DETERMINATION OF UNCULTURED ENDOPHYTIC BACTERIAL COMMUNITY FROM RICE ROOT

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Abstract

Culture-independent approaches were developed for rapid analysis of microbial community diversity in various environments. Direct analysis based on 16S rDNA as the phylogenetic markers is the most ordinary, conventional and suitable methods for bacterial diversity analysis. The objective of this study is to investigate the microbial diversity from the rice root tissues using culture-independent approach by 16S rDNA library construction. The 16S rDNAs were directly extracted from a total genomic DNA by polymerase chain reaction (PCR) amplification using with the bacteria-specific primer set. The 16S rDNAs were subsequently analysed by cloning and restriction digestion. The amplified ribosomal DNA restriction analysis (ARDRA) clustered the 16S rDNAs into eight majority patterns. These predominant patterns were analysed by DNA sequencing. A better understanding at microbial diversity level is critical to potentiate the endophyte as plant growth promoters.

Abstrak

Pendekatan tanpa-kultur dibangunkan untuk menganalisa secara pantas kepelbagaian komuniti mikrob di dalam persekitaran yang berbeza. Analisa secara langsung berdasarkan kepada 16S rDNA sebagai penanda filogenetik adalah kaedah yang paling konvensional dan sesuai digunakan untuk menganalisa kepelbagaian bakteria. Objektif kajian ini adalah untuk mengkaji kepelbagaian diversiti bakteria dari tisu akar padi dengan menggunakan kaedah tanpa-kultur melalui pembinaan perpustakaan 16S rDNA. 16S rDNA telah diekstrak daripada seluruh DNA genom dengan tindak balas rantaian polymerase (PCR) menggunakan set primer yang khusus untuk bakteria. 16S rDNA ini seterusnya dikaji melalui pengklonan dan pencernaan sekatan. Analisa amplifikasi pembatasan DNA ribosom (ARDRA) mengklasifikasikan 16S rDNA kepada lapan corak majoriti. Corak-corak yang utama ini dipilih untuk penjujukan DNA. Pemahaman yang mendalam terhadap kepelbagaian diversiti mikrob adalah penting bagi peningkatan potensi endofit sebagai faktor penggalak pertumbuhan tumbuhan.

Keywords: Bacterial diversity, 16S rDNA, ARDRA

INTRODUCTION

Endophytic bacteria are those bacteria that colonize the internal tissue of plant showing no external sign of infection or not causing disease symptoms (Schulz and Boyle, 2006). Some are known to play important roles in plant yield and growth promotion, plant health, and protection (Hallmann and Berg, 2006). It has been reported that endophytic bacteria have dependent upon cultivation, and phenotypic and morphological characterisations for their identification (Fisher et al., 1992). They have been used to control plant parasitic nematode, antagonise bacterial pathogens and display strong anti-fungal activity. In biotechnology applications the endophytic bacteria have been manipulate for, such as plant-growth promotion or enhanced pest control, but have only recently been considered in relation to degradative capacity as part of a phytoremediation strategy. It is an

emerging bioremediation technology which uses plants to stabilise and clean contaminated soil and becoming accepted as a commercial opportunity to more conventional physicochemical methods.

Previously, the traditional culture dependent methods only enable characterisation of approximately 1% of the soil bacterial population (Torsvik et al., 2008). As such, majority of the microorganisms from natural communities as observed under the microscope may not grow under laboratory conditions, even though they are metabolically active (Jong and Pauline, 2008). This indicates that the traditional culture dependent methods for diversity measurements enable exploration of only a tiny fraction of the genetic information in a microbial community. Culturing problem can be overcome by applying growth independent molecular methods otherwise changed the research strategies from analysing purified isolates to analysing the entire community (Seckbach, 2000). In this study, we employed the culture independent approach based on 16S rDNA library to analyse the endophytic bacterial community in the rice root. The research data obtained would provide comprehensive information of the bacterial populations that formulate the intimate endophytic relationship with the rice plant.

METHODOLOGY

Bulk DNA extraction

Samples were obtained from rice root tissue. The bulk DNA of endophytic bacteria was extracted using PowerMax® Soil DNA Isolation kit as instructed by the manufacturer (Mo Bio Laboratory, Carlsbad, U.S.A).

PCR amplification of total 16S rDNA

PCR amplification targeting the 16S rRNA genes was carried out. The PCR reaction mixture consisted of 200 µM of each deoxynucleoside triphosphate, 1X PCR reaction buffer, 0.25 µM of 27 forward and 1492 reverse primers and 2.5 U *Taq* DNA polymerase (Yeastern Biotech Co., Ltd, Taipei, Taiwan). The reaction mix was placed in a MyCycler™ Thermal Cycler System with Gradient Option (BioRad, Hercules, U.S.A) at single step of initial denaturation at 95°C for 3 minutes followed by 35 cycles of 30s at 95°C, 30s at annealing temperature of 52°C, 60s elongation at 72°C and final extension of 15 minutes at 72°C.

Construction and analysis of 16S rDNA library

Cloning

A commercial kit CloneJET™ PCR Cloning Kit (Fermentas, Lithuania) was used for the purpose. The blunting reaction consisted of 5 µl of 2 × reaction buffers, 4 µl of PCR product, 0.1 µl of water and 0.3 µl of DNA blunting enzyme. The mixture was briefly vortexed and centrifuged for 3-5 s. The mixture was then incubated at 70°C for 5 min and briefly chilled on ice. The ligation reaction consisted of 0.3 µl pJET1.2/Blunt cloning vector (50 ng/µl) and T4 DNA ligase (5 U/µl) each were then added to the blunting reaction mixture (9.4 µl). The ligation mixtures (10 µl) were incubated at 22°C for 2 hours. The ligation mixture was used directly for bacterial transformation.

Transformation of recombinant plasmids into Escherichia coli DH5α competent cells

The ligated PCR products were transformed into *E. coli* DH5*α* (ECOS 101) competent cells (Yeastern Biotech Co., Ltd, Taipei, Taiwan). The cells were spread plated onto pre-chilled (4°C)

Luria Bertani agar plate containing 50 $\mu g ml^{-1}$ of ampicillin. The plates were incubated at 37°C for 16 hours. The colonies were screened for insert sizes of approximately 1.5 kb by performing colony PCR with the vector primers pJET1.2 forward primer (5'-CGA CTC ACT ATA GGG AGA GCG GC-3') and pJET1.2 reverse primer (5'-AAG AAC ATC GAT TTT CCA TGG CAG-3').

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

ARDRA was performed to analyse the diversity of positive 16S rDNA clones. Digestion of the PCR product (5 µl) was done at 37°C for 16-18 h. The restriction fragments were separated on a 3% MetaPhor® Agarose gel (Cambrex Bio Science Rockland, East Rutherford, U.S.A) in 1× TAE buffer at 100 V for 40 minutes and visualized via ethidium bromide staining and UV illumination. In the analysis of ARDRA patterns, bands with the same gel mobility were considered equivalent and dependent of their relative intensity. Sequencing reactions were then carried out commercially for plasmid clones that represent the different ARDRA patterns. The rRNA gene sequences obtained were subjected to National Center for Biotechnology (NCBI) BLAST searches to identify their closest relatives.

RESULTS AND DISCUSSION

PCR amplification

Polymerase chain reaction (PCR) was carried out on *E. coli* DH5α transformants carrying the endophyte 16S rDNA. Conceptually, PCR is performed based on the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. At the end of a PCR reaction, the specific sequence will be accumulated in billions of copies (amplicons). In this study, a total of 475 colonies were screened by PCR. A 16S rDNA clone library was constructed in order to identify the diversity of endophytic bacteria from rice root. Among the screened transformants, 341 clones containing positive inserts with band size of approximately 1,500 bp each were obtained. The representative clones were demonstrated in Figure 1.

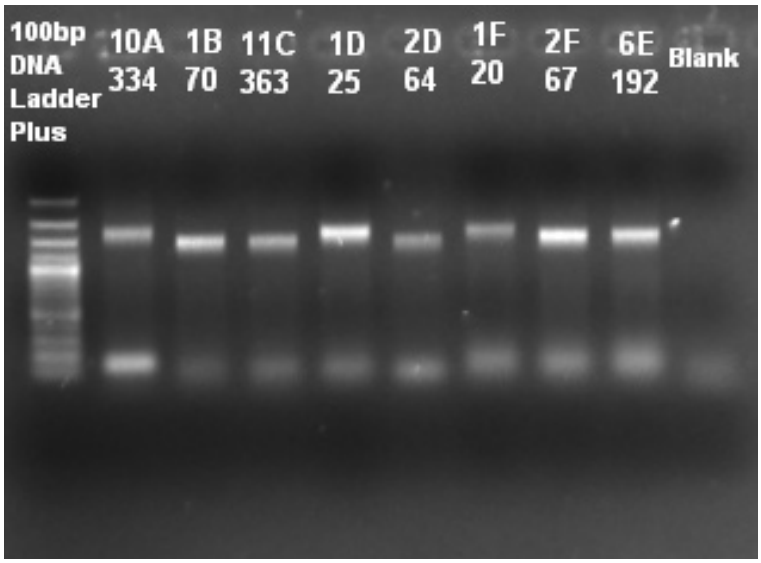

Figure 1 PCR-amplified 16S rDNA fragments of representative *E. coli* DH5α clones.

Analysis of ARDRA patterns of endophytic bacteria 16S rDNA

ARDRA was performed to analyse the diversity of positive 16S rDNA clones. In the analysis of ARDRA patterns, bands with the same gel mobility were considered equivalent, taking into consideration their relative intensity. The restriction patterns were sorted and grouped. Each restriction pattern was predicted to represent an individual bacteria species. In this study, ARDRA was performed by double-digesting each rDNA with two restriction endonucleases (REs), *Hae*III and *Hin*fI (New England Biolabs, United Kingdom) which were originated from *Haemophilus aegyptius* and *Haemophilus influenza*, respectively. From the ARDRA analysis, 51 ARDRA patterns were identified in the endophytic community. Two predominant populations were observed. These are Pattern 1B and Pattern 1F which constituted 48.41% and 29.68% of the total analysed community. Besides, six ARDRA patterns recorded more than 2 representative clones. Patterns 1D and 2D contributed about 2.12% and 1.41% abundance of the total library, while patterns 2F, 6E, 10A, and 11C shared similar percentage abundances which were 0.71% of the total library. Other patterns were each represented by only one bacterial clone. The representative ARDRA patterns (major populations) are demonstrated in Figure 2.

Table 1 The predominant ARDRA patterns in the endophytic root community.

No.	Pattern type designation	No. of representative clones	%
1	1B 70	137	48.41
2	1F 20	84	29.68
3	1D 28	6	2.12
4	2D 64	4	1.41
5	10A 334	2	0.71
6	11C 363	2	0.71
7	2F 67	2	0.71
8	6E 192	2	0.71
9	Others	44	15.55

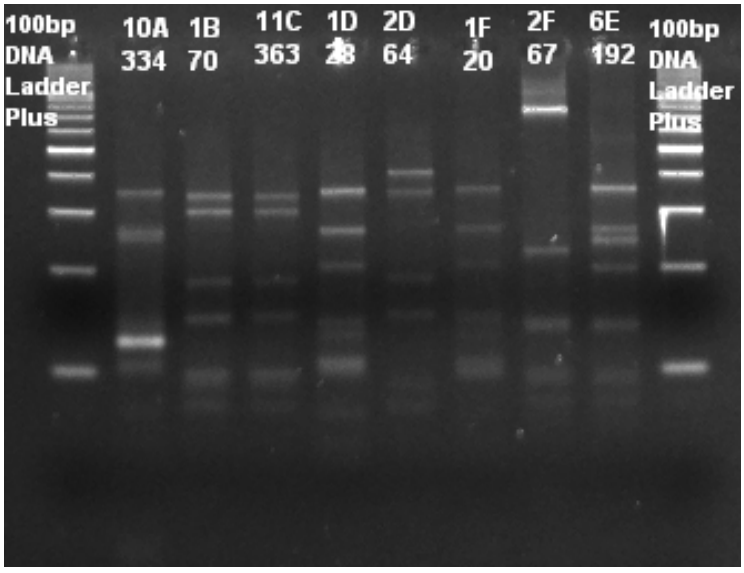

Figure 2 ARDRA patterns of the predominant endophytic 16S rDNAs.

CONCLUSIONS

The detection of 51 ARDRA patterns in the endophytic rDNA library indicated a highly diverse bacterial community in the rice root tissue. Further works involve DNA sequencing analysis would be carried out for the representative *E.coli* DH5*α* clones. The sequencing analysis will reveal the genus of the each representative clones.

REFERENCES

Fisher, P.J., Petrini,O. and Lappin-Scott, H.M., 1992. The distribution of some fungal and bacterial endophytes in maize (*Zea mays L.), New Phyt.* **122**: 299-305.

Hallmann, J. and Berg, G., 2006. Spectrum and Population Dynamics of Bacterial Root Endophytes. Springer-Verlag, Berlin.

Jong, B.C. and P.W.Y. Liew., 2008. Application of rDNA-PCR Amplification and DGGE Fingerprinting for Detection of Microbial Diversity in a Malaysian Crude Oil. J. Microbiol. Biotechnol. **18**(5), 815-820.

Schulz, B., and Boyle, C., 2006. What Are Endophytes? Springer-Verlag, Berlin.

Seckbach, J., 2000. Journey to diverse microbial worlds: adaptation to exotic environments. Netherlands: Kluwer Academic Publisher.

Torsvik, V., Daae, F.L., Sandaa, R.-A., and Ovreas, L., 1998. Review article: novel techniques for analysing microbial diversity in natural and perturbed environments. J. Biotechnol. **64**, 53-62.