Methylobacterium zatmanii, A PINK PIGMENTED FACULTATIVE METHYLOTROPHIC (PPFM) BACTERIUM ISOLATED FROM THE HUMAN ORAL CAVITY

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ABSTRACT

A pink pigmented facultative methylotrophic (PPFM) bacterial isolate (P1UM) was obtained from the oral cavity of a male patient diagnosed with periodontitis and dental caries. The bacterial isolate exhibited glistening, smooth, circular, pink colored colonies in minimal medium supplemented with 0.5% methanol. Microscopic morphological examination showed that the isolate is a Gram-negative rod-shaped bacterium with polyß-hydroxybutyrate (PHB) granules. Phylogenetic analysis using its16S rDNA sequence revealed that the isolate is closely related to *Methylobacterium zatmanii*.

INTRODUCTION

The human oral cavity is a complex biological system where diverse microbial flora interact with each other as well as with the host structures (Zambon, 1985). Several reports (Kroes et al., 1999; Paster et al., 2001; Actis et al., 2003) revealed the there are approximately 500 bacterial species that colonize the human oral cavity, but most of them are still poorly characterized. One interesting group of bacteria residing the human oral cavity are the methylotrophs as demonstrated by Anesti et al. (2005). Volatile compounds are generated in the oral cavity such as methanethiol and dimethylsulfide which can be tapped by this bacterial group as carbon and energy sources. A subset of these methylotrophs is the pink pigmented facultative methylotrophic (PPFM) bacteria. These bacteria generate reddish to pinkish colonies when grown in a minimal medium supplemented with one-carbon organic compound.

PPFM bacteria are ubiquitous in nature as they were isolated from various sources (Lee, 2007). They were reported to be isolated from plants, soil, water and air (Jourand et al., 2004, Gallego et al. 2005, De Marco et al., 2004, Lo and Lee 2007; Weon et al. 2008). Moreover, Anesti et al. (2004,

2005) demonstrated the presence of these bacteria as part of the normal human microbiota. Nonetheless, they were also isolated in human clinical specimens such as blood from AIDS patient (Gilardi and Faur, 1984; Gilchrist et al. 1986) and urinary tract infections among immunocompetent patients (Lee et al., 2004). Hence, these can be considered as opportunistic pathogens.

This study demonstrated the presence of PPFM bacterium from the upper molars of a male patient diagnosed with periodontitis and dental caries. Moreover, the PPFM isolate was identified using phenotypic and genotypic characteristics. The presence of this bacterial group may influence the oral health of an individual. More studies however, may need to be done to prove such contention.

MATERIALS AND METHODS

Isolation of PPFM samples from the Upper Molars

Samples were taken by swabbing with sterile cotton the upper molars of fifteen patients with dental caries and periodontitis as diagnosed by a certified doctor of dentistry. Oral swabs were streaked on Mineral Salt Medium supplemented with 0.5% methanol. Inoculated plates were incubated for one (1) week at 37°C. Pink colonies that developed on the plates were picked and re-streaked in non-selective medium until pure isolates were obtained.

Phenotypic Characterization

Colonial morphology of the isolate was described after growing in minimal medium with 0.5% methanol for one week at 37°C. Microscopic morphology was described after Gram-staining and polyß-hydroxybutyrate (PHB) staining (Weyant et al.,1996). Oxidase, catalase and urease tests (Benson, 2002) were performed on the isolate. Additional biochemical tests using the API 20 NE ID system (BioMérieux, Marcy-I'Etoile, France) was done.

Genotypic Characterization - 16S rDNA sequence analysis

DNA extraction was done by boiling method (Ivanov et al., 1987). Two to three colonies were suspended in TE buffer and subjected to centrifugation at 1000 rpm for 3 minutes at 4°C. The suspension was then boiled at 100 °C for 10 minutes. The crude DNA extract was used as template for the PCR amplification of the 16Sr RNA gene. PCR was performed using the PTC-100 Peltier Thermal Cycler with the following forward 5'CAG GCC TAA CAC ATG CAA GTC 3' and reverse primers 3'GGG CGG WGT GTA CAA GGC 5'. The primers used and PCR condition were based on Marchelis et al. (1998). The amplicon has an approximate size of 1,300 bp. The amplicons were sent to First Base Pte. Ltd in Malaysia for sequencing.

Identification of PPFM bacterial isolate

The obtained phenotypic characteristics of the isolate P1UM were compared with the published characteristics of PPFM bacterial species described in the Bergy's Manual of Determinative Bacteriology (Bergy and Holt, 2000), as well as with in the studies of Anesti et al. (2004) and Kato et al. (2005). Moreover, the sequence was subjected to Nucleotide Basic Local Alignment Search Tool (BLAST). Phylogenetic analysis included *Rhodopseudomonas palustris* DSM 123 as an outgroup as this belong to the same division with *Methylobacterium* (Alpha-proteobacterial group) (Kato et al.,2005). Methods for phylogenetic tree construction found in Molecular Evolutionary Genetic Analysis (MEGA) software version 4 (Tamura et al., 2007) were employed. The obtained sequence was deposited to GenBank with accession number EU855844.

RESULTS AND DISCUSSION

One (1) out of the fifteen patients surveyed demonstrated the presence of PPFM bacteria. The isolate was designated as P1-UM. This observation was consistent with the report of Anesti et al. (2005) that methylotrophic bacteria can thrive in the human mouth. The presence of methylotrophic bacteria may be due to the methylated compounds produced in the human cavity, which can be used by the microorganism as carbon and energy source.

The colonial morphology of the isolate was distinctly pink, small-sized (approximately 1 mm in diameter) round, raised, and opaque with entire margin when cultivated in minimal salt medium with 0.5% methanol (Figure 1a). Gram staining revealed that the isolate is a Gram-negative bacilli with some vacuolated structures within their cells (Figure 1b). The bacterial cells was measured approximately 1.0 um long by 0.5 um wide. Moreover, the cells of the isolate contain sudanophilic granules as shown after PHB staining. The result of the biochemical tests showed that the isolate is urease-, catalase-, oxidase- and nitrate reductase-positive. According to Green (2001), these biochemical features conform to the characteristics of the genus *Methylobacterium*. Isolate P1UM was also able to assimilate D-glucose, L-arabinose, D-mannose, D- mannitol, L-arginine, adipic acid, potassium gluconate, and malic acid (Table 1). The percent similarities of isolate P1UM in terms of the phenotypic characteristics considered in this study with the reported type strains of *Methylobacterium* range from 38% to 77%. These low percent similarities suggest that the isolate is phenotypically distinct from strains reported in previous studies (Anesti et al. 2005; Kato et al., 2005). This observation reflects the phenotypic diversity of genus *Methylobacterium* as discussed by Kato et al., (2005) and Green (2001). This underscores that biochemical features may not be sufficient as bases for definitive species determination.

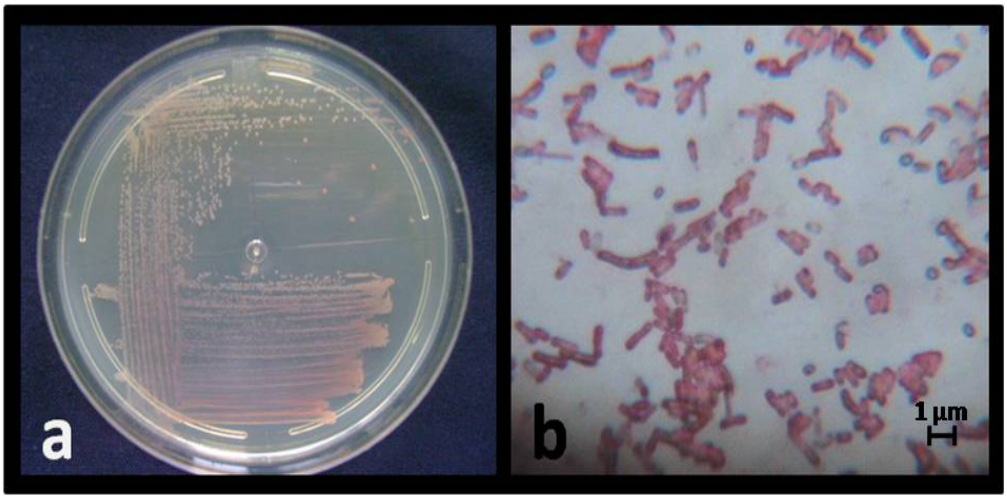

Figure 1. (a) Colonial morphology of the isolate after growing in minimal medium with 0.5% methanol at 37°C for one week and **(b)** Microscopic morphology of the isolate P1-UM after Gram staining

Genotypic characterization using 16S rDNA analysis revealed that the isolate showed 99.0% homology to the reported sequences of *Methylobacterium zatmanii* in the NCBI database. Phylogenetic analysis also revealed that isolate P1-UM is closely related to the type specimen *M. zatmanii* DSM5688 (AB175647) (Figure 2). Pair-wise analysis of the sequence of the isolate showed that there is a 99.7% similarity not only with *M. zatmanii* but also with *M. extorquens* and *M. chloromethanicum.* This could account for the observed low bootstrap value between the isolate and the type strain, *M. zatmanii* DSM5688 (AB175647). Due to the 16S rDNA sequence similarity among *M. extorquens, M. chloromethanicum* and *M. zatmanii*, Kato et al. (2005) proposed that these species may be lumped into one cluster or species complex. Hence, other genotypic methods such as DNA-DNA hybridization and Multi-locus Sequence Typing (MLST) may be performed to yield conclusive species identification.

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Table 1. Comparison of P1-UM from 15 Methlyobacterium isolates from the characterization based on the Bergy's Manual of Determinative Bacteriology (2000), Anesti et al. (2004) and Kato et al (2005).

CHARACTERISTICS	P1-UM	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+	W	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	W	-	-	-	-	-	-	+	-	W	-	-	-	+	-
Assimilation of:																
Malic Acid	+	+	+	+	+	+	-	+	+	+	-	+	-	+	+	+
Glucose	+	-	-	-	W	+	W	W	-	+	-	W	+	W	+	+
Arabinose	+	-	-	-	W	-	-	-	-	-	-	-	+	+	+	+
D-mannose	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	W
Fructose	-	+	+	+	+	-	+	-	-	+	+	+	-	+	-	-
Xylose	-	-	-	+	-	-	-	-	-	+	-	-	+	-	+	+
Potassium gluconate	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
Adipic Acid	+	-	-	-	-	-	-	-	-	-	-	-	-	W	+	+
Citrate	-	-	-	-	-	-	-	-	-	-	+	-	W	+	+	+
% Similarity	100	54	46	38	62	62	46	62	62	46	38	54	54	69	77	69

1 M. lusitanum; 2 M. rhodesianum; 3 M. thiocynatum; 4 M. zatmaani; 5 M. chloromethanicum; 6 M. dichloromethanicum; 7 M. extorquens; 8 M. aminovorans; 9 M. rhodinum; 10 M. suomiense; 11 M. organiphilum; 12 M. mesophilicum; 13 M. organiphilum 2; 14 M. radiotolerans; 15 M. Fujiwaense

Note: (+) positive; (-) negative; (W) weakly positive; and (nd) not determined

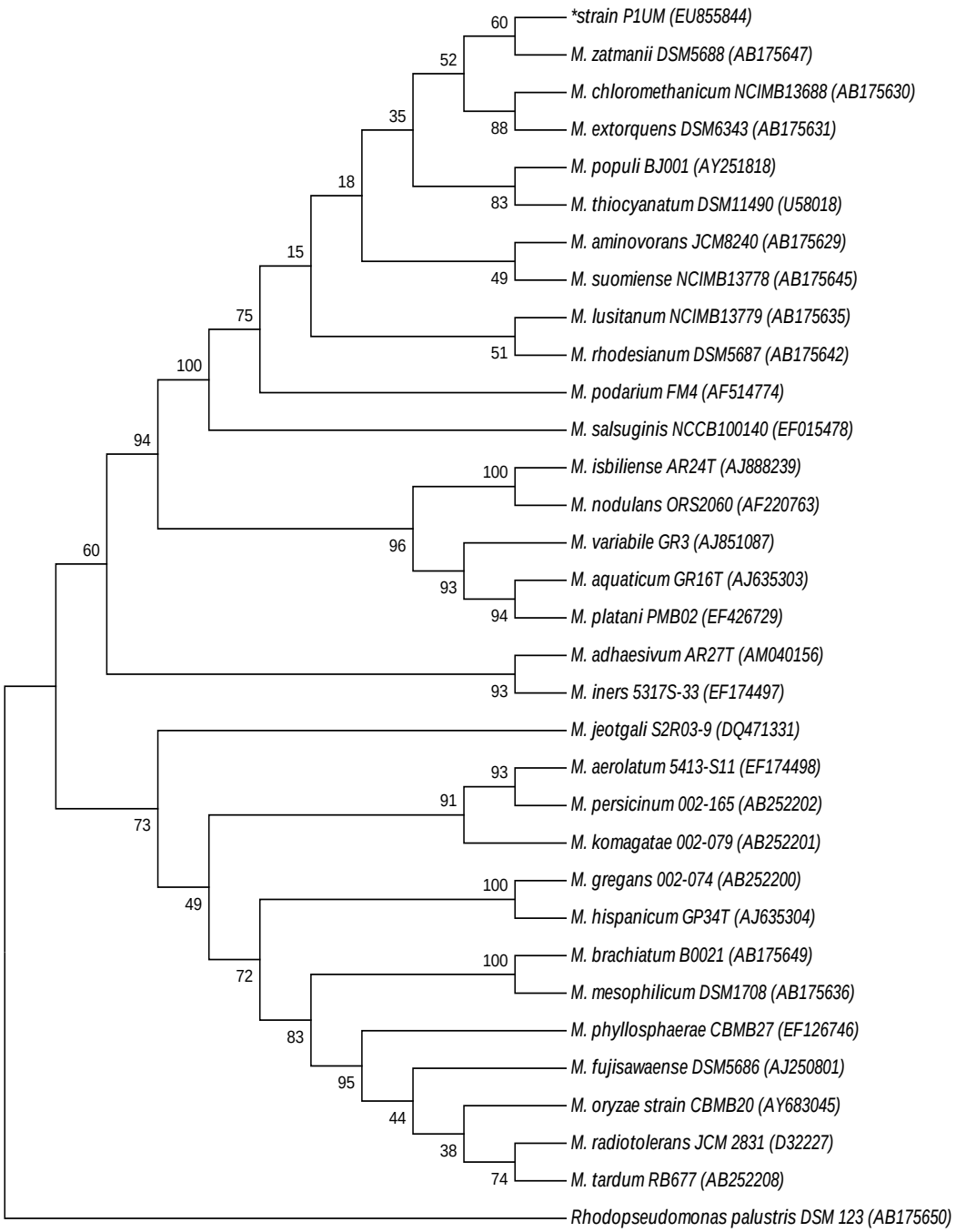

Figure 2. Phylogenetic tree showing the relationship of isolate P1-UM with 32 valid species and type strains of *Methylobacterium* using Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates. There were a total of 1176 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

This paper demonstrated the presence of *M.zatmanii* in the human oral cavity. This adds to the list of PPFM bacterial species isolated as reported by Anesti et al. (2005). There have been several case-reports (Hornei et al., 1999; Rice et al., 2000) of *M. zatmanii* isolated in clinical specimens. Nonetheless, these studies did not establish the role of these bacteria in disease causation. Likewise, this present work only demonstrated the presence of PPFM isolate from the oral cavity of a patient from dental caries and periodontitis. However, the influence of this bacterium on oral health still needs to be investigated.

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