PHENOTYPIC CHARACTERIZATION OF PINK PIGMENTED FACULTATIVE METHYLOTROPHIC BACTERIA FROM SOIL EXPOSED TO VEHICULAR SOOT

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ABSTRACT

Twenty three (23) pink pigmented facultative methylotrophic bacterial isolates were obtained from soil collected along the island pavement of Taft Avenue fronting De La Salle University-Manila campus. They were described in terms of morphological and biochemical properties, as well as, responses to selected antimicrobials. All isolates were gram-negative rod shaped cells with sudanophilic cysts. Colonial morphologies of the isolates were described after growing them in both minimal and enriched media. All bacterial isolates showed circular, entire, opaque, raised to convex colonies regardless of the media used. Differences however, in terms of intensity of pink pigmentation and consistency were observed when the isolates were grown in different media. In terms of biochemical characteristics, all isolates exhibited urease, catalase, amylase and oxidase activities. Variations in terms of their ability to oxidize different sugars and citrate as carbon and energy sources were observed among the isolates. All isolates yielded negative to blood hemolysis test, indole production methyl red and Voges Proskauer tests. The temperature for the optimum growth of the bacterial isolates was at 30°C. Some strains however, were observed to grow at 37°C and 4°C. All isolates were susceptible to imipinem, β-lactams and β-lactam-β-lacatamase inhibitor formulations, tetracycline but resistant to meropenem. Based on the phenotypic characteristics observed, the isolates are assigned to the genus *Methylobacterium*.

Keywords: PPFM bacteria, *Methylobacterium*, soil bacteria, air pollution.

INTRODUCTION

Soil is a good source of microorganisms which have potential for industrial and agricultural applications. Some are being tapped in agriculture for their ability to improve the nitrogen content of the soil. These are exemplified by diazotrophic or nitrogen fixing bacteria. Some toxins produced by some strains of soil bacteria are used for bio-control of pests. These are represented by the various strains of *Bacillus thuringiensis* (Nicholls *et al.*, 1989).

One interesting group of soil bacteria is the pink-pigmented facultative methylotrophic (PPFM) bacteria. They can utilize C1 compound as well as

multi-carbon compounds which explain their ubiquitous distribution. They are involved in many biological interactions as some strains can enhance plant growth (Ivanova *et al.*, 2001; Koenig *et al*., 2001; Lidstrom and Chistoserdova, 2002) and protect some plants against fungal pathogens (Madhaiyan *et al.*, 2004). Green (2001) observed that PPFM bacteria can be isolated from vehicular exhaust and soot, as these bacteria are able to degrade aliphatic and aromatic hydrocarbons. Furthermore, Popp *et al.* (2006) revealed that PPFM bacteria may be part of the consortia of microorganisms involved in the degradation of mineral oil in soil. These characteristic features give PPFM bacteria as possible pollution indicators. Considering the potential of this group of microorganisms to be utilized in bioremediation and biotechnology, study on this group is needed (Lee, 2007). Hence, this study was conducted to answer the dearth of information regarding local PPFM bacteria. The objective of this study was to isolate and characterize PPFM bacterial isolates from soil samples exposed to vehicular soot. Specifically, it was aimed at describing PPFM bacteria from soil collected along the island pavement of Taft Avenue fronting DLSU-Manila campus.

MATERIALS AND METHODS

Isolation of PPFM Bacteria

All of PPFM bacterial isolates were isolated from surface soil at the island pavement island along Taft Avenue facing DLSU-Manila campus. Since PPFM bacteria are obligate aerobes, only top soil was collected using a shovel and placed in a clean plastic bag. Collected soil samples were then transported and processed at the DLSU microbiology laboratory. Soil suspension was prepared by adding 9ml of sterile distilled water to 1g of soil sample. The suspension was then subjected to decimal serial dilution. An aliquot of 0.1 ml of dilution was inoculated on ammonium mineral salts agar with 0.5% methanol (AMS). Inoculated plates were then incubated for one week at room temperature. Pink colonies observed from AMS agar plates were then fished out and streaked on fresh media until pure isolates were obtained. Colonies were also streaked on non-selective media (glycerol-peptone agar) to confirm the purity of isolates. Pure isolates were maintained in glycerol-peptone agar slant at 4°C. Sub-culturing was done after every three weeks.

Morphological Characterization of PPFM Bacterial Isolates

Colonial morphology of PPFM bacterial isolates was described after growing them in the in minimal media namely; AMS agar and methanol mineral salt agar (MMS). The chemical compositions of these media were formulated by Green (2001). Isolates were also grown in enriched media namely; glycerol-peptone agar, nutrient agar and tryptic-soy agar plates. Colonies were described after growing them for one week at 30°C. Description of the colonies was based on

size, shape, color, optical density, elevation, margin and consistency. Microscopic morphology of PPFM bacterial isolates was described after performing gram staining and Poly-β-hydroxybutyrate staining.

Physiological Characteristics of PPFM Bacterial Isolates

Temperature tolerance of the PPFM bacterial isolates was determined by growing them on AMS agar plates in 3 different environments: in locker (25°C), refrigerator (4°C) and incubator (37°C). The presence of growth as indicated by development of pink colonies was checked daily for one week.

Biochemical Characterization of PPFM Bacterial Isolates

The following biochemical tests were performed on all of the PPFM bacterial isolates: urease test, methyl red test, Voges-Proskauer test, indole test, catalase test, gelatin hydrolysis test, starch hydrolysis test, blood hemolysis test, oxidase test and citrate utilization test. Isolates were inoculated in appropriate test media and incubated for one week at 25°C. Moreover, API-50CH (Biomerieux) was employed to determine the ability of these isolates to oxidize selected carbohydrates. Sterile peptone water with phenol red indicator was used as the suspension medium for inoculation of the API strips.

Antimicrobial Susceptibility Testing of Isolates

The Kirby Bauer disk diffusion technique was employed to determine the response of the isolates to the different antimicrobials. Considering that PPFM bacteria are oxidase positive, gram negative bacilli, the guidelines set by the Clinical Laboratory Standards Institute (CLSI, 2006) for *Pseudomonadaceae* were adapted. The following antimicrobials of varying chemical groups as recommended by CLSI (2006) were used: aminoglycosides represented by gentamicin (10μg), and amikacin (30μg); β-lactam group of antibiotics such as ampicillin (10 μg), piperacillin (100 μg) and cefoperazone (70μg); β-lactam antibiotics with β-lactamase inhibitor formulations such as amoxycillin (20 μg)/clavulanic acid (10μg), sulbactam (10 μg)/ampicillin (10 μg) and piperacillin (100 μg)/tazobactam (10 μg); tetracycline (30 μg); and carbapenems represented by imipenem (10 μg) and meropenem (10 μg).

Due to the differences in the growth requirements of the PPFM bacteria with pseudomonads, modifications were done on the CLSI protocol. These include extending the incubation period from 24 hours to 3-5 days; as well as an incubation temperature of 30°C; as well as the use of tryptic soy agar as test medium.

It should be noted that the PPFM bacteria did not yield the required lawn growth in Mueller-Hinton II agar, as recommended by CLSI (2006). Several

culture media were therefore screened where PPFM bacteria can generate the required lawn growth. Among the various media tested, only tryptic soy agar with an incoulum adjusted to McFarland standard #1 yielded the lawn growth needed for antimicrobial susceptibility testing.

Inoculum was prepared by suspending colonies from 3-5 day old cultures sterile tryptic soy broth. Turbidity of the suspension was then adjusted to McFarland standard #1.0 and inoculated in tryptic soy agar. The surface of the plates was then inoculated with a sterile cotton swab. Antimicrobial disks were applied aseptically to the inoculated plates and incubated at room temperature (30°C) for 3 to 5 days. A clinical isolate of *Pseudomonas aeruginosa* was included in the antimicrobial testing to serve as control.

The diameters of the zones of inhibition were measured to the nearest millimeter and recorded. The CLSI Zone Diameter Interpretive Standards for *Pseudomonadaceae* were used to interpret the data gathered. Antimicrobial susceptibility testing on all isolates was done in triplicates

RESULTS AND DISCUSSION

Number of Obtained PPFM Bacterial Isolates

Pink colonies started to appear in selective media (MMS agar plates and AMS agar plates) after 3 to 4 days of incubation. The growth in non-selective media (nutrient agar plates and glycerol-peptone agar plates) was performed to confirm the purity of PPFM bacterial isolates. Moreover, growth of the isolates in these media indicated that they exhibit facultative methylotrophy. A total of twenty three (23) bacterial isolates were obtained. They were maintained in glycerol peptone-agar slants at 4°C. They were sub-cultured after every 3 weeks of incubation. All of these isolates are currently being kept at the DLSU Microbiology laboratory.

Microscopic Morphology of PPFM Bacterial Isolates

Microscopic morphology of all of the isolates showed gram negative rod shaped cells that occurred singly or in rosettes. They were non-spore and non-capsule formers. The isolates however generate lipid cysts that appeared black after staining with Sudan Black. These inclusion bodies are responsible for the resistance of PPFM bacteria to desiccation (Green 2001, Trotsenko *et al.*, 2001), as well as functions in the storage of energy in the absence of external carbon and energy sources (Madigan *et al.*, 2003).

Colonial Morphology of PPFM Bacterial Isolates

All twenty three isolates were able to grow in both minimal media with methanol (AMS and MMS), as well as enriched media. Their ability to grow in the enriched media imply that they can tap organic compounds other than methanol as sources of carbon and energy. All of the isolates exhibited circular, entire, opaque, raised to convex colonies regardless of the growth media used. They however, differ in the intensity of pink pigmentation. Faint pigmentation was generally observed in the minimal media. Intense pink chromogenesis was however observed when the isolates were grown in glycerol peptone agar, nutrient agar and tyrptic soy agar. This observation suggests that pigment production could be nutrient dependent. Fasim *et al.* (2003) observed that exposure to high concentration of metal salts may hinder pigment production among bacterial strains. The manifestation of pink pigmentation in PPFM bacteria indicates the presence of carotenoids (Trotsenko *et al.*, 2001). This is known to protect PPFM bacteria from intense exposure to sunlight and ultraviolet radiation (Liu *et al.*, 1993; Rheinheimer, 1974).

Biochemical Properties of the PPFM Bacterial Isolates

All of the isolates have biochemical features consistent with the genus *Methylobacterium* as observed by Weyant *et al.* (1996) and Green (2001). They all exhibited oxidase, catalase and amylase activities. Moreover, all of the isolates were urease positive, though they vary in terms of their degree of enzymatic activity. This was demonstrated by the varying rate and intensity in the color change of the chemical indicator in urea broth. The ability to oxidize different carbohydrates as well as citrate utilization varies among the different isolates. Based on these differences, the bacterial isolates were categorized into 13 groups. Table 1 summarizes the biochemical properties of the collected isolates.

Temperature Tolerance of the Isolates

Temperature-sensitivity test revealed that all PPFM bacterial isolates have the optimum temperature for growth at 25^{o}C. Only three groups (Groups V, VI and VII) were able to grow at 37^{o}C. It is interesting to note that these are the isolates that showed positive for citrate utilization. All except for one strain did not grow at 4^{o}C. This observation indicates that majority of the collected isolates are psychrotrophic. Rate of growth was much slower however, when they were grown in these temperatures. Daily observations have shown that PPFM bacteria grow faster at 25^{o}C, as they develop visible pink colonies after three days of incubation, while at 37^{o}C and 4^{o}C, colonies started to appear on the fifth day of incubation. This finding is in contrast with the isolates obtained by Lo (2006) from air. None of the aerial isolates from the study were able to grow at 37^{o}C and 4^{o}C.

Responses of PPFM Bacterial Isolates to Antimicrobials

All of the PPFM bacterial isolates were susceptible to the β-lactam antibiotics, β-lactam-β-lactamase inhibitor formulations, tetracycline, aminoglycosides. This finding is similar to the previous studies on PPFM clinical isolates (Lee *et al.*, 2004; Hornei *et al*., 1999 and Korvick *et al.*, 1989).

Discordant carbapenem susceptiblility was observed in all of the isolates. They were found to be susceptible to imipinem, but resistant to meropenem. This observation is similar to the study of Zaharatos *et al.* (2001). They reported their isolates to be extremely susceptible to imipenem and highly resistant to meropenem, a unique characteristic of *Methylobacterium* species that can be utilized to distinguish this genus from other pink pigmented bacterial genera like *Roseomonas*.

CONCLUSION AND RECOMMENDATIONS

The phenotypic characteristics observed on the isolates were consistent with the description of the genus *Methylobacterium* as described by Green (2001). Enzymatic activities such as catalase, amylase, oxidase and urease as well as discordant carbapenem susceptibility are considered diagnostic feature of this genus. Nonetheless, identification of the isolates up to the species level may require genotypic characterization, such as 16S rDNA sequencing. Further studies can be done on their tolerance of pollutants derived from vehicular emission if they are to be used as pollution indicator or for bioremediation.

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