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ISOLATION AND CHARACTERIZATION OF PINK-PIGMENTED, FACULTATIVE METHYLOTROPHIC (PPFM) BACTERIA FROM LEAVES OF NEEM, *Azadirachta indica* A. Juss.

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ABSTRACT

A total of twenty isolates of pink-pigmented, facultative methylotrophic bacteria were obtained from the leaves of neem. All isolates exhibited pink to orange-pink pigmentation, entire margin, round colonies with a smooth glistening surface, and convex elevation. Most of the colonies were opaque with butyrous consistency. Staining revealed rod to coccobacilli shaped, Gram negative cells, containing poly-β-hydroxybutyrate granules. Biochemical analyses showed that all were catalase positive; majority of them were positive for citrate utilization, urease and oxidase activities but were negative for amylase activity. They can be cultivated on ammonium mineral salt (AMS) agar with methanol, glycerol peptone agar (GPA) and tryptic soy agar (TSA) with variations in colonial morphology. Based on the observed characteristics, the isolates obtained belong to the genus *Methylobacterium*.

Keywords: leaf bacteria, PPFM bacteria, *Methylobacterium*

INTRODUCTION

Pink-pigmented, facultative methylotrophic (PPFM) bacteria are an interesting group of prokaryotic eubacteria. Their ability to metabolize C-1 compounds like methanol and a variety of organic compounds makes them highly ubiquitous in distribution. Moreover, their distinctive pink pigmentation due to carotenoids; render them to be tolerant to extreme light conditions and radiations. These features could explain their occurrence in diverse ecological systems such as soil, plants, air, water and even humans. (Anesti *et al.,* 2005; Rice *et al.,* 2000; Barbeau, 1996).

PPFM bacteria have been isolated from a variety of plants, ranging from mosses and ferns to seed producing plants (Lee, 2007). However, Hirano and Upper (2000) reported that some plants like olive trees may not harbor PPFM bacteria.

It is worthwhile to note that the relationship of PPFM bacteria to plants is not completely understood. However, there are studies to show that

PPFM bacteria promote plant growth and development by generating vitamins, phytohormones as well as supply nitrogen to the plant through diazotrophy (Madhaiyan *et al.,* 2005; Van Aken *et al*., 2004; Koenig *et al*., 2002; Basile *et al.,*1985)

Neem (*Azadirachta indica* A.Juss.)**,** a relative of the mahogany, is a native of South East Asian countries. Various parts of the tree have been used to effectively treat or control a wide range of diseases, thus called a "wonder plant". The antibacterial properties are attributed to the presence of substances like azadirachtin, salannin, meliantriol, and nimbin. As such, neem extracts were found effective against the multi-drug-resistant *Vibrio cholerae* (Thakurta *et al.,* 2007), pathogenic bacteria of fish (Das *et al.*,1999), *Propionibacterium acnes* (Jain and Basal, 2003) and as a molluscicide for different kinds of snails (Ebenso, 2004). Its biodegradable nature makes azadirachtin a potential eco-friendly biopesticide.

Various studies done on neem prove its economic value for potential drugs, pesticides and remedies. However, no studies have been done to explore the presence of PPFM bacteria in the neem tree (*Azadirachta indica* A.Juss.). While neem is well known for its antimicrobial and insecticidal properties, it is worthwhile to investigate whether this plant harbors PPFM bacteria. Hence, this study determined the presence of PPFM bacteria in the leaves of neem.

METHODOLOGY

PPFM Bacterial Isolation

A neem tree within a household in Dasmariñas Village, Makati City, Philippines, served as source of leaf samples. The imprint method of Holland *et al.* (2000), using ammonium mineral salt (AMS) agar with 0.5% methanol, was employed to isolate PPFM bacteria from the neem leaves. Leaf samples were washed with sterile water to remove dirt and soil that may adhere to the surface. A total of fifteen leaf imprints were done. Inoculated plates were incubated at 25°C for one week. Observation was done daily from the time of inoculation. Pink colonies that appeared on the plates were then picked and re-streaked on fresh media, until pure cultures were obtained. Pure isolates were then maintained in slants at 4^{o}C. These isolates are currently kept at the DLSU-Microbiology Laboratory.

Characterization of the Bacterial Isolates

Colonial morphology of the isolates was described after growing them on glycerol peptone agar (GPA) after one week of incubation at 25^{o}C. Isolates were also streaked on Tryptic Soy Agar (TSA) to check their ability to grow in the said medium. Microscopic morphology ws described after performing Gram, background and polyβ-hydroxybutyrate (PHB) staining. All of the isolates were subjected to the following biochemical tests: oxidase, catalase, urease, citrate utilization and starch hydrolysis tests.

RESULTS AND DISCUSSION

Out of the fifteen leaf imprints, twenty (20) PPFM bacterial isolates were obtained in this study. Pink colonies were observed by the 6^{th} to 7^{th} day of incubation. Other bacterial and fungal colonies also appeared during the one week observation period. This suggests that a plethora of microorganisms may reside in the leaves of neem. The twenty isolates were categorized into seven groups based on their morphological and biochemical characteristics. Table 1 summarizes the phenotypic characteristics of the PPFM bacterial isolates. The colonial morphology of the isolates in GPA was similar in terms of shape, margin, elevation and optical density. They possessed round, raised, smooth margined and transluscent colonies. Variations were observed in terms of intensity of pigmentation, size and consistency of the colonies as shown in Figure 1. Based on these morphological differences, the isolates were classified into seven colonial types (A-G). Variations in the colonial morphology among PPFM bacteria isolated from various sources were also observed in previous studies (Jang and Lee 2008; Lo and Lee, 2007; Carvajal *et al*.,2006).

Among the media tested, GPA seemed to be a better culture medium compared to AMS and TSA. It was observed that colonies of PPFM bacteria start to appear around three days after inoculation in GPA while it took a week before growth was observed in AMS. This could be due to the fact that AMS is a minimal medium, while GPA is an enriched medium. Faster and more luxuriant growth is expected when bacteria are grown in enriched medium. Nonetheless, there are isolates that showed little or no growth in TSA. This observation was consistent with the report of Carvajal *et al*. (2006).

Examination of microscopic morphology revealed that all isolates yielded Gram negative rods to cocco-bacilli. Figure 2 shows the microscopic appearance of representative PPFM bacterial isolates. The observed microscopic appearance is similar with the description of PPFM bacteria by Green (2001). Moreover, it was observed that all isolates showed PHB granules. Studies by Corpel *et al.* (1986) indicate occurrence of poly-β-hydroxybutyrate and polyphosphate bodies in all the strains of PPFM they isolated from plant surfaces. The different carbon sources, such as glycerol, methanol and formate are used to form these inclusion bodies, which may serve as nutrient reserves.

Biochemical analyses of the bacterial isolates revealed that all of them were catalase and urease positive. Majority of them were positive for oxidase and amylase activities and have the ability to utilize citrate as alternative carbon source. Variations however, were observed in terms of intensity of these activities as indicated by differences in the color change of chemical indicators of the test media used. Extension of the incubation period for one to

two weeks was done in order to observe the enzymatic activities in some isolates. Previous reports (Jang and Lee, 2008; Idris *et al*. 2006; Gallego *et al.,* 2005; Madhaiyan *et al*, 2005; Jourand *et al.,* 2004) on the biochemical properties of PPFM bacteria support these findings. The biochemical features of these isolates may help PPFM bacteria thrive in the leaves of plant (Holland and Pollaco, 1994).

During the course of the study, it was noted that some isolates were able to grow at low temperatures, while incubating the isolates at higher temperature tend to slow down their growth. This observation was similar to the previous studies by Jang and Lee (2008) and Carvajal *et al.,*(2006). They noted that some PPFM bacterial strains are psychrotropic.

It is worthwhile to note that some isolates, although were derived from the same leaf imprint, vary in terms of phenotypic characteristics. This suggests that there is diversity of PPFM bacterial strains residing within a leaf of the plant.

CONCLUSION AND RECOMMENDATIONS

The study demonstrated the occurrence of PPFM in the leaves of the neem tree. Based on the phenotypic characteristics of the isolates, they can be assigned to the genus *Methylobacterium* based on the minimum criteria set by Green (2001). Molecular techniques, such as, 16S rDNA sequence analysis and multi-locus sequence typing (MLST), may be employed to further identify the isolates up to the species level. Variations on the morphological and biochemical properties of the different isolates underscore the diversity of PPFM bacterial strains residing the leaves of the plant. As neem is known to have antimicrobial properties, it is interesting to note that this plant harbor PPFM bacteria. The ability of these bacteria to thrive in this plant therefore necessitates further investigation.

More studies are required to explain the association of PPFM bacteria with the neem leaves. Furthermore, it is recommended that that other plant parts be explored for the presence of these ubiquitous bacteria.

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Table 1. PPFM isolates grouped based on similarities in biochemical and morphological characteristics.

*** Group number**	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**
Isolate Designation	1b,1c,2a, 9b, 14a	2b, 7c, 8b	3a,3b	3c,4a, 8a	5a,5b, 12a	11a, 11b	7b, 13a
Number of isolates	5	3	2	3	3	2	2
Colonial Type in GPA	A	B	C	D	E	F	G
GROWTH in:							
AMS	+	+	+	+	+	+	+
GPA	+	+	+	+	+	+	+
TSA	+	LG	+	NG	+	+	+
BIOCHEMICAL							
Urease	+	w+	w+	+	w+	+	+/ w+
Amylase	– / w+	–	– / w+	–	–	–	–
Citrate	+	–/+	+	+	w+ / –	+	– / w+
Catalase	+	+	+	+	+	+	+
Oxidase	+	+	+	+/ w+/ –	-	–	w+
STAINING							
Gram's stain	–	–	–	–	–	–	–
PHB	+	+	+	+	+	+	+

Legend

* Isolate designation with same number indicate isolates obtained from the same leaf print. The letter in the isolate designation indicates that it is a different colony picked from the leaf print of the designated number.

A- Pink, medium to large size, entire margin, convex elevation, butyrous to brittle consistency, opaque density, smooth and glistening to dull appearance.

B- Light pink to pink, medium to small size, entire margin, convex elevation, butyrous consistency, opaque density, smooth and glistening to dull appearance.

C- Pink, small sizes, entire margin, convex elevation, butyrous consistency, opaque density, smooth and glistening appearance.

D- Pink, large to small size, entire margin, convex elevation, butyrous to brittle consistency, opaque density, smooth and dull to glistening appearance.

E- Light pink, medium to small size, entire margin, convex elevation, butyrous to sticky and watery consistency and transluscent density, smooth and glistening appearance

F- Light pink, medium to small size, entire margin, convex elevation, watery consistency, opaque density, smooth and glistening appearance

G- Light pink, medium to very small size, entire margin, convex elevation, butyrous consistency, opaque density, smooth and glistening appearance

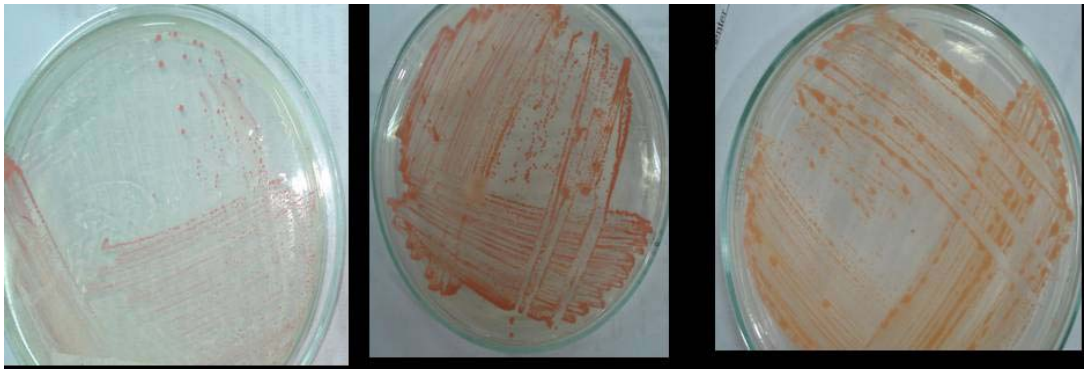
\+ = Positive

– = Negative

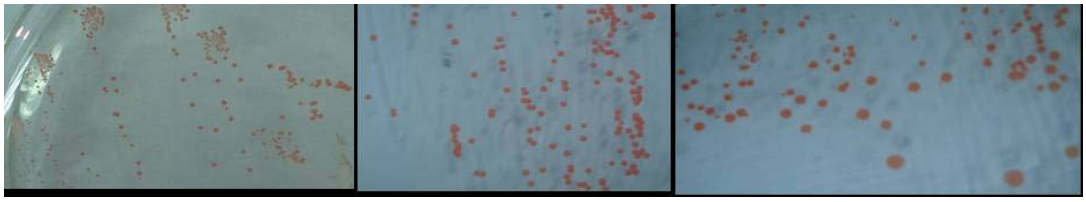
w+ = Weakly positive

NG = No growth

LG = Limited growth

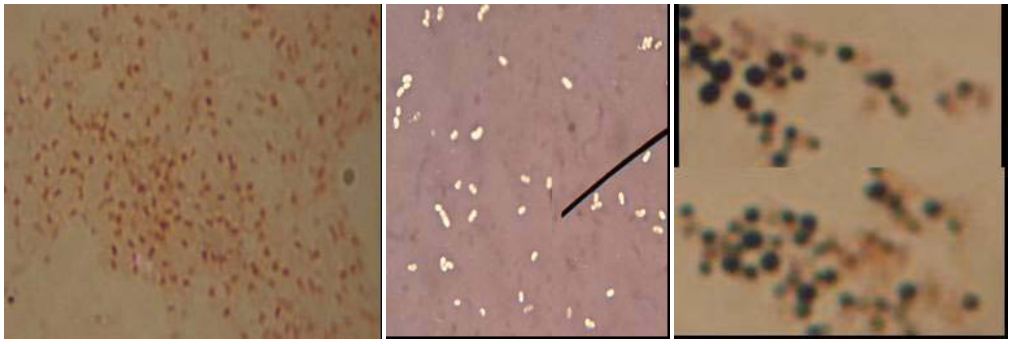


a. Isolate 2b:light pink pigmentation **b.** Isolate 3a: pink pigmentation **c.** Isolate 11a: orange/pink pigmentation



d. Isolate 3a: small size colonies **e.** Isolate 3c: medium size colonies
f. Isolate 9b: large size colonies

Figure 1. Variations in terms of colonial morphology of the PPFM bacterial isolates grown in GPA after one week of one week incubation at 25^{o}C. Images a-c, show the differences in the intensity of pigmentation, while images d-f shows the range of colony size, at the quaternary streak of the inoculated plate. Description on the size of the colonies was based on the following: diameter of colony; **small** (<1mm), **medium** (~1mm) and **large** (>1mm) colonies.



a. gram staining **b.** background staining **c.** PHB staining

Figure 2. Microscopic morphology of the PPFM bacterial isolates. a. Gram staining revealed gram negative rod shaped cells, b. Background staining showed the isolates are rod to cocco-bacilli in shape c. Cells showing PHB granules (black dots).