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PRESENCE OF *hemA*-LIKE AND *hemT*-LIKE GENES IN A NUMBER OF ANOXYGENIC PHOTOSYNTHETIC BACTERIAL ISOLATES FROM INDONESIA AND SOIL SAMPLES FROM BOGOR AREA

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ABSTRACT

The *Rhodobacter sphaeroides hemA* and *hemT* are known to encode a distinct 5-aminolevulinic acid (ALA)-synthase isozyme. This enzyme catalyzes the first and rate limiting step in ALA biosynthesis through the C_4 pathway. This study was carried out to detect *hemA*-like and *hemT*-like genes in twenty Anoxygenic Photosynthetic Bacterial (APB) isolates from several wetland areas in Indonesia, and four DNA samples that were isolated from four soil samples obtained from Bogor area. Hybridization techniques of Southern and dot blot were used, using *hemA* and *hemT* fragment as probes. Southern hybridization analyses indicated the presence of *hemA*-like gene in five of APB isolates, i.e., MB15, MB16, MB21.2, MB55 and MB6, whereas *hemT*-like gene was detected only in MB15. Dot blot hybridization analyses suggested that the soil samples from waterlogged paddy-field, dry paddy-field as well as a mud pond were predominantly occupied by prokaryotic organisms which harboured *hemA*-like gene. However, *hemT*-like sequences were also found in soil sample from dry paddy-field.

Key words: *hemA*-like gene / *hemT*-like gene / Southern hybridization analysis / dot blot hybridization analysis.

INTRODUCTION

The *Rhodobacter sphaeroides hemA* and *hemT* genes encode a distinct 5-aminolevulinic acid (ALA) synthase isozyme (Neidle & Kaplan 1993). ALA-synthase catalyzes the first and rate-limiting step in ALA biosynthesis through the C_4 pathway. ALA is the first committed precursor in the common tetrapyrrole pathway (Goodwin & Mercer 1986; Beale & Weinstein 1991; Beale 1995). Recently, ALA has received attention as a new biodegradable herbicide (Sasaki *et al.* 1987) and insecticide (Sasaki *et al.* 1990).

The DNA sequences of *hemA* and *hemT* genes and their location on *R. sphaeroides* physical map have been determined. The *hemA* gene is located on the large chromosome whereas *hemT* gene is found on the small chromosome (Neidle & Kaplan 1993a). The *hemA* and *hemT* genes encode peptides that are 53% similar to each other, and these peptides are also significantly similar to ALA-synthase from several bacteria and eucaryotic species (Neidle & Kaplan 1993). The *hemA* fragment has been cloned in *R. sphaeroides* (Tai *et al.* 1988) as well as in *Escherichia* *coli* (Werf & Zeikus 1996). The cloned *hemA* fragment is expressed well in *E. coli* and able to enhance ALA production (Werf & Zeikus 1996). This evidence shows that *hemA* can be used as a genetic material for enhancing ALA production.

Since *hemA* and *hemT* genes have high homology to gene encoding ALA-synthase from other organisms, *hemA*-like and *hemT*-like genes might be found in other bacteria that form ALA through the C_4 pathway. In this study, we detected the presence of *hemA*-like and *hemT*-like genes in Anoxygenic Photosynthetic Bacterial (APB) isolates, because most member of APB use the C_4 pathway to produce ALA. Soil, especially paddy-field soil, is known as a common habitat of APB (Habte & Alexander 1980: Gest *et al.* 1985). Therefore, we also carried out an experiment to detect these genes in four soil samples. The results of this study would be expected to generate some insights on the distribution and population density of APB as well as *R. sphaeroides* strains. In addition, the specificity of *hemT* would be assessed to be used as a specific molecular marker for *R. sphaeroides* isolates.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions and soil samples

The bacterial strains and plasmids used are listed in Table 1. APB isolates were grown photoheterotrophically in Sistrom's minimal medium (Lueking *et al.* 1978) in full filled screw-cap tubes, pH 7.2. *E. coli* were grown at 37°C in Luria Bertani (LB)

Table 1. Bacterial strains and plasmids

Bacteria and strains or plasmids	Relevant characteristics	Reference
APB isolates:		
MB2	Wild type, from Central Kalimantan	Irawan *et al.* (1998)
MB6	Wild type, from Central Kalimantan	Irawan *et al.* (1998)
MB7	Wild type, from Central Kalimantan	Irawan *et al.* (1998)
MB11.1	Wild type, from Central Kalimantan	Irawan *et al.* (1998)
MB12.2	Wild type, from Central Kalimantan	Irawan *et al.* (1998)
MB15	Wild type, from Bogor	Irawan *et al.* (1998)
MB16	Wild type, from Pangandaran	Irawan *et al.* (1998)
MB18	Wild type, from Ciwaluya	Irawan *et al.* (1998)
MB19	Wild type, from Bogor	Irawan *et al.* (1998)
MB21.1	Wild type, from Ciamis	Irawan *et al.* (1998)
MB21.2	Wild type, from Ciamis	Irawan *et al.* (1998)
MB22	Wild type, from Pontianak	Irawan *et al.* (1998)
MB23	Wild type, from Ujung Kulon	Irawan *et al.* (1998)
MB28	Wild type, from Ujung Kulon	Irawan *et al.* (1998)
MB31	Wild type, from Ujung Kulon	Irawan *et al.* (1998)
MB39	Wild type, from Merauke	Irawan *et al.* (1998)
MB54	Wild type, from Jambi	Irawan *et al.* (1998)
MB55	Wild type, from Jambi	Irawan *et al.* (1998)

Table 1. Continued

Bacteria and strains or plasmids	Relevant characteristics	Reference
MC1	Wild type, from Kotorayo	Irawan *et al.* (1998)
BW12	Wild type, from Central Kalimantan	Irawan *et al.* (1998)
R. spaeroides 2.4.1	Wild type, from USA	Suwanto & Kaplan (1989)
S. E. coli	supE44 Δ*lac*U169	Sambrook *et al.*, 1989
Plasmids:		
pUC19	*Amp*^{r},*lacI*,*lacZ*	Sambrook *et al.*, 1989
pUI 1004	pUC18, 1.8 kb *hemT*	Neidle & Kaplan, 1993a
pUI1014	pUC18, 2 kb *hemA*	Neidle & Kaplan, 1993a
pUI612	pUC18, 1.08 kb *pucBA*	Kiley & Kaplan, 1987
pHF1.1	pHE3, 2.2 kb 16S & 23S rrna	Schleifer *et al.*, 1985

(Sambrook *et al.* 1989) supplemented as needed with antibiotics. Antibiotics were added at the following concentrations: 100μg Ampicillin/ml (for maintaining pUC-19, pUI1004, pUI1014, and pUI612), 25μg Chloramphenicol/ml (for maintaining pHF1.1). Soil samples were taken from waterlogged paddy-field, dry paddy-field, LSI pond and Grawida yard, Bogor Agricultural University, Darmaga Campus. All sampling areas were located at Darmaga, Bogor. The description of the soil samples are listed in Table 2.

Table 2. Soil samples

No.	Sampling Location	Soil Description
1.	Waterlogged paddy field	Dark brown, waterlogged, sandy, t: 27°C
2.	Dry paddy field	Dark brown, dry, finely granulated, porous, t: 29°C.
3.	LSI pond	Dark brown, silt, t: 26°C
4.	Grawida Yard	Red brick, massive, clayey, t: 29°C

DNA isolation

Plasmids DNA were isolated by using Wizard Miniprep DNA Purification System (Promega, Wisc.) according to the manufacturer's instruction. Genomic DNA was extracted from each APB isolate using the phenol extraction method with slight modification as follows. The cell pellet was suspended in EDTA solution containing 15 mg lysozyme/ml, and incubated at 37°C for 1 hour. The lysis was accomplished by adding 300 μl SDS buffer (0.1 M NaCl; 4% SDS; 0.5M Tris-HCl, pH 8). The extract was freeze-thawed. The DNA was phenol-extracted and ethanolprecipitated as in standard protocol (Sambrook *et al.* 1989). The *E. coli* genomic DNA was isolated as described previously (Leach *et al.* 1994).

The DNA was extracted from soil by using modified Tiedje Method (Keller 1997, unpublished). The soil sample (10 g) was finely grinded. The 5 g grinded soil was mixed with 13.5 ml Tiedje buffer (100 mM Tris-HCl, pH 8; 100 mM Na-EDTA, pH 8; 100 mM Na_2PO_4, pH 8; 1.5 M NaCl; 1% CTAB) in 100 ml centrifuge tube, and freeze-thawed 3x. After freeze-thawing, 100 µl proteinase-K (20 mg/ml) (Sigma, Singapore) was added and incubated at 37°C for 30 minutes, then 10 ml 10 % SDS was added and incubated at 65°C for 2 hours. The mixture was centrifuged at 6000 g for 10 minutes. The supernatant was extracted with 1 volume of chloroform (Merck, Jakarta) and centrifuged at 6000 g for 1 minute. The aqueous phase was transferred to a new 100 ml centrifuge tube and 0.6 volume of isopropanol (Merck, Jakarta) was added at room temperature. The DNA was recovered by centrifugation at 16,000 g for 20 minutes at 4°C. The supernatant was discarded and the pellet was washed with 70 % ethanol. The DNA was dried at room temperature and dissolved in 100 µl TE buffer (0mM Tris-HCl, pH 8; 1mM EDTA).

Southern hybridization analysis

The 1.2 kb *Bam*HI (NEB, Singapore) fragment from pUI1014 and 1.8 kb *Bam*HI (NEB, Singapore) fragment from pUI1004 were isolated for preparing *hemA* and *hemT* probes. The fragments were purified from agarose gel using the Gene Clean Kit (Bio 101 Inc, La Jolla, Calif.), and labeled with biotin-14-ATP using Nick Translation System (GIBCO/BRL, Grand Island, NY) according to the manufacturer's instruction. The unincorporated nucleotides were removed from the probes with nuctrap push columns (Stratagene, La Jolla, Calif.).

The genomic DNAs extracted from APB isolates were digested with *Eco*RI (NEB, Singapore), except the DNA from MB15 isolate, which was digested with *Bam*HI (NEB, Singapore). Digestions were performed in appropriate buffer at 37°C for 12 hours. The digested DNA was electrophoretically separated. The DNA fragments were transferred to a nylon membrane (Photogene, GIBCO/BRL, Grand Island, NY) by capillary action with standard method (Sambrook *et al.* 1989). Hybridization was carried out as described previously at 42°C for 12 hours (Sambrook *et al.* 1989), followed by washing at 37°C for 2x 15 minutes each, and detection using a chemiluminescent method (Photogene Detection System, GIBCO/BRL, Grand Island, NY).

Dot blot hybridization analysis

Four biotinilated DNA probes, i.e. *hemA*, *hemT*, *pucBA*, and 16S rRNA genes were used. The probes were prepared as described above. To avoid bias in calculation, the population density of prokaryotes was based on the same amount of soil samples, and not on the same amount or standardization of DNA concentration. DNA isolated from soil samples were denatured as described previously (Keller and

Manak 1992), and applied to nylon membrane (Photogene, GIBCO/BRL, Grand Island, NY) by spotting directly onto the membrane. High stringency hybridization and washing conditions were used. Hybridization was carried out at 42°C for 12 hours, with washing temperature of 55°C (Sambrook *et al.* 1989). Detection was done using the Photogene Detection System (GIBCO/BRL, Grand Island, NY).

RESULTS AND DISCUSSION

Southern hybridization analyses

Southern hybridization analyses were performed to determine the presence of *hemA*-like and *hemT*-like genes in twenty APB isolates from Indonesia (Table 1). *R. sphaeroides* 2.4.1 (Rsp 2.4.1) *hemA* and *hemT* genes were used as probe. The analyses identified some homologous regions the homology to *hemA* in the genomic DNA of MB6, MB15, MB16, MB21.2 and MB55. The region of homology to *hemA* probe in the genomic DNA of each APB isolate is shown in Figure 1a, lanes 3-7. The *hemT* probe hybridized only to 4.8 kb *Bam*HI fragment in MB15 genomic DNA (Fig. 1b, lane 3).

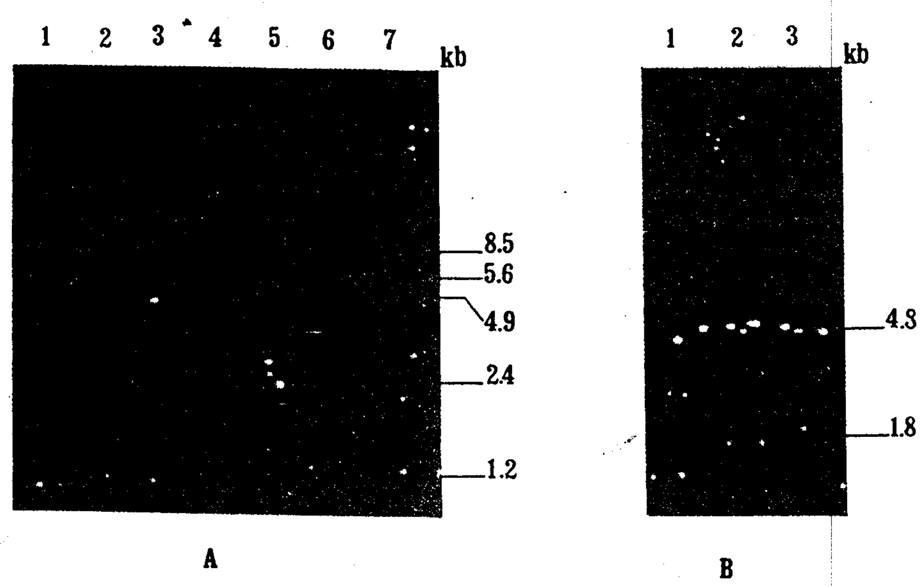

Figure 1. A. Southern hybridization analyses using *hemA* probe.
Lanes (1) *Rhodobacter sphaeroides* 2.4.1, (2) λ*Bst*EII, (3) MB15, (4)MB16, (5) MB21.2, (6) MB55, and (7) MB6

B. Southern Hybridization analyses using *hemT* probe
Lanes (1) *Rhodobacter sphaeroides* 2.4.1, (2) λ*Bst*EII, and (3) MB15

The results implied the presence of *hemA*-like gene in MB6, MB15, MB16, MB21.2 and MB55, whereas *hemT*-like gene was only implied in MB15. The presence of *hemA*-like and/or *hemT*-like genes in the five APB isolates indicates the ALA biosynthesis in these APB isolates is employed through the C_4 pathway, because the C_4 pathway is mediated by ALA synthase which encoded by *hemA* and/or *hemT* genes. Moreover, the presence of *hemA*-like and/or *hemT*-like genes in the five APB isolates indicates that these APB isolates could be classified under sub group α-proteobacteria. In photosynthetic bacteria, C_4 pathway is utilized by purple non-sulfur bacteria, especially sub group α-proteobacteria (Avissar *et al.* 1989; Beale 1995). The sub group α-proteobacteria contains species of genera *Rhodospirillum, Rhodopila, Rhodopseudomonas, Rhodomicrobium* and *Rhodobacter* (Imhoff 1995).

Neither *hemA*-like gene nor *hemT*-like gene was identified in the other 15 APB isolates. The data suggested that the C_4 pathway is not utilized by these APB isolates to produce ALA. The 15 APB isolates might produce ALA through C_5 pathway, which does not require ALA synthase. These APB isolates used are not the member of sub group α-proteobacteria, although all APB isolates studied here belong to purple non-sulfur bacteria.

The *hemT*-like gene was only identified in MB15. This APB isolate also carries *hemA*-like gene. Interestingly, the *hemA*-like gene in MB15 was detected on the same locations with Rsp. 2.4.1 *hemA*, i.e., at 1.2 kb and 2.4 kb *Bam*HI fragments (Fig. 1a, lane 1 and 3). The strong intensity of the hybridization signals revealed that the similarity between MB15 *hemA*-like gene and Rsp.2.4.1 *hemA* was very high. Moreover, the color comparison of MB15 culture with Rsp. 2.4.1 culture also showed a high similarity. Based on these findings, it is very likely that MB15 was *Rhodobacter sphaeroides*. We tentatively conclude that *hemT*-like gene harbors specifically in *R. sphaeroides*. Neidle and Kaplan (1993) reported that the *R. sphaeroides* is the only bacterial species that produces two ALA synthase isozymes. However, previously ALA synthase isozymes are found in some vertebrates (Dierks 1990), while no previous information on bacterial ALA synthase isozymes have been reported.

Dot blot hybridization analysis

Dot blot hybridization analysis was employed to detect the presence of *hemA-like* and *hemT-like* genes in DNA extracted from four soil samples (Table 2). Four DNA probes, i.e., *hemA, hemT, pucBA,* and 16S rRNA gene were used. This analysis also revealed the relationship of the activity of prokaryote and APB in the soil samples with the presence of *hemA*-like and *hemT*-like genes.

Figure 2 shows the results of dot blot hybridization using the four probes. Interpretation of these results is described in Table 3. Based on the hybridization using *hemA* probe (Fig. 2a), the presence of *hemA*-like gene was detected in soils taken from waterlogged paddy field, dry paddy field and LSI pond. The presence of *hemA*-like gene in these soil samples indicates the activity of organisms producing

ALA through the C_4 pathway. The C_4 pathway is utilized by animals, fungi, protozoa and sub group α-proteobacteria (Avissar *et al.* 1989; Beale & Weinstein 1991; Beale 1995).

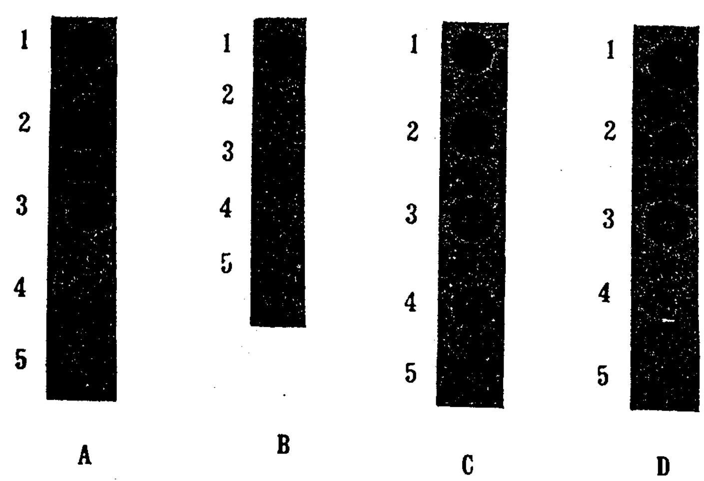

Figure 2. Dot blot hybridization analyses using *hemA* (A), *hemT* (B), 16S rRNA (C), and *pucBA* (D) as probes. Dots (1) *Rhodobacter sphaeroides* 2.4.1., (2) soil sample from waterlogged paddy field, (2) soil sample from dry paddy field, (3) soil sample from LSI pond, and (5) soil sample from Grawida yard

Table 3. Interpretation of the result of dot blot hybridization

No.	Soil Samples	Interpretation of Dot Blot Hybridization Results			
		hemA	***hemT***	**Prokaryote**	**APB**
1	Waterlogged paddy field	++	-	++	++
2	Dry paddy field	+++	++	++++	+++
3	LSI pond	+	-	+++	+
4	Grawida Yard	-	-	+	+

\+ : Detected
_ : Not detected

The number of (+) represent the degree of intensity of hybridization signal

Using 16S rRNA probe, we identified the presence of prokaryotic micro-organisms in all four soil samples (Fig. 2c). The results of hybridization to *pucBA* probe indicated that the APB could be detected in all soil samples (Fig. 2d). Thus, it is possible to find the α-proteobacteria in the soil samples, because some members of α-proteobacteria are classified as APB group. The possibility for the α-proteobacteria to exist in the four soil samples correlates with the presence of *hemA*-like gene in these soil samples. However, in spite of the detection of the APB activities in soil from Grawida yard, *hemA*-like gene was not found in this soil. The intensity of hybridization signal to both 16S rRNA (Fig. 2c) and pucBA (Fig. 2d) probes shows that the population densities of both prokaryote and APB in soil from Grawida yard are much lower than those found in the other three soil samples. The α-proteobacteria might not exist in the soil from Grawida yard or the α-proteobacteria were actually present in this soil, but the population density is very low. Therefore, presence of *hemA*-like gene could not be detected. Soils obtained from waterlogged paddy field, dry paddy field and LSI pond were found to have high population density of prokaryotes and APB. The high population density of APB enables the α-proteobacteria to proliferate. The presence of *hemA*-like gene in these soil samples is related to the population density of APB. The population density of APB as well as other microorganisms in the soil is dependent on the availability of growth nutrient, O_2 and water (Brock & Madigan, 1991). The fertility and the soil texture are responsible for the availability of these factors. Therefore, the presence of *hemA*-like gene in soil is indirectly affected by the fertility and the soil texture

Based on the results of hybridization to *hemA* probe, the *hemA*-like gene might be present in the soil taken from APB habitat. In nature, APB occur in moist soil, paddy field, sewage water, fresh water, brackish water, waste water, marine habitat and in extreme condition of the Antartic (Sasikala *et al.*1985). The presence of *hemA*-like gene in the soil is related to the fertility of the soil, because the availability of growth nutrient affects APB growth.

The dot blot hybridization using *hemT* probe identified the presence of *hemT*-like gene only in soil taken from dry paddy field (Fig. 2b, dot 3). By comparing the intensity of hybridization signal to *hemT* probe with the hybridization signal to *hemA* probe, it is clear that the homologous sequence to *hemT* probe is present in relatively fewer amount than the homologous sequence to *hemA* probe. The results indicate that not all organisms carrying *hemA*-like gene also carry the *hemT*-like gene. These findings confirm the data obtained from Southern hybridization analyses that the presence of *hemT*-like gene is more specific than that of *hemA*-like gene. The *hemT* or *hemT*-like gene might be specific to *R. sphaeroides*. However, further studies to determine the nature of specificity of *hemT* or *hemT*-like gene to *R. sphaeroides* should be carried out to develop *in situ* hybridization method to examine directly the distribution and density of *R. sphaeroides* in the soil or water ecosystem.

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