THE USE OF PULSE FIELD GEL ELECTROPHORESIS FOR MEGAPLASMID ISOLATION OF *Pseudomonas solanacearum*

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Ringkasan

Strain-strain bakteri *Pseudomonas solanacearum* E.F. Smith penyebab layu tanaman telah dilaporkan mempunyai plasmid-plasmid berukuran besar. Pada plasmid tersebut diduga terdapat gen-gen yang menentukan patogenisitas bakteri tersebut. Megaplasmid mempunyai ukuran yang sangat besar dan jumlah kopi yang rendah. Ekstraksi plasmid dengan metode lisis di luar gel menyebabkan kerusakan struktur plasmid dan tidak menghasilkan pita DNA pada gel setelah elektroforesis. Pada eksperimen ini diuji penggunaan *Pulse Field Gel Electrophoresis* (PFGE) untuk mengisolasi megaplasmid strain 2305L (biovar 2 berasal dari tomat di Lembang) dan strain JGP (biovar 3 berasal dari kacang tanah di Jambegede, Malang). PFGE dilarikan dengan 'ramping time' 40 – 60 detik, 'running time' 26 jam pada voltase 200 volt. Hasilnya menunjukkan bahwa kedua strain yang dicoba mempunyai megaplasmid yang dapat dipisahkan dari DNA genomnya. Ukuran megaplasmid tersebut lebih besar dari 770 kb.

Abstract

The strains of *Pseudomonas solanacearum* E.F. Smith have been reported to have megaplasmids. The plasmid was suspected to carry genes of pathogenicity of the bacterium. The plasmids have quite big size and low copy number. Extraction of the plasmid out of gel causes the damage of the plasmid and no band appears on gel after the electrophoresis. This experiment was done to isolate megaplasmid of two strains of *P. solanacearum* strain 2305L (biovar 2 tomato isolate from Lembang) and strain JGP (biovar 3 peanut isolate from Jambegede, Malang) using Pulse Field Gel Electrophoresis (PFGE). The PFGE was run at the ramping time of 40 – 60 seconds, the running time of 26 hours and the voltage was set at 200 volt. The results suggested that the two strains of *P. solanacearum* have megaplasmids which could be separated from the genom. The size of the megaplasmids were bigger than 770 kb.

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Introduction

Research on molecular level of *P. solanacearum* has been started in the past five years. Cook *et al.* (1989) reported the diversity of the bacterium based on the RFLP pattern. Subandiyah *et al.*, (1991) reported the DNA fingerprinting of the Indonesian isolates compared to the Australian isolates. Seal *et al.* (1992) developed the DNA-probes and primers of PCR for detecting the strains of *P. solanacearum*. Ma (1990) found a sequence of 12.8 kb appeared only in *P. solanacearum* strain which was virulent on peanut and not in strains which were not virulent on peanut. Holloway (1992) (personal com.) suggested that pathogenicity of *P. solanacearum* was controlled by the genes located both in chromosome and megaplasmid, however, the genetic of the bacterium pathogenicity is remain unclear.

Some reports show that strains of *P. solanacearum* have megaplasmid which move very slowly on the gel. The plasmid appeared only when cells lysis was conducted on the gel before electrophoresis. Using a regular electrophoresis apparatus with the position of the gel horizontal or vertical and by manipulating the current voltage and running time, the megaplasmid bands were located between the genom bands and the wells (Morales and Sequeira, 1985; Bominathan and Mahadevan, 1988). Morales and Sequeira (1985) found megaplasmid with one or two copies on 22 strains from 39 strains of *P. solanacearum*. The size of the plasmid was ranged from 7.5 to 750 kb.

Cell lysis out of gel for isolating megaplasmid of banana strain of *P. solanacearum* was done, however the plasmids were disappeared after phenol: chloroform extraction (Bominathan and Mahadevan, 1988). The disappearance of megaplasmid band on the gel after the extraction process was due to the high sensitive characteristic of the plasmid to friction and movement during the extraction process. The conventional agarose gel electrophoresis only separate DNA fragment with the size less than 50 kb or work best in the 0.2 – 20 kb size range of DNA molecules. Therefore when the plasmid is bigger than 50 kb it does not appear on the gel. PFGE is able to separate DNA with the size ranged from 100 – 10.000 kb and resulting undamaged plasmid or chromosomes as the lysis is occurred on gel except when digestion is needed (Suwanto, 1992).

Methods

1. Bacterial Cultivation

Isolates 2305 L (biovar 2) obtained from tomato in Lembang and JGP (biovar 3) originated from peanut in Malang were used in

this experiment. The isolates were grown in CPG broth (casamino acid 0,1%, peptone 10%, and glucose 0,5%, Kelman, 1954) for 12 – 18 hours at room temperature with gentle shaking. The cells were harvested from 20 ml culture by pelleting in a centrifuge and washed in PIV (10 mM Tris-Cl pH 7.5 and 1 M NaCl). The pellet was resuspended in PIV to get the density of 1.8 × 10^9 cells/ml.

2. Insert Preparation and Cell Lysis

The volume of 0.6 ml cell suspension in an eppendorf tube at 37°C was mixed with 0.4 ml of 2% low melting point extra pure agarose in PIV at the same temperature. The mixture was mixed well and filled into the insert mold with 100 μl per hole. The insert mold with the samples was put on ice or in a refrigerator for 10 – 15 minutes. The insert samples were pushed out of the insert mold when they have set and put in a bottle with EC lysis solution (6 mM Tris pH 7.6, 1 M NaCl, 100 mM EDTA, 0.5% Brij-58, 0,5% sarkosil and 0.2% sodium deoxycholate) which contain 20 μg/ml DNase-free RNase and 1 mg/ml lysozyme. The volume of EC lysis solution was 5 times of the insert volume. The samples in the lysis solution were incubated at 37°C overnight with gentle shaking. The EC lysis solution was replaced with the same volume of ESP solution (0.5 M EDTA ph 9, 1% sodium lauryl sarkosil) containing 200 μg/ml proteinase K. The samples in the ESP solution were incubated at 50°C for 2 × 24 hours with gentle shaking.

3. Electrophoresis Condition

The samples were washed in 1 × TE buffer at 50°C for 30 minutes and then at room temperature for 2 hours. The volume of the TE buffer was 5 – 8 times of the insert volume. The insert samples were cut in 2 mm width using a cover slip glass and ready to be inserted to the agarose wells. The agarose slab was made of 1% extra pure agarose in 0.5 × TBE.

The samples were inserted into the agarose wells and then sealed with 1% low melting point agarose. The running buffer used in the experiment was 0.5 × TBE. Two combinations of pulse were set in the experiment, the first set was with the ramping time of 1 – 15 second running for 12 hours at 170 volt and the second set was with the ramping time of 40 – 60 second running for 26 hours at 200 volt. The temperature was set at 7°C or became 12°C in the tank. The PFGE model CHEF-DR Tm II Electrophoresis Cell from Biorad was used in the experiment and conducted at the Biochemistry Laboratory of IUC-Biotechnology, Gadjah Mada University.

Results and Discussion

The size at which agarose gel electrophoresis mobility become independent of molecular weight depends strongly on the electrical field strength and the gel concentration because the DNA molecules tend to remain oriented along the direction of the applied electrical field. In practice it is difficult to achieve useful resolution for DNA molecules larger than 50 – 100 kb in conventional electrophoresis. The new generation of electrophoresis, PFGE is promising to get a better result for separating large DNA molecules, large plasmid DNA or chromosomes in agarose gel at finite field strengths. In this method, pulsed, alternating, orthogonal electric fields are applied to a gel. Large DNA molecules become trapped in their reptation tube every time the direction of the electric is altered and can make no further progress through the gel until they have reoriented themselves along the new axis of the electric field. The larger the DNA molecule, the longer the time required for this realignment. Molecules of DNA whose reorientation time are less than the period of the electric pulse will therefore be fractionated according to the size (Olson, 1989).

Using the ramping time of 1 – 15 second running for 12 hours at 170 volt there was only one band appeared on both isolates of 2305 L and JGP. It suggested that the megaplasmid was not able to be separated from the genom. On the other hand, using another combination with the ramping time of 40 – 60 second running for 26 hours at 200 volt both of the isolates showed two bands on every track (Figure 1 and 2). The megaplasmid bands are located under the genom bands. The isolate 2305 L (track 3 and 4) has a little higher megaplasmid band than that of JGP isolate (track 5 and 6). The yeast standard chromosome showed 12 bands instead of the complete 15 bands (track 1 and 8) with the size of 245 kb to 770 kb (second band). Based on the position of the megaplasmid bands compared to the position of yeast standard chromosome bands, the size of megaplasmid of *P. solanacearum* was bigger than 770 kb.

A further experiments should be done to get better separation of the genom and the megaplasmid and to isolate the megaplasmid DNA from the gel. Different combination of ramping time, running time, and the voltage which are higher than those used in this experiment is needed. Furthermore it is worth to clone the genes located in the megaplasmid and to investigate the role of the genes. By understanding more on the genetic of the bacterium it is possible to get a better technique for controlling the pathogen in the field.

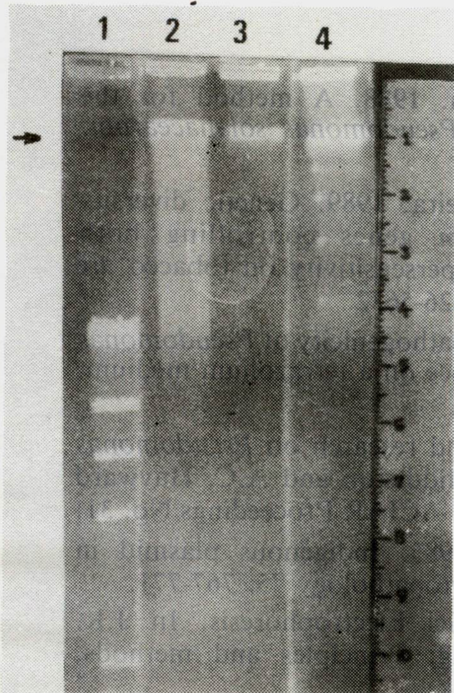

Figure 1.

PFGE result of *P. solanacearum* with the combination of ramping time of 1-15 sec., running time of 12 h, at 170 volt
1 : lamda DNA-Hind III
2 : strain 2305 L
3 : strain JGP
4 : Yeast Chromosome.

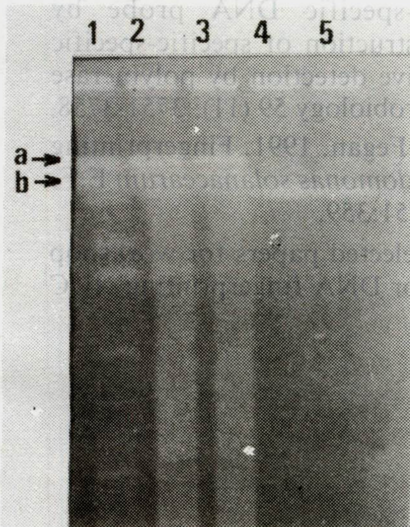

Figure 2.

PFGE result of *P. solanacearum* with the combination of ramping time of 40-60 sec., running time of 26 h, at 200 volt
1 : Yeast Chromosome.
2 and 3 : strain 2305 L
4 and 5 : strain JGP
a : genomic DNA
b : megaplasmid DNA.

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KARAKTERISTIK *Aeromonas hydrophila* PADA IKAN LELE (*Clarias sp*) DI DAERAH ISTIMEWA YOGYAKARTA DAN JAWA TENGAH SELATAN

(CHARACTERISTICS OF *Aeromonas hydrophila* ON CATFISH, *Clarias sp*, IN YOGYAKARTA SPECIAL TERRITORY AND SOUTH CENTRAL JAVA PROVINCE)

Oleh:

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Intisari

Aeromonas hydrophila adalah bakteri penyebab penyakit ikan air tawar yang terpenting di Indonesia. Bakteri ini sangat ganas dan dapat menyebabkan kematian lebih dari 60% dalam waktu sekitar 7 hari. Penggunaan vaksin memberi harapan cukup baik, tetapi untuk pengembangannya masih mengalami hambatan, karena karakteristik bakteri ini belum banyak diketahui dan adanya heterogenisitas antigenik.

Tujuan penelitian adalah untuk mengetahui sifat morfologi, biokimia, serologi dan patogenisitas beberapa isolat *A. hydrophila* dari Daerah Istimewa Yogyakarta dan Jawa Tengah bagian selatan.

Hasil penelitian menunjukkan bahwa *A. hydrophila* ternyata dapat merupakan penyebab infeksi primer pada ikan lele. Bakteri ini bersifat Gram negatif, berbentuk batang, koloni bulat, cembung, berwarna kekuning-kuningan, dan mempunyai variasi biokimia. Dari 23 isolat yang memfermentasi glukosa dan membentuk gas 82,61%, laktosa 73,9%, sukrosa 100%, monitol 4,76%, dulcitol 8,69%, sorbitol 8,69%, arabinosa 60,69%, adonitol 13,04% dan raffinosa 26,09%. Sedang uji serologi menunjukkan bahwa diantara isolat ada yang bersifat reaksi silang dan mempunyai *common antigen*. Dosis letal (LD_{50}) bakteri berkisar antara ($5{,}78 \times 10^4$) – ($3{,}35 \times 10^7$) sel/ml, dengan rata-rata waktu kematian 7,5 – 9,7 hari. Suntikan eksotoksin ternyata dapat menimbulkan kematian dan gejala yang sama dengan infeksi oleh sel utuh secara alami atau laboratoris.

Abstract

Aeromonas hydrophila is one of the most important bacterial pathogen in fresh-water fishes in Indonesia. The bacteria can cause mortality more than 60% in cultured fish in approximately 7 days. Vaccination has a good prospect to solve the disease problem cause by MAS. However, there are some problems to develop mass production of *A. hydrophila* vaccine. Because the bacteria has antigenic heterogenicity and the characteristics are not well understood.

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The objectives of the study were to know the characteristics of *A. hydrophila* especially the morphology, biochemical, serology, and pathogenicity of some isolates from Yogyakarta Special Territory and South Central Java Provinces.

The results indicated that *A. hydrophila* could become a primary pathogen on catfish. The bacteria were rod, Gram negative, round, convex and yellowish collonies, they had various biochemical characteristics. From 23 isolates, 82.61% was fermentative on glucose and produced gas, lactose 73.9%, sucrose 100%, mannitol 4.76%, dulcitol 8.69%, sorbitol 8.69%, arabinose 60.69%, adonitol 13.04%, and raffinose 26.09%. Some isolates had serological cross reaction and it indicated that the bacteria had common antigens. The LD_{50} ranging from 5.78×10^4 cell/ml to 3.35×10^7 cell/ml, with mean time to death between 7.5 days and 9.7 days. Exotoxin injection caused death and produced the same signs as natural infection.

I. Pengantar

Bakteri *Aeromonas hydrophila* merupakan bakteri gram negatif yang bersifat oportunistik patogen terutama pada ikan yang luka maupun yang stres (Wakabayashi dkk., 1981). Penyakit bakterial yang disebabkan oleh bakteri tersebut dikenal dengan nama *MAS (Motil Aeromonas Septisemia).* Penyakit ini cukup ganas, khususnya pada benih ikan lele. Hasil penelitian menunjukkan bahwa penyakit ini dapat menyebabkan tingkat kematian lebih dari 60% (Supriyadi dan Rukyani, 1990) dalam waktu sekitar 7 hari (Triyanto, 1990).

Penanggulangan penyakit oleh *A. hydrophila* dapat dilakukan antara lain dengan sanitasi lingkungan, meningkatkan nutrisi yang diberikan, penggunaan obat-obatan dan dengan vaksinasi. Usaha vaksinasi untuk mencegah penyakit yang disebabkan bakteri *A. hydrophila* menurut Plumb (1984) mempunyai prospek yang baik karena efektif dan tidak mempunyai dampak sampingan. Tetapi pengembangan vaksin masih menghadapi beberapa masalah, karena karateristik bakteri ini belum banyak diketahui dan adanya heterogenitas antigenik. Untuk itu penelitian tentang sifat bakteri *A. hydrophila* sangat diperlukan.

Tujuan umum penelitian adalah meningkatkan pengetahuan tentang *A. hydrophila* yang merupakan salah satu bakteri penyebab penyakit ikan air tawar terpenting di Indonesia. Sedang tujuan khusus adalah untuk : Mengetahui sifat biokimia dan serologi bakteri *A. hydrophila* yang menyerang ikan lele di Daerah Istimewa Yogyakarta dan Jawa Tengah bagian selatan. Gejala penyakit dan patogenisitas serta berapa lama gejala dan kematian ikan terjadi.

Hasil penelitian ini akan bermanfaat sebagai dasar mencari alternatif penanggulangan penyakit MAS secara tepat dan berdampak sampingan kecil, serta terjangkau oleh petani. Sedang bagi industri akan menjadi bahan pertimbangan dalam memproduksi vaksin.