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Biochemical Diversity of Bacterial Isolates from Paddy Soils of Peninsular Malaysia

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ABSTRACT

In this study, the diversity of bacterial isolates from paddy soil located in several rice growing areas of Peninsular Malaysia was evaluated. Phenotypic and physiological characteristics of the isolates were recorded to categorize and identify the bacteria. Ten strains of bacteria were recovered from six different locations (Alor Bakat, Kg. Seligi, Bachok, Kelantan; Sekinchan, Selangor; Kobah, Kedah; and Sg. Batu Pahat, Perlis). The spread-plate technique on nutrient agar at pH 7.0 was used to isolate and purify all the strains. The characteristic of the bacteria strains were determined using the Gram staining, motility test, as well as the shape and size of the single colony on solid media. From the various tests conducted, nine isolates were identified as Gram-positive rods, and only one was Gram-negative cocci. Further biochemical tests were carried out to determine the ability of these bacteria strains to hydrolyze starch, casein and gelatine, ferment carbohydrate (glucose, lactose and sucrose), enzyme production (catalase and oxidase), MR-VP tests and growth under anaerobic condition; the elevated NaCl was also examined. On the basis of these tests and the biochemical characteristics, nine of the strains belonged to the same genus, *Bacillus*, with three potentially different species. Meanwhile, only one strain showed the characteristics related to *Proteus mirabilis*. This study also showed that the dominant bacteria genera are generally limited, despite the contrasting geographical location and soil characteristics in which the strains were isolated.

Keywords: Bacteria diversity, biochemical tests, morphology, paddy soils

INTRODUCTION

Rice is the only major grain crop grown almost exclusively as human food. Indeed, rice constitutes half of the diet of 1.6 billion people and another million rely on it for more than one fourth of their diet (De Datta and Brady, 1987). It is expected that in the year 2020, an additional 300 million tonnes will be needed to feed the rapidly expanding human population. To meet this demand, its production must increase by 65% within the 30 years and must be achieved with only minimal expansion of the cultivated area (Saito and Watanabe, 1978).

In low input traditional rice cultivation, plant N originated from the soil, and replenished from the atmosphere by spontaneous biological nitrogen fixation (BNF). Research on rice nutrition has shown that even when high amounts of inorganic N fertilizer are applied, rice plant obtain 60 – 70% of their N from the soil (Reddy

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et al., 2002). Therefore, crop intensification may affect rice soil fertility if proper N input does not replenish the N taken up from the soil and replenishment can be achieved by increasing chemical fertilizers and biological N sources, such as green manure crops, enhancing N_2 fixation by indigenous BNF agents (free living bacteria) and decreasing N loss by proper N application and water management (O'Hara *et al*., 2002).

N_2-fixing agents in soil and water can be considered as natural "fertilizer factories". Promoting their growth and N_2-fixing activity is an important strategy in sustaining rice production. Biological nitrogen fixation technologies are especially important for long-term maintenance of soil fertility. The technologies are environmentally safe and reduced environmental pollution is achieved. Fertilizer savings, improved soil properties, reduced pests and diseases, as well as often being economically justifiable are the other advantages related to the BNF (Ladha and Reddy, 2003).

The determination of the composition of microbial communities in soil is not necessary for a better quantification of nutrient transformation. However, the biodiversity of the soil micro-organism is important in relation to the maintenance of soil ecosystem function. The presence of free-living bacteria, within the rice rhizophere, is also an indication of the importance of rhizobacteria in contributing to the nitrogen requirements of the paddy plant. In Malaysia, however, very limited study has been done on the ecological and diversity of rhizobacteria from wetland paddy soil. The purpose of this study was to determine the biochemical biodiversity of rhizobacteria from wetland paddy soil and investigate the distribution of dominant bacterial groups from different geographical locations in Peninsular Malaysia.

MATERIALS AND METHODS

Bacteria Sources

Ten strains were obtained from several sources of six main geographical locations: Alor Bakat, Kg. Seligi, Bachok, Kelantan; Sekinchan, Selangor; Kobah, Kedah, and Sg. Batu Pahat, Perlis. The strains used in this study are listed in Table 1, while the location of each site is indicated in *Fig. 1*. The description and characteristics of the paddy soils are given in Tables 2 and 3, respectively.

Isolates P1, P13, S1, 22, 23, 40, 44 and 47 were recovered from the rhizosphere area (within 3 mm of the root structure) in paddy soil. Isolate P2 was recovered from the paddy soil around the rhizosphere area, while isolate P3 was recovered from the paddy soil between the rhizosphere areas.

TABLE 1
The source of strains

Strain	Location/source
P1	Alor Bakat, Bachok, Kelantan (rhizosphere)
P2	Alor Bakat, Bachok, Kelantan (around rhizosphere)
P3	Alor Bakat, Bachok, Kelantan (between rhizosphere)
P13	Alor Bakat, Bachok, Kelantan (rhizosphere)
S1	Kg. Seligi, Bachok, Kelantan (rhizosphere)
22	Kg. Seligi, Bachok, Kelantan (rhizosphere)
23	Pasir Puteh, Kelantan (rhizosphere)
40	Sekinchan, Selangor (rhizosphere)
44	Kobah, Kedah (rhizosphere)
47	Sg. Batu Pahat, Perlis (rhizosphere)

Fig. 1: The location of sampling sites in Perlis, Kedah, Selangor and Kelantan, which represent the major rice growing areas in Peninsular Malaysia

Preliminary Tests

Colony morphology was determined after 2 to 4 days of growth on the nutrient agar plates incubated at 35 – 37˚C. Each isolate was submitted to Gram staining and was examined for cellular morphology and arrangement. One loop of distilled water was put on a clean slide. After that, one loop of the broth culture was added onto it after 16 hours of incubation, and this was spread until it was well mixed. The slide was subsequently heated to get a dry smear. The crystal-violet solution was then poured on the entire smear for 1 minute. The slide was washed gently with running water, and iodine solution was then added for 1 minute. Then, some drops of 95% alcohol were put on the particular smear to decolourize the blue stain. Safranin gram solution was then added to stain the negative bacteria in red colour for 30 seconds. The dried and coloured smear was examined under oil immersion microscope (Blazevic and Grace, 1975).

The isolates were also examined for endospore formation using the endospore staining technique. A heat smear from 24 hours Schaeffer's sporulation agar of culture was prepared. The smear was covered with 5% aqueous solution of malachite green and steamed over boiling water for 5 minutes. After the slide has cooled sufficiently, it was rinsed with distilled water and counterstained with 0.5% safranin solution for about 20 seconds. The slide was then briefly rinsed with distilled water and blotted dry with tissue paper, before it was examined under oil immersion (Blazevic and Grace, 1975).

Biochemical Tests

The following tests were carried out for all the strains: starch, casein and gelatin hydrolysis; oxidase, catalase, indole and hydrogen sulphite production; Citrate utilization; tyrosine degradation; acid production from carbohydrates (glucose, lactose and sucrose); reduction of Nitrate to Nitrite; MR – VP test; and phenylalanine deamination (Blazevic and Grace, 1975).

Growth on NaCl and Oxygen Requirement

Each isolated colony was streaked on 7% and 10% sodium chloride (NaCl) agar and incubated at 37˚C for 7 days, so as to examine the requirement for NaCl. The isolates were also examined for requirement of oxygen. The culture was streaked on a nutrient agar and placed into an anaerobic jar. Oxygen was evacuated from the jar using the gas-generating kits. The 'gas-kit' (Oxoid) was disposible H_2 and CO_2 generator envelopes. The introduction of water, along with the generator envolope in the lid, induces the generation of hydrogen and carbon dioxide gases. The hydrogen combined with the oxygen in the jar to produce water.

RESULTS AND DISCUSSION

The isolated strains were examined for their cell morphology and Gram reaction. Table 4 shows that nine out of 10 strains were Gram positive bacteria. Therefore, the isolation of bacteria from the rice rhizosphere of different geographical locations in Peninsular Malaysia showed that 90% of the strains are Gram positive rods bacteria and 10% are Gram negative cocci bacteria. No other morphological characteristics were obtained and this indicated a generally low diversity of bacteria in paddy soil. This study indicates that bacteria associated with rice rhizosphere in the Peninsular are widespread, but limited to a couple of genera especially to the *Bacillus* species. This result concurs with the report by Wartiainen *et al*. (2008), indicating

TABLE 2
Description of paddy soil where bacteria were isolated

Soil Series	Location	Taxonomic Class	Parent Material
Kangar	Sg. Batu Pahat, Perlis	Typic Endoaquert, very fine clayey, mixed, Isohyperthermic	Riverine alluvium
Kundor	Kobah, Kedah	Typic Tropaquept, very fine clayey, mixed, acid, Isohyperthermic	Marine alluvium
Briah	Sekinchan, Selangor	Typic Endoaquepts, fine, mixed, isohyperthermic	Mixed riverine and estuarine deposits
Jabil	Bachok, Kelantan	Typic Paleeaqults, fine to very fine, kaolinitic, Isohyperthermic	Recent riverine alluvium
Lubok	Pasir Puteh	Typic Fluvaquent, fine, mixed, isohyperthermic	Recent riverine alluvium

TABLE 3
Selected physical and chemical characteristics of paddy soil series

Properties	Kangar	Kundor	Briah	Jabil	Lubok Itik
Texture class	Silty Loam	Silty Loam	Clayey	Clayey	Clayey
Total (%)					
Clay	16	21	55	83	94
Silt	62	66	34	13	2
Sand	22	23	11	1	1
pH(H_2O)	6.80	4.68	4.60	4.70	4.70
CEC (cmol kg^{-1})	8.85	14.85	15.66	12.66	15.02
Total N (%)	0.05	0.13	Nd*	0.25	0.28
Exchangeable					
K (cmol kg^{-1})	0.06	0.22	0.30	0.14	0.22
Mg (cmol kg^{-1})	0.30	0.86	4.11	0.40	1.65
Ca (cmol kg^{-1})	7.11	6.82	5.42	0.27	2.10
Organic matter	1.25	5.26	Nd*	3.31	5.17

*Nd = Not determined

that the diazotrophic community from a paddy field in China centred around the *Azoarcus* genera. Furthermore, Gram-positive bacteria and actinomycetes were the dominant microbes in rice soils, as reported by Kimura and Asakawa (2006).

TABLE 4
Results of the morphology test

Strain	Gram staining	Cells morphology
P1	Purple, Gram +	Rods
P2	Purple, Gram +	Rods
P3	Purple, Gram +	Rods
S1	Purple, Gram +	Rods
22	Purple, Gram +	Rods
23	Purple, Gram +	Rods
40	Purple, Gram +	Rods
44	Purple, Gram +	Rods
47	Purple, Gram +	Rods
P13	Red, Gram -	Coccus

The presumptive test for the Gram positive bacteria showed that these strains were possibly *Bacillus* species, due to the presence of endospore. This was done using the Schaeffer–Fulton method. The malachite will stain endospores green, whereas the vegetative cells will only be stained by the safranin. Endospore is different from vegetative cells because they have a greater resistance to heat and other destructive agents. All the Gram positive isolates have the ability to degrade hydrogen peroxide by producing the enzyme catalase. Bubbles of free oxygen were released in the presence of catalase (Blazevic and Grace, 1975). Thus, catalase test is important to distinguish the *Bacillus* species from genus *Clostridium*, which is also an endospore-forming, but is catalase negative (*Bergey's Manual of Systematic Bacteriology*, 2005).

P13, the only strain of Gram negative bacteria, exhibited a swarming motility on the solid media, which might be classified in *Proteus* species. The important characteristic which distinguishes these genera from other *Enterobacteria* is their ability to deaminate phenylalanine by changing the media to dark green colour after several days of incubation (*Bergey's Manual of Systematic Bacteriology, 2005*). The production of deaminase phenylalanase by strain P13 enables it to deaminize the amino acid phenylalanine into phenylpyruvic acid.

The ability of each strain to produce enzyme for the hydrolysis of starch, casein and gelatin is given in Table 5. All the isolates have the ability to hydrolyze starch by producing the amylase enzyme. This indicated that the entire Gram positive and Gram negative strains can utilize starch as a carbon source. In the presence of amylase, these macromolecules will be hydrolyzed into dextrins, maltose and glucose molecules. All the Gram positive strains also showed the ability to utilize casein, as compared to the Gram negative strain, P13, which is incapable of hydrolyzing casein as a nitrogen source. This is because these bacteria produce more soluble and transparent derivatives as their nitrogen source. For gelatine liquefaction test, strain S1, 40 and 47 were unable to hydrolyze gelatine into amino acid because they lack gelatinase enzyme. They might be in a same group due to their similar reactions in starch, casein and gelatin hydrolisis test.

Microbes ferment many organic compounds including carbohydrates to obtain their energy source. The fermentation of carbohydrate by the isolates were 100% for glucose, 50% for lactose and 50% for sucrose. Table 6 shows the results derived from the fermentation of carbohydrate for each strain. All the isolates showed their ability to generate energy through the bio-oxidation of simple sugars (glucose), by producing acetic acid, lactic acid, formic acid, ethanol and carbon dioxide as common products. The production of these acids cause the medium to change colour, i.e. red to yellow. However, the only Gram-negative bacteria, P13 and other 4 strains of Gram-positive bacteria (P1, P2, P3 and 23) demonstrated a weak fermentation capability because they were unable to ferment a more complex structure of carbohydrate, such as lactose and sucrose, which are a combination of two monosaccharide molecules. Therefore, these strains might be of the same species (*Bergey's Manual of Systematic Bacteriology*, 2005).

Table 7 shows the results gathered from the other biochemical tests. All the strains

TABLE 5
Hydrolisis tests for starch, casein and gelatine

Test	Strain									
	P1	P2	P3	S1	22	23	40	44	47	P13
Starch	+	+	+	+	+	+	+	+	+	+
Casein	+	+	+	+	+	+	+	+	+	-
Gelatin	+	+	+	-	+	+	-	+	-	+

+ positive reaction
- negative reaction

TABLE 6
Results of carbohydrate fermentation

Fermentation	Strain									
	P1	P2	P3	S1	22	23	40	44	47	P13
Glucose	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	+	+	-	+	+	+	-
Sucrose	-	-	-	+	+	-	+	+	+	-

+ positive reaction
- negative reaction

were capable of utilizing sodium citrate when incorporated in a mineral medium as the only carbon source. Oxaloacetate and acetate were the primary products which were subsequently converted to pyruvate and carbon dioxide by an oxaloacetate decarboxylase (O'Brien and Geisler, 1974).

A considerable number of bacteria can be differentiated on the basis of the end products produced, when they ferment glucose in MR-VP medium. Some of the bacteria, such as genus *Proteus* and *Aeromonas* ferment glucose, were used to produce large amounts of acetic, lactic and formic acids, together with carbon dioxide, hydrogen and ethanol. The accumulation of these acids will lower the pH of the medium to five or less, and the indicator will turn red when methyl red is added (Baker and Silverton, 1986). This indicates that the organism is a mixed acid fermenter (e.g *Proteus*, *Citrobacter*). As for butanediol fermenter (e.g *B. cereus*, *B. polymyxa*), they produced little or none of these acids, as compared to acid fermenter which produced a large amount of polar butylene glycol. Acetoin, which is an intermediate in the production of butylene glycol, is responsible for the development of red colour in the VP test instead of acids. The results indicated that strain P13 is a mixed acid fermenter, while strains 22 and 44 are butanediol fermenters. As for the remaining strains, they cannot be differentiated using the MR-VP test because it was found to give positive results for both tests.

Many facultative bacteria are able to use oxygen in nitrate as hydrogen acceptor. Nitrate (NO_3) will be converted into nitrite (NO_2) when it is utilized by facultative bacteria (Doelle, 1975). This enzymatic reaction is controlled by an inducible enzyme known as nitratase. Among the isolates, only strain S1 and P13 were able to utilize nitrate. Although strain S1, 40 and 47 were suspected to be in the same group, S1 showed dissimilarity in the reduction of nitrate, since the presence of free oxygen might prevent nitrate reduction (Revsbech *et al*., 2006). Thus, this factor might be the major cause of the dissimilarity.

The results presented in Table 7 also show that the Gram-negative bacteria, P13, is able to degrade tyrosine and produce hydrogen sulphide. The capability of this strain to produce hydrogen sulphide from amino acid cysteine is dependent on the enzyme cysteine desulfurase, which works in conjuction with the coenzyme pyridoxyl phosphate. Hydrogen sulphide is the initial product of cystein deamination.

Table 8 shows the differences in the results gathered on the growth ability in 5% and 7% sodium chloride (NaCl). Amongst these strains, P1, P2, P3 and 23 showed the ability to grow in a medium which contained 7% NaCl. This shows that the strains are more resistant to the

TABLE 7
Results of biochemical tests

Test	Strain									
	P1	P2	P3	S1	22	23	40	44	47	P13
Oxidase	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-
Hydrogen sulphite	-	-	-	-	-	-	-	-	-	+
Citrate	+	+	+	+	+	+	+	+	+	+
Tyrosine	-	-	-	-	-	-	-	-	-	+
Nitrate	-	-	-	+	-	-	-	-	-	+
MR	+	+	+	+	-	+	+	-	+	+
VP	+	+	+	+	+	+	+	+	+	-

+ positive reaction
- negative reaction

TABLE 8
Growth ability in NaCl and in anaerobic condition

Growth ability	Strain									
	P1	P2	P3	S1	22	23	40	44	47	P13
7% NaCl	+	+	+	-	-	+	-	-	-	-
10% NaCl	-	-	-	-	-	-	-	-	-	-
Anaerobic	+	+	+	+	+	+	+	+	+	+

+ positive reaction
- negative reaction

high osmotic pressure than the others. From the physical test, all the isolates were shown to be able to grow anaerobically.

CONCLUSIONS

From this study, 9 out of the 10 strains isolated from the different locations of rice rhizosphere were Gram positive rods bacteria. They are suspected to be *Bacillus* species due to the endospore-formation and series of presumptive test. According to the biochemical tests, strain P1, P2, P3 and 23 might be of the same species, while strain S1, 40 and 47 were listed in the same group. Meanwhile, Strains 22 and 44 are classified in another group. The only Gram-negative bacteria, P13, was presumably *P. mirabilis,* as it posed some important characteristics with this species of bacteria. The study indicated that bacterial diversity from the rhizosphere of paddy soil in Peninsular Malaysia is narrow but it is widespread throughout the geographical location of the country.

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