Molecular characterization of a bacterial consortium enriched from an oilfield that degrades phenanthrene

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Abstract

Characterization of functional and phylogenetic genes was carried out on a bacterial consortium, enriched from a water treatment system of an oilfield, that could use phenanthrene as the sole carbon source. The mixed culture degraded 130 mg phenanthrene l⁻¹ in 16 days, which is significantly faster than previously reported pure cultures. The existence of catabolic genes (*nahAc*, *C23O*) in the mixed culture was quantitated by most probable number PCR. The plasmid encoding phenanthrene catabolic genes increased relative to the chromosome genes. Heterogeneous bacteria were present according to both PCR denaturing gradient gel electrophoresis and cloning methods, suggesting the possible existence of cooperation between different biochemical PAH-transforming pathways.

Introduction

Many microbes with the ability to degrade polycyclic aromatic hydrocarbons (PAHs) have been isolated and PAH degradation mechanisms, enzymes and genes have been widely studied using these isolates (Tuomi *et al.* 2004). However, PAH biodegradation by pure cultures may not represent the actual behavior of environmental microbes since the cooperation of indigenous strains is damaged. Ghazali *et al.* (2004) constructed a microbial consortium by mixing together several known strains to degrade hydrocarbons. However, it is difficult to maximize cooperation among different species using a synthetic microbial consortium, and a more reasonable way to acquire an efficient degradation system would be by enriching microbes from environments using the target compounds as the carbon source.

In this study, an enriched culture system for phenanthrene degradation was acquired from the

aerobic sludge of a water treatment plant within an oilfield. The consortium was analyzed by both PCR denaturing gradient gel electrophoresis and a cloning method. Changes of phenanthrene degradation genes, including naphthalene-2,3-dioxygenase (*nahAc*) and catechol-2,3-dioxygenase (*C23O*) that are responsible for the biotransformation of naphthalene and phenanthrene, were followed using most probable number PCR.

Materials and methods

Selective culture

The minimal basal salt (MBS) medium used in this study contained, per liter of water, 1000 mg (NH₄)₂SO₄, 800 mg K₂HPO₄, 200 mg KH₂PO₄, 200 mg MgSO₄·7H₂O, 100 mg CaCl₂·2H₂O, 5 mg FeSO₄·7H₂O, and 1 ml of vitamin solution (Zhang & Young 1997). The culture was carried

out at 150 rpm and 37 °C for 20 days with 10 mg phenanthrene as the sole carbon source in 100 ml MBS medium.

Phenanthrene degradation and analysis

Degradation was performed in flasks containing 100 ml MBS medium and 13 mg phenanthrene after inoculation of 1 ml fresh culture (10^4 copies of 16S rDNA). Heat-killed cells (autoclaved) and non-inoculated flasks were used as negative and blank controls, respectively. Cultures were grown in the dark with shaking at 150 rpm and 37 °C. A series of identical vessels, in triplet, were taken for analysis at given times.

The samples were extracted twice by 100 ml hexane. After being concentrated to dryness under a gentle flow of N_2 , the extracts were dissolved in an appropriate amount of hexane and analyzed in duplicate by GC/MS using an Agilent 6890 GC equipped with an HP-5 MS column (Gabriel *et al.* 2005). For the extraction, the recovery of the external standard ranged from 92 to 100%, with a mean value of 96% and a relative standard deviation of 4%.

DNA extraction and most probable number-PCR (MPN-PCR)

One milliliter culture was taken for genomic and plasmid DNA extraction using the Unix-10 column DNA Preps Kit (Sangon, China) and EZ-10 Spin column Plasmid Mini-preps Kit (BBI, Canada) following the manufacturer's instructions, respectively.

Both DNA extracts were serially diluted 10-fold, and three samples of each dilution were subjected to PCR. The PCR primers are shown in Table 1. The programs of PCR were optimized as follows: first denaturation was carried out at 94 °C for 4 min, then 40 cycles were completed with denaturation at 94 °C for 40 s. Annealing was performed at 60 °C for *EUB* and *C23O* genes, 54 °C for *nahAc* and 55 °C for *EUB-GC* clamp. Elongation was conducted at 72 °C for 40 s and a final elongation occurred at 72 °C for 10 min.

DGGE and cloning of 16S rDNA

The V3 region of the 16S rRNA gene was amplified from genomic DNA, and provided for DGGE analysis on a Bio-Rad Dcode system (Muyzer *et al.* 1993). For cloning, another pair of primers (Holger *et al.* 1997) was used to amplify the 16S rRNA gene with a different final extension at 72 °C for 30 min. T/A cloning and blue/white screening were conducted using a T-vector PCR product clone Kit (Sangon, Shanghai). After restriction enzyme analysis, foreign fragments with a unique profile were sequenced.

Results and discussion

Phenanthrene degradation and cell growth

As shown in Figure 1, 130 mg phenanthrene l^{-1} was degraded by the mixed culture of bacteria to 0.013 mg l^{-1} within 16 days. This was higher

Table 1. Primers used in this study.

Primer	Target	Sequence (5'–3')	Expected size (bp)	Reference
<i>EUBF</i>	16s rDNA	GCACAAGCGGTGGAGCATGTGG	455	Iwamoto <i>et al.</i> (2000)
<i>EUBR</i>		GCCCGGGAACGTATTCACCG		
<i>EUB341F^a</i>	16s rDNA	GCCCGGGAACGTATTCACCG	230	Muyzer <i>et al.</i> (1993)
<i>EUB518R</i>		ATTACCGCGGCTGCTGG		
<i>F27</i>	16s rDNA	AGAGTTTGATC(A/C)TGGCTCAG	1374	Holger <i>et al.</i> (1997)
<i>R1378</i>		CGGTGTGTACAAGGCCCGGAACG		
<i>nahAcF</i>	<i>nahAc</i>	TGGCGATGAAGAAGCTTTTC	992	Laurie & Jones (2000)
<i>nahAcR</i>		AACGTACGCTGAACCGAGTC		
<i>C23OF</i>	C23O	AAGAGGCATGGGGGCGCACCGGTTTCGATCA	377	Sei <i>et al.</i> (2004)
<i>C23OR</i>		CCAGCAAACACCTCGTTGCGGTTGCC		

The target gene of *EUB* and *EUB-GC* is 16s rDNA.

GC-clamp: CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G was attached to EUB341F.

than the rate achieved by *Pseudomonas aeruginosa*, which degraded 100 mg phenanthrene l⁻¹ to 0.2 mg l⁻¹ in 30 days (Romero *et al.* 1998). During the degradation, the cell mass in terms of DNA copies increased from 10⁴ copies ml⁻¹ to about 10⁸ copies ml⁻¹.

Variations of target catabolic genes

Figure 2 shows that both *nahAc* and *C23O* encoded, respectively, by plasmid and chromosome existed in the mixed culture. However, the number of copies of the plasmid encoding genes increased with the degradation of phenanthrene while the

Fig. 1. Phenanthrene degradation and cell growth. Phenanthrene concentration was detected by GC-MS. The number of 16S rDNA copies was used to indicate the cell mass. (◆) concentration of phenanthrene; (■) copies of 16S rDNA (EUB).

Fig. 2. Changes of the catabolic genes during phenanthrene degradation. The copies of *nahAc* and *C23O* encoded by chromosome and plasmid, respectively, were quantified using MPN-PCR (□) *nahAc* encoded by chromosome; (▨) *C23O* encoded by chromosome; (▤) *nahAc* encoded by plasmid; (■) *C23O* encoded by plasmid.

number of chromosome encoding genes stayed almost unchanged. As shown in Figure 3, the number of copies of plasmid encoding catabolic genes relative to each DNA copy increased during degradation of phenanthrene, suggesting the increase of plasmid numbers in each bacterial cell. The catabolic genes harbored by a plasmid can be transferred to other bacteria, even taxonomically divergent hosts in the environment, and function well (Bhadbhade *et al.* 2002). This indicates that plasmid-mediated bioaugmentation might be a possible strategy, especially for systems not suitable for the survival of isolated bacteria.

Biodiversity of genotypes

Heterogeneous diversity of the consortium was studied by both PCR-DGGE and 16S rDNA cloning methods, and the sequencing results are shown in Table 2, which indicate the existence of bacteria of different genera in the consortium. Compared to the sequencing results of DGGE bands, cloning gave a more decisive result. Among the 7 clones sequenced, three clones belonged to *Sphingomonas* sp., two to *Burkholderia* sp., and one to *Pandora* sp. Chiara *et al.* (2004) reported that *Pseudomonas* sp. encodes the *nah*-homologous pathway (salicylate-catechol formation), while *Burkholderia* sp. encodes a *phn*-homologous pathway (phthalate-protocatechuate formation) during the degradation of phenanthrene. The existence of three species of *Sphingomonas* and *Pandora* sp. indicates that phenanthrene might also be metabolized through other pathways. The cooperation among diverse genotype bacteria and possible plural degradation pathways might be the main reasons for the fast degradation of phenanthrene by the consortium. Furthermore, such a biological system should be stable since the ecological relationship among native microbes could be maintained. So a consortium enriched from environment is more applicable than pure culture for pollutant removal.

Conclusions

Different species and genera in the consortium with different pathways performed well in

Fig. 3. Relative ratios of catabolic genes to EUB during phenanthrene degradation. The ratios were obtained by dividing the logarithm of catabolic gene copies by the logarithm of EUB copies. (a) *nahAc*; (b) *C23O*. (◆) chromosome; (■) plasmid.

Table 2. Genotype diversity in the consortium.

Highest similarity (DGGE)	Homology ^a (%)	Acces. No. ^b	Highest similarity (clone)	Homology (%)	Acces. No.
Uncultured bacterium	97	DQ225130	<i>Burkholderia</i> sp.	99	DQ256752
Uncultured <i>Pseudomonas</i> sp.	98	DQ225131	<i>Pandora</i> <i>pnomenusa</i>	99	DQ256753
Uncultured bacterium	97	DQ225132	<i>Sphingomonas</i> sp. <i>A24</i>	97	DQ256754
Uncultured bacterium	98	DQ225133	<i>Burkholderia</i> sp. <i>B7</i>	99	DQ256755
Uncultured bacterium	96	DQ225134	Uncultured <i>sphingobium</i> sp. <i>B14</i>	97	DQ256756
Uncultured <i>Dyella</i> sp.	96	DQ225135	Uncultured <i>sphingomonas</i> sp. <i>B22</i>	98	DQ256757
Uncultured bacterium	95	DQ225136	Uncultured bacterium clone <i>B9</i>	98	DQ256758
Uncultured <i>Pseudomonas</i> sp.	98	DQ225137			

The left part is the sequencing results of DGGE and the right is the sequencing results of cloning.

^aHomology was the result of BLAST in Genbank.

^bThe Accession No. for Genbank.

phenanthrene degradation, indicating that the coexistence and cooperation of indigenous bacteria might promote phenanthrene degradation. The catabolic genes harbored by the plasmid contributed more than those by the chromosome to the degradation of phenanthrene.

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