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Citation

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Accessibility
Family- and Genus-Level 16S rRNA-Targeted Oligonucleotide Probes for Ecological Studies of Methanotrophic Bacteria

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Received 9 April 2001/Accepted 17 July 2001

Methanotrophic bacteria play a major role in the global carbon cycle, degrade xenobiotic pollutants, and have the potential for a variety of biotechnological applications. To facilitate ecological studies of these important organisms, we developed a suite of oligonucleotide probes for quantitative analysis of methanotroph-specific 16S rRNA from environmental samples. Two probes target methanotrophs in the family Methylocystaceae (type II methanotrophs) as a group. No oligonucleotide signatures that distinguish between the two genera in this family, Methylocalcis and Methylosinus, were identified. Two other probes target, as a single group, a majority of the known methanotrophs belonging to the family Methylococcaceae (type I/X methanotrophs). The remaining probes target members of individual genera of the Methylococcaceae, including Methylobacter, Methylothermus, Methylobacterium, Methylococcus, and Methylocaldum. One of the family-level probes also covers all methanotrophic endosymbionts of marine mollusks for which 16S rRNA sequences have been published. The two known species of the newly described genus Methylosarcina gen. nov. are covered by a probe that otherwise targets only members of the closely related genus Methylobothrius. None of the probes covers strains of the newly proposed genera Methylocaldula and “Methylocaldum,” which are polyphyletic with respect to the recognized methanotrophic families. Empirically determined midpoint dissociation temperatures were 49 to 57°C for all probes. In dot blot screening against RNA from positive- and negative-control strains, the probes were specific to their intended targets. The broad coverage and high degree of specificity of this new suite of probes will provide more detailed, quantitative information about the community structure of methanotrophs in environmental samples than was previously available.

MATERIALS AND METHODS

Bacterial cultures. The reference cultures used in this study were obtained from various sources, as indicated, and are available from either the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, United Kingdom) or the American Type Culture Collection (ATCC, Manassas, Va.). Refer-
Oligonucleotide probe design. The oligonucleotide probes developed and/or optimized in this study are listed in Table 1. The mismatch position in probe designation represents the forward position of the homologous base in the *E. coli* 16S rRNA gene. By use of the SEQLAB sequence editor in the Wisconsin Package (Genetics Computer Group, Madison, Wis.), 16S rRNA sequences (>1,300 bp) were aligned initially using the PILEUP function within the editor and then adjusted manually with secondary-structure considerations as described previously (2). With the help of computer-generated consensus sequences, the alignments were scanned visually for signature sequences of 18 to 30 nucleotides that distinguished methanotrophs at the family or genus level. Candidate oligonucleotide sequences were then examined for specificity using the basic BLAST search and Probe Match functions of GenBank and the RDP-II, respectively (5, 42). Except as described below, only sequences exhibiting high specificity for methanotrophs and retrieving a majority of the sequences in their target groups were pursued further.

**RESULTS AND DISCUSSION**

**Overview.** In recent years, interest in the physiology, ecology, and evolution of methanotrophs has intensified, and there is high demand for tools to facilitate quantitative studies of in situ methanotroph community structure (21, 34, 46, 50). Our objectives were to develop phylogenetic oligonucleotide probes for analysis of methanotrophs at the family and genus levels and to optimize the probes for use in quantitative hybridization through empirical determination of their *T*_m under standard hybridization conditions.

Visual comparison of aligned 16S rRNA reference sequences initially revealed 36 potential probe sequences for fur-
**Table 1. Oligonucleotide probes targeting methanotrophic bacteria**

<table>
<thead>
<tr>
<th>Target group</th>
<th>Probe sequence (5’→3’)</th>
<th>Reference strain(s)</th>
<th>Positive control(s)</th>
<th>Negative control (no. of mismatches)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Methanotrophs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylosinus trichosporium OB3b (1)</td>
<td>AGTCCAGGGAGCCG</td>
<td>S-F</td>
<td>Mlm-0732-a-A-19</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>GGTGCTTCTATAGGTAATGT</td>
<td>Mlm-0732-b-A-19</td>
<td>Methylomonas rubra Methylomonas methanica</td>
<td></td>
</tr>
<tr>
<td>Methylocaldum gracile (1)</td>
<td>51</td>
<td>S-F</td>
<td>Mml-0732-a-A-19</td>
<td>54</td>
</tr>
<tr>
<td>Degradative methanotrophs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylococcus capsulatus BG8 (1)</td>
<td>AGGTAATGT</td>
<td>Mlm-0732-a-A-19</td>
<td>Methylomonas rubra Methylomonas album</td>
<td></td>
</tr>
<tr>
<td>Methylococcus capsulatus Bath</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylococcus capsulatus C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylocaldum gracile</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylocaldum gracile (1)</td>
<td>51</td>
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<td>Mml-0732-a-A-19</td>
<td>54</td>
</tr>
</tbody>
</table>

**FIG. 1.** Typical $T_d$ curves illustrating the ability of the probes to discriminate quantitatively between target and nontarget rRNA with a 1- or 2-base mismatch.
two family-level probes (Gm633 and Gm705) covered 82% of the available probes. For each probe.

Three probes covered all representatives of the two recognized thermophilic genera, Methylococcus and Methylocaldum. Mlc123 and Mlc1436 each matched all Methylococcus strains in environmental samples. Probe Mcd77 covered the three recognized strains of the recently described genus Methylocaldum. The target region was unique, and a Probe Match analysis retrieved no sequences with fewer than four mismatches from non-Methylocaldum species.

The complete suite of γ-methanotroph probes covered 97% of the strains listed in Fig. 3; only two sequences were not covered. One is that of Methylocaldum sp. strain HB. Because it is the only known γ-methanotroph that is phylogenetically close to the family Methylococccaceae (8), this result was expected.

**Probe specificity and optimization for quantitative hybridization.** The probes described here are intended to quantify 16S rRNA from specific microbial populations against a background of many unknown populations in environmental samples. The probes must discriminate against non-target 16S rRNA that may have a difference of only 1 base from the intended target. The primary factor for achieving stringent specificity and quantitative hybridization of 16S rRNA from environmental samples is accurate determination of the melting characteristics of the probe-target duplex. Hence, empirical determination of the $T_m$ is essential (32, 54). We have optimized the probes presented here for stringent discrimination against nontarget RNA and also for quantitative hybridization by empirically determining the $T_m$ for each probe.

The $T_m$s of the probes ranged from 49 to 57°C (Table 1). When Northern blots were hybridized overnight and then washed at the appropriate $T_a$, target and nontarget rRNAs were visually distinguishable on blots and yielded quantitatively distinct results when analyzed using a scintillation counter (Fig. 1) or a phosphorimager (data not shown). These results verify that the use of known concentrations of reference rRNA as standards will permit quantitative analysis of environmental rRNA possessing the target sequence, as demonstrated previously (49, 54).

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**Fig. 2.** Range of strain coverage for oligonucleotide probes targeting α-methanotrophs (Am), % Ambiguity, percentage of positions within the entire sequence that indicate ambiguous bases, shown as an index of overall sequence quality. Under "Probes," solid fill indicates identity between the probe and a target sequence; cross-hatching indicates identity between the probe and a nonmethanotroph; numbers of mismatched bases between the probe sequence and the corresponding 16S rRNA sequence. Where a number is shown in white on a solid background, the apparent mismatches were disregarded based on criteria outlined in Materials and Methods. An open diamond denotes the occurrence of one or more ambiguous bases in the probe target region that are consistent with the probe sequence. For example, if the probe has an A corresponding to a Y (International Union of Pure and Applied Chemistry [IUPAC] ambiguity code for C or T), then the possible T is consistent with the probe sequence. ns, no sequence available in probe target region.

Two family-level probes (Gm633 and Gm705) covered 82% of the available γ-methanotroph 16S rRNA sequences (Fig. 3). Gm705 had the broadest coverage, including representatives of six γ-methanotroph genera and the methanotrophic endosymbionts of marine mussels. Gm633 was more limited, but it provided better coverage of Methylobacter and Methylocaldum spp. The genera Methylomicrobium and Methylomarina eluded these two probes. However, almost complete coverage of the family can be achieved by combining these family-level probes with two or more of the genus-level probes described below.

Several probes provide genus-level detection of the closely related γ-methanotroph genera Methylobacter, Methylomicrobium, and Methylomarina (Fig. 3). Together, probes Mlb482 and Mlb662 covered all representatives of the genus Methylobacter. An indicated 6-base mismatch between Mlb482 and the 16S rRNA sequence for Methylobacter sp. strain T20 (AF131868) stems from seemingly errant insertions at positions 497 and 505 (E. coli numbering), as judged by the level of within-genus sequence conservation in the probe region and the fact that the indicated base change would violate the secondary structure of the 16S rRNA molecule. If the two apparent insertions are disregarded, the sequence matches Mlb482 perfectly. Probes Mmb482 and Mmb1007 each matched all available Methylomicrobium sequences. Mmb1007 also covered both strains of the newly described genus Methylomarina, which are closely related to Methylomicrobium spp. (58). Three other probes covered all of the recognized Methylomonas isolates. Representatives of this genus fell into two groups that differ by an A or C at position 746 (E. coli numbering). We designed two probes (Mlm732a and Mlm732b) to distinguish between the two subgenus groups. Mlm482 provided the broadest coverage of Methylomonas spp., but all representatives of the genus were covered only when the three Mlm probes were combined.

Three probes covered all representatives of the two recognized thermophilic genera, Methylococcus and Methylocaldum. Mlc123 and Mlc1436 each matched all Methylococcus sequences available. PCR primers corresponding to these two probes might be ideal for specific amplification of nearly complete (~1,300-bp) 16S rRNA genes from Methylococcus strains in environmental samples. Probe Mcd77 covered the three recognized strains of the recently described genus Methylocaldum. The target region was unique, and a Probe Match analysis retrieved no sequences with fewer than four mismatches from non-Methylocaldum species.
Probes Am445, Mmb1007, Mlm482, Mlm732b, Mlc123, and Mcd77 each exhibited at least two base mismatches against any nonmethanotroph sequence, whereas probes Gm633, Gm705, Mlb482, Mmb482, Mlm732a, and Mlc1436 each exhibited at least one base mismatch with any nonmethanotroph sequence. Probes Mlb662, Mmb482, and Mmb1007 matched sequences from one to four /H9253-methanotrophs outside their respective target genera (Fig. 3). Although we consider this problem to be minor, these probes could yield ambiguous results for fine-scale descriptions of /H9253-methanotroph communities. All other genus-level probes were specific to their intended target genera. The α- and γ-methanotroph probes had no cross-family hybridization potential.

FIG. 3. Range of strain coverage for oligonucleotide probes targeting γ-methanotrophs (Gm). Mlc, Methylococcus; Mcd, Methylocaldum. % Ambiguity, percentage of positions within the entire sequence that indicate ambiguous bases, shown as an index of overall sequence quality. The unpublished 16S rRNA sequence for Methylosinus methane strain S1 (marked with a star in the “GenBank accession number” column) is available as RDP sequence Mlm.metha1 (C. R. Woese, 1991). Under “Probes,” solid fill indicates identity between the probe and a target sequence; diagonal hatching indicates identity between the probe and a nontarget /H9253-methanotroph strain; cross-hatching indicates identity between the probe and a nonmethanotroph; numbers are numbers of mismatches between the probe sequence and the corresponding 16S rRNA sequence. Where a number is shown in white on a solid background, the apparent mismatches were disregarded based on criteria outlined in Materials and Methods. An open diamond denotes the occurrence of one or more ambiguous bases in the probe target region that are consistent with the probe sequence. For example, if the probe has an A corresponding to a Y (IUPAC ambiguity code for C or T), then the possible T is consistent with the probe sequence. ns, no sequence available in probe target region.
ment approaches, such as monitoring of CH₄ enrichment cultures, use as PCR primers in cases where amplified products are to be sequenced for identification, or analysis of community composition in environmental samples where the nontarget organisms with which the probes hybridize should be minor components of the community. For instance, because marine *Cycloclasticus* spp. were the only nonmethanotrophs that matched Mlb662 (Fig. 3), this probe might be appropriate for probing nonmarine samples.

**Probing the database.** The GenBank database contains thousands of bacterial 16S rRNA gene sequences from cultures and environmental clones (6). Hence, “probing” this database should provide a powerful assessment of a probe’s ability to select specifically for methanotroph sequences against a background of myriad nonmethanotroph sequences. We subjected each probe sequence to a basic BLAST search (5) and examined sequences retrieved with an identical match. Only those sequences identified as 16S rRNA genes were considered. The organism identifications were based solely on information provided in the accession records or in publications cited therein.

Eleven of 14 probes retrieved only sequences that were identified as methanotrophs (Table 2). Probes Am976, Mlb662, and Mlc1436 retrieved a number of sequences representing a narrow range of nonmethanotrophic taxa. The first two of these probes matched environmentally restricted taxa, such as obligate pathogens (*Afipia* spp.) and obligate marine bacteria (*Cycloclasticus* spp.). If used strategically, therefore, these probes are likely to be useful for studying methanotroph communities. From the data in Table 2, it would be premature to conclude that Mlc1436 is nonspecific. All but one of the nonmethanotroph sequences retrieved by this probe were nearly identical clones of putative β-Proteobacteria from an activated sludge reactor. However, no cultured organisms belonging to the β-Proteobacteria were retrieved, and no published data were cited in the accession records to confirm the phylogenetic position of these environmental clones. Overall, the data in Table 2 suggest that at least 11 and possibly 12 of the probes presented here are highly specific to methanotrophic bacteria and that the two clearly nonspecific probes should hybridize to a phylogenetically limited range of nonmethanotrophs with restricted environmental distributions.

**Summary and conclusions.** The breadth and specificity of the probes reported here are unprecedented, providing 97% coverage of the 87 methanotroph 16S rRNA sequences examined (Fig. 2 and 3). Several new methanotroph genera that have been proposed recently following the isolation of novel strains are covered. Of the three strains apparently not covered by the probes, one is no longer extant and the available 16S rRNA sequence is of low overall quality, bringing into question whether the indicated probe mismatches are correct. The other two strains (Methylcocella palustris sp. strain K and *Methylothemus* sp. strain HB) that did not match any probe are polyphyletic with respect to the *Methylomicrobaceae* and *Methylococcaceae*, thus reflecting the high specificity of the probes to the phylogenetic clades they were designed to target. Initial results from studies with several soils indicate that the probes are effective for studying methanotroph communities in soil (unpublished data), perhaps the most difficult substrate on which to perform quantitative hybridization assays (4). Hence, all of the methanotroph taxa that have become well known through years of laboratory studies, as well as several recently described taxa, can now be studied at both the family and genus levels in environmental samples by using the probes reported here.

**ACKNOWLEDGMENTS**

J. Gulledge and A. Ahmad contributed equally to this work.

We gratefully acknowledge the following individuals: M. Polz for training and helpful discussions on designing oligonucleotide probes; A. J. Auaman, A. M. Costello, and M. E. Lidstrom for updated 16S rRNA sequences and a protocol for extracting nucleic acids from methanotrophs; G. M. King, R. Knowles, J. C. Murrell, J. S. Poindexter, and J. D. Semrau for providing reference cultures; and A. A. DiSpirito for reference genomic DNA.

This work was supported by the U.S. National Science Foundation (award DEB9708092) and was initiated while J. Gulledge was a DOE-Energy Biosciences Research Fellow of the Life Sciences Research Foundation.

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