

Genetic Diversity of Methylophilic Bacteria from Human Mouth Based on Amplified Ribosomal DNA Restriction Analysis (ARDRA)

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Methylophilic bacteria inhabit the human mouth. In this study, methylophilic bacteria were isolated from the human mouth microflora of 63 subjects, especially from the tongue, gingival, and subgingival area using minimal agar supplemented with 1% methanol. The obtained isolates were subjected to biochemical assays, continued with antibiotics susceptibility testing using ampicillin (10 µg), tetracycline (20 µg), kanamycin (30 µg), trimethoprim (5 µg), and streptomycin (10 µg). Genetic diversity was analyzed using ARDRA method. Isolates varying in morphology characteristics were amplified for 16S rRNA gene and continued with DNA sequencing. As many as 21 methylophilic bacterial isolates were purified and divided into seven groups with different phenotypic profiles. A majority of the isolates were resistant to trimethoprim but sensitive to kanamycin, streptomycin, and tetracycline. Resistance to ampicillin was variable in each isolate. ARDRA showed nine different digestion profiles. DNA sequencing analysis of the 16S rRNA gene showed that six isolates with different phenotypic and digestion profiles were closely related to *Methylobacterium radiotoleran* (94%), *Microbacterium esteraromaticum* (99%), *Pseudomonas* sp. (100%), and three of them were exhibited 99, 99, and 98% sequence similarity with *Gordonia* sp., respectively. The results of this study revealed diversity among methylophilic bacteria particularly in human mouth.

Key words: methylophilic bacteria, molecular characterization, restriction profile

INTRODUCTION

Human mouth is a habitat which is likely to be colonized by bacteria. More than 500 species of bacteria lived in human mouth (Foster *et al.* 2003). Those bacteria form complex interaction among them and also interact with host tissue. The oral bacteria such as streptococci, lactobacilli, staphylococci, corynebacteria, and anaerobic bacteria, bacteroides usually called as normal mouth microbiota (Roger 2008).

Methylophilic bacteria are a group of bacteria which is also found in human mouth (Anesti *et al.* 2005). Methylophilic bacteria are able to use one carbon compounds such as methane, methanol, halogenated methane, and methylated amine as sources of carbon and energy for their growth (Nishio *et al.* 1997). The availability of one carbon compounds in human mouth, including methylated sulphides (such as methanethiol and dimethylsulphide) supports the growth of methylophilic bacteria. These compounds, together with hydrogen sulphide, are product of microbial degradation of free sulphur amino acids, thiol and disulphide groups which are released from proteins (Anesti *et al.* 2005). Volatile sulphur compound (VSC) in oral cavity caused the production of halitosis or bad breath (Ratcliff 1999).

Methylophilic bacteria isolated from human mouth are suggested to have ability in reducing the odour of

human mouth since it can oxidize some volatile sulphur compounds such as dimethylsulphide and methyl mercaptan (Anesti *et al.* 2005; Brownlee 2005).

Methylophilic bacteria produce some enzymes that involved in the metabolism of specific one carbon compound, such as methane monooxygenase (MMO), methanol dehydrogenase (MDH), methylamine dehydrogenase, methyltransferase, methanesulphonic acid monooxygenase (Chistoserdova *et al.* 2005), and dimethylsulphide oxygenase (Anesti *et al.* 2005). Those enzymes play important role in the oxidation of C1 compounds to formaldehyde which is further be assimilated.

The presence of methylophilic bacteria in human mouth is diverse. It is important to investigate the diversity of methylophilic bacteria in human mouth since it can provide more information about various methylophilic bacteria lived in human mouth. Thus, it helps to study the strength and the weakness of candidate bacteria that potential to be applied in many sectors.

In this study, we analyzed the genetic diversity of methylophilic bacteria from human mouth based on Amplified Ribosomal DNA Restriction Analysis (ARDRA). The isolates were also identified and tested for their susceptibility to some antibiotics.

MATERIALS AND METHODS

Isolation and Characterization of Methylophilic Bacteria from Human Mouth with Biochemical Assay.

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Samples were obtained from subgingivalis area (S), gingivalis area (G), and tongue (L). Samples were collected by cotton swabs and suspended into 2 ml of sterile 0.85% sodium chloride solution, for gingivalis and tongue and with sterile toothpick for subgingivalis. The samples were inoculated in minimal medium supplemented with 1% (v/v) methanol and incubated at 28 °C for approximately one week. Colonies of bacteria were then isolated. Catalase test and oxidase test were used to identify the isolates. Gram staining was also performed to differentiate the isolates.

Antibiotics Susceptibility Testing. Antibiotics susceptibility test was carried out by the agar disk diffusion method (Bauer *et al.* 1996). The following disks were used: ampicillin (10 µg), tetracycline (30 µg), kanamycin (30 µg), trimethoprim (5 µg), and streptomycin (10 µg). The diameters of zones of inhibition were measured to the nearest millimeter. Isolates were categorized as susceptible, intermediate and resistant based on interpretive criteria developed by the National Committee for Clinical Laboratory Standards (NCCLS, 1999).

Amplification of 16s rRNA Gene by PCR. The isolates are enriched in Luria Agar medium at 28 °C for 2 days. Genomic DNA was extracted using the CTAB method and amplified by PCR with primers 63f (52-CAGGCCTA ACACATGCAAGTC-32) and 1387r (52-GGGCGGWTGT ACAAGGC-32, W=A or T) (Marchesi *et al.* 1998). The reaction mixture contained 2.5 µl enzyme buffer, 0.5 µl dNTP, 1 µl 25 pmol primer 63f, 1 µl 25 pmol primer 1387r, 0.5 µl (2.5 U) Taq polymerase (New England Biolabs, Mass., USA), and 1 µl DNA sample. PCR amplification was performed in a total volume of 25 µl using GeneAmp® PCR system 2400 (Perkin Elmer, USA). The PCR conditions were as follows: initial denaturation for 5 minutes at 94 °C and 25 cycles consisting of denaturation at 94 °C for 30 seconds, primer annealing at 55 °C for 30 seconds, and

elongation at 72 °C for 1 minute. The PCR product was checked by electrophoresis (Mini-Sub Cell GT, Bio-Rad, Richmond, CA) on a 0.8% agarose gel at 80 V for 1 hour.

Genetic Diversity Analysis with ARDRA. PCR products of the isolates were digested with restriction enzyme *Sau3AI* (New England Biolabs, Mass., USA). The digestions were carried out in a total volume of 20 µl for 24 hours at 37 °C. The mixture contained 3 µl PCR product, 2 µl of 10× restriction buffer, 14.5 µl ddH₂O and 0.5 µl unit restriction enzyme *Sau3AI* (New England Biolabs, Mass., USA). Digestion of 16s rRNA gene *Escherichia coli* DH5α was also done to compare with other isolates. The digested amplicon was visualized by electrophoresis on a 2% agarose gel at 45 V for 2 hours. Treecon computer program (Yves-Van de Peer of Department of Biochemistry, www.informatik.uni-erlangen.de) was employed to determine the phylogenetic tree based on the restriction fragment pattern.

DNA Sequencing Analysis. DNA fragment from six isolates were chosen to be sequenced based on the dominant group that isolated from human mouth habitat. PCR product was purified using DNA Gel Extraction Kit (V-Gene, Hangzhou, China). Purified products were sequenced using big dye terminator with ABI PRISM™ model 3130 Genetic Analyzer. The sequence data of bacterial isolates were compared to sequence from GenBank in National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) using BLASTN program to identify the isolates.

RESULTS

Isolation and Characterization of Methylophilic Bacteria from Human Mouth. From 63 subjects, a total of 21 isolates of methylophilic bacteria were successfully isolated using one carbon compound medium as selective medium (Table 1). The isolates showed various gram

Table 1. Characteristics and antibiotic susceptibility of the isolates

Isolates	Group	Colour	Gram	Morphology	Catalase	Oxidase	Antibiotic susceptibility test*				
							AMP	K	S	W	TC
GB1	I	pink	+	coccus	+	-	S	S	S	R	S
GB2	I	pink	+	coccus	+	-	S	S	S	R	S
GB31	I	pink	+	coccus	+	-	S	S	S	R	S
GB4	I	pink	+	coccus	+	-	S	S	S	R	S
GB5	I	pink	+	coccus	+	-	S	S	S	R	S
GB6	I	pink	+	coccus	+	-	S	S	S	R	S
LAU1	II	pink	-	bacil	+	+	R	S	S	R	S
LAU2	II	pink	-	bacil	+	+	I	S	S	R	S
LAU3	II	pink	-	bacil	+	+	R	S	S	R	S
ST1	III	orange	+	coccus	+	-	R	S	S	R	S
GT1	III	orange	+	coccus	+	-	R	S	S	R	S
GAW1	III	orange	+	coccus	+	-	R	S	S	R	S
SBE1	III	orange	+	coccus	+	-	S	S	S	R	S
SBF1	III	orange	+	coccus	+	-	S	S	S	R	S
LO2	III	orange	+	coccus	+	-	S	S	S	R	S
LX1	III	orange	+	coccus	+	-	S	S	S	R	S
GX1	III	orange	+	coccus	+	-	S	S	S	R	S
LAJ1	IV	light yellow	+	bacil	+	-	S	S	S	S	S
LAO1	V	yellow	+	coccobacil	+	-	R	S	S	I	S
SAV1	VI	white-blue	+	coccus	+	-	R	S	S	S	S
LBB1	VII	white	-	bacil	+	+	R	R	R	R	R

*AMP: ampicillin, K: kanamycin, S: Streptomycin, W: Trimethoprim, TC: Tetracycline, R: Resistant, S: Sensitive, I: Intermediat.

staining result, morphology, and pigmentation. All isolates were catalase-positive, showing that all isolates were aerobic bacteria whereas only 4 isolates were oxidase-positive. Based on its characteristic, the isolates were divided into 7 groups (Table 1).

Antibiotics Susceptibility Testing. Most of the isolates were resistant to trimethoprim, except isolate LAJ1, LAO1, and SAV1 (Table 1). Only isolate LBB1 was resistant to kanamycin, streptomycin, and tetracycline whereas other isolates were sensitive. Ampicillin susceptibility test showed various result among the isolates (Table 1).

Extraction of Genomic DNA and Amplification of 16S rRNA Gene. After the isolates were purified, the genomic DNA of the isolates was extracted using CTAB method (Marchesi *et al.* 1998). Genomics DNA of the isolates were used as template in 16S rRNA gene amplification. The expected amplicon size is approximately 1400 bp.

Genetic Diversity Analysis with ARDRA. Digestion with restriction enzyme *Sau3AI* produced different profile among the isolates (Figure 1). The isolates with different phenotypic profiles showed different restriction profile.

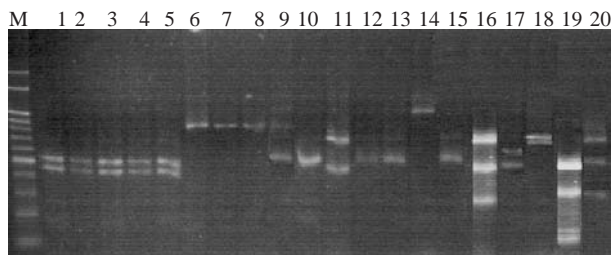


Figure 1. Restriction pattern of PCR-amplified fragment of 16S rDNA genes digested with *Sau3AI* (Lanes M: 1 kb DNA ladder, 1: GB2, 2: GB3, 3: GB4, 4: GB5, 5: GB6, 6: LAU1, 7: LAU2, 8: LAU3, 9: ST1, 10: GT1, 11: GAW1, 12: SBE1, 13: SBF1, 14: LO1, 15: GX1, 16: SAV1, 17: LBB1, 18: LAJ1, 19: LAO1, 20: *E. coli* DH5 α).

The isolates with similar characteristic also showed similar profile, such as isolate GB2-GB6 and isolate LAU1-LAU3. It is possible that the isolates were collected from one subject in the same isolation area. Therefore, a few isolates showed similar 16S rRNA gene restriction profile.

However, the seven orange isolates were shown to be different (Figure 1; lane 9-15). Although the isolates have similar phenotypic profiles, only 5 isolates showed similar digestion profile, whereas 2 isolates were shown to have different profile. These isolates are isolate GAW1 (lane 11) and isolate LO1 (lane 14).

The restriction fragment pattern was used to construct phylogenetic tree (Figure 2). Phylogenetic tree showed nine groups of bacteria were separated into 2 clusters with bootstrap 100 (from 100 phylogenetic tree constructed by the program, it always separated into 2 clusters). However, the characteristic to distinguish the clusters have not yet been fully known.

DNA Sequencing Analysis. Isolate LAU3, GX1, LAJ1, LBB1, GB31, and ST1 were chosen to be identified based on phenotypic and digestion profile. These isolates represented the dominant group of bacteria that successfully isolated in this study. The results of DNA sequencing were partial sequence of 16S rRNA gene (data was not shown). Sequence similarity of the isolates using BLASTN program was shown in Table 2. Based on sequencing analysis, some isolates were successfully identified as *Methylobacterium radiotolerans*, *Gordonia* sp., *Microbacterium esteraromaticum*., and *Pseudomonas* sp.

DISCUSSION

Methylophilic bacteria are a group of bacteria that capable in using C1 compounds such as methanol as their only carbon and energy sources (Dien *et al.* 2003). In this

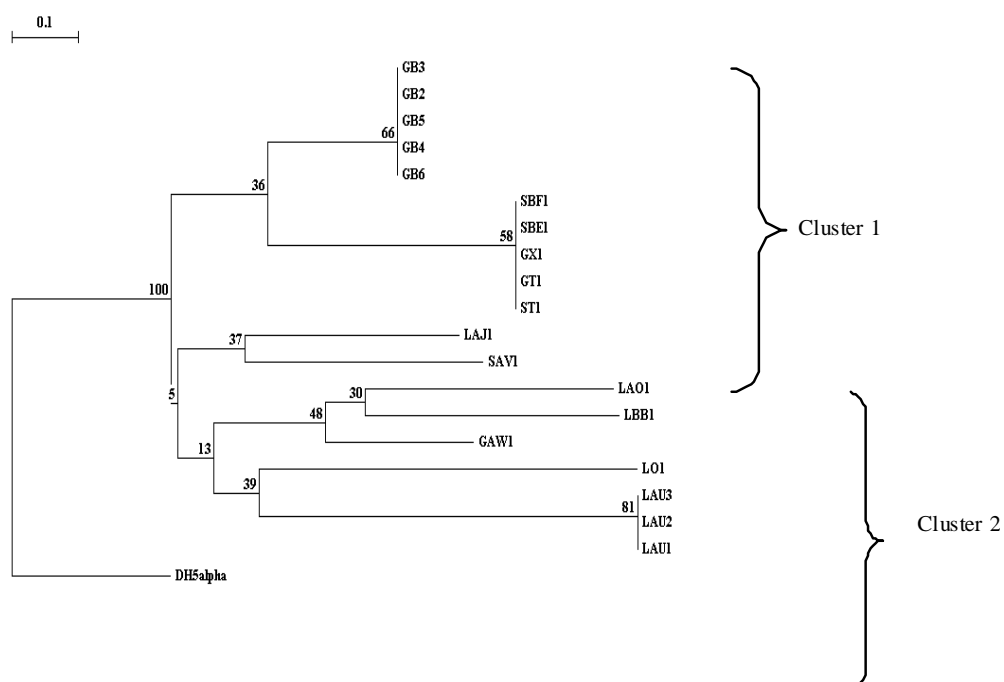


Figure 2. Phylogenetic relationships among the isolates based on restriction pattern of PCR-amplified fragment of 16S rDNA genes.

Table 2. Sequence analysis results with BLASTN program

Isolates	Group	Sequence similarity		
		Organism	Accession number	Percent of identity (%)
LAU3	II	<i>Methylobacteriaceae radiotolerans</i>	EU851880	94
GX1	III	<i>Gordonia</i> sp.	EU851878	99
ST1	III	<i>Gordonia</i> sp.	EU851881	98
GB31	I	<i>Gordonia</i> sp.	EU851878	99
LAJ1	IV	<i>Microbacterium esterarmaticum</i>	EU851882	99
LBB1	VII	<i>Pseudomonas</i> sp.	EU851883	100

study we used one carbon compound medium (minimal medium supplemented with 1% methanol) as selective medium for these bacteria and biochemical assays oxidase and catalase assay (Anesti *et al.* 2005).

Seven groups of the isolates with different phenotypic profiles showed the diversity of methylotrophic bacteria in human mouth (Table 1). Further, the diversity of methylotrophic bacteria in human mouth was also shown by ARDRA analysis. ARDRA analysis could also distinguish the orange coloured isolates which have similar phenotypic profile. These results suggested that ARDRA technique could be used to show the genetic diversity among the isolates with same phenotypic profiles. However, alternative techniques could be used to increase the discriminative, such as amplification of 16S-23S rDNA intergenic spacer or genome analysis with Macrorestriction Fragment Length Polymorphism (MFLP). Both of these techniques have been used by Riupassa and Suwanto (2004) to analyze the genetic diversity of pink pigmented facultative methylotroph isolated from some Indonesian edible leafy plants.

The phylogenetic tree showed that isolate GB2-GB6 was distinct from isolate LAU1-LAU3 although they produced the same pigment, indicating that the metabolism of pigment synthesis is not related to the relationship among the isolates. Isolate GAW1 and isolate LO1 were also separated into different branch in phylogenetic tree although the isolates have similar characteristics. Moreover, these isolates were distinct from the other orange isolates. These results suggested that 16S rRNA gene of these isolates were difference from 16S rRNA gene of the other orange isolates. It may possible that the species or genus of these isolate were different from the other isolates.

Further, the isolates were examined using DNA sequencing analysis. In this study partial 16S rDNA sequence was used to identify the isolates. The fact that isolate LAU3 produced pink pigment lead to the initial hypothesis that isolate LAU3 was a pink pigmented facultative methylotroph (PPFM) bacteria. Further, isolate LAU3 showed similar characteristics and morphology with PPFM bacteria. Moreover, this isolate was closely related to *Methylobacterium radiotolerans* (94%). Previously, a group of PPFM bacteria has been isolated from human mouth by Anesti *et al.* (2005). This study also supported the successful of PPFM bacteria isolation from human mouth.

Besides involved in metabolism of one carbon compounds in human mouth, PPFM bacteria also produced

micronutrient *pyrrolo-quinoline quinone* (PQQ). PQQ was a cofactor redox reaction and have characteristic as vitamin (Bishop *et al.* 1998). Thus, it is possible to apply PPFM bacteria for human being. However, for its application as odour reducing bacteria, it is still provide much more studies, such as the efficacy of the metabolism of VSC in oral cavity and its safety for human considering that PPFM bacteria has been reported as pathogen opportunistic (Hornei *et al.* 1999).

Isolate GX1, isolate ST1, and isolate GB31 were closely related to *Gordonia* sp. (99, 98, and 99%), which previously called *Rhodococcus*. Phenotypic profiles of isolate ST1 and GX1 were similar to *Gordonia* that isolated by Lesens *et al.* (2000), which is salmon-orange coloured. Moreover, *Gordonia* isolated by Lesens *et al.* (2000), also showed the same result as isolate GX1. Both isolates showed resistant to trimetoprim and sensitive to aminoglycoside group such as kanamycin and gentamycin, and to β -lactam group such as penicillin and ampicillin.

There still quite a few publications that studied the C1 metabolism of *Gordonia* genus. Presumptively, isolate GX1, ST1, and GB31 use alcohol dehydrogenase enzyme while growing on minimal media supplemented with 1% (v/v) methanol. This enzyme is an enzyme possessed by *Rhodococcus* genus, which plays a role in alcohol metabolism, both the short chain and long chain alcohol (Schenkels & Duine 2000). However, it should still be confirmed by detecting the presence of alcohol dehydrogenase coding gene.

Gordonia sp. has never been successfully isolated from human mouth. All this time, those bacteria are mostly isolated from clinical specimens, such as blood and hospital-origin culture, especially from patient catheters and nurses (Lesens *et al.* 2000), indicating that *Gordonia* sp. belongs to an opportunistic bacteria group.

Isolate LAJ1 was closely related to *Microbacterium estearomaticum* (99%) which is a rod shaped Gram positive bacteria, possesses yellow or orange coloured pigmentation, and catalase-positive (Funke *et al.* 1995). The metabolism of C1 compounds in *Microbacterium* sp. has not paid much attention in the past, but a few species, for example, *M. lacticum*, have been known for their ability to grow on methanol substrate (Mokashi & Paknikar 2002). However, no further explanation about the metabolism is stated.

There was also no report about the isolation of *Microbacterium* sp. in the human mouth. Previously, this bacteria has been successfully isolated from soil, corn

liquid waste, and clinical specimens such as blood, hospital air, and wounds (Funke *et al.* 1995), indicating that *Mircobacterium* is an opportunistic pathogen. Susceptibility testing done by Funke *et al.* (1995) also showed a compatible result with this study, that this bacteria is tetracycline-sensitive.

Isolate LBB1 was identified as *Pseudomonas* sp. (100% sequence similarity). A few species from *Pseudomonas* sp. have been reported to be successfully isolated from the human mouth, such a *P. alcalophila* and *P. aleovorans* (Hutter *et al.* 2003). Most of *Pseudomonas* is not included in methylophilic bacteria. However, a few *Pseudomonas* have alcohol dehydrogenase which are able to oxidize methanol, such as *P. aeruginosa*, and *P. putida* (Vangnai *et al.* 2002). Moreover, *P. fluorescens* has also been reported to effectively degrade VSC compounds, dimethyl disulphide (Nakagawa *et al.* 2007).

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