

# Polyaromatic Hydrocarbon Degradation and Dioxygenase Gene Detection from *Alteromonas alvinellae* Bt05

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## Abstract

Bt05 is marine bacterium which was isolated from the Jakarta Bay, Indonesia. The aim of this study was to characterize PAHs-degrading property and molecular identification by partial analysis of 16S rRNA gene, and to partially analyze dioxygenase gene of Bt05 isolate. Our further study on this isolate revealed that it could degrade three PAHs (phenanthrene, dibenzothiophene, fluorene) between 60%–90% within 11 days at 100 ppm level. This finding indicated the potential of the isolate for bioremediation of PAHs. The isolate was identified as *Alteromonas alvinellae* by phylogenetic analysis of 16S rRNA gene sequence. Sequence analysis of the PCR product of PAH dioxygenase genes amplified using two primer set (iiDA and ppAH) of the isolate were identified 97% as naphthalene dioxygenase gene (*phaAc*) and 58% as 1,2-dioxygenase.

**Keywords:** polyaromatic hydrocarbon, dioxygenase, *Alteromonas*

## Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of compounds composed of two or more fused aromatic rings that are important components of crude oil, creosote, and coal tar (Head *et al.*, 2006). Based on the molecular weight of these hydrocarbons, PAHs can be classified into two broad groups: (i) the low molecular weight (LMW) PAHs that contain 2-3 benzene rings, such as naphthalene, fluorene, and phenanthrene, and (ii) the high-molecular weight (HMW) PAHs, such as fluoranthene, pyrene, and chrysene (Wilson & Jones, 1993; Kanaly & Harayama, 2000). They are the cause of great environmental concern because of their persistence, toxicity, mutagenicity, and carcinogenicity (Xue & Warshawsky, 2005). Due to their properties, they are listed on priority-pollutant lists in most countries (Cerniglia, 1993; Collins *et al.*, 1998). The stability, persistency, and carcinogenic index of PAHs increase due to an increase in the number of aromatic rings, structural angularity and hydrophobicity (Marston *et al.*, 2001).

Bioremediation is considered as the most significant and influential for degradation or detoxification of xenobiotic compounds (Sutherland *et al.*, 1995).

Several reports on the isolation of PAHs-degrading bacteria and the biochemical and genetic analyses of PAHs degradation have been reported (Cerniglia, 1992; Kasai *et al.*, 2002; Okazaki *et al.*, 2006; Kumar & Khanna, 2009; Juckpecha *et al.*, 2012). PAH-degrading bacteria from marine habitats have been extensively studied, reports of PAH-degrading bacteria such as *Cycloclasticus* (Dyksterhouse *et al.*, 1995; Geiselbrecht *et al.*, 1998), *Neptunomonas* (Hedlund *et al.*, 1999), *Halomonas* (Melcher *et al.*, 2002), *Pseudoalteromonas* (Hedlund *et al.*, 2006), *Novosphingobium* (Sohn *et al.*, 2004), *Pseudomonas* (Tagger *et al.*, 1990; Murniasih *et al.*, 2009), and *Alteromonas* (Zaidi *et al.*, 2003) have appeared.

One of the bacteria that play an important role in PAH degradation is *Alteromonas*. Members of the genus *Alteromonas* are increasingly recognized as globally distributed heterotrophic marine bacteria (Martinez *et al.*, 2008; McCarren *et al.*, 2010). *Alteromonas* was reported as a key agent of PAHs biodegradation in crude oil-contaminated coastal sediment (Jin *et al.*, 2012). Complete genome and annotation of *Alteromonas* strains SN2 are now available (Jin *et al.*, 2011).

Microbial degradation of PAHs is an oxidative attack (Butler & Mason, 1997). The enzymes that catalyze insertion of molecular oxygen into aromatic benzene rings are termed oxygenases (Mason & Cammack, 1992). They require transition metals, such as iron and heme, as catalytic

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centers. Oxygenases that utilize non-heme Fe(II) are called Rieske-type non-heme iron aromatic ring-hydroxylating oxygenase (RHO) whereas others that use heme are cytochrome P450s (Feig & Lippard, 1994; Raag & Poulus, 1989). The term RHO is used herein to denote the Rieske-type non heme iron ring-hydroxylating oxygenase. The RHO enzyme system has been extensively studied in many different microorganisms since the initial reaction mostly determines the aromatic substrate for degradation (Gibson *et al.*, 1995; Chang & Zylstra, 1998; Saito *et al.*, 1999; Khan *et al.*, 2001). Kweon *et al.* (2008) presented a scheme for classification of RHOs reflecting new sequence information and interactions between RHO enzyme components. This phylogenetic classification scheme was converted to a new systematic classification consisting of 5 distinct types.

Primers for dioxygenase genes (*nahAc*, *phnAc*, *nidA*) and the conserved segments of RHOs were designed and used for the detection of novel dioxygenase genes in PAH-degrading bacterial isolates and environmental samples (Hedlund *et al.*, 1999; Stach & Burns, 2002; Brezna *et al.*, 2003; Zhou *et al.*, 2006). However these primers were not able to detect the dioxygenase gene from some PAHs-degrading bacterial species. This fact indicates that it is important to study the functional gene from PAHs degrading bacteria (Meyer *et al.*, 1999).

Most of the research works on bacterial diversity and functional genes related to PAHs degradation were conducted in subtropical areas. In the tropical sea environment, especially in Indonesia, the isolation and characterization of PAH-degrading bacteria were reported (Harwati *et al.*, 2009; Murniasih *et al.*, 2009; Riffiani, 2010; Teramoto *et al.*, 2010; Lisdiyanti *et al.*, 2011), but detection of their functional genes are limited. The diversity of oil-degrading bacteria

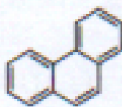
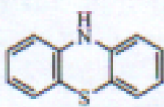
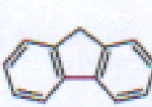
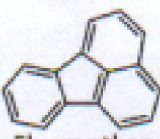
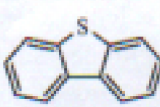
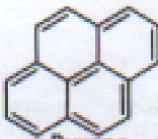
of Muara Kamal, the Jakarta Bay, was reported by Yopi *et al.* (2006). It was stated that the microbial isolate from Muara Kamal, Jakarta, has the ability to degrade PAH compound. One of the isolates that have high activity to degrade PAHs by sublimation test is Bt05. However, the PAHs degradation ability of Bt05 isolate is unknown. Therefore, it is necessary to analyze the ability of this isolate to degrade PAHs compounds and the existence of dioxygenase gene. This paper aims to characterize PAHs-degrading property and molecular identification by partial analysis of 16S rRNA gene, and to partially analyze dioxygenase gene of Bt05 isolate.

## Materials and Methods

**Bacteria strain.** The Bt05 is the collection of marine bacterium from Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), and NITE Japan.

**PAHs degrading test.** PAHs degrading bacteria were characterized by using sublimation test (Alley & Brown, 2000). A bacterium was incubated for 2–4 week after sublimation. Sublimation test was carried out on solid ONR7 media (Dyksterhouse *et al.*, 1995) and PAHs (phenanthrene, dibenzothiophene, fluorene, fluoranthene, phenothiazine and pyrene) (Table 1). ONR7 medium contained 22.79 g NaCl, 11.18 g MgCl<sub>2</sub>·6H<sub>2</sub>O, 3.98 g Na<sub>2</sub>SO<sub>4</sub>, 1.46 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 1.3 g 3-[N-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid (TAPSO), 0.72 g KCl, 0.27 g NH<sub>4</sub>Cl, 89 mg Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 83 mg NaBr, 31 mg NaHCO<sub>3</sub>, 27 mg H<sub>3</sub>BO<sub>3</sub>, 24 mg SrCl<sub>2</sub>·6H<sub>2</sub>O, 2.6 mg NaF, dan 2.0 mg FeCl<sub>2</sub>·4H<sub>2</sub>O per liter. Sublimation test conditions (melting point and time) for each compound were shown in Table 1. Bacteria which grow and give clear zone were anticipated to represent PAH degrading bacteria.

Table 1. Sublimation Test Condition

Conditions	PAHs Compounds					
						
	Phenanthrene	Phenothiazine	Fluorene	Fluoranthene	Dibenzothiophene	Pyrene
Melting point (°C)	100	100	115	130	130	130
Time (min)	5	3	3	30	30	30



**Phenanthrene, dibenzothiophene, and fluorene biodegradation test.** Bt05 isolate was analyzed for its ability to utilize phenanthrene, dibenzothiophene, and fluorine. PAHs degrading activity was tested using growth test. The growth test was set up with 50 ml of ONR7 medium in 300-ml Erlenmeyer flasks aseptically supplemented with 100 ppm of PAHs as a sole source of carbon. The culture was incubated on 200 rpm rotary shaker and 30°C temperatures. The growth of bacteria in the all substrates was evidenced by spectrometrically monitoring an increase in bacterial mass and by measuring a decrease of substrate by Gas Chromatography (GC). Metabolites were extracted from culture grown using dichloromethane and analyzed by GC Shimadzu 17A. For the detector, *flame ionization detector* (FID) was applied, with a 30-cm silica capillary column (HP1) with 0.35 mm diameter, 0.33 µm thickness of film, while the oven temperature was 60°C and increased to 280°C, with 6 ml/minute flow rate. The incubation time was 15 minutes. The temperature of the detector and injector were 300°C and 240°C, respectively. Nitrogen was used as gas carrier. Degradation activity was measured based on phenanthrene remaining.

**16S rRNA gene analysis.** Sequence analysis of the partial 16S rDNA gene was performed by amplifying the gene by PCR using 9F and 1492R primers (Calvaca *et al.*, 1999). The amplification were conducted with reaction condition of PCR was 95°C, 2 min (1 cycle); 95°C, 30 sec; 65°C, 1 min; 72°C, 2 min (10 cycles); 95°C, 30 sec; 55°C, 1 min; 72°C, 2 min (30 cycles); and 72°C, 2 min (1 cycle). The PCR products were analyzed by electrophoresis on 0.8% agarose gel. The PCR products were

also purified using *AGENCOURT® CLEANSEQ® Dye-Terminator Removal* (Beckman Coulter-USA) according to the manufacturer's recommendations for sequence analysis.

**PAHs dioxygenase gene detection.** The gene fragments for PAHs dioxygenase were amplified by PCR with degenerate primers (pPAH-F/pPAHNR700, NAH-F/R, iiDA-F/R, and Kasuga-F/R) (Table 2). PCR amplified fragment was cloned by pGEMT-Easy vector. Then the plasmid recombinant was transformed in *E. coli* JM109 competent cell (Inoue *et al.*, 1990). The plasmid pGEMT-Easy recombinant was transformed in *E. coli* JM109 detected by PCR colonie.

**DNA sequencing and sequence analysis.** Purified plasmids containing the appropriate insert DNA were sequenced using DNA sequencer ABI 310 (Pharmacia) at NITE-Japan. The obtained nucleotide sequences were analyzed using BioEdit. BLSTX (Altschul *et al.*, 1997) was used for homology searching. Multiple alignment and phylogenetic tree were produced by CLUSTAL X (Higgins & Sharp, 1988), and the phylogenetic tree was visualized by the NJ plot program (Thompson *et al.*, 1994) and Mega 3.1 ABI sequencer software (Kumar *et al.*, 2004).

## Results

### Biodegradation of PAHs by Bt05 Isolate

The biodegradation ability of Bt05 isolate was tested by providing PAHs as the source of carbon and energy. We used six different PAHs compounds

Table 2. PCR Primers were used in this study.

Primers	Sequence (5' to 3')	Reference
9F	AGRGTTTGATCMTGGCTCAG	Cavalca <i>et al.</i> (1999)
1492R	TACGGYTACCTTGTTAYGACTT	
pPAH-F	GGYAAAYGCNAAAGAATTCGTNTGYWSHTAYCAY	Geiselbrecht <i>et al.</i> (1998)
pPAHNR700	CCAGAATTCNGTNGTRTTHGCATCRATSGGRTKCCA	
NAH-F	CAAAA (A/G) CACCTGATT (C/T) ATGG	Jones <i>et al.</i> (1999)
NAH-R	(C/T) (A/G) CG (A/G) G (C/G) GACTTCTTTCAA	
iiDA-F	TGYHSNTAYCAYGGNTGG	Iida <i>et al.</i> (2002)
iiDA-R	TCNRCNGCNARYTTCCARTT	
Kasuga-F	TGYCGBAYCGBGGSAWG	Kasuga <i>et al.</i> (2001)
Kasuga-R	CCAGCCGTGRTARSTGCA	
pGMT-M13F	CGCCAGGGTTTTCCAGTCACGAC	Knoche & Kephart (1999)
pGMTM13R	TCACACAGGAAACAGCTATGAC	

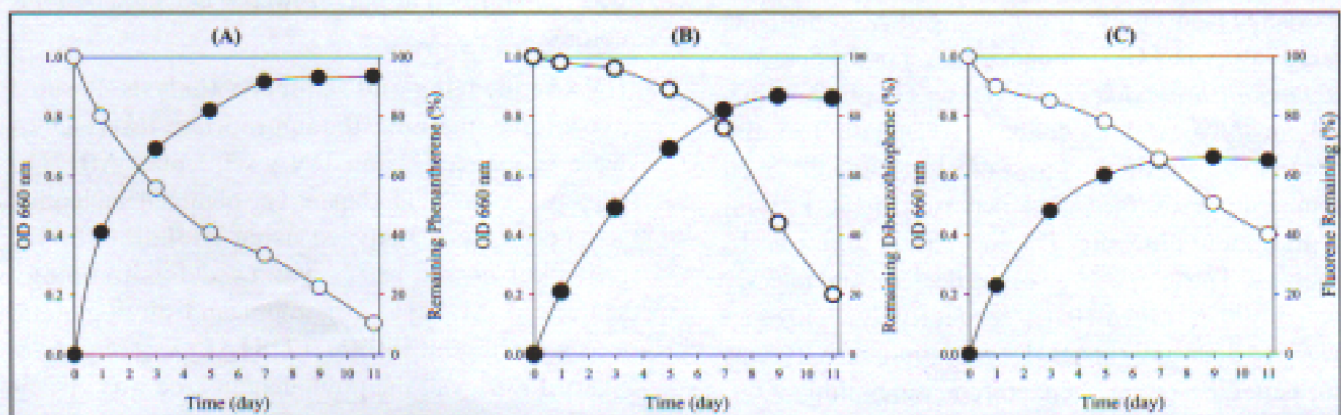


for this purpose. As shown in Table 3, the culture was able to utilize phenanthrene, dibenzothiophene, and fluorene. The PAH degradation ability of isolate Bt05 was evaluated in ONR7 medium and containing 100 ppm of each individual PAH compound (phenanthrene, dibenzothiophene, or fluorene). Figure 1 shows that the individual PAH concentration profiles with respect to incubation time. PAH losses in uninoculated control experiments were negligible. Bt05 isolate was able to degrade all of phenanthrene, dibenzothiophene, and fluorene. Phenanthrene, dibenzothiophene, and fluorene were

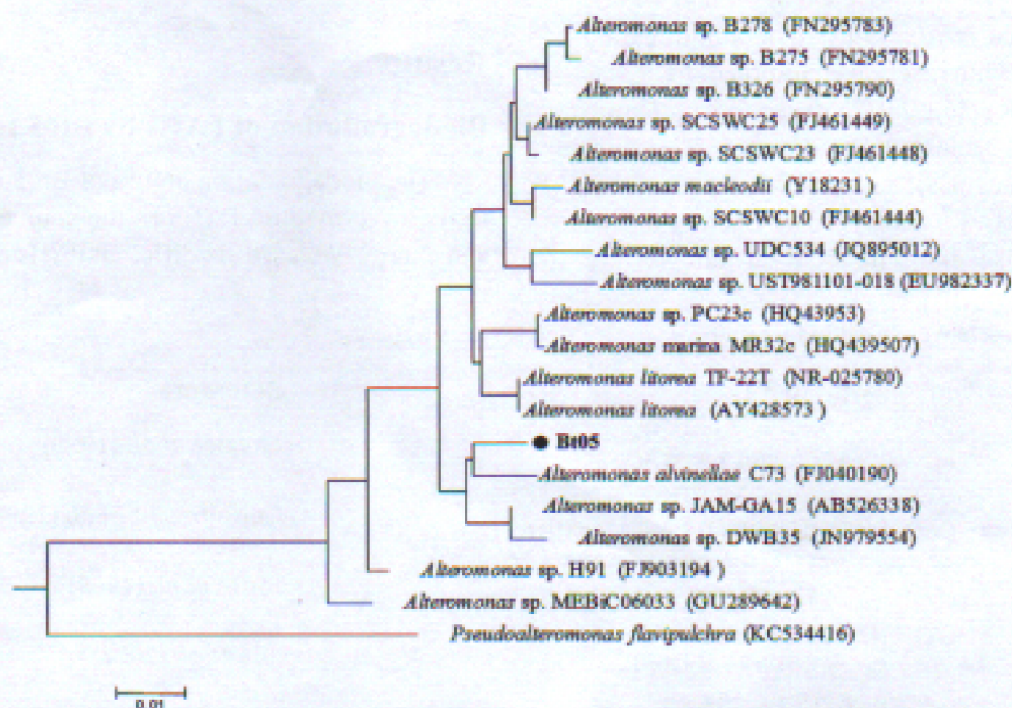
degraded around 90%, 80%, and 60%, respectively, after 11 days of incubation. Figure 1 shows that the PAHs degradation rate is sharper at stationary phase.

### Phylogenetic analysis

Isolate Bt05 was identified based on 16S rDNA sequence analysis. Approximately 1,114 bp of Bt05 isolate 16S rRNA gene was sequenced. After using BLAST analysis, Bt05 isolate has 99% identity with *Alteromonas alvinellae* C73 (FJ040190). The phylogenetic tree for Bt05 isolate is shown in Figure 2.



**Figure 1.** Biodegradation of (A) phenanthrene, (B) dibenzothiophene, and (C) fluorene by BT05 isolate. (●) cell growth and (○) remaining of PAHs compound



**Figure 2.** Phylogenetic tree derived from 16S rDNA gene sequence of Bt05 isolate and sequences of closest phylogenetic neighbours obtained by NCBI BLAST(n) analysis. The NJ-tree was constructed using neighbour joining algorithm with Kimura 2 parameter distances in MEGA 3.1 software. *Pseudoalteromonas flavipulchra* (KC534416) has been taken as an out-group. Bar, 1% estimated sequence divergence.



### Detection of PAH dioxygenase gene

Amplification of the initial PAH dioxygenase genes in Bt05 isolate was performed by PCR using four degenerated primer sets (pPAH-F/R, NAH-F/R, iiDA-F/R and Kasuga-F/R). We obtained 2 PCR products of PAH dioxygenase gene from iiDA and pPAH primers. When amplified using iiDA-F/R primer set, Bt05 showed a PCR product of 300 bp by using the degenerated primer set of pPAH-F/R gave a 630-bp amplification product (Figure 3); whereas, we did not obtain PCR products of PAH dioxygenase gene from NAH and Kasuga primers. These primers were not able to detect the dioxygenase gene from Bt05 isolate. Summary of these results was shown in Table 3.

Sequence analysis of the PCR product that was amplified using primer set, pPAH-F/NR700, and subsequent homology search analysis using BLASTX program showed that the deduced amino acid sequence of 97% is most closely related to amino acid sequence with naphthalene dioxygenase gene (*phaAc*) from *P. aeruginosa* (D84146). Sequence analysis of the dioxygenase gene from Bt05 isolate, amplified using primer set of iiDa-F/R, revealed that its deduced amino acid sequence showed 58%

identity to that of 1,2-dioxygenase from *P. cepacia* 2CBS (Haak *et al.*, 1995).

### Discussion

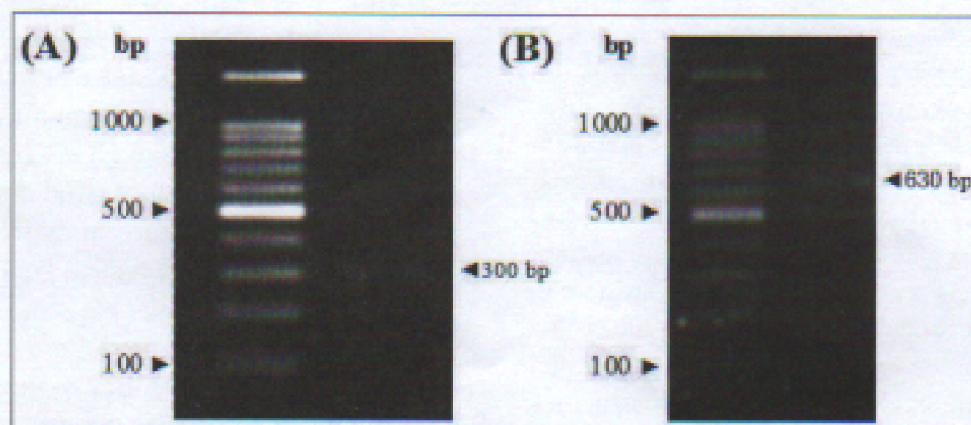
The ability of PAHs compounds degradation by Bt05 isolate was known using the sublimation and growth tests. Using sublimation test, positive PAHs degradation is shown by the formation of a clearing zone around a colony or by a change in a colony's color due to transformation of PAHs into other substances (Kiyohara *et al.*, 1982; Alley & Brown, 2000). Bt05 isolate could degrade only LMW PAH compounds, such phenanthrene, fluorene and dibenzothiophene. Otherwise, this isolate could not degrade HMW PAH compounds, such as fluoranthene and phenothiazine (Table 3). The growth test results of Bt05 isolate in several PAHs also were shown in Table 3. Similar with sublimation test result that Bt05 isolate could growth on LMW PAHs (phenanthrene, fluorene, and dibenzothiophene). This result indicated that Bt05 use these compounds as sole carbon and energy source.

PAHs degradation of Bt05 isolate was monitored until 11 days (Figure 1). As expected, PAH compounds with more rings were degraded more

**Table 3.** Phenotypic and genotypic characterization of Bt05 isolate

Sublimation Test						Growth Test					PCR by using			
Phn	Fln	Fuo	Dbz			Fln	Fuo	Dbz	Pyr		pPAH	NAH	iiDA	Kasuga
+	-	+	-	+	-	+	-	+	-	+	+	-	+	-

Note: sign (+) indicates the formation of clear zone around the isolate Bt05 after sublimation test able to grow in the substrate test with a value of OD  $\geq 0.5$ . PCR product of PAH dioxygenase gene detection. Sign (-) showed the opposite. Phn: phenanthrene; Ptz: phenothiazine; Fln: fluorene; Fuo: fluorene; Dbz: dibenzothiophene; and Pyr: pyrene



**Figure 3.** PAHs dioxygenase gene PCR amplification for Bt05 isolate. Detection of PAH dioxygenase gene used (A) iiDA, and (B) pPAH primers.



slowly, thus fluorene degradation was very slow. The cell growth of Bt05 for all substrates showed its maximum at 7 days after incubation. However, the higher of cell growth was shown at phenanthrene as a carbon source followed by dibenzothiophene and fluorene. Maximum degradation of phenanthrene was 11 days. Phenanthrene remained at the concentration of 10.5% after 11 days cultivation. Phenanthrene degradation activity of Bt05 isolate was higher than *Pseudomonas* sp. Kalp-3b22 (Murniasih *et al.*, 2009). Maximum degradation of phenanthrene by *Pseudomonas* sp. Kalp-3b22 was only 49.5% after 29-day cultivation period. Using the same condition, *Pseudomonas* sp. Kalp-3b22 degraded dibenzothiophene leaving only 20% of this compound at the end of cultivation. In addition to its ability to degrade phenanthrene and dibenzothiophene, Bt05 could utilize fluorene as a sole source of carbon and energy. The degradation of fluorene could reach as high as 60% at day 11 of incubation.

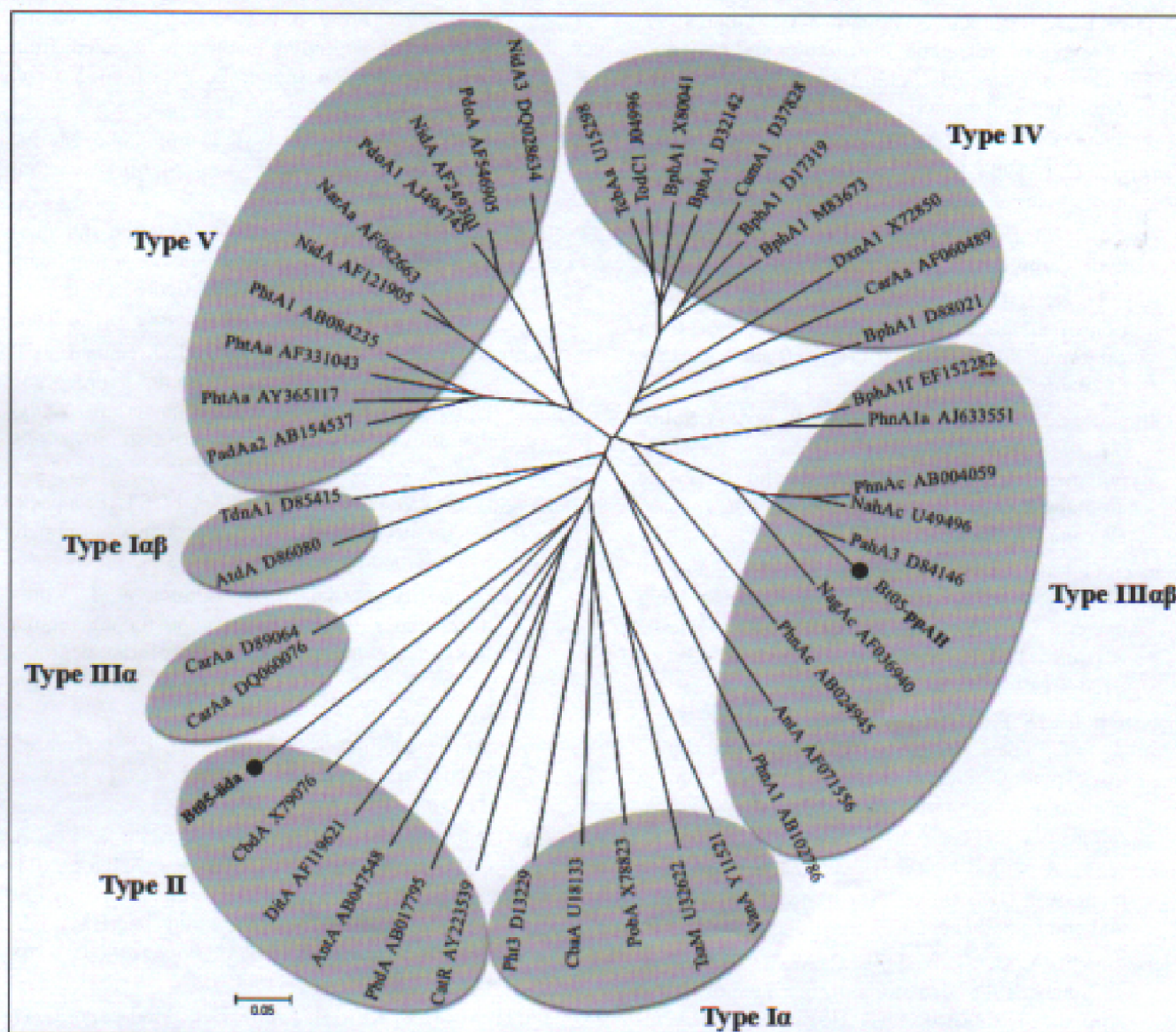
Bt05 isolate was identified as *Alteromonas alvinellae* (Figure 4). Genus of *Alteromonas* belongs to Class  $\gamma$ -proteobacteria and Family *Alteromonadaceae*. This genus is known as heterotrophic marine bacteria and is known for their versatile metabolic activities (Ng *et al.*, 2013). The genus *Alteromonas* is also able to use polyaromatic hydrocarbons as carbon source (Cui *et al.*, 2008; McCarren *et al.*, 2010). Thus, *Alteromonas* species are functionally important in recovering marine habitats after oil pollution. Since the past decades, PCR amplification using gene specific primers or degenerate primers has been used to study the diversity of aromatic-dioxygenase genes in PAH degrading bacterial isolates and environmental samples (Hedlund *et al.*, 1999; Zhou *et al.*, 2006). The primers were designed based on the dioxygenase genes including *nahAc* and *phnAc* (Jones *et al.*, 1999), *nidA* (Brezna *et al.*, 2003), and the conserved segments of RHO (Kahl & Hofer, 2003). Their use has resulted in the detection of novel dioxygenase genes from environmental samples. In this study, we used four sets of primers to detect genes that play role in the PAHs degradation. Only two primers sets, *iiDa* and *ppAH* were able to give good PCR products. Widada *et al.* (2002) also reported the similar results about it. It is suggested that this bacteria have diverse catabolic capacities for PAH (Meyer *et al.*, 1999).

Bt05-*iiDa* and Bt05-*ppAH* sequences were aligned with another RHO genes based on recent classification. These sequences were classified into type II for Bt05-*iiDa* and type IIIa $\beta$  for Bt05-*ppAH* of RHO enzyme (Figure 4). Type II contains two-component RHO systems that consist of an oxygenase and an FNRN-type reductase. The type system enzyme is also organized at 2-halobenzoate 1,2-dioxygenase (*P. cepacia* 2CBS), anthranilate dioxygenase (*Acinetobacter* sp. ADP1), and benzoate 1,2-dioxygenase (*Acinetobacter* sp. ADP1). Type III represents a group of three-component RHO systems that consist of an oxygenase, a [2Fe-2S]-type ferredoxin and an FNRN-type reductase. This type is also organized at 3,4-dihydroxyphenanthrene dioxygenase (*Acinetobacter faecalis* AFK2), Naphthalene dioxygenase (*Pseudomonas* sp. 9816-4), and PAH dioxygenase (*P. putida* OUS82) (Kweon *et al.*, 2008).

Two types of Bt05 PAH dioxygenase genes were identified. These results strengthened the assessment that Bt05 isolate has PAH dioxygenase gene complex. This was confirmed by several reports. Genome of *Alteromonas* sp. SN2 has a circular chromosomal genome of 4,972,148 bp with a GC content of 43.5% and no plasmid. Twenty-one dioxygenase genes, known to be essential for metabolizing recalcitrant organic compounds (Gibson & Parales, 2000; Kim *et al.*, 2008), were present in the genome (Jin *et al.*, 2011). Likewise on its 6.5 Mb genome of *Mycobacterium vanbaalenii* PYR-1 contains 194 chromosomally encoded genes likely associated with degradation of aromatic compounds (Kim *et al.*, 2008). These observations suggest that bacteria have diverse catabolic capacities for PAH.

Bt05 was identified as *Alteromonas alvinellae* by phylogenetic analysis of 16S rDNA sequence. Result of this study indicated that Bt05 isolate could degrade phenanthrene, dibenzothiophene, and fluorene. This result also indicated that Bt05 was potential for bioremediation of PAHs. The future study from this isolate is the study on dioxygenase gene and also enzymes. These enzymes play an important role in the environmental biodegradation of organic pollutants and in the biotechnology industry for the production of value-added chemicals.





**Figure 4.** The phylogenetic tree, inferred by the neighbor-joining method, shows the relative positions of Bt05-ppAH and Bt05-iiDA among other RHOs based on the recent classification (Kweon *et al.*, 2008). Gene ID (GI) numbers were provided for RHO sequences the detail information of which can be found in the NCBI database. The bar indicates 5% sequence divergence.

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