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[ENV06] molecular biological analysis of hydrocarbon degraders from oily waste polluted sites

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Introduction

Oily waste has been identified to be the major pollutant in the environment. The sources may come from palm oil mills, refineries, oleo chemical plants, food and beverage industries and also petrochemical industries. Application of physicochemical methods in clean up process do not completely destroy the contamination and costly. These physicochemical technologies are often inadequate because they require the collection and concentration of the pollutants, which is in many instances is dispersed in the environment. Natural treatment utilizing microorganism as degrader or accumulator is currently receiving much favorable attention as a promoting method because microorganisms can take over in situ mineralization of pollutants.

Naphthalene has been identified as a major compound in oily waste. Naphthalene which is the simplest Polyaromatic hydrocarbon (PAH) has been studied widely because of their health risk and toxicity in the environment. Many bacteria has been identified capable to degrade naphthalene in nature especially Pseudomonads. The *nah* genes which encode enzymes for naphthalene degradation in bacteria are organized into two separate operons (encoding genes of the upper and lower pathway). The upper pathway constitutes *nah*AaAbAcAdBCFDE (Eaton and Chapman, 1992) while the lower pathway (*sal* operon) constitutes *nah*GHIJK (Yen and Gunsalus, 1982). A single regulatory protein, NahR (Lys-type transcriptional regulator) encoded by *nah*R links the induction of both operons (upper and lower operons).

Material and Methods

Bacterial strain.

The bacterial strains used in this study are listed in Table 1. Most of the bacteria were isolated from hydrocarbon contaminated sites.

TABLE 1 List of bacterial strain that has been used in this study.

Bacterial strain	Source / Reference
Escherichia coli (DS941)	D.J. Sheratt (Oxford University)
Acinetobacter calcoaceticus (BD 413)	Nielsen *et al*., 2000
Pseudomonas aeruginosa	Shafiza, 1997
Acinetobacter sp.	Merican *et al*., 2002
Pseudomonas sp.	Shafiza, 1997
Aeromonas sp.	Shafiza, 1997

Sampling

Samples that had been contaminated with petroleum hydrocarbon were obtained from oily waste contaminated site near car workshops at Pantai Hills Park, Kuala Lumpur, Malaysia. The sites had been contaminated for a long period of time with used motor oil and other petroleum derivatives. The sample soils were taken from two sites; site I and site II. Site I is located two meters from site II and the contamination rate is much lower. Site II is where the soil is highly contaminated. It is located near the contamination source.

Bacterial growth media

Luria-Bertani (LB) medium was used for cultivation of bacteria.

DNA extraction

Two type of DNA extraction were performed, which are DNA extraction from pure bacterial culture (laboratory collection) and direct DNA extraction from contaminated soils.

DNA extraction from bacterial culture was adopted from Midi-Prep Technical Bulletin (Promega, USA).

Total DNA was extracted from hydrocarbon-contaminated soil samples using a modified procedure based on the method of McDonald (1999). The soil sample (approximately 2 g dry weight) was suspended in 5 ml extraction buffer (McDonald, 1999) and incubated at 37°C for 2 h, mixing every 20 min. Further cell lysis was achieved by adding 0.5 ml of 10% (w/v) SDS and performing three cycles of freeze-thawing in ice and a 70°C water bath for 5 min. The sample was then centrifuged at 5,000 rpm for 5 min and the supernatant was collected and held at 4°C. The pellet was resuspended in 5 ml of extraction buffer (Selenska and Klingmuller, 1991), and 0.5 ml proteinase K (10 mg/ml) was added and incubated at 37°C for 30 min and 65°C for 1 h, with mixing every 20 min. The sample was then centrifuged at 5,000 rpm for 5 min and the supernatant held at 4°C. After one further extraction with 5 ml of fresh extraction buffer at 65°C for 1 h, the three supernatant fractions were pooled and centrifuged at 5,000 rpm for 30 min. PEG 6000 was added to the supernatant to a final concentration of 15% (w/v) and 1.5 ml of 5 M NaCl was also added gradually with shaking. After overnight precipitation at 4°C and centrifugation at 5,000 rpm for 5 min, the pellet was resuspended in 1 ml TE. Resulting DNA extracts were stored at -20oC until analyzed. The DNA extracts was then further purified using spin column method. 400 µl extract samples were added to NucleoSpin column (Clontech). The sample was centrifuged for 1 min at 14,000 rpm. DNA was rinsed 3 times with 400µl of 100% ethanol, and centrifuged for 1 min at 14,000 rpm. DNA was eluted with 50 µl TE.

PCR amplification

HotStart PCR was conducted to avoid unwanted amplifications such as non-specific amplification and primer dimer formation, which can occur at room temperature once all reaction components are mixed. In general, the HotStart technique limits the availability of one necessary reaction component until a temperature >60ºC is reached. The reaction cycle (denaturing- 92˚C for 1 minute; annealing- 40˚C for 30 second and extension- 70˚C for 2minutes) was repeated for 35 times. PCR was performed using universal primer (pA-pH) and the designed primers.

Computational method for sequence analysis

Computer-based analysis was used for alignment of sequence, construction of phylogenetic trees and PCR primer design. Computational analysis was mainly used the Seqweb version 2.02 of GCG (Genetic Computer Group) in Wisconsin package by Accelrys Inc. GCG-SeqWeb software which was provided by Bioinformatics Tool @ NBBNet; a web portal of resources under the National Biotechnology and Bioinformatics Network (NBBNet) by National Biotechnology Directorate, Malaysia (www.nbbnet.ukm.my).

Results

Sequences alignment and phylogenetic analysis of nah gene

The multiple alignments of *nah* gene were performed and the phylogenetic tree was constructed. The phylogenetic tree show that *nah*A genes can be clustered into four major groups (Figure 1).

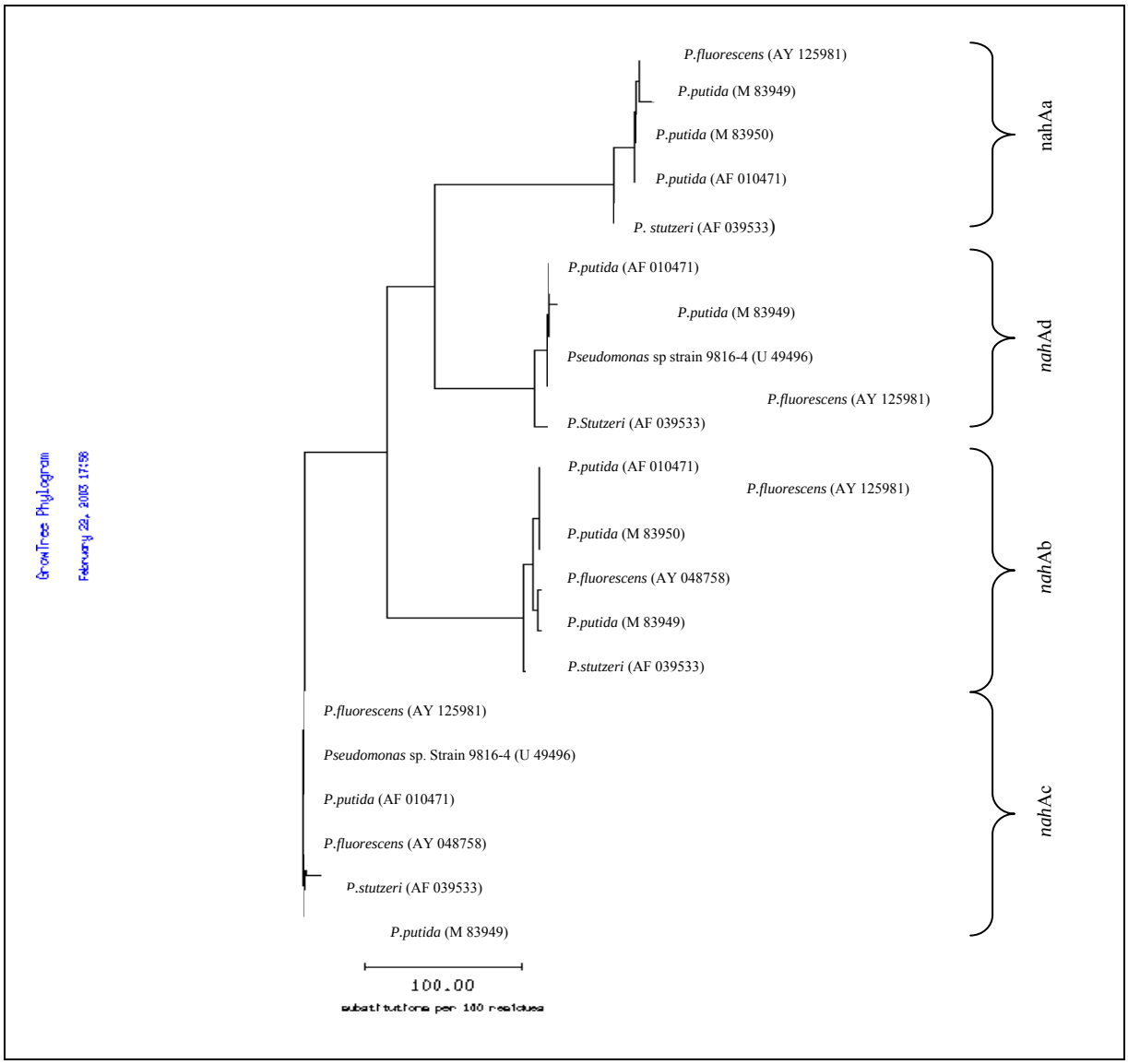


FIGURE 1 The phylogenetic tree of *nah*A genes constructed by GrowTree of SeqWeb-GCG, Wisconsin Package (Accelrys Inc. USA).

This is because *nah*A gene product is a naphthalene dioxygenase complex, which comprised of *nah*Aa, *nah*Ab, *nah*Ac and *nah*Ad that encode naphthalene dioxygenase reductase, naphthalene dioxygenase ferredoxin, naphthalene dioxygenase Fe-S large subunit (α subunit) and naphthalene dioxygenase Fe-S small subunit (β subunit), respectively.

The *nah*Aa genes show high differences with other *nah*A genes. *nah*Aa gene gave 48% differences with nahAd in the second cluster. *nah*Aa and *nah*Ad genes show 32% differences with *nah*Ab while *nah*Ac genes in fourth cluster show 52% differences from other *nah*A genes. Based on clusters obtained from phylogenetic tree of *nah*A genes, the multiple alignments of peptide sequences were performed individually for each *nah*A genes. The consensus sequence was displayed by Pretty program. Figures 2, 3, 4 and 5 show the selected regions of conserved sequences obtained in multiple alignments of *nah*A genes. The conserved sequences for nucleotide and amino acids, which indicated by the frame, were considered as oligonucleotide candidate for PCR primer design. List of PCR primer derived from phylogenetic trees and aligned sequences in this study were shown in Table 2

***nah*Aa gene**

Forward

Reverse

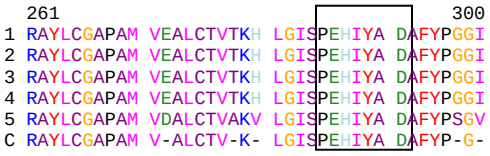
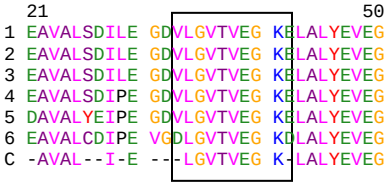


FIGURE 2 The peptide and nucleotide alignment of the *nah*Aa genes. Box indicates the primer sequence designed.
Sequence designations:
1= *Pseudomonas flourescens* (AY 125981)
2= *Pseudomonas putida* (M 83950)
3= *Pseudomonas putida* (AF 010471)
4= *Pseudomonas putida* (M 83949)
5= *Pseudomonas stutzeri* (AF 039533)

***nah*Ab gene**

Forward



Reverse

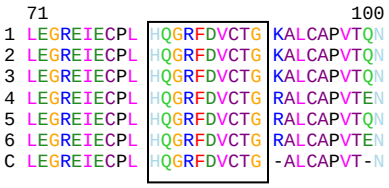


FIGURE 3 The peptide and nucleotide alignment of the *nah*Ab genes.
Sequence designations:
1= *Pseudomonas flourescens* (AY 125981)
2= *Pseudomonas putida* (M 83950)
3= *Pseudomonas putida* (AF 010471)
4= *Pseudomonas putida* (M 83949)
5= *Pseudomonas stutzeri* (AH 039533)
6= *Pseudomonas flourescens* (AY 048758)

***nah*Ac gene**

Forward

Reverse

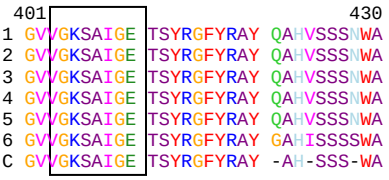
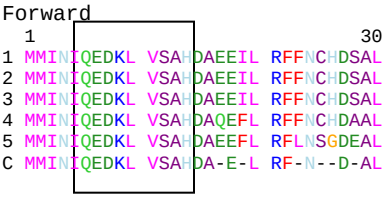


FIGURE 4 The peptide and nucleotide alignment of the *nah*Ac.
Sequence designations:
1= *Pseudomonas fluorescens* (AY 125981)
2= *Pseudomonas* sp. (U 49496)
3= *Pseudomonas putida* (AF 010471)
4= *Pseudomonas fluorescens* (AY 048759)
5= *Pseudomonas putida* (M 83949)
6= *Pseudomonas stutzeri* (AF 039533)

***nah*Ad gene**

Forward



Reverse

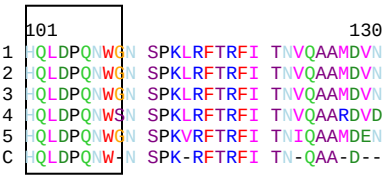


FIGURE 5 The peptide and nucleotide alignment of the *nah*Ad genes for selected conserved region as PCR primer.
Sequence designations:
1= *Pseudomonas* sp. (U 49496)
2= *Pseudomonas putida* (AF 010471)
3= *Pseudomonas fluorescens* (AY 125981)
4= *Pseudomonas putida* (M 83949)
5= *Pseudomonas stutzeri* (AF 039533)

TABLE 2 List of PCR primer derived from phylogenetic trees and aligned sequences in this study

Primer		Primer sequence	Location
*nah*A1	F	5'-G CCA GAT GAC CAA GAA ATG GAG TTC C –3'	447-472
(520bp)	R	5'-GGC ATC GGC ATA AAT ATG TTC GGG –3'	943-966
*nah*A2	F	5'-CTC GGC GTG ACY GTC GAA GGY AAG -3'	100-123
(171bp)	R	5'-GCC TGT RCA AAC RTC AAA YCW ACC TTG -3'	244-270
*nah*A3	F	5'-CAA AAG CAC CTG ATT CAT GGC GAY G-3'	108-132
(1185bp)	R	5'-CTC GCC GAT CGC CGA TTT GCC -3'	1272-1292
*nah*A4	F	5'-CAA GAA GAC AAA CTK GTY TCC GCC CAC-3'	16-43
(309bp)	R	5'-CCA GTT TTG CGG ATG CAG TTG ATG-3'	301-324

DNA extraction

Extracted DNA from both samples (pure bacterial strain and hydrocarbon contaminated soils) was visualized and photographed using DCode GelDoc systems (Biorad Inc. USA). Figure 6 shows the extracted DNA both from bacterial strain collection and environmental samples under agarose gel.

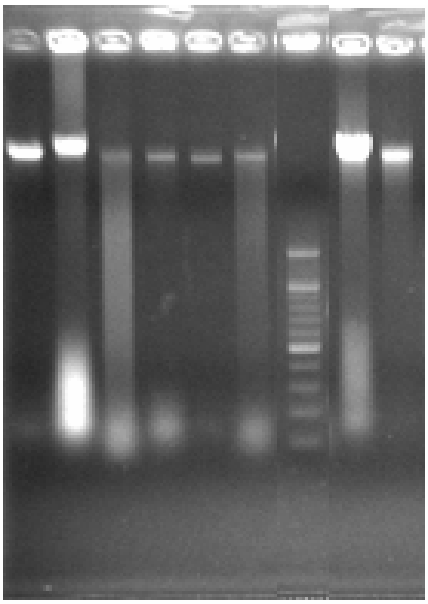


FIGURE 6 Ethidium Bromide-stained (1% w/v) of crude DNA extracted from laboratory strains (Lane 1-6) and Environmental sample (lane 8-9). Lane 1*: Acinetobacter* sp. (PCS), lane 2: *Pseudomonas aeruginosa* (PAS), lane 3*: Escherichia coli* strain DS941, lane 4: *Acinetobacter calcoaceticus* BD413, lane 5: *Pseudomonas* sp., lane 6: *Aeromonas* sp., lane 7: DNA marker, lane 8: Environmental sample (site 1), lane 9: Environmental sample (site 2).

PCR amplification

Extracted DNA from pure bacterial strain was amplified using universal primer (FIGURE 7) and specific-designed PCR primer (FIGURE 8-11).

PCR amplification was also performed on contaminated soil sample (FIGURE 12).

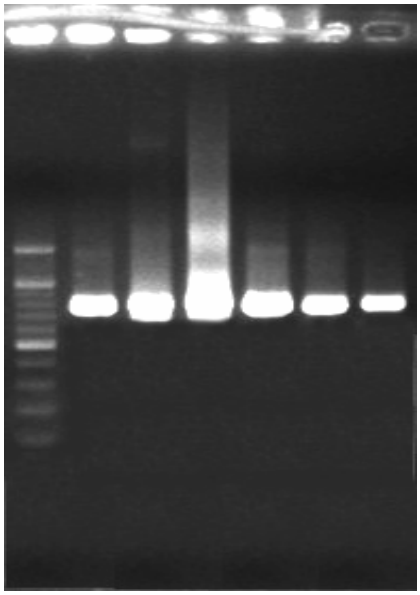


FIGURE 7 Ethidium bromide- stained agarose gel (1% w/v) displaying PCR product of DNA extracted from laboratory strains using universal primer pA and pH.

Lane1: DNA marker (200bp DNA step ladder), lane 2: *Acinetobacte*r sp., lane3: *Pseudomonas aeruginosa*, lane 4: *Escherichia coli* (DS 941), lane 5: *Acinetobacter calcoaceticus* (BD 413), lane 6: *Pseudomonas* sp., lane 7: *Aeromonas* sp.

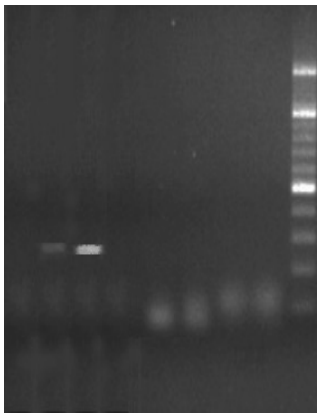


FIGURE 8 Ethidium bromide-stained agarose gel (1% w/v) of PCR product of DNA extracted from laboratory bacterial strains collection amplified using *nah*A1 primer set.
Lane 1: *Acinetobacter* sp., lane2: *Pseudomonas aeruginosa*, lane 3: *Escherichia coli* (DS 941), lane 4: *Acinetobacter calcoaceticus* (BD 413), lane 5: *Pseudomonas* sp., lane 6: *Aeromonas* sp, lane 9: DNA marker (200bp DNA step ladder

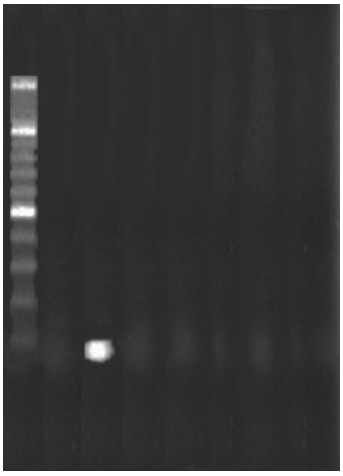


FIGURE 9 Ethidium bromide-stained agarose gel (1% w/v) of amplified PCR product of DNA extracted from laboratory strains using *nah*A2 primer set.
Lane1: DNA marker (200bp DNA step ladder), lane 2: *Acinetobacter* sp., lane3: *Pseudomonas aeruginosa*, lane 4: *Escherichia coli* (DS 941), lane 5: *Acinetobacter calcoaceticus* (BD 413), lane 6: *Pseudomonas* sp., lane 7: *Aeromonas* sp

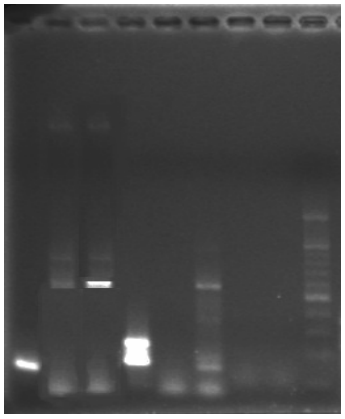


FIGURE 10 Ethidium bromide-stained agarose gel (1% w/v) displaying amplified PCR product using designed PCR primer *nah*A3.
Lane 1: *Acinetobacter* sp., lane2: *Pseudomonas aeruginosa*, lane 3: *Escherichia coli* (DS 941), lane 4: *Acinetobacter calcoaceticus* (BD 413), lane 5*:* *Pseudomonas* sp., lane 6: *Aeromonas* sp, lane 9: DNA marker (200bp DNA step ladder

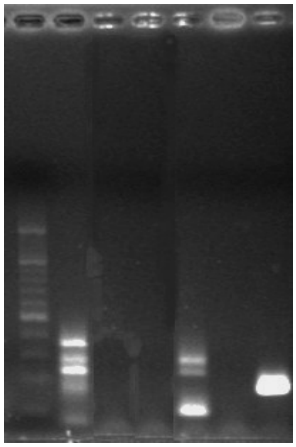


FIGURE 11 Ethidium bromide- stained agarose gel (1% w/v) displaying PCR product of DNA extracted from laboratory strains using designed PCR primer *nah*A4.
Lane1: DNA marker (200bp DNA step ladder), lane 2: *Acinetobacter* sp., lane3: *Pseudomonas aeruginosa*, lane 4: *Escherichia coli* (DS 941), lane 5: *Acinetobacter calcoaceticus* (BD 413), lane 6: *Pseudomonas* sp., lane 7: *Aeromonas* sp

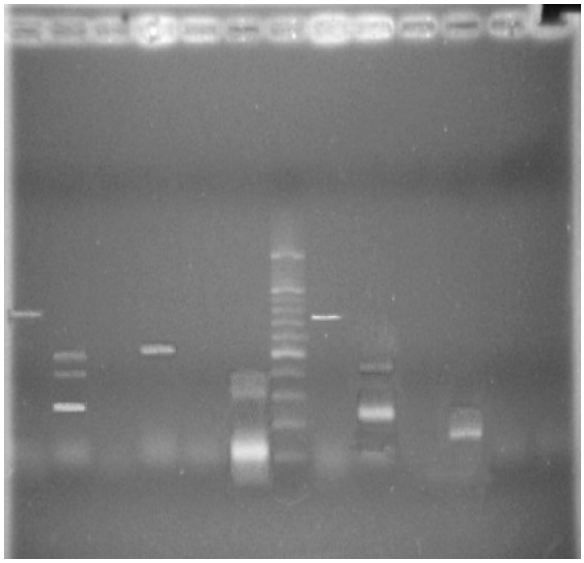


FIGURE 12 Ethidium bromide-stained agarose gel (1% w/v) of amplified PCR product using universal primer and designed specific PCR primer for naphthalene degradation genes.
Lane 1: Environmental sample from site I (Env1) using universal primer pA-pH, lane 2: Env1 with nahA1primer, lane 3: Env1 with nahA2 primer, lane 4: Env1 using nahA3 primer, lane 5: Env1 with nahA4 primer, lane 6: Env1 with nahR primer, lane 7: DNA marker (200bp DNA step ladder), lane 8: Environmental sample from site II (Env2) using universal primer pA-pH, lane 9: Env2 with nahA1 primer, lane 10: Env2 with nahA2 primer, lane 11: Env2 with nahA3 primer, lane 12: Env2 with nahA4 primer, and lane 13: Env2 amplified using nahR primer.

Discussion

The amplification of *nah*Aa gene from laboratory strains using nahA1 primer set shows positive result in *Pseudomonas aeruginosa* and *Escherichia coli*. *Pseudomonas aeruginosa* shows a blur DNA fragment while *Escherichia coli* show a very clear and high concentration of DNA fragment.

The appearance of this DNA fragment in *Pseudomonas aeruginosa* is not undoubtedly because *Pseudomonas* sp. is an important soil bacteria in naphthalene degradation and had been reported to produce many biodegradative enzymes (Tortora *et al*., 1992). The smear DNA may due to the low DNA concentration or low DNA yield of *Pseudomonas aeruginosa*. It is in opposite situation with *Escherichia coli*, which is never reported as naphthalene degrader. The DNA fragment obtained from *Escherichia coli* may be due to the low specificity of the designed PCR primer. The other possibility is cross-contamination from other naphthalene degraders during incubation time of the laboratory strains.

Only one laboratory strain show positive result when PCR amplification of *nah*Ab gene was performed using nahA2 primer. *Pseudomonas aeruginosa* show that *nah* genes are usually carried by Pseudomonads (Simon *et al*., 1993; Eaton and Chapman, 1992; Harayama *et al*., 1989; Yen and Gunsalus, 1983). Even when using PCR primer with small product (~170 base pairs), *Pseudomonas aeruginosa* still show a very clear DNA fragment that cannot be indicated in other laboratory strains used in this study especially non-naphthalene degraders.

The results obtained are varying when amplification of *nah*Ac gene was performed with laboratory strains using nahA3 primer. Positive results were obtained from *Pseudomonas aeruginosa, Escherichia coli* and *Aeromonas* sp., while *Acinetobacter* sp. and *Acinetobacter calcoaceticus* (BD413) show unexpected DNA fragments. Multiple DNA fragments which were obtained from *Aeromonas* sp. and *Acinetobacter calcoaceticus* may be due to low specificity of the PCR primer even though the PCR primer is designed for degenerated sequences. Other explanation is the fragmentation of DNA template during the DNA extraction and purification processes may cause the multiple DNA fragments pattern (McCoy, 2000). The appearance of DNA fragment by *Escherichia coli* is unexpected.

Amplification of *nah*Ad gene, which encodes naphthalene dioxygenase Fe-S small subunit using nahA4 primer set were performed and only *Aeromonas* sp. show positive result. This indicates that *Aeromonas* sp. might carry *nah*Ad gene and also able to degrade naphthalene. Previous study by Tawfiq (2003) shows that *Aeromonas* sp. is one of the naphthalene degraders. Both *Acinetobacter* sp. show multiple DNA fragments pattern but none of these bands indicate the expected DNA fragment size.

DNA from two locations (site I and site II) was extracted directly for the amplification of

naphthalene degradation genes. Site I and II show a DNA fragment with expected size when amplify using universal eubacterial primer pA and pH. This indicates that the DNA extracted directly from site I and site II are amplifiable using Polymerase chain reaction. Amplification of DNA template from site I with designed nah genes PCR primer sets show positive results in nahA1, nahA3 and nahR primers. This indicates that the bacteria in this environmental sample may carry *nah*Aa and *nah*Ac genes, which encode naphthalene dioxygenase reductase, naphthalene dioxygenase Fe-S large subunit (Iron-Sulfur protein) and regulatory enzyme respectively. The finding of these fragments can be first indicator to show that the naphthalene degradation process might happen in this environmental sample.

Site II only show positive result when amplify using nahA1 primer for detection of nahAa gene that encode naphthalene dioxygenase reductase. This was indicated by the appearance of DNA fragment with size 500 base pairs approximately. The unexpected size of DNA fragment was indicated with nahA3 primer.

The different result show in site I and site II may be due to its soil component where the soil in site II contains more hydrocarbon than site I. The high concentrations of hydrocarbon in site II may slow down or inhibit the bacterial grow in the soil that can cause low DNA yield in the extraction process.

Acknowledgements

The authors wished to thank the Ministry of Science, Technology and Innovation (MOSTI), Malaysia for the financial support under National Science Fellowship and IRPA grant 08-02-03-0761 awarded to Amir Feisal Merican.

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