacter* sp as previously claimed[27].

2.2 Preliminary studies of protocatechuate degradation in strain NCIMB 10467

In a previous report, strain NCIMB 10467 had been found to use protocatechuate as a sole carbon and energy source to grow, and no induction was required for the oxidation of protocatechuate[11]. In this study, the whole-cell biotransformation experiment showed that protocaetchuate could be degraded without a lag phase even using succinate-grown cells. This is consistent with what had been previously described. The cell extracts from different substrates-grown cultures, however, exhibited protocatechuate dioxygenases at considerably different rates. The cell extracts from protocatechuate-grown bacteria were found to contain protocatechuate dioxygenase with a specific activity of 403 U/g, which was at least 35 times higher than that from other substrates-grown bacteria, including 3-hydoxybenzoate, salicylate and naphthalene, as shown in Table 2. In addition, the cell extracts from these non-protocatechuate substrates-grown cultures did not show protocatechuate dioxygenase activities until Fe^{2+} was added (Fig. 2-B). This is in contrast to the cell

Table 2 Specific activities of protocatechuate dioxygenase in crude extracts of strain 10467 grown on various substrates

Inducer	Activities	
	SA	RA
Salicylate	3.62	0.9
3HBA	10.74	2.7
Naphthalene	9.08	2.2
Gentisate	ND	0
Protocatechuate	403.78	100

SA: Specific activities (U/g protein); RA: Relative activities (%); ND: Not detected. The rate of enzyme activity was assigned a value of 100% when its substrate was protocatechuate

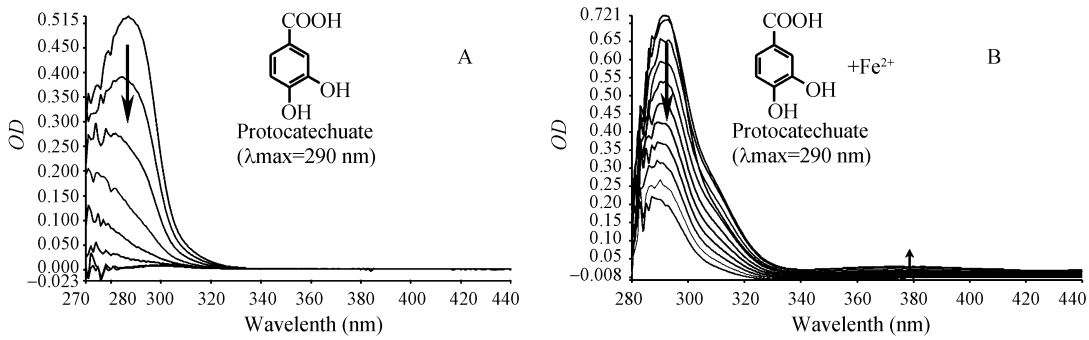

Fig. 2 Spectral changes of degradation of protocatechuate by crude extracts of *Burkholderia* sp. NCIMB 10467 grown on different substrates

A: From protocatechuate-grown cells; B: From other substrates-grown cells

extracts from protocatechuate-grown culture, in which a much higher activity was present in the absence of Fe^{2+}. No protocatechuate dioxygenase activity was detected from the cell extracts of gentisate-grown cells even after the addition of Fe^{2+}. Interestingly, a tiny broad peak between 360 nm to 420 nm was formed during the reaction in Fig. 2-B, which did not exist in Fig. 2-A. This phenomenon may be due to formation of unknown products from protocatechuate by certain induced-enzymes when grown on above non-protocatechuate substrates.

2.3 Cloning and sequence analyses of the protocatechuate catabolic genes

A pair of primers based on a conserved region of the protocatechuate 3,4-dioxygenase gene wai initially used to amplify a PCR product with an anticipated size of 298 bp. Subsequently, a DNA fragment of 9505 bp extending from this 298 bp region was obtained and sequenced after several cycles of genome walking. Nine complete ORFs and two truncated ORFs (ORF1 and ORF5) were identified as shown in Fig. 3 and their annotations were completed based on the results of blast analyses (Table 3). Of these ORFs, *pcaG* and *pcaH* were proposed to encode the α and β subunits of protocatechuate 3,4-dixygenase respectively. Furthermore, the DNA sequences of this 9.5 kb fragment showed high identities (more than 95%) with the corresponding fragment from chromosome 2 of *Burkholderia xenovorans* LB400, which was through genome sequencing but its function has not been experimentally determined. The gene organizations were also conserved in these two strains and no other protocatechuate catabolic genes flanking *pcaGH* were identified.

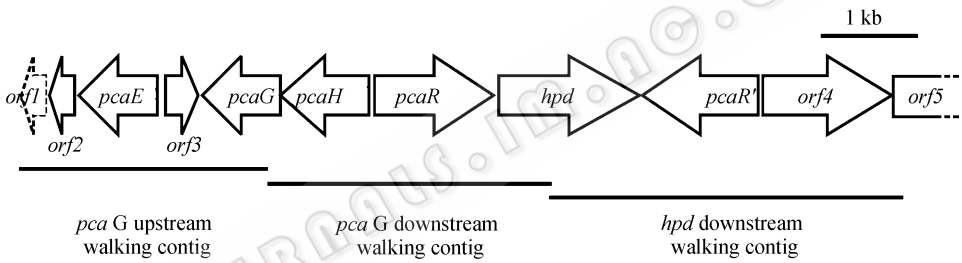

Fig. 3 Organization of the ORFs in protocatechuate *ortho* catabolic pathway from *Burkholderia* sp. NCIMB 10467

The arrows indicate the size and the direction of each ORF. The lines below represent the contigs acquired from three genome walking. The italics refer to the names of each gene. Dotted outlines indicate that only partial sequences have been obtained for these genes

Table 3 Blast homology search results for deduced amino acid sequences of genes flanking *pcaGH* gene

Putative ORFs	Functional description of closest relative	Identity(%)	Accession No.
Orf1	Hypothetic protein	98	YP552564
Orf2	Cold-shock DNA-binding domain protein	100	YP552565
PcaE	Amino acid efflux pump, RhtB family, LysE superfamily	96	YP552566
Orf3	Hypothetical protein	100	YP552568
PcaG	Protocatechuate 3,4-dioxygenase, alpha subunit	95	YP552569
PcaH	Protocatechuate 3,4-dioxygenase, beta subunit	98	YP552570
PcaR	Transcriptional regulator, LysR family	97	YP552571
Hpd	4-hydroxyphenylpyruvate dioxygenase	96	YP552572
PcaR′	Transcriptional regulator, LysR family	95	YP552573
Orf4	Hypothetic protein	97	YP552574
Orf5	Putative FAD-dependent oxidoreductase	95	YP552575

2.4 Expression and enzyme assay of PcaGH

After induction with IPTG, cell extracts of *E. coli* Rosetta containing plasmid pZWLS005 were found protocatechuate 3,4-dioxygenase with a specific activity of 1010 U/g against protocatechuate as substrate. SDS-PAGE of the same extracts showed elevated levels of polypeptide of about 23 kD (shown in Fig. 4). The molecular masses of PcaG and PcaH were 21.5 kD and 23 kD respectively, deduced from the amino acid sequences. Therefore it was probable that

the SDS-PAGE failed to distinguish these two polypeptides of similar size from each other. Neither activity nor enhanced 23 kD polypeptide band was detectable in controls where the expression vector pET5a contained no insert. Fig. 5 showed the rapid transforma- tion by the cell extract of protocatechuate (λ_{max} = 290 nm) to β-carboxy-*cis, cis*-muconic acid (λ_{max} = 270 nm) as described originally by Gibson[23]. Protocatechuate 3,4-dioxygenase activity was also detected in the cell extract of strain NCIMB10467 grown on protocatechuate (403 U/g).

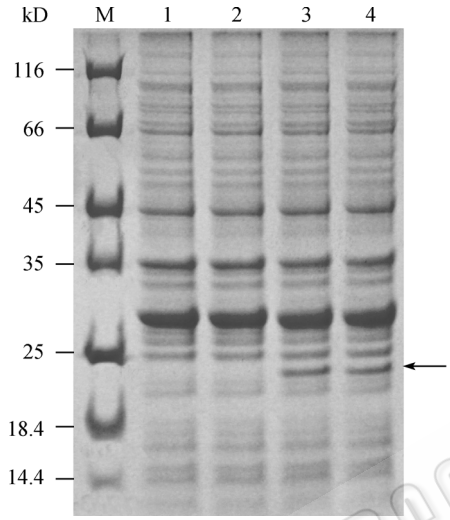

Fig. 4 The SDS-PAGE of overexpressed PcaGH in Rosetta on a 10% gel

M: Molecular weight marker; 1-2: Blank control (Rosetta containing pET5a); 3-4: Cell extracts containing PcaGH induced with IPTG. The molecular mass of the overexpressed polypeptide (indicated by an arrow on the right) is about 23 kD.

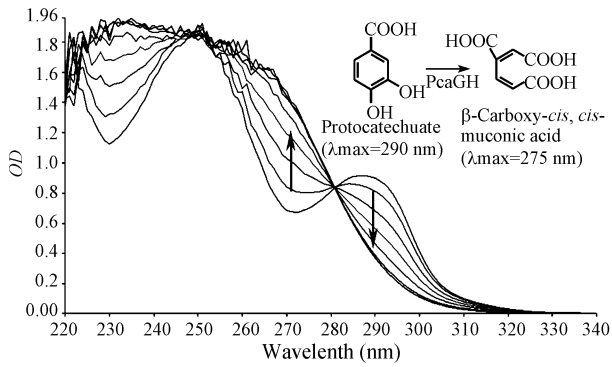

Fig. 5 Spectral changes during formation of β-carboxy-*cis, cis*-muconic acid from protocatechuate by cell extracts of *E. coli* Rosetta [pZWLS005] expressing *pcaGH*

Sample and reference cuvettes contained 50 mmol/L phosphate buffer (pH 7.4) and cell extracts of strain *E. coli* Rosetta [pWZLS005] in 0.5 mL volumes. The reaction was initiated by the addition of protocatechuate to 0.22 mmol/L and the spectra were recorded every 30 s after the addition of protocatechuate.

2.5 *pcaH* was essential for protocatehcuate degradation in strain NCIMB 10467

To investigate the possible involvements of *pcaH* gene in degradation of protocatechuate *in vivo*, *pcaH* gene disrupted mutant of strain NCIMB 10467 was constructed and functionally analyzed. Strains 1441 (*pcaH*-disrupted NCIMB 10467) completely lost the ability to grow on protocatechuate, and the unutilized substrate was oxidized into a compound of red color. On the other hand, the disruption of *pcaH* gene had no impact on the metabolism of 3-hydroxybenzoate and salicylate in strain NCIMB 10467. The assay was done three separate times with similar results obtained, and a representative example is depicted in Fig. 6.

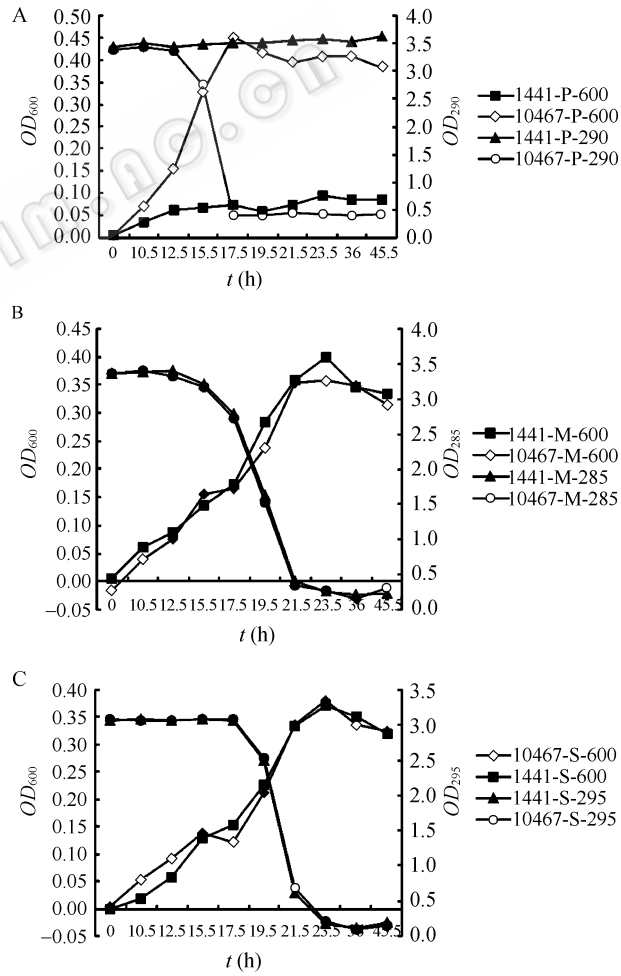

Fig. 6 Growth and degradation curves of strains 1441 and 10467 on different substrates

A: Strains grew on protocatechuate (P, λ_{max}= 290 nm); B: Strains grew on 3-hydroxybenzoate (M, λ_{max}= 285 nm); C: strains grew on salicylate (S, λ_{max}= 295 nm)

3 Discussion

Previous biochemical evidence on the strain NCIMB 10467 suggested that it could oxidize the protocatechuate without induction using whole cells[11]. However, this study has shown that protocatechuate was metabolized differently with various substrates induction when the cell extracts were used. Together with the physiological character of the *pcaH* knockout mutant, it was found that protocatechuate could induce the *ortho* cleavage of itself by P34D, while 3-hydroxybenzoate, salicylate and naphthalene might induce some unspecific enzymes. But these enzymes exhibited activities only in the presence of ferrous iron and metabolize protocatehcuate at a much lower rate than specific protocatechuate 3,4-dioxygenase induced by its native substrate.

Six enzymatic steps encoded by eight genes complete the conversion of protocatechuate to tricarboxylic acid cycle intermediates in various strains[28–30]. It is unexceptional that these eight genes were located in two or more distinct genetic loci in bacteria, such as *Pseudomonas putida* KT2440, *Silicibacter pomeroyi* DSS-3 and ect[31]. Similar organization was also evident in *Burkholderia xenovorans* LB400 according to the annotation from its genome sequence[32], although no experimental characterization has been performed. Within the 9.5 kb DNA fragment obtained from this study, only *pcaG* and *pcaH* genes were involved in the protocatechuate catabolism, and it was reasonable to speculate that protocatechuate catabolic cluster was also dispersedly distributed in the genome of strain NCIMB 10467.

P34D is normally composed of two polypeptides chains: the α-chain encoded by the *pcaG* and the β-chain encoded by *pcaH*[33]. Brown et al had supplied alignments of structure and sequence determined P34D α-chain and β-chain among more than 26 bacterial strains, and the results revealed that the β-chain showed higher sequence identities than the α-chain due to the β-chain provided most of the active sites[5]. This was supported by the research of P34D from marine bacteria *Rosebacter*, in which the nonheme Fe^{3+} coordinating residues were all located in the β-chain [31]. Therefore, the *pcaH* was chosen to be disrupted in this study. Disruption of this gene in strain NCIMB 10467 lead to loss of its ability to catabolize protocatechuate, while there was no effect on its catabolism of 3-hydroxybenzoate. The latter must undergo different routes from protocatechuate cleavage pathway, presenting an example of co-existence of versatile metabolic pathways for aromatics in strain NCIMB 10467.

4 Conclusion

The strain NCIMB 10467 was reclassified as the genus of *Burkholderia* in this study. The *pcaGH* genes cloned from it were proved to encode a functional protocatechuate 3,4-dioxygenase, which was essential for the metabolism of prtotocatechuate in this strain.

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