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GENETIC DIVERSITY ANALYSIS OF THERMOPHILIC BACTERIA FROM CANDRADIMUKA CRATER IN CENTRAL JAVA EMPLOYING PCR-RFLP OF 16S-rRNA GENE

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ABSTRACT

The specific primers for bacteria (63f and 1387r) were used to amplify the 16S-rRNA genes from total community genomic DNA of thermophilic bacteria. The total community genomic DNA was obtained from muds and water samples of Candradimuka crater, Dieng Plateau, Central Java. PCR products were cloned into vector pCR®2.1-TOPO (3.9 kb) and transformed into *Escherichia coli* TOP10. Two tetrameric restriction endonucleases *RsaI* and *HhaI* were employed to generate Restriction Fragment Length Polymorphisms (RFLP) paterns. These enzymes yielded 10 and 9 groups of 16S-rRNA profiles or OTU (Operational Taxonomic Units) from 27 16S-rRNA gene clones. *RsaI* was found to be more discriminative in differentiating the clones than *HhaI*. *RsaI*-RFLP indicated that OTU 7 and OTU 3 represented the most abundant clones, i.e. 6 and 5 clones respectively. The distribution of 16S-rRNA gene clones could indicate relative distribution of specific groups of thermophilic bacteria in their natural habitat. Analysis of diversity at the DNA level could represent both culturable and unculturable bacteria in the environment. Similarity analysis showed that at level 0.600 there were 8 different groups from 10 RFLP profiles generated by *RsaI* digestion. This study indicated that there were at least 8 groups of different thermophilic bacteria occupying Candradimuka crater.

Key words: Thermophiles, 16S-rRNA, Candradimuka crater.

INTRODUCTION

Sustainability of the biosphere on our planet depends on microbial activities. However, we know very little about the microbial world. The reason for this poor understanding lies in the fact that microbes are tiny and individually invisible to the eye. Until recently, microbial identification required isolation of pure cultures followed by a series of physiological tests and biochemical characterization. The pure culture approach has limited our view of microbial diversity in the world, since

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it has been known that approximately only 1% of microorganisms in the environment could be cultured (Amann *et al.* 1995; Pace 1997; Borneman *et al.* 1996). Microorganisms are involved in many important activities such as soil formation, toxin removal, biogeochemical cycles of carbon, nitrogen, phosphor and others, conservation of more complex species, and to serve as valuable materials to many industries. Environmental stresses can alter microbial population and therefore endanger biosphere health.

In the absence of systematic approaches to studying and ensuring survival of diverse microorganisms, perhaps the best approach is to preserve as many habitats as possible, especially those from extreme environment which may have unique microorganisms (Fox 1994; Borneman *et al.* 1996). One of the extreme environments is thermal environment which could be found in natural and artificial systems such as volcanic region, coal refuse piles, hydrothermal vents and geothermal power plants (Stetter 1995). Species diversity of the thermophilic organisms is found in both the Domain of Bacteria and Archaea. Thermophilic bacteria exhibit a wide range of nutritional capability such as phototrophy and chemotrophy; autotrophy and heterotrophy; chemolitotrophy and chemo-organotrophy; aerobiosis and anaerobiosis (Brock 1986).

The application of molecular approaches to assess bacterial diversity is now entering the exponential growth. These approaches have overcome the requirement for prior cultivation. The most widely used technique to amplify the gene coding for 16S-rRNA from environmental samples relies on the application of PCR (Service 1997; Marchesi *et al.* 1998). For identification purposes, hypervariable regions of rRNA molecule are particularly useful due to relatively high differences between species but relatively low variability within species. Currently, there are more than 4000 16S-rRNA entries in the database, covering about 1800 species which continue to grow (Böttger 1996; Amann *et al.* 1994). Hypervariable regions are also the regions with the most concentrated polymorphism for restriction fragment length polymorphisms (RFLP) purposes (Green 1998). The highly conserved regions of the rRNA molecule can serve as primer binding sites for *in vitro* amplification by PCR (Amann 1994). PCR primers designed and evaluated by Marchesi *et al.* (1998) i.e. 63f (5'-CAG GCC TAA CAC ATG CAA GTC) and 1387r (5'-GGG CGG WGT GTA CAA GGC) could amplify 1300 bp of a consensus 16S-rRNA genes from bacteria.

In our study, we attempt to explore genetic diversity of thermophilic bacteria with PCR-RFLP of 16S-rRNA genes from environmental DNA. RFLP analysis was conducted using tetrameric endonucleases *Rsa*I (5'-GT↓AC) and *Hha*I (5'-GCG↓C). These enzymes along with *Bst*UI were described by Moyer *et al.* (1996) as the most efficacious at detecting and differentiating bacterial Small Sub Unit rRNA genes on the basis of their ability to correctly classify Operational Taxonomic Units (OTU). In this study, we have also analyzed partial 16S-rRNA sequences of two dominant OTU in Candradimuka crater.

MATERIALS AND METHODS

Sample collection

Candradimuka crater is located in Dieng Plateau (above 2000 m from sea level), Central Java, Indonesia. Sample was collected from a small pool (C1 site) characterized by almost boiling hot spring with temperature of 88°C and pH7 (Fig. 1). Water and sandy-mud were collected in a 250 ml sterile bottle, tightly sealed, and transported within 24 hours for immediate processing.

Figure 1. Profile of C1 Site in Candradimuka crater, Dieng Plateau, Central Java.

Extraction and purification of genomic DNA

Approximately 5 g of sample was extracted as described by Zhou *et al.* (1996) and modified by Tiedje (1997, unpublished). The crude DNA obtained was purified with the Prep-A-Gene kit (Biorad, Richmond, CA).

Amplification and cloning of 16S-rRNA genes

The 16S-rRNA genes were PCR-amplified by specific primers for Bacteria (63f and 1387r) from purified genomic DNA (200 ng) using Ready-To-GO PCR

Beads (Pharmacia-Biotech). Total volume of PCR reaction contained 1.5 U *Taq* DNA Polymerase, 10mM Tris-HCl (pH 9 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTPs and stabilizer including BSA. The reaction was incubated in Gene Amp PCR System 2,400 Thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.). The PCR protocol was: pre-PCR at 94°C for 2 min, denaturation at 92°C for 30 s, annealing at 55°C for 30 s, elongation at 75°C for 1 min and post-PCR at 75°C for 5 min with a total of 30 cycles (Marchesi *et al.* 1998).

The PCR product was cloned into pCR®2.1-TOPO (3.9 kb), and transformed into competent *E. coli* TOP10 using TOPO TA Cloning kit (Invitrogen Corp.). The positive clones were screened for α-complementation and selected as white colonies.

RFLP analysis of 16S-rRNA genes

Recombinant plasmids were isolated using the Wizard Plus SV Miniprep System (Promega, Madison, WI). *Eco*RI and *Rsa*I were employed to digest the plasmids in order to verify the cloned DNA and to classify according to their restriction patterns. The reason for selecting these two enzymes was based on the fact that the insert DNA was cloned between two sites of *Eco*RI and there are 3 *Rsa*I sites in the vector plasmid. Clones that contained approximately 1,300 bp of 16S-rRNA genes were chosen for RFLP analysis. These clones were subsequently PCR-amplified, restricted by tetrameric endonuclease *Rsa*I and *Hha*I to detect the different restriction profiles. Restriction enzyme digestion was performed at 37°C for 6 hours before they were separated by gel electrophoresis in 2% agarose gel, stained with 0.5 μg of ethidium bromide per ml and visualized by UV transilluminator (Sambrook *et al.* 1989). RFLP profiles obtained from *Rsa*I digestion were used to generate dendrogram reflecting their genetic relationship employing UPGMA clustering method from NTSys program (Rohlf 1990).

DNA Sequencing of 16S-rRNA genes

Genes for 16S-rRNA from two dominant OTUs were partially sequenced to infer the closest related organism from RDP database. The sequencing reactions were done using the Big Dye Ready Reaction Dye Deoxy Terminator kit and purification using ethanol-sodium acetate precipitation. The reactions were run on an ABI PRISM 377 DNA Sequencer (Perkin-Elmer Cetus, Norwalk, Conn.).

RESULTS AND DISCUSSION

Screening for α–complementation yielded 36 white colonies (transformants). Recombinant plasmids from all of these transformants were extracted. Verification of the clones containing an insert DNA (16S-rRNA gene) using *Rsa*I generated 19 groups of restriction profiles. Thus, we used these 19 groups for subsequent RFLP analysis. A total of 16 groups (representing 27 clones) contained the entire 1.3 kb to 1.35 kb 16S-rRNA insert after PCR-amplification using amplimers 63f and 1387r.

Two tetrameric restriction endonucleases were employed to differentiate RFLP profile from 16S-rRNA genes of 16 groups mentioned above in order to identify every object to be classified as OTU or a strain in most bacteriological works (Logan 1994). The RFLP profiles resulted from *Rsa*I digestion generate 10 OTUs (Fig. 2) which were: OTU 1 (clone number 1, 2, 5, 31), OTU 2 (number 3), OTU 3 (number 4, 10, 14, 21, 32), OTU 4 (number 6), OTU 5 (number 9), OTU 6 (number 7, 8), OTU 7 (16, 18, 19 25, 26, 34), OTU 8 (number 17, 20), OTU 9 (number 12, 15, 27, 36) and OTU 10 (number 30). Two dominant OTUs are OTU 7 (6 clones) and OTU 3 (5 clones). In evaluating these profiles, we identified discrete patterns that are specific for a clone and could be distinguished one from another as an OTU. Results from these data infer that bacteria belonging to OTU 7 and OTU 3 were distributed relatively wide and dominant at the habitat of Candradimuka crater.

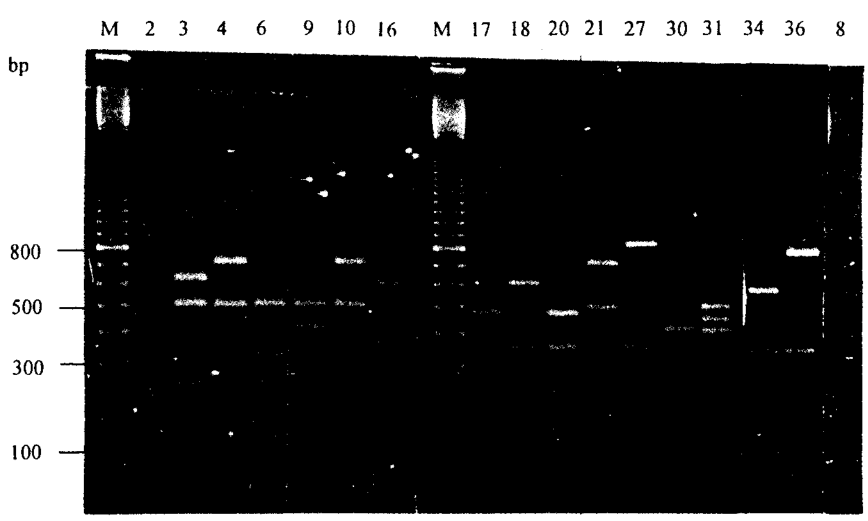

Figure 2. RFLP profile of 16S-rRNA gene digested with *Rsa*I. Molecular standard is *100 bp DNA Ladder* (M).

The second digestion of 16S-rRNA gene using *Hha*I produced 9 OTU (Fig. 3). The grouping of restriction profiles was the same as *Rsa*I except that OTU 7 and OTU 8 (from *Rsa*I-RFLP) were in the same group in *Hha*I-RFLP. The result of RFLP using these two enzymes indicated that *Rsa*I was more discriminative than *Hha*I to differentiate the clones, in this case for bacterial strains from Candradimuka crater belonging to OTU 7 and OTU 8. This result was in agreement with the one conducted by Moyer *et al.* (1996) that *Hha*I, *Rsa*I and *Bst*UI were the most

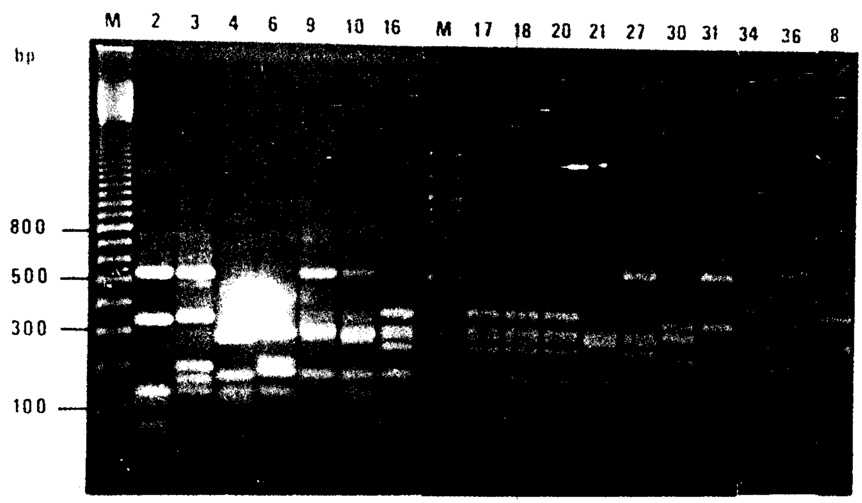

Figure 3. RFLP profile of 16S-rRNA gene digested with *HhaI*. Molecular standard is *100 bp DNA Ladder* (M).

efficacious enzymes at detecting and differentiating 16S-rRNA genes originated from environmental DNA.

Our result indicated that at least 10 OTUs were present in Candradimuka site C1 which reflected the relative abundance of bacteria in this extreme habitat, in contrast to only two culturable clones obtained on Thermophilic Maintenance medium incubated at pH 7 and temperature of 70°C under aerobic condition. Research conducted by Huber *et al.* (1991) with samples taken from three sites of Candradimuka crater obtained two hyperthermophilic Archaea, i.e. *Thermoproteus* and *Desulfurococcus* and novel anaerobic obligate bacteria having chemolitotrophy H_2/NO_3^-. The use of amplimers 63f and 1387r in this study would limit the amplification of 16S-rRNA genes only for Bacteria. Consequently, one should keep in mind that assessment of Prokaryote diversity in this study did not include members from Archaea. The reduction of 16 groups of restriction profiles to 10 OTUs from *RsaI*-RFLP profiles could be explained with the possibility that the gene insert may have two different orientations when ligated to the vector. The difference could be determined from recombinant plasmid restriction profiles, but not for intact 16S-rRNA genes obtained from PCR. The 10 OTUs of thermophilic bacteria were analysed further using cluster analysis (Fig. 4). This dendrogram showed that at level 0.600 there were 8 groups from 10 OTUs, in that OTU 4 and OTU 5 were in one cluster as well as OTU 7 and OTU 8.

From this dendrogram, it could also be shown that at level 0.205, there were two groups of genetic relationship. One group consisting of OTU 7, 8, 9 and 10, were in the same group with IT-08 isolate obtained from Gunung Pancar, West Java

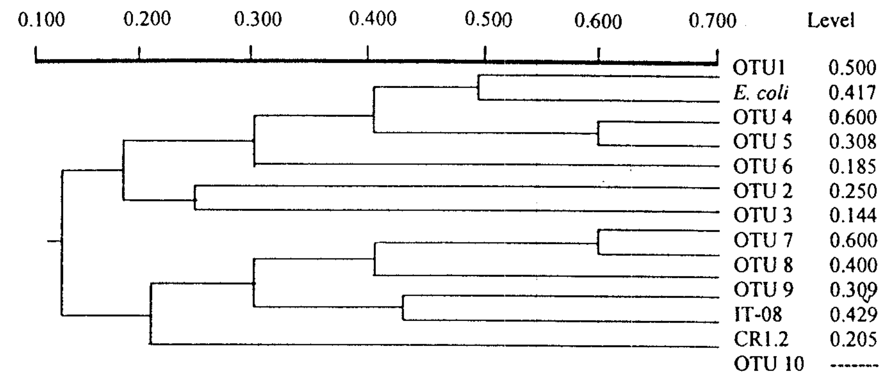

Figure 4. Dendrogram of RFLP profile of 16S-rRNA gene after digestion with *Rsa*I. The number at horizontal axis represented % of similarity based on Jaccard coefficient.

(Tan 1999) and CR1.2 (isolate from 16S-rRNA gene library of Candradimuka crater at Site 4). Isolate of IT-08 has the DNA sequence similar to *Bacillus thermoleovorans* (98%) whereas CR1.2 has the DNA sequence similar to *Brevibacterium thermoruber* (99%). The other group shows that OTU 1 was closely related to *E. coli* (16S-rRNA genes in pKK3535 constructed by Brosius *et al.*, 1981 *in* Herawati, 1996). One possible explanation is the construction for OTU 1 *and E. coli* involved 3 restriction fragments which have two similar band sizes. Another explanation is that OTU 1 is a new bacterial isolate which yielded DNA profile similar to *E. coli* when restriction enzyme *Rsa*I was employed in this study.

In a habitat like Candradimuka crater, there may be some dominant bacteria occupying that extreme habitat. They are physiologically active among the slow grower and dormant bacteria. Therefore, estimating genetic diversity through isolation of DNA from environmental sample is able to represent all of the organisms including unculturable ones. Although there are some biases such as different cell lysis methods and amplimer sets resulting in limited comparability to other studies, the data of 16S-rRNA genes from environmental libraries demonstrated the presence of hitherto unidentified bacteria. Only a minority of sequences retrieved from directly isolated soil DNA were reported to be closely related to cultured organisms so that bacterial communities in the environment were composed mainly of uncultured species (Felske *et al.* 1998).

Partial sequencing of OTU 3 (520 bp) and comparison to Ribosomal Database Project (RDP) database from the University of Illinois indicate that OTU 3 demonstrated 96% similarity to *Ocrobactrum anthropi* (IAM 14119) whereas OTU 7 (450 bp) had 86% similarity to *Pseudomonas flavescens* (B62(T)). DNA sequencing provides more accurate data because nucleotides are the basic unit of information, straightforward approaches for inferring phylogenetic history and very reliable to compare specific DNA fragment. Besides the advantages, this technique carries inherent drawbacks, such as relatively high cost operation and impractical for routine application long DNA molecule. On the contrary, PCR-RFLP technique is simple, rapid, low cost, for a 50 – 3000 bp DNA fragment range could be assayed, and especially more appropriate when many individuals need to be sampled (Hillis *et al.* 1996). To assess the diversity of an environmental sample using PCR-RFLP study, we do not have to use the most accurate technique like DNA sequencing. Therefore, the simpler and faster method could be valuable in taxonomic study for bacterial *in situ* detection and routine investigation.

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