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Detection of *Methylobacterium* Species by 16S rRNA Gene-Targeted PCR

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We designed PCR primers for specific amplification of the 16S rRNA genes of seven species of the genus *Methylobacterium*. All of the pairwise species tested were successfully differentiated by PCR detection with a combination of five primer sets, with the exception of *M. extorquens* and *M. rhodesianum*. These primers did not cross-react with closely related bacteria in the alpha subclass tested.

Pink-pigmented bacteria are found in oligotrophic freshwater environments such as the surface water of reservoirs and drinking tank water (7–11, 14, 22, 23). These bacteria isolated from water distribution systems were found to be highly resistant to chlorine (8–10, 12, 22, 23). More attention has been paid to the pink-pigmented bacteria as possible pathogens, because isolation of such bacteria from clinical specimens has been reported (15, 16, 29). These bacteria were previously identified as members of the genus *Pseudomonas* on the basis of physiological traits (11, 12, 15, 16, 29) but should have been classified in the genus *Methylobacterium*, due to their methylotrophic properties (9, 17, 18, 27, 28, 31).

Methylotrophic bacteria are organisms that are able to utilize single-carbon compounds more reduced than carbon dioxide as the sole carbon source for growth and are divided into two groups, those which can utilize methane as the sole carbon source and those which cannot (19, 20). Methylotrophs that do not utilize methane are further classified into two groups that use the ribulose monophosphate and serine pathways for formaldehyde assimilation. Members of the genus *Methylobacterium* are methanol utilizers possessing the serine pathway for formaldehyde assimilation. Intensive studies on 16S rRNA gene sequence analysis by others have shown that *Methylobacterium* is phylogenetically an alpha subclass of the *Proteobacteria*, whereas ribulose monophosphate methylotrophs are of the beta subclass (2, 5, 30). On the other hand, the type II methanotrophs possessing the serine pathway fall into the alpha subclass of the *Proteobacteria* and compose a distinct cluster from the serine methylotrophs (3, 5).

These methylotrophic bacteria isolated from chlorinated water are relatively slow growers and require prolonged incubation time to form detectable colonies on conventional media such as nutrient agar (9, 23). Although medium containing lincomycin to select pink-pigmented bacteria from other predominant heterotrophic bacteria has been described previously (13), confirmation by numerous physiological tests is still required. Because these bacteria are slow growers, identification by physiological testing is laborious.

The objective of the present study was to develop a procedure to identify *Methylobacterium* species by PCR. As the mechanisms and the genes responsible for chlorine resistance in *Methylobacterium* spp. are currently unknown (23), we amplified the 16S ribosomal DNA (rDNA) sequence by PCR (26, 28). Primers that amplify 16S rDNA of *Methylobacterium* spp. were designed primarily to differentiate *Methylobacterium* spp. from type II methanotrophs as well as from *Pseudomonas* spp., most of which belong to the gamma subclass. Species-specific primers for *Methylobacterium* species were also explored, because the intensive phylogenetic studies of Hiraishi et al. (23) revealed a wide distribution of chlorine-resistant species within the genus *Methylobacterium*, and the pink-pigmented bacterial species isolated from chlorinated water is not always *Methylobacterium extorquens* as has been thought.

Selection of primers. All the 16S rDNA sequence data were obtained from the DDBJ, EMBL, and GenBank databases. The accession numbers are listed in Table 1. The 16S rDNA sequence data of seven *Methylobacterium* species were available. The 16S rDNA sequences of *Methylobacterium* and of other bacteria in the alpha subclass of *Proteobacteria*, including type II methanotrophs *Methylocystis parvus*, *Methylocystis minimus*, *Methylocystis pyriformis*, *Methylosinus sporium*, and *Methylosinus trichosporium*, were aligned with the personal computer software Clustal V (21). PCR primers were designed with the personal computer software Oligo (National Biosciences, Plymouth, Minn.). Oligodeoxynucleotide primers were synthesized with a Cyclon plus DNA synthesizer (Millipore, Milford, Mass.) and purified with an Oligo-Pak PS2 column (Millipore).

The V1, V2, and V3 regions (nomenclature of Neefs et al. [25]) were chosen for designing targets of the PCR primers, because large deletions, which are characteristic of the alpha subclass of the *Proteobacteria*, are located in these regions and large gaps appeared in these regions upon multiple sequence alignment. Thus, *Methylobacterium* species were differentiated from the bacteria in the beta and gamma subclasses including serine methylotrophs such as *Methylobacillus* and *Methylosinus* spp. Two sets of primers with multiple mismatches in this region of the 16S rDNA sequence of type II methanotrophs were designed and designated Mb1 and Mb2.

Brusseau et al. (5) reported that an oligonucleotide corresponding to positions 990 to 1008 (*E. coli* International Union of Biochemistry numbering [4]) can be used as a Northern hybridization probe for serine methylotrophs. Thus, we designed a primer set that can amplify a 16S rDNA sequence including this region and designated it Mb3.

As those regions are less variable among members of the genus *Methylobacterium*, interspecies differentiation of *Methylobacterium* species by PCR amplification is not expected. Other regions were explored, and a region corresponding to positions 650 to 670 and the V6 region were found to have

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TABLE 1. Sources of bacterial strains and the accession numbers of 16S rDNA

Strain	Strain[a]	Accession no.[b]
M. extorquens	IAM 1081	NA
	IAM 12623	NA
	IAM 12631^T	D32224
	IAM 12632	M29027
	ATCC 43645^T	NA
	NU[c]	M95656
M. rhodesianum	JCM 2815	D32228
M. zatmanii	JCM 2819	D32230
M. rhodinum	JCM 2811^T	D32229
M. organophilum	JCM 2833^T	D32226
M. mesophilicum	JCM 2829^T	D32225
M. radiotolerans	IAM 12098^T	D32227
	IAM 12099	NA
Methylobacillus glycogenes	JCM 2850^T	M95652
R. rubrum	IFO 3986	D30778
A. tumefaciens	IAM 13155	X67223
	NU	D14506
	NU	M11223
B. diminuta	IAM 12691^T	M59064
	JCM 2788^T	M59064
R. palustris	JCM 2524	D25312
R. sphaeroides	IFO 12203^T	D16425
P. aeruginosa	ATCC 9027	NA
P. stutzeri	IAM 12931	NA
P. fluorescens	IAM 12022	NA
A. faecalis	IFO 13111^T	M22508
A. xylosoxidans subsp. *denitrificans*	IAM 12370^T	M22509

[a] A superscript T indicates a type strain. ATCC, American Type Culture Collection, Rockville, Md.; IAM, Institute of Applied Microbiology, Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Japan; JCM, Japan Collection of Microorganisms, Wako, Japan..

[b] NA, not available.

[c] NU, the strain corresponding to the 16S rDNA sequence was not used in this study.

relatively high interspecies sequence variability. Two forward (4F and 5F) and four reverse (4R, 6R, 7R, and 8R) primers were selected, and five combinations of the primer sets 4F-4R, 4F-7R, 4F-8R, 5F-6R, and 5F-8R were designated Mb4a, Mb4b, Mb4c, Mb4d, and Mb4e, respectively. The sequences of the designed oligodeoxynucleotide primers are listed in Table 2. The differences in the nucleotide sequence between target regions of the tested bacteria and the primers are summarized in Table 3.

Reactivities of the primer sets. Bacteria used in this study and their sources are listed in Table 1. We isolated pink-pigmented bacteria, strains N10 and N11, from water containing over 0.2 mg of chlorine liter^{-1}. These bacteria were routinely maintained on nutrient agar (Difco Laboratories, Detroit, Mich.). Cell extracts were prepared by heating frozen cells at 95°C for 90 s and then treating the cells with proteinase K (Wako, Osaka, Japan) at 55°C for 60 min. The optical density at 260 nm of the extract was adjusted to 1. The PCR assay was performed in 40 μl of reaction mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphate, 2 μl of template DNA, 16 pmol of each primer, and 0.5 U of *Taq* DNA polymerase (Takara, Ohtsu, Japan). Preheating at 94°C for 30 s was followed by 30 thermal cycles of 90 s each at 94, 60, and 72°C, with a 4-min final extension at 72°C on a programmed DNA incubator, Zymoreactor AB1800 (ATTO, Tokyo, Japan). To prevent nonspecific annealing of the primers, a hot start was performed with Ampli Wax (Perkin-Elmer). PCR products were electrophoresed on a 2% agarose gel (LO3; Takara) with TEA buffer, containing 40 mM Tris, 1 mM EDTA, and 20 mM sodium acetate (pH 8.0), at 100 V for 26 min and then were detected by ethidium bromide staining.

Reactivities of the eight primer sets were examined at annealing temperatures of 58 and 60°C, and the results are summarized in Table 4. Since the reactivity profiles of all of the tested *M. extorquens* strains were the same, the results for strain IAM12631 are shown in Table 4. None of the primer sets gave PCR products with any of the species belonging to the beta or gamma subclass, including *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Pseudomonas fluorescens*, *Alcaligenes faecalis*, and *Alcaligenes xylosoxidans* subsp. *denitrificans* (data not shown). We also examined the reactivity of other heterotrophic bacteria belonging to the alpha subclass with the primer sets. *Rhodobacter sphaeroides* (alpha-3) and *Brevundimonas diminuta* (alpha-2) reacted with neither of the primer sets under our conditions. *Agrobacterium tumefaciens* (alpha-2) and *Rhodospirillum rubrum* (alpha-1) reacted only with Mb3 at 60°C. The reactivity of *A. tumefaciens* diminished when the annealing temperature was raised to 62°C, while that of *R. rubrum* was slightly reduced at 62°C. Even under stringent conditions (annealing temperature of 64°C), *Rhodopseudomonas palustris* (alpha-2) gave a positive reaction with Mb1, Mb2, and Mb3. There are eight mismatches in the forward primer of Mb1, but they are located in the AT-rich part of the 5′ terminus of the forward primer. This might allow the primer to amplify under stringent conditions. Hiraishi et al. (23) showed that the phylogenetic positions of *Methylobacterium* species are in group 2 of the alpha subclass of the *Proteobacteria*. The results of the PCR imply a closer phylogenetic relationship of *Methylobacterium* species to *R. palustris* than to *A. tumefaciens*. The physiological characteristics of *R. palustris* are reported to be closely related to those of *Methylobacterium* species (24, 32). Although discrimination of *Methylobacterium* species from other bacteria in the alpha subclass was not satisfactory with the Mb1, Mb2, and Mb3 primers, the PCR primer sets in the Mb4 region successfully discriminated *Methylobacterium* from other strains in the alpha-2 subgroup tested.

Primer sets Mb1, Mb2, and Mb3 reacted with seven species of *Methylobacterium* at annealing temperatures of 58 and 60°C. As there are three or four mismatches in the nucleotide sequences of the forward primer of Mb1 and Mb3 with the target regions in *Methylobacterium organophilum* and *Methylobacterium radiotolerans*, the annealing temperature was raised from 58 to 64°C. The reactivity of the primer sets was reduced with

TABLE 2. Sequences of primers and their corresponding positions of 16S rDNA in *E. coli*

Primer[a]	Sequence	Position in *E. coli*	Tm[b] (°C)
1F	5′-CTT-CGG-GTG-TCA-GTG-GCA-GAC-3′	88–108	56.8
1R	5′-TAT-CGT-CCC-GGA-CAA-AAG-AGC-3′	432–452	55.7
2F	5′-GAT-CGG-CCC-GCG-TCT-GAT-TAG-3′	226–246	60.4
2R	5′-CCG-TCA-TTA-TCG-TCC-CGG-ACA-3′	439–459	59.2
3F	5′-CGC-CGT-AAC-GCA-TTA-AGC-ATT-3′	856–876	56.8
3R	5′-GGC-TTA-TCA-CCG-GCA-GTC-TCC-3′	1153–1173	57.4
4F	5′-CTT-GAG-ACC-GGA-AGA-GGA-C-3′	650–668	48.4
4R	5′-CCG-ATC-TCT-CGA-GGT-AAC-A-3′	1001–1019	47.3
5F	5′-CTT-GAG-TAT-GGT-AGA-GGT-T-3′	650–668	37.5
6R	5′-GGA-ATC-TCT-CCC-CAT-AAC-A-3′	1001–1019	46.0
7R	5′-CAA-GTC-TCC-CTG-GGT-AAC-A-3′	1001–1019	47.0
8R	5′-CAA-ATC-TCT-CTG-GGT-AAC-A-3′	1001–1019	41.8

[a] F, forward primer; R, reverse primer.

[b] Melting point calculated by the nearest neighbor method (3, 6) with the computer software Oligo. (salt, 50 mM; DNA, 250 pM).

TABLE 3. Number of mismatches in sequences between targeted regions of 16S rDNA and PCR primers designed

Strain[d]	No. of mismatches[a]							
	Mb1[b] (1F-1R)[c]	Mb2 (2F-2R)	Mb3 (3F-3R)	Mb4a (4F-4R)	Mb4b (4F-7R)	Mb4c (4F-8R)	Mb4d (5F-6R)	Mb4e (5F-8R)
M. extorquens	1-0	0-0	0-0	0-0	0-6	0-4	6-7	6-4
M. rhodesianum	1-0	0-0	0-0	0-0	0-6	0-4	6-7	6-4
M. zatmanii	0-0	0-0	0-0	0-2	0-4	0-6	6-9	6-6
M. rhodinum	1-0	0-0	0-0	0-6	0-0	0-2	6-8	6-2
M. organophilum	3-0	0-0	4-0	3-4	3-2	3-0	3-6	3-0
M. mesophilicum	1-0	0-0	0-0	6-7	6-8	6-6	0-0	0-6
M. radiotolerans	4-1	0-1	3-0	6-3	6-3	6-1	0-7	0-1
R. rubrum	5-7	1-7	2-2	7-10	7-14	7-9	7-13	7-12
A. tumefaciens	7-6	4-6	2-1	5-11	5-8	5-8	2-11	2-10
B. diminuta	3-5	1-4	2-3	7-10	7-11	7-9	3-12	3-10
R. palustris	8-1	1-2	2-0	6-13	6-12	6-12	2-12	2-13
R. sphaeroides	5-7	2-6	4-1	6-8	6-13	6-8	6-11	6-11

[a] Number of mismatches in forward and reverse primers.
[b] Set of primers.
[c] Primer names; F and R indicate forward and reverse primers, respectively.
[d] No 16S rDNA sequence data are available for strains N10 and N11, and the number of mismatches cannot be analyzed.

increasing annealing temperature, but interspecies differentiation with these primers was incomplete. The primer set Mb1 barely reacted with *M. radiotolerans* at 62°C and reacted not at all at 64°C, whereas it reacted with *M. organophilum* even at 64°C. Primer set Mb3 reacted only slightly with *M. radiotolerans* at 60°C but reacted not at all at over 62°C for both strains. Conditions for interspecies differentiation with Mb4 primer sets were further investigated. Many of the *Methylobacterium* strains did not react at 60°C when there were more than three mismatches in either of the Mb4 primer sets. *M. organophilum* reacted with neither of the primers in the Mb4 region. Mb4c, Mb4d, and Mb4e exhibited species-specific reactivity with *Methylobacterium rhodinum*, *M. radiotolerans*, and *Methylobacterium mesophilicum*, respectively. Mb4a could not differentiate *M. extorquens*, *Methylobacterium rhodesianum*, and *Methylobacterium zatmanii*, and Mb4b could not differentiate *M. rhodinum* and *M. zatmanii*. We lowered the annealing temperature when there was no amplification at 60°C, despite fewer than three nucleotide mismatches in the primer sets. *M. organophilum* reacted only slightly with Mb4e but did not react with Mb4b and Mb4c even at 58°C. We raised the annealing temperature when amplification occurred at 60°C, despite more than three nucleotide mismatches in the primer sets. *M. zatmanii* did not react with Mb4b at 62°C.

Hiraishi et al. elucidated that species within the genus *Methylobacterium* can be divided into three subclusters (23). *M. zatmanii*, *M. rhodinum*, and *M. organophilum*, together with *M. extorquens* and *M. rhodesianum*, make up subcluster I, in which the latter two species are most closely related. The former three species can be differentiated by PCR reactivity with Mb4a, Mb4b, and Mb4c primers. *M. zatmanii* reacted with the primer set Mb4a; however, it can be distinguished from *M. extorquens* and *M. rhodesianum* by the reactivity with Mb4b. *M. rhodinum* also reacted with Mb4b, but *M. zatmanii* can be distinguished from it because the former strain did not react with Mb4c, whereas the latter did. However, *M. extorquens* and

TABLE 4. PCR reactivity of primer sets at annealing temperatures of 58, 60, and 62°C

Strain	PCR reactivity[a] of primer set at indicated temp (°C)																							
	Mb1			Mb2			Mb3			Mb4a			Mb4b			Mb4c			Mb4d			Mb4e		
	58	60	62	58	60	62	58	60	62	58	60	62	58	60	62	58	60	62	58	60	62	58	60	62
M. extorquens	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—
M. rhodesianum	+	+	+	+	+	+	+	+	+	+	+	+	—	+w	+w	—	+w	—	—	—	—	—	—	—
M. zatmanii	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+w	+w	+w	—	—	—	—	—	—	—
M. rhodinum	+	+	+	+	+	+	+	+	+	+w	+w	—	+	+	+	+	+	+	—	—	—	—	—	—
M. organophilum	+	+	+	+	+	+	+w	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
M. mesophilicum	+	+	+	+	+	+	+w	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—	—	—
M. radiotolerans	+	+w	—	+	+	+	+w	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+w
R. rubrum	—	—	—	—	—	—	+w	+w	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A. tumefaciens	—	—	—	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
B. diminuta	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
R. palustris	+	+	+	+	+	+	+w	+w	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
R. sphaeroides	—	—	—	—	—	—	+w	+w	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
N10	+	+	+	+	+	+	+	+w	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+w
N11	+	+	—	+	+	+	+	+w	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+w

[a] +, substantial band; +w, faint band; —, no reaction.

M. rhodesianum were hardly differentiated with either of the primer sets in the Mb4 region examined, because there is no difference in the target region of the 16S rDNA sequences between the two. These species will be indistinguishable by the PCR reactivity of the primers for the 16S rDNA fragments, because only nine differences were scattered throughout the 1,407 nucleotide positions of the 16S rDNA. Other techniques such as ligase chain reaction (1), which can discriminate a single base mismatch, are needed to differentiate these strains.

The chlorine-resistant pink-pigmented bacteria, which were originally isolated from a potable water treatment system, have been classified into subcluster I together with *M. extorquens*, *M. rhodesianum*, *M. zatmanii*, *M. rhodinum*, and *M. organophilum* (22, 23). On the other hand, the profiles of primer reactivity of our isolates were the same as that of *M. radiotolerans*, which is classified into subcluster II together with *M. mesophilicum* from the 16S rDNA study. As some clinical isolates of pink-pigmented bacteria have been reported as *M. mesophilicum* (15, 16, 29), studies on the pathogenicity of *M. radiotolerans* are required.

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