REVIEW

CURRENT RESEARCH STATUS ON THE BIOLOGY OF PINK PIGMENTED FACULTATIVE METHYLOTROHPHIC (PPFM) BACTERIA BELONGING TO THE GENUS *METHYLOBACTERIUM* IN THE PHILIPPINES+

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ABSTRACT

This paper outlines the current state of knowledge on *Methylobacterium* spp. or commonly known as Pink Pigmented Facultative Methylotrophic (PPFM) bacteria in the Philippines. This review deals with its isolation and cultural properties, colonial, microscopic, biochemical, physiological and genotypic characterization, diversity and systematics, plant- and human-association, and lastly, the research thrusts and directions applicable in the Philippines.

KEYWORDS: Anthony Lee, Pink Bacteria, Systematics, Plant-association, Bioremediation

INTRODUCTION

Pink Pigmented Facultative Methylotrophic (PPFM) bacteria belonging to the genus *Methylobacterium* are considered to be ubiquitous in nature (Lee, 2007). Research endeavour on this microorganism was initiated by Dr. Anthony Lee in the country and because of his mentorship, there have been numerous studies done emphasizing its diversity. *Methylobacterium* has been isolated in different species of plants (Dizon and Esquivel, 2002; Kumar and Lee, 2009; Lee, 2010), water types (Carvajal *et al.*, 2006; dela Cruz, 2007; Palines *et al.* 2012), soil (Carvajal *et al.*, 2006; Jang and Lee, 2008; Johnson *et al.*, 2015), air (Lo and Lee, 2007; Kwong *et al.*, 2011), food (Amparado and Lee, 2010) and different parts of the human body (Carvajal *et al.*, 2006; Carvajal *et al.*, 2011; Uy *et al.*, 2013; David and Diongzon, 2013). Several studies have also further investigated its biological characteristics such as their generation time (De Guzman and Pamaong, 2007), antibiograms

+*This paper is dedicated to the late Dr. Anthony C. Lee, a professor, mentor and colleague, who guided us in pursuing and studying this very complex but interesting microorganism; always reminding us to be "in the Pink of health".*

(Pasamba *et al.*, 2007), antibiotic susceptibility (Ong, 2008), and specific genes (Anyog et. al., 2008; Tan and Grecia, 2009; Lee, 2010; Uy and Uy, 2011; Johnson *et al.*, 2015). Moreover, there are few studies which emphasized on plant-association (Celestino and Leonardo, 2002; and Gapuz *et al.* 2005). With this, the primary aim of this paper is to consolidate all its findings and provide guidance to future researchers who will be undertaking an interest to study this group of microorganisms. The paper highlights a synthesis of the different research outputs about the biology of *Methylobacterium* in the Philippines. The cultural properties, colonial and microscopic characteristics, biochemical and physiological attributes, antibiograms and antibiotic susceptibility, genotypic characterization, diversity and systematics and its plant- and human-association are outlined in this paper. In the end, it emphasizes the new areas that can be investigated and its potential use for biotechnology applicable in the Philippines. Also, the references section was divided into two categories namely; local and international in order to guide researchers where to obtain and search for studies on *Methylobacterium*.

Cultural Properties

The enrichment, isolation procedures and its cultural features of *Methylobacterium* are very well-studied (Garrity *et al.*, 2005; Green, 2001). These microorganisms are relatively easy to isolate because of their ability to grow on methanol as their sole carbon source. Thus, it is recommended to isolate this microorganism using a selective media called Methanol Mineral salts (MMS) media.

It has been observed that *Methylobacterium* spp. are fairly slow growers. It takes approximately 2 – 3 days at either 25^{o}C or 30^{o}C in order to view visible colonies or confluent growth. Sometimes it will take more than 7 days for colonies to reach their maximum size (Garrity *et al.*, 2005; Green, 2001). However, the doubling time of *Methylobacterium* remained uncertain until De Guzman and Pamaong (2007) investigated its doubling or generation time in three different culture media. Results of their study revealed that it takes approximately one (1) hour in all media tested. Their study further supports the slow growth of *Methylobacterium* either in minimal or enrich media.

Colonial and Microscopic Morphology

Colonial morphology was described after growing in minimal medium with 0.5% methanol for one week at either 25^{o}C or 37^{o}C on environmental or human source respectively. All locally reported isolates had shown similar colonial morphology which exhibited entire, convex, butyrous consistency, which raised opaque colonies when grown in an enrichment

media, Glycerone Peptone Agar (GPA). However, the size and pigmentation of the colony differ. Lee (2010) also made use of Spectral Imaging to view the colonial morphology in higher magnification. Pigmentation of the colony ranges from link pink to orange in color. Although orange pigmentation is included in the range of possible *Methylobacterium* strains, it is cautioned by the present researchers that the orange pigmentation grown in MMS with 24-48 hours growth may indicate a different species, *Gordonia*. David and Diongzon (2013) verified as such using 16S rDNA. Thus, the colonial morphology grown in MMS may only serve as a presumptive identification of the microorganism.

Methylobacterium spp. are gram-negative rod-shaped or bacillus microorganisms. Most of the studies conform to this observation, however some reports observed to be gram variable. The size of the bacterium was measured to be approximately 1.0 μm long by 0.5 μm wide (Carvajal *et al.*, 2011). Moreover, bacterial cells of *Methylobacterium* spp. are observed to have vacuolated structures identified as poly-β-hydroxybutyrate granules using PHB granule staining (Carvajal *et al.*, 2006; Jang and Lee, 2008; Kumar and Lee, 2009; Lee, 2010; Carvajal *et al.*, 2011).

Physiological and Biochemical Characterization

Numerous culture media were tested for the growth of *Methylobacterium*. It was observed that there is limited growth on Brain Hear Infusion Agar, Tryptic Soy Agar, Mueller Hinton Agar while no growth was observed Mannitol Salt Agar, Mac Conkey Agar, Eosin Methylene Blue (David and Diongzon, 2013; Lee, 2010; Pasamba *et al.*, 2007; Carvajal *et al.* 2006). Such limited or absence of growth may be due to the presence of bacteriostatic dyes and bile salts that can lead towards the inhibition of growth to *Methylobacterium* (Lee, 2010; David and Diongzon, 2013). This observation conforms to Brown *et al.* (1992) and Garrity *et al.* (2005).

Methylobacterium are said to grow optimally 25^{o}C to 30^{o}C for environmental samples (Lee, 2010) while human-derived samples grow optimally at 30^{o}C -37^{o}C (Diongzon and David, 2013). It was reported that certain strain may grow as low as 4^{o}C indicating they are some psychrotrophic strains (Lee, 2010; Kumar and Lee, 2009; Jang and Lee, 2008). It has been observed that *Methylobacterium* can tolerate salinity up to 2% (Lee, 2010) and no growth was already observed from more than 7.5% salinity. This is accordance to Garrity *et al.*, (2005) wherein *Methylobacterium* species do not exhibit growth at 6% NaCl and beyond. Lee (2010) also investigated its *Methylobacterium* isolates to have a tolerance of 2% methanol, while a number of isolates could tolerate up to 5% methanol.

Cellular fatty acid content revealed that octadecenoic acid, pentadecanoic acid, octadecanoic acid and hexadecanoic acid were the

predominant fatty acids in all isolates (Anyog *et al.* 2008; Lee 2010). These findings were consistent with the report of Hiriashi *et al.* (1995) and Weyant *et al.* (1996) that these fatty acids are found in different species of *Methylobacterium*. Moreover, Anyog *et al.*, (2008) investigated further the fatty acid profiles of the different *Methylobacterium* isolates from identified known species strains. They observed that identified isolates and known species strains did not did not yield the same fatty acid profiles. Thus this method is not sensitive enough to discriminate different species of *Methylobacterium*.

Biochemical assay kits such as API-20 NE, API-50 CH and BBL crystal Enteric/Non-fermenter have been employed to describe the biochemical characteristics of *Methylobacterium* isolates and, as well as, use for identification (Palines and Teves, 2011; Carvajal *et al.*, 2011; Lee, 2010). However, there are limitations in using these assays. With regards to bacterial identification, studies by Carvajal *et al.* (2011) and Lee (2010) have shown very low similarity percentages (40-75%) of their isolates to specimen types as listed by Kato *et al.* (2005). This indicates that the results of the biochemical assays may not reflect characters notable for species discrimination. The findings in these assays can only demonstrate the diversity of strains of *Methylobacterium*. Also, the database of species of these assay kits is very limited in *Methylobacterium* wherein only one is listed. In selected local studies, only oxidase, catalase and urease tests were performed because these are known to be diagnostic to the identification of the genus *Methylobacterium* (Green, 2001). Other biochemical tests such as citrate utilization test, casein and starch hydrolysis tests, methyl red, Voges-Proskauer, and SIM (sulfide, indole and motility), triple sugar iron, carbohydrate fermentation tests were also performed in several studies to describe different *Methylobacterium* strains.

Antibiograms and Antibiotic Susceptibility

Brown *et al.* (1992) and Garrity *et al.* (2005) have reported and described the antimicrobial susceptibility of *Methylobacterium* sp. Ongbin (2007) and Pasamba *et al.* (2007) investigated the antibiograms and antibiotic susceptibility patterns of *Methylobacterium* strains. It was observed that *Methylobacterium* have varying sensitivity to β-lactam antibiotics, sensitivity to tetracyclines, imipinem, chloramphenicol and aminoglycosides while there is resistance to meropenem. It was note-worthy to mention that the work of Pasamba *et al.* (2007) observed a discordant carbapenem (highly susceptible to imipinem but resistant to meropenem) susceptibility. Therefore, such observation may be a distinguishing feature of *Methylobacterium* (Zarahatos *et al.*, 2001). Ongbin (2007), on the other hand, determined the Minimum Inhibitory Concentration (MIC) of selected antimicrobials. The results, in most antimicrobials, were inconsistent with the findings of Brown *et al.* (1992). It was further discussed that the variation found yielded may be due to the

difference in culture medium used in the antimicrobial testing. Furthermore, the inconsistency in the results with previously reported data could be explained by strain differences. Brown *et al.* (1992) tested strains derived from clinical specimens, while Ongbin (2007) tested isolates derived from various non-clinical sources.

Genotypic Characterization

The sequencing of the 16S Ribosomal RNA (*16S rRNA*) gene has been widely utilized by researchers in order to trace phylogenetic relationships between bacterial groups or species and to identify bacterial isolates from various sources, such as environmental or clinical specimens (Woese, 1987; Clarridge, 2004). Because of the phenotypic plasticity of *Methylobacterium* for species identification, the use of the 16S *rRNA* has been preferred by the present researchers. The use of *16S rRNA* gene sequence provides accurate, elucidated identification and definition of the difficult bacterial isolates than with phenotypic method (Clarridge, 2004). This section outlines also the methodologies in the extraction and amplification of the 16S rDNA. Primer sequences used in the genotypic characterization are listed in Table 1.

Because of limited resources at the early stages of *Methylobacterium* research, the method utilized for DNA Extraction was the boiling method (Ivanov *et al.*, 1987). However, it yielded very low DNA concentration and it suggested the use the commercial DNA Extraction kits to obtain very high DNA concentration. In amplifying the *16S rRNA* gene, universal primers for Eubacterial species designed by Kawai et Al. (2005) were used. The PCR conditions were optimized to the following conditions using the MJ Research Thermocycler and the Corbette Palm Cycler: initial denaturation at 94°C, then 35 cycles of denaturation at 94°C for 1 minute, annealing temperature at 50°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes. This primer and condition yields an amplicon appriximately 1,300 bp.

Screening genes that are involved in the biological processes undergone by different species of *Methylobacterium* were also done. These are *1-aminocyclopropane-1-Carboxylate Deaminase (ACC) deaminase* (Tan and Grecia, 2009), *miaA* gene (Anyog et. al., 2008; Uy and Uy, 2011; Lee, 2010), *mxaF gene* (Uy and Uy, 2011; Lee, 2010; Johnson *et al.*, 2015), mmoX gene and pmoA gene (Johnson *et al.*, 2015). PCR conditions for *miaA* gene are highlighted by Koenig *et al.* (2002). The primer pair of *miaA gene* and PCR condition yielded an amplicon size of 300bp which corresponds to an internal segment found within the gene. In the study of Lee (2010), nearly 10% of the isolates were positive for the presence of the 300bp amplicon showing the potential of these isolates in producing cytokinin.

Table 1. Primers used for *Methylobacterium* genotypic characterization.

Gene	Forward Primer	Reverse Primer	Expected amplicon size
16S rRNA	5'- GCA CAA GCG GTG GAG CAT GTGG - 3'	5'- GCC CGG GAA CGT ATT CAC CG - 3'	1300 bp
miaA	5'- GAA TTC GTC TAC GCC GAC CT - 3'	3'- GAA TTC GTC GTC CCG TCG AGA T - 5'	300 bp
mxaF	5'- GCG GCA CCA ACT GGG GCT GGT - 3'	3'- GGG CAG CAT GAA GGG CTC CC - 5'	1,800 bp
mmoX	5'- GGC TCC AAG TTC AAG GTC GAG C - 3'	5'-TGG CAC TCG TAG CGC TCC GGC TCG - 3'	450 bp
pmoA	5'- GGN GAC TGG GAC TTC TGG - 3'	5'- GAA SGC NGA GAA GAA SGC - 3'	530 bp

Table 2. Reported and Identified *Methylobacterium* species based on their 16S rDNA sequence in the Philippines.

***Methylobacterium* species**	**Source**
M. zatmaani	Mouth (Carvajal *et al*., 2011)
M. rhodesianum	Scalp (Uy *et al*., 2013)
M. radiotolerans	Human belly button (David and Diongzon, 2013), Ferns (Lee, 2010), Plants (Anyog *et al*., 2008)
M. thiocynatum	Ferns (Lee, 2010), Plants (Anyog *et al*., 2008)
M. lusitanum	Ferns (Lee, 2010), Air (Anyog et al., 2008)
M. komagatae	Ferns (Lee, 2010)
M. aminovorans	Ferns (Lee, 2010)
M. hispanicum	Plants (Anyog *et al*., 2008)
M. populi	Ferns (Lee, 2010), Air (Anyog *et al*., 2008), Soil (Anyog *et al*., 2008)
Methylobacterium sp.	Nasal Cavity (Uy *et al*., 2013), Water (Palines *et al*., 2012), Ferns (Lee, 2010)

On the other hand, Uy and Uy (2011) investigated the presence of *mxaF* gene that encodes for the alpha subunit of the enzyme methanol dehydrogenase (MDH). This enzyme is responsible oxidizing methanol to formaldehyde (McDonald and Murrell, 1997). The subunit is approximately 1.8 kb in size and encodes a 66-kDa polypeptide. The *mxaF* gene has been reported to contain the active site of MDH and to be possibly highly preserved in methylotrophs (McDonald and Murrell, 1997). PCR conditions used were followed from the studies of McDonald and Murrell (1997) and Inagaki *et al.* (2004). Uy and Uy (2011) reported that all of its isolates were positive for the *mxaF* gene. Their results were consistent with previous studies (Anesti *et al.*, 2004; McDonald and Murrell, 1997).

Johnson *et al.*, (2015) amplified the *mxaF*, *mmoX* and *pmoA* genes from soil isolates. The *mmoX* gene codes for the alpha subunit of the hydroxylase of soluble methane monooxygenase enzyme, which constitutes the active site (Ali, 2006); while the *pmoA* gene, on the other hand, is a particulate methane monooxygenase gene which is usually found in methanogens (McDonald, et. al. 1997). PCR conditions were followed from Inagaki *et al.* (2004). Their results revealed the presence of these three genes from the soil isolates, thus, consistent with previous studies of Theisen, et al. (2005) and Horz et al. (2001).

Diversity, Taxonomy and Systematics

To date, there are 51 known *Methylobacterium* species (Green, 2014). Earlier works on its identification were limited based on their colonial, microscopic and selected biochemical tests and assays. The biochemical tests, namely: urease, oxidase and catalase, are known to be diagnostic to genus *Methylobacterium.* Furthermore, discordant carbapenem is a distinguishing feature of the genus apart from its relatives. Because of the limitations with regards to phenotypic tools, the species identification of local isolates have been unknown. However, with the advent of DNA technology, identification was easily achieved by comparing *16S rRNA* from known and validated strains. Table 2 presents the reported and identified *Methylobacterium* species using *16S rRNA* sequencing and their source. Unidentified *Methylobacterium* spp. in some reports were considered to be novel sequences.

Liwanag and Carvajal (2011) studied the phylogeny of 32 *Methylobacterium* type species from Green (2010) and also used different published *Methylobacterium* strains obtained from GENBANK. The results of their study conform to the phylogeny of what Kato *et al.*, (2005) presented. Moreover, the study explored "hypervariable" regions of the 16 rDNA of validated *Methylobacterium* species from Green (2010), however, no noticeable pattern of nucleotides can be discriminated from these microorganisms into their appropriate species.

Plant Association

The earlier works on *Methylobacterium* focused on its potential application in agriculture for increasing crop yield. Most of the reported *Methylobacterium* strains were isolated from leaf surfaces of plants and have been shown to produce phytohormones, such as cytokinins and auxins (Long *et al.*, 1997; Ivanova *et al.* 2000; 2001; Lidstrom and Chistoserdova, 2002). Thus, they can stimulate seed germination and enhance plant growth. Celestino and Leonardo (2002) and Gapuz *et al.* (2005) inoculated *Methylobacterium* to different plants to promote growth rate. Both studies found significant growth rate of plants inoculated with the microorganism compared to the control. Nowadays, investigations focused on elucidating the genetics behind the production of these plant hormones in these bacteria. Two of which are the *miaA gene* and the *1-aminocyclopropane 1 carboxylate (ACC) deaminase* enzyme.

Methylobacterium spp. have the ability to produce cytokinin, hence it may the *miaA* gene, which encodes for the enzyme isopentyltransferase. The mechanism and form of cytokinin produced by *Methylobacterium* are different as observed in plant pathogens such as *Agrobacterium tumefaciens*. Lee *et al.* (2005), Anyog *et al.* (2008) and Lee (2010) investigated positively-screen *miaA* gene *Methylobacterium* isolated into a plant growth assay. Neither root- nor seed-enhanced germination was significantly observed from different test plant organisms. Lee (2010) further discussed that even though *miaA* gene is present in the microorganism, it may not be expressed largely in order for cytokinin activity to happen. A more sensitive assay may be employed to evaluate the cytokinin activity of the *Methylobacterium* bacterial isolates. Certain plant associated methylotrophs, such as *Methylobacterium*, can regulate the ethylene levels in plants by generating *1-aminocyclopropane 1 carboxylate (ACC) deaminase* enzyme. In controlling the release of ethylene in plants, it may influence plant growth and help them cope with environmental stressors such as hypo-osmotic stress and herbivory (Madhaiyan *et al.* 2006; 2007). The study of Tan and Grecia (2009) investigated positively-screened *ACC deaminase Methylobacterium* isolates into a plant growth assay and showed significant root elongation growth. With the advent of high throughout technology, Knief *et al.*, (2012) characterized the microbiota of the phyllosphere and rhizosphere of rice cultivars using the metaproteogenomic approach. Their findings showed that the majority of the proteins for methanol-based methylotrphy microbiota were linked to the genus *Methylobacterium* in the phyllospehere of the rice cultivars.

Human Association

It was thought that PPFM bacteria only exist and thrive in environmental sources, however, there are documented reports that have isolated this microorganism in different parts of the human body such as the

oral cavity (Anesti *et al.*,2005), and feet (Anesti *et al.*, 2004). Furthermore, there has been an increase in published case-reports and studies that implicates this microorganism causing infection (Fanci *et Al.*, 2010; de Cal *et Al.*, 2009; Hogues *et al*., 2008; Abdel-Haq and Asmar, 2008; Anesti *et al.*, 2004 & 2005; Lee *et al.*, 2004; Engler and Norton, 2001; Sanders *et al.*, 2000; Kaye *et al.*, 1992; Gilchrist *et al.* 1986; Gilardi and Faur, 1984). Locally, *Methylobacterium* spp. have been isolated from the feet (Carvajal *et al.*, 2006), oral cavity (Carvajal *et al.*, 2011), nasal cavity, scalp (Uy *et al.*, 2013) and the human navel (David and Diongzon, 2013). The existence of this group of microorganisms in the human body may be considered as either transient microflora or opportunistic pathogens (Green 2001; Anesti *et al.*, 2004 & 2005). No studies yet have reported any possible infection case caused by *Methylobacterium* or determining the relationship of certain diseases to its presence.

Bioremediation

Due to the diverse metabolic features of genus *Methylobacterium*, the ability of some strains to metabolize long chain aliphatic hydrocarbons and thrive in polluted environment, make them potential pollution indicators (Green, 2001). It has been demonstrated that genus *Methylobacterium* is part of the consortia of microorganisms involved in the degradation of mineral oil in soil (Popp *et al.*, 2006; Siciliano *et al.*, 2001). Johnson *et al.*, (2015) investigated this metabolic feature by using the crude oil degradation assay. Isolates were screened positive for *mxaF*, *mmoX* and *pmoA* genes and it was shown that this microorganism was able to utilize crude oil after 21 days of incubation. The findings support previous studies wherein *Methylobacterium populum* showed petroleum-degradation in petroleum-contaminated soils (Siciliano *et al*., 2001).

Future Directions of *Methylobacterium* Research in the Philippines

In the past decade, studies in the Philippines have concentrated mostly in the immense diversity of *Methylobacterium* from different sources and its characterization. Although there is rich information of its diversity, there are novel species in our local environment waiting to be explored and identified. Furthermore, Lee (2010) and Carvajal (2011, 2012, 2013) recommends to use current methodologies to further discriminate and identify *Methylobacterium* species by employing Multi-Locus Sequence Type (MLST) analysis or Genome-wide analysis using Next Generation Sequencing technology.

Other research avenues and opportunities can focus in the areas of plant association and biotechnology. There have been preliminary studies done

to assess the association of plants and the microorganism for plant growth with the corresponding genes involved.

The present researchers suggest that a more thorough investigation in identifying the different metabolic pathways. This involves the combination of the microorganism's physiology and genetics that produces plant hormones. This is monumental in the agricultural setting if the characteristics of *Methylobacterium* are utilized as a biological agent to increase crop growth and yield other than fertilizers. Current molecular DNA technology approaches namely functional genomics, transcriptomics, proteomics, metabolomics may provide a wider and better understanding of the interaction between microorganism and plants. Bioremediation potential of *Methylobacterium* can be highlighted in their carotenoid pigments and sudanophilic cysts. Lee (2007) had pointed out the potential of carotenoid pigments produced by these microorganisms as colorant in food industries (Green, 2001). Moreover, sudanophilic cysts in *Methylobacterium* are suggested to be stores of poly-β-hydroxybutyrate, a raw material for the synthesis of biodegradable plastics (Yezza, 2007). Further work is needed to determine the poly-β-hydroxybutyrate's productivity of the microorganism in different biomass substrates.

ACKNOWLEDGEMENTS

The authors would like to extend their sincere gratitude to their former students who worked on this organism and also, more importantly, to the former students of the late Dr. Anthony Lee for the sheer hard work and dedication on studying this microorganism. Also the Biology Department of De La Salle University which supported the studies conducted on *Methylobacterium* by providing the laboratory facilities, materials and reagents. We would also like to acknowledge Mr. Glenn Oyong for the molecular aspects of the different studies, Mr. Ramonito Oroceo, Mr. Giovanni Perez and Ms. Roni Cuasay for technical assistance in the laboratory and Dr. Esperanza Cabrera for her unwavering support and guidance to the present researchers and former students who worked on this microorganism.

LITERATURE CITED

Abdel-Haq N.M and B.I. Asmar. (2008). *Methylobacterium mesophilicum* cellulitis and lymphangitis following a dog bite. Journal of Pediatric Infectious Diseases Vol. 3(4):279-28.

Amparado K.M.F. and A.C. Lee. (2010). Presence of Facultative Methylotrophic Bacteria in Shrimp Paste Bagoong Alamang. Proceedings of the Osaka University-De La Salle University Academic Workshops, 15:

Anesti, V., McDonald, I., Ramaswamy, M., Wade, W., Kelly, D., and Wood, A. (2005). Isolation and molecular detection of methylotrophic bacteria occurring in the human mouth. Environmental Microbiology, 7: 1227–1238.

Anesti, V., Vohra, J., Goonetilleka, S., McDonald, I.R., Straubler B., Stackebrandt, E., Kelly, D.P., and A.P. Wood. (2004). Molecular detection and isolation of facultatively methylotrophic bacteria, including Methylobacterium podarium sp. nov., from the human foot microflora. Environmental Microbiology. 6:820.

Anyog, M.T.E., Bautista, G.C.D., Ilustre, A.K.P., and Mativo, C.A.(2008). Characterization of selected strains of pink pigmented facultative methylotrophic (PPFM) bacteria deposited at the DLSU-Manila Microbiology laboratory (Undergraduate Thesis). De La Salle University-Manila.

Brown, W., Sautter, R., and Crist, A Jr. (1992). Susceptibility Testing of Clinical Isolates of Methylobacterium Species. Antimicrobial Agents and Chemotherapy 36(8): 1635-1638.

Carvajal T.M., Tan R.L. and A.C. Lee. (2011). Pink Pigmented Facultative Methylotrophic (PPFM) bacteria from the Human oral cavity. Philippine Journal of Systematic Biology Vol. 5: 1-9.

Carvajal, T.M., Dolleton, M.A.M., Estrella, D.S., Gagui O.A., Peralta E.C., Valera M.C.D.,and A.C. Lee. (2006). Phenotypic characterization of Pink Pigmented Facultative Methylothrophic (PPFM) bacteria from Various Sources. Proceedings of the Osaka University-De La Salle University Academic Workshops, 7:5-7.

Celestino J. and J. Leonardo.(2002). Assessment Of Growth Rate Of Mungbean (*Phaseolus Radiatus*) Cultivated With Pink-Pigmented Facultative Methylotrophic (PPFM) Bacterial Isolates (Undergraduate Thesis). De La Salle University – Manila

Chanama, S. (n. d.). Comparative 16S rRNA sequence analysis. URL: http://www.cph.chula.ac.th/J%20Health%20Res/files/FullText/.../Suchat_107.pdf. Retrieved: January 2010.

Clarridge, J. E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews*, 17 (4), 840-862.

David A.K.S. and N.E.M. Diongzon. (2013). Phenotypic and genotypic characterization of pink pigmented facultative Methylotrophic (PPFM)

bacteria isolated from the human belly button (Undergraduate Thesis). De La Salle University – Manila.

De Cal M, Cazzavillan S, Cruz D, Nalesso F, Brendolan A, Rassu M, Ronco C.(2009). Methylobacterium radiotolerans bacteremia in hemodialysis patients. G Ital Nefrol. 5: 616-20.

De Guzman M. and J. Pamaong. (2008). Generation Time of *Methylobacterium extroquens* UPLB Strain Grown In Selected Commercial Media (Undergraduate Thesis). De La Salle University – Manila

De la Cruz, I. (2007). Isolation and Characterization of Pink Pigmented Facultative Methylotrophic Bacteria from polluted water Leveriza creek (Undergraduate Thesis). De La Salle University – Manila.

Dizon R. and V. Esquivel.(2002). Isolation and Characterization of Pink-Pigmented Facultative Methylotrophic (PPFM) Bacteria From Selected Phylospheres (Undergraduate Thesis). De La Salle University – Manila.

Engler C. and R. Norton. (2001). Recurrent Methylobacterium Mesophilicum Sepsis Associated With Haemodialysis. Pathology Vol. 33(4): 536-537.

Fanci, R., Corti, G., Bartoloni, A., Tortoli, E., Mariottini, A., and Pecile, P. (2010). Unusual *Methylobacterium fujisawaense* infection in a patient with acute leukaemia undergoing hematopoietic stem cell transplantation: first case report. *Case Reports in Medicine*, 2010: 1-3.

Gapuz, K.T., Mending, F.D., Mendoza, A.K., Que, E.C. and Tan, M.S. (2005). Bioactivities of pink pigmented facultative methylotrophic bacteria isolated from *Coleus amboinicus*, *Muntingia calabura, Pilea cadierei, Pseuderanthemum reticulatum*, and *Swietenia macrophylla* (Undergraduate Thesis). De La Salle University – Manila.

Garrity GM., Bell JA. And T. Lilburn. (2005). Genus I. *Methylobacterium*: In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2 (*The Proteobacteria*), Part C (The Alpha-, Beta-, Delta-, and Epsilonproteobacteria. pp. 567–571. Edited by D. J. Brenner, N. R. Krieg, J. T. Staley & G. M. Garrity. New York: Springer.

Gilardi G.L. and Y.C. Faur. (1984). Pseudomonas mesophilica and an unnamed taxon, clinical isolates of pink-pigmented oxidative bacteria. J Clin Microbiol 20: 626–629.

Gilchrist MJR, Kraft JA, Hammond JG, Connelly BL, and MG Myers.(1986). Detection of Pseudomonas mesophilica as a source of nosocomial

infections in a bone marrow transplant unit. J Clin Microbiol 23: 1052–1055.

Green P.N.(2014). List of Prokaryotic Names with Standing in Nomenclature. http://www.bacterio.net/methylobacterium.html. Accessed on November 15, 2014.

Green, P.N. (2001). The genus Methylobacterium, *In* The Prokaryotes ed. by M. Dwokin. Release 3.5, 1994-2004, Springer –Verlag, New York, LLC. Available at: Hiraishi, A., Furuhata, K., Matsumoto, A., Koike, K. A., Fukuyama, M., & Tabuchi, K. (1995). Phenotypic and genetic diversity of chlorine-resistant *Methylobacterium* strains isolated from various environments. *Applied and Environmental Microbiology*, 61, 2099-2107.

Hogue R., Graves M., Moler S., and J.M. Janda. (2007). Pink-pigmented Non-fermentative Gram-negative Rods Associated with Human Infections: a Clinical and Diagnostic Challenge. Infection 35: 126 – 133.

Inagaki, F., Tsunogai, U., Suzuki, M., Kosaka, A., Machiyama, H., Takai, K.,…Horikoshi, K. (2004). Characterization of C1 – metabolizing prokaryotic communities in methane seep habitats at the Kuroshima Knoll, Southern Ryukyu Arc, by analyzing pmoA, mmoX, mxaF, mcrA, and 16S rRNA genes. Applied and Environmental Micobiology, 70(12), 7445-7455.

Ivanova, E. G., N. V. Doronina, A. O. Shepeliakovskaia, A. G. Laman, F. A. Brovko, and Yu. A. Trotsenko 2000. Facultative and obligate aerobic methylobacteria synthesize cytokinins. Mikrobiologiya69**:**764-769.

Ivanova, E. G., N. V. Doronina, and Y. A. Trotsenko. 2001. Aerobic methylobacteria are capable of synthesizing auxins. Microbiology 70**:**392-397.

Jang, S.B. and A.C. Lee (2008). Phenotypic characterization of pink-pigmented facultative methylotrophic bacteria from soil exposed to vehicular soot. Philippine Journal of Systematic Biology Vol. II. 1: 32-39.

Johnson S.L., Sosa G.A., and Galvez J.B. (2015). Detection of *mxaF*, *mmoX*, *pmoA*,and *mcrA* genes in Methylotrophic bacteria Isolated from Miriam College and Screening for Crude Oil Degradation and Heavy Metal Resistance (Undergraduate Thesis). Miriam College, Quezon City, Philippines.

Kato, Y., Asahara, M., Arai, D., Goto, K., and Yokota, A. (2005). Methylobacterium dichloromethanicum as later subjective synonyms of

Methylobacterium extorquens and of Methylobacterium lusitanum as a later subjective synonym of Methylobacterium rhodesianum. Journal of General and Applied Microbiology 51, 287-299.

Kawai M, Matsutera E, Kanda H, Yamaguchi N, Tani K, Nasu M. 2002. 16S ribosomal DNA-based analysis of bacterial diversity in purified water used in pharmaceutical manufacturing processes by PCR and denaturing gradient gel electrophoresis. Appl Environ Microbiol. ;68:699–704

Kaye KM, Macone A, and P.H. Kazanjian.(1992).Catheter infection caused by Methylobacterium in immunocompromised host: report of three cases and review of the literature. Clin Infect Dis 14: 1010–1014.

Knief, C., Delmotte, N., Chaffron, S., Stark, M., Innerebner, G., Wassmann, R., Mering C.V. & Vorholt, J. A. (2012). Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. The ISME journal, Vol. 6 (7), 1378-1390.

Koenig, R. L., Morris, R. O., & Polacco, J. C. (2002). tRNA is the source of low-level trans-zeatin production in *Methylobacterium* spp. *Journal of Bacteriology*, 184 (7), 1832-1842.

Kumar R. and A.C. Lee. (2009). Isolation and characterization of Pink Pigmented, Facultative Methylotrophic (PPFM) bacteria from leaves of Neem, *Azadirachta indica* a. Juss. Philippine Journal of Systematic Biology 3(1):8-16.

Kuykendall DL, Young JM, Martinez-Romero E., Kerr A. and H. Sawada. 2005. Genus I. *Rhizobium*: In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2 (*The Proteobacteria*), Part C (The Alpha-, Beta-, Delta-, and Epsilonproteobacteria. pp. 325–339. Edited by D. J. Brenner, N. R. Krieg, J. T. Staley & G. M. Garrity. New York: Springer.

Kwong, K. M., Garcia G.M., and J.C. Zarsuelo. (2011). Isolation and characterization of pink-pigmented facultative methylotrophic bacteria from vehicular exhaust pipes (Undergraduate Thesis). Xavier University-Ateneo de Cagayan, Cagayan de Oro City

Lee A.C. (2010). Phenotypic and genotypic diversity of pink pigmented facultative methylotrophic bacteria from selected pteridophytes within De La Salle University-Manila Campus (Dissertation Thesis). De La Salle University – Manila

Lee, A.C. (2007). Pink Pigmented Facultative Methylotrophic Bacteria: Common Yet Unexplored Locally. Philippine Journal of Systematic Biology 1(1):61-72.

Lee, A.C., V.V.C. Esquivel and R.M. C. Dizon. (2005). Potential of Pink Bacteria in Biotechnology: Preliminary Assessment of Cytokinin Activity of Local Isolates of Pink Pigmented Facultative Methylotrophic Bacteria. Proceedings of the Osaka University- De La Salle University Science Research Workshops, 5:52-54.

Lee, C., Y. Tang, and J. Liu. (2004). Underdiagnosis of urinary tract infection caused by Methylobacterium species with current standard processing of urine culture and its clinical implications. J. Clin. Microbiol. 53: 755–759.

Lidstrom, M.E. and L. Chistoserdova. 2002. Plants in Pink: Cytokinin production by Methylobacterium. Journal of Bacteriology, 184(7): 1818.

Liwanag, A.J.M. and T.M. Carvajal. (2011). A Phylogenetic Analysis of Validated Bacterial Species of Genus *Methylobacterium* Using 16S rRna sequences deposited in the GENBANK. Poster presented at the 40th PSM Annual Convention and Scientific Conference and 6th Asia Pacific Biotechnology Congress on May 10 – 14, 2011 Philippine International Convention Center, Pasay City Philippines

Lo, J.M. and A. C. Lee. (2007). Phenotypic Characterization of Air-Borne Pink Pigmented Facultative Methylotrophic Bacteria from a High Vehicular Traffic Density Environment In Manila, Philippines. Philippine Scientist 44:25-34.

Long, R., Morris, R., and Polacco, J., Cytokinin Production by Plant-associated Methylotrophic Bacteria, Am. Soc. Plant Physiol., 1997, Abstr. no. 1168.

McDonald, I. & Murrell, J. (1997). The methanol dehydrogenase structural gene mxaF and its use as a functional gene probe for methanotrophs and methylotrophs. Applied and Environmental Microbiology, 63(8), 3218-3224.

McDonald, I. R., Doronina, N. V., Trotsenko, Y. A., McAnulla, C., & Murrell, J. C. (2001). *Hyphomicrobium chloromethanicum* sp. nov. and *Methylobacterium chloromethanicum* sp. nov., chloromethane-utilizing bacteria isolated from a polluted environment. *International Journal of Systematic and Evolutionary Microbiology*, 51, 119-122.

Ongbin T. (2008). Susceptibility Testing Of Pink-Pigmented Facultative Methylotrophic (PPFM) Bacterial Isolates To Selected Antimicrobials (Undergraduate Thesis). De La Salle University – Manila.

Palines J.A.V., Teves J.C.P., Carvajal T.M., and A.C. Lee.(2012).Pink Pigmented Facultative Methylotrophic (PPFM) Bacteria Isolated From Water Samples In Taal Lake, Talisay, Batangas. The Journal of Tropical Biology 10: 3-7.

Pasamba, E., R. Demegillo and A.C. Lee. (2007). Antibiograms of Pink Pigmented Facultative Methylotrophic Bacterial Isolates from Various Sources. Philippine Scientist 44:47-56

Popp, N., Schlömann, Mau, M. (2006). Bacterial diversity in the active stage of a bioremediation

Sanders, J. W., J. W. Martin, M. Hooke, and J. Hooke. (2000). *Methylobacterium mesophilicum* infection: case report and literature review of an unusual opportunistic pathogen. Clin. Infect. Dis. 30:936–938.

Tan J. and S. Grecia. (2009). Polymerase Chain Reaction Based Detection of 1-Aminocyclopropane-1-Carboxylate Deaminase Gene In Selected Strains Of Pink Pigmented Facultative Methylotrophic Bacteria (Undergraduate Thesis). De La Salle University Manila.

Theisen, A., & Murrell, J. (2005). Facultative methanotrophs revisited. Journal of Bacteriology, 187(13), 4303-4305. doi:10.1128/jb.187.13.4303-4305.2005.

Uy M.M. and J. Uy. (2009). Characterization and identification of pink-pigmented facultative methylotrophic bacteria from human scalp and nasal cavity (Undergraduate Thesis). De La Salle University Manila

Uy M.M., Uy J., Castro C.Z.R., Carvajal T.M., Ho H.T. and A.C. Lee. (2013). Pink Pigmented Facultative Methylotrophic (PPFM) Bacteria Isolated From The Human Scalp And Nasal Cavity. Philippine Journal of Systematic Biology.

Weyant, R.S., C.W. Moss, R.E. Weaver, D.G. Hollis, J.G. Jordan, E.C. Cook, and M.I. Denashvar. (1996). Identification of Unusual Pathogenic Gram-Negative Aerobic and Facultatively Anaerobic Bacteria,2nd ed. Center for Disease Control, Williams & Wilkins. pp. 388-389.

Woese, C. R. (1987). Bacterial evolution. *Microbiological Reviews*, 51, 221-271.

Yezza, A., Halasz, A., Levadoux, W., Hawari, J., (2007). Production of poly--hydroxybutyrate (PHB) by Alcaligenes latus from maple sap. Appl. Microbiol. Biotechnol. 77, 269–274.

Zaharatos GJ, Dascal A, Miller MA. (2001). Discordant carbapenem susceptibil-ity in Methylobacterium species and its application as a method for phenotypic identification. J Clin Microbiol 39: 2037–2038.