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Comparison of the functional characterization between alkane monooxygenases for low-molecular-weight polyethylene biodegradation

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ABSTRACT

AlkB1 of the *Pseudomonas aeruginosa* strain E7 is known as an enzyme that is used in alkane degradation. It has been reported that AlkB1 is active for degradation of low molecular weight polyethylene (LMWPE-1) and that degradation increased in the presence of RubA1, RubA2, and RubB. As an extension of these results, this study investigated the activity of AlkB2 toward LMWPE-1 degradation and the mutual interaction between AlkB2 and RubA1/RubA2. The activity of AlkB1 in LMWPE-1 degradation was compared with that of AlkB2. An examination of the transcription levels of *alkB1* and *alkB2* induced by *n*-dodecane and *n*-hexadecane indicated that the transcription of *alkB1* was induced by *n*-hexadecane but not by *n*-dodecane, whereas that of *alkB2* was induced by both *n*-dodecane and *n*-hexadecane. The transcription level of *alkB2* induced by *n*-hexadecane was about 7 times higher than that of *alkB1*. The transcription of *alkB2* was also higher and persisted longer than that of *alkB1* in the presence of LMWPE-1. The results of LMWPE-1 biodegradation by BCA (inserted *alkB1*), BCA2 (inserted *alkB2*), and BCA2R (inserted *alkB2*, *rubA1* and *rubA2*) recombinant strains under controlled compost conditions revealed that LMWPE-1 biodegradation by the BCA2 strain was higher than that by the BCA strain, and the biodegradation activity of the BCA2 strain was similar to that of the strain cloned with *alkB1, rubA1, rubA2*, and *rubB*. LMWPE-1 biodegradation by the BCA2R strain was not higher than that by the BCA2 strain, even though the BCA2R strain contains RubA1 and RubA2. Therefore, it was concluded that AlkB2 is more effective than AlkB1 toward LMWPE-1 degradation, and that the regulation mechanism of AlkB2 is different from that of AlkB1.

1. Introduction

Polyethylene (PE) is widely used for various applications ranging from single use disposables to industrial materials and agricultural plastics, but it is considered as a troublesome material because it is highly recalcitrant to biodegradation in the natural environment due to its high molecular weight and hydrophobicity (Sen and Raut, 2015). Thus, discarded PE waste accumulation defiles the natural landscape, disturbs the ecosystem, and devastates soil and marine environments (Sen and Raut, 2015; Roy et al., 2011).

A great deal of research has been devoted to isolation of PE degrading microorganisms from various microbial sources. Quite a limited number of microorganisms that were isolated could grow using PE as the sole carbon source, *Bacillus cereus, Bacillus megaterium, Bacillus subtilis* (Abrusci et al., 2011), *Rhodococcus* sp., *Delftia* sp. (Koutny et al., 2009), *Acinetobacter baumannii* (Nowak et al., 2011), *Brevibacillus parabrevis* (Pramila et al., 2012), *Chryseobacterium gleum*, (Jeon and Kim, 2014), and *Pseudomonas aeruginosa* (Jeon and Kim, 2015) being one instance. However, the activity of any identified microorganisms toward PE degradation was so low that they cannot be utilized for actual bioremediation of a natural environment polluted with PE waste.

Though studies on genes and mechanism associated with PE degradation are extremely scarce, it has been reported that the enzymes involved in the alkane hydroxylase system pathway take part in PE degradation (Jeon and Kim, 2015). The alkane hydroxylase system was investigated focusing mostly on *Pseudomonas putida* GPo1, which can degrade alkanes with an alkane monooxygenase that participates in the first step in the pathway and functions by hydroxylation of the terminal carbon of alkanes (Rojo,

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2009). Alkane monooxygenase requires two soluble electron transferring proteins, rubredoxin and rubredoxin reductase. *P. putida* GPo1 possesses two rubredoxins and a rubredoxin reductase. The latter transfers electrons from NADH to rubredoxin, which then transfers electrons to alkane monooxygenase (Rojo, 2009; Smits et al., 2002).

Unlike *P. putida* GPo1 that has one alkane monooxygenase (Rojo, 2009), other bacteria may have two or more alkane monooxygenases, in which case alkane monooxygenases of the bacteria show distinctly different characteristics. It has been reported that *Alcanivorax borkumensis* SK2 possesses two alkane monooxygenases, i.e. AlkB1 and AlkB2. The former is usually able to degrade C_5–C_{12}, while the latter can degrade C_8–C_{16} hydrocarbons (Miri et al., 2010). According to Takei et al. (2008), *Rhodococcus* sp. TMP2 has 5 alkane monooxygenases (AlkB1, AlkB2, AlkB3, AlkB4 and AlkB5). The transcription of *alkB1* and *alkB2* is induced by 0.1% *n*-hexadecane and pristine, whereas that of *alkB3*, *alkB4*, and *alkB5* is neither affected by *n*-hexadecane nor by pristine, indicating that the conditions for induction of those genes are different from each other.

P. aeruginosa is commonly detected in soil and water. This strain can use a wide range of substrate as a source of carbon and energy. It has two alkane monooxygenases (AlkB1 and AlkB2), two rubredoxins (RubA1 and RubA2), and one rubredoxin reductase (RubB). All of the enzymes contribute to the degradation of *n*-alkanes (Marín et al., 2003). *P. aeruginosa* has two alkane monooxygenases, AlkB1 and AlkB2, whose activity toward LMWPE-1 degradation and the conditions for the induction of the relevant genes may differ from each other.

Jeon and Kim (2015) examined whether LMWPE-1 could be degraded by the alkane hydroxylase system of *P. aeruginosa* E7 that showed activity for LMWPE-1 degradation. *A. borkumensis* SK2 has two AlkBs, and the gene encoding AlkB1 exists as a cluster and degrades hydrocarbons together with accessory proteins such as RubA1, RubA2, and RubB, whereas the gene encoding AlkB2 does not form a cluster (Miri et al., 2010). Jeon and Kim (2015) presumed that *P. aeruginosa* E7 expressing AlkB1 and AlkB2 could exhibit similar activities as *A. borkumensis* SK2.

In this study, we compared the degradability of LMWPE-1 by AlkB1 and AlkB2 in *P. aeruginosa* E7 and attempted to determine the gene encoding the enzyme that shows higher activity toward LMWPE-1 degradation. The transcription patterns of *alkB1* and *alkB2* induced by *n*-dodecane, *n*-hexadecane, and LMWPE-1, respectively, and the conditions for induction of the respective genes and their transcription level were examined. Recombinant strains of BCA (inserted *alkB1*), BCA2 (inserted *alkB2*), and BCA2R (inserted *alkB2*, *rubA1* and *rubA2*) were prepared and their activities of LMWPE-1 biodegradation were compared under controlled compost conditions.

2. Material and methods

2.1. Chemicals and culture media

n-Dodecane and *n*-hexadecane (all $\geq$ 99% pure) were purchased from Sigma. LMWPE-1 was prepared by thermal degradation of a commercial high-density PE in a strict nitrogen atmosphere. The number- and weight-average-molecular-weight of LMWPE-1 were 790 and 1700, respectively. The basal medium was composed of K_2HPO_4, 2.34 g; KH_2PO_4, 1.33 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 1 g; NaCl, 0.5 g; yeast extract, 0.06 g and 1 mL of trace element solution ($CoCl_2$, 11.9 mg L^{-1}; $NiCl_2$, 11.8 mg L^{-1}; $CrCl_2$, 6.3 mg L^{-1}; $CuSO_4$, 15.7 mg L^{-1}; $FeCl_3$, 0.97 g L^{-1}; $CaCl_2$, 0.78 g L^{-1} and $MnCl_2$, 10.0 mg L^{-1}) in 1 L D.W. (Kim and Park, 2010)).

2.2. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was applied to compare transcription patterns between *alkB1* and *alkB2* in *P. aeruginosa* E7 when exposed to alkanes or LMWPE-1. *P. aeruginosa* E7 grown overnight in a nutrient broth was inoculated into 40 mL basal medium containing 0.5% LMWPE-1 and then incubated for 22 days. *P. aeruginosa* E7 was also inoculated into 60 mL basal medium containing 1% *n*-dodecane or *n*-hexadecane and then incubated for 7 days. The same bacterium incubated in a nutrient broth (Difco) in the absence of the alkanes and LMWPE-1 was used as a negative control. The incubation was carried out at 37 °C and 120 rpm. Total RNA was isolated from culture medium using RiboEx solution (GeneAll, Korea) and then separated by electrophoresis on 1% agarose gel. Respective RNA samples were placed on a UV/Vis spectrophotometer (DU730, BeckMan Coulter) to measure their UV absorbance at wavelengths of 260 nm and 280 nm to verify their purity and quantitation. The cDNA was synthesized so that the final concentration of the respective RNAs was adjusted to be 0.1 μg μl^{-1} by 5 × All-In-One RT MasterMix (Applied Biological Materials, Canada).

For real-time PCR using SYBR, the cycling condition established consists of pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 63 °C for 40 s, extension at 72 °C for 40 s, and final extension at 72 °C for 5 min. The denaturation, annealing and extension steps were repeated 30 times. The 16S rRNA gene was used as a reference, 2p*alkB1*-F and *alkB*-R were used for *alkB1*, P*alkB2*-F and *alkB2*-R for *alkB2*, and 16SRT-F and 16SRT-R primers were used for the 16S rRNA gene (Table 1). The specificity of the PCR products to be used for the PCR reaction was verified using a melting curve plotted through 80 cycles after the completion of the PCR: 1 min at 95 °C, 1 min at 55 °C, with the rest of cycles by incrementing by 0.5 °C with every cycle starting to elapse 10 s from 55 °C. CFX96 real-time PCR system software Version 1.0 (Bio-rad) was used to obtain the threshold cycle (C_T) of respective reactants and the method of $2^{-\Delta\Delta ct}$ was used for fold change values with respect to the number of genes (Livak and Schmittgen, 2001).

2.3. Cloning of alkB2, rubA1 and rubA2

P. aeruginosa E7 exhibiting high activity toward LMWPE-1 biodegradation isolated from Manripo beach (Jeon and Kim, 2015), was used to extract genomic DNA by using the DNeasy Blood and Tissue Kit (Qiagen). Then, *alkB2* was amplified through PCR by using ez *alkB2*-F, ez *alkB2*-R and *alkB2*-R, each of *rubA1*, *rubA2* was amplified with ez a2rA1-F and *rubA1*-R, and Ez *rubA2*-F and Ez *rubA2*-R, as shown in Table 1. The cycling conditions for the PCR were pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing for 40 s (at 63 °C in the case of *alkB2* and 58 °C in the cases of *rubA1* and *rubA2*), extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. The denaturation, annealing and extension steps were repeated 30 times. Those amplified genes were purified using Expin PCR SV (GeneAll) and then ligated together with pET21a cleaved with XhoI(C↓TCGAG) and HindIII(A↓AGCTT) (Enzynomics, Korea) at 37 °C for 15 min by using EZ-cloning kit (Enzynomics), and then inserted into *Escherichia coli* DH5-a competent cell. Recombinant vector extracted from white large colony was inserted into *E. coli* BL21 competent cell. Inserted genes of white-colored circular colony were analyzed for genes sequence (Macrogen, Korea).

2.4. Biodegradation test

Biodegradability of LMWPE-1 was measured at 37 °C for 50 days according to the KS M3100-1:2002; MOD ISO 14855:1999 standard. Each recombinant strain was cultivated overnight in 20 mL Luria