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Analysis of *fae* and *fhcD* Genes in Mono Lake, California

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Genes for two enzymes of the tetrahydromethanopterin-linked C_1 transfer pathway (*fae* and *fhcD*) were detected in hypersaline, hyperalkaline Mono Lake (California), via PCR amplification and analysis. Low diversity for *fae* and *fhcD* was noted, in contrast to the diversity previously detected in a freshwater lake, Lake Washington (Washington).

Methylotrophic bacteria are a group of organisms that consume a wide range of C_1 compounds, such as methane, methanol, methylated amines, methylated glycines, halomethanes, and methylated sulfur species (1, 17). They are found in a variety of environments, such as freshwater, marine, and terrestrial habitats, as well as habitats characterized by extreme conditions, such as highly saline, alkaline, or acidic habitats (2, 4–6, 8, 11, 18, 21, 24, 25, 28). Along with the classic cultivation approaches, molecular tools have been in use for culture-independent detection and characterization of natural methylotroph populations. The traditional molecular tools include oligonucleotide probes and PCR primer sets targeting genes conserved among specific groups of methylotrophic bacteria, such as 16S rRNA genes (3, 8, 23, 24, 26, 27), or functional genes encoding specific methylotrophic functions, such as particulate and soluble methane monooxygenases (5, 8, 18, 20), methanol dehydrogenase (8, 19, 24), corrinoid-linked methyltransferase (21), or methanesulfonic acid monooxygenase (15). The phylogenetic probes have been used successfully to uncover the diversity of α- and γ-proteobacterial methylotrophs (3, 8, 23, 24, 25, 27), and the functional probes have been used for detecting a range of methylotrophs possessing respective primary oxidation genes (5, 8, 15, 18–21, 24). Recently, Kalyuzhnaya and colleagues have developed a suite of novel primer sets designed for a broader detection of C_1-oxidizing capacity in the environment (12, 13). These primer sets target four genes in the tetrahydromethanopterin (H_4MPT)-linked pathway, the pathway widespread in methylotrophic bacteria (30) but also found in nonmethylotrophs: *fae*, *mtdB*, *mch*, and *fhcD*. These new tools, tested on microbial populations in a freshwater lake (Lake Washington, Washington), uncovered a broad diversity of *fae*, *mtdB*, *mch*, and *fhcD* phylotypes belonging to α-, β-, and γ-proteobacteria, including methanotrophs, nonmethanotrophic methylotrophs, and bacteria not known for methylotrophic ability, such as *Burkholderia* spp. (12, 13). In addition, sequences belonging to highly divergent bacterial groups, such as *Planctomycetes*, and yet unaffiliated divergent species have been uncovered (12, 13). The broadest range of divergent sequences was detected using two of the primer sets, those targeting *fae* and *fhcD.* Most of the phylotypes detected in Lake Washington were not closely related to known organisms, suggesting that the majority of the population potentially involved in C_1 metabolism in this environment remain unidentified (12, 13). Mono Lake (California) is an extreme environment characterized by high rates of methane production and methane oxidation (10). Initial insights into the methane-oxidizing bacterial population in the site were recently obtained via fluorescence in situ hybridization and denaturing gradient gel electrophoresis employing oligonucleotide primers specific for known methanotroph groups (4). The goal of this work was the assessment of the diversity of C_1-utilizing bacteria in Mono Lake by use of tools with a broader detection range, i.e., PCR primers targeting *fae* and *fhcD*.

TABLE 1. Data for water samples from different depths[a]

Depth (m)	Concn (μM) of:		Oxidation rate (nmol liter^{-1} day^{-1})	Recovery of:	
	O_2	CH_4		*fae*	*fhcD*
5	137.0	0.3	0.0	+	+
9	128.9	0.2	0.0	+	+
10	115.8	0.4	0.1	+	+
11	82.8	0.5	0.2	+	+
12	71.3	0.7	1.4	+	+
13	60.2	0.6	1.5	+	+
13.5	36.9	0.4	0.0	+	+
14	30.2	0.5	11.3	+	+
15	24.9	0.6	11.7	+	+
16	9.9	1.3	32.5	−	+
17	9.2	2.4	8.1	−	+
18	0.0	3.5	5.4	−	+
20	0.0	7.1	2.0	−	+

[a] Depth of water column versus dissolved oxygen (O_2) and methane (CH_4) concentrations, biological methane oxidation rate, and recovery of *fae*- and *fhcD*-specific PCR products.

Water samples from Mono Lake were collected from 20 discrete depths, between 5 and 38 m, near a permanently moored buoy in the central basin of Mono Lake (station 6; 37°57.822′ N, 119°01.305′ W) by using 5-liter Niskin bottles deployed from a small boat in August 2002. Dissolved methane concentrations were determined using headspace extraction followed by gas chromatography (10). Dissolved oxygen concentrations were determined using an O_2 sensor (Yellow Spring Instruments) (4). Rates of methane oxidation were determined using a [^{3}H]CH_4 tracer technique (4). Oxygen concentrations in the upper water column were high (>100 μM), while methane concentrations were low (Table 1). Rates of

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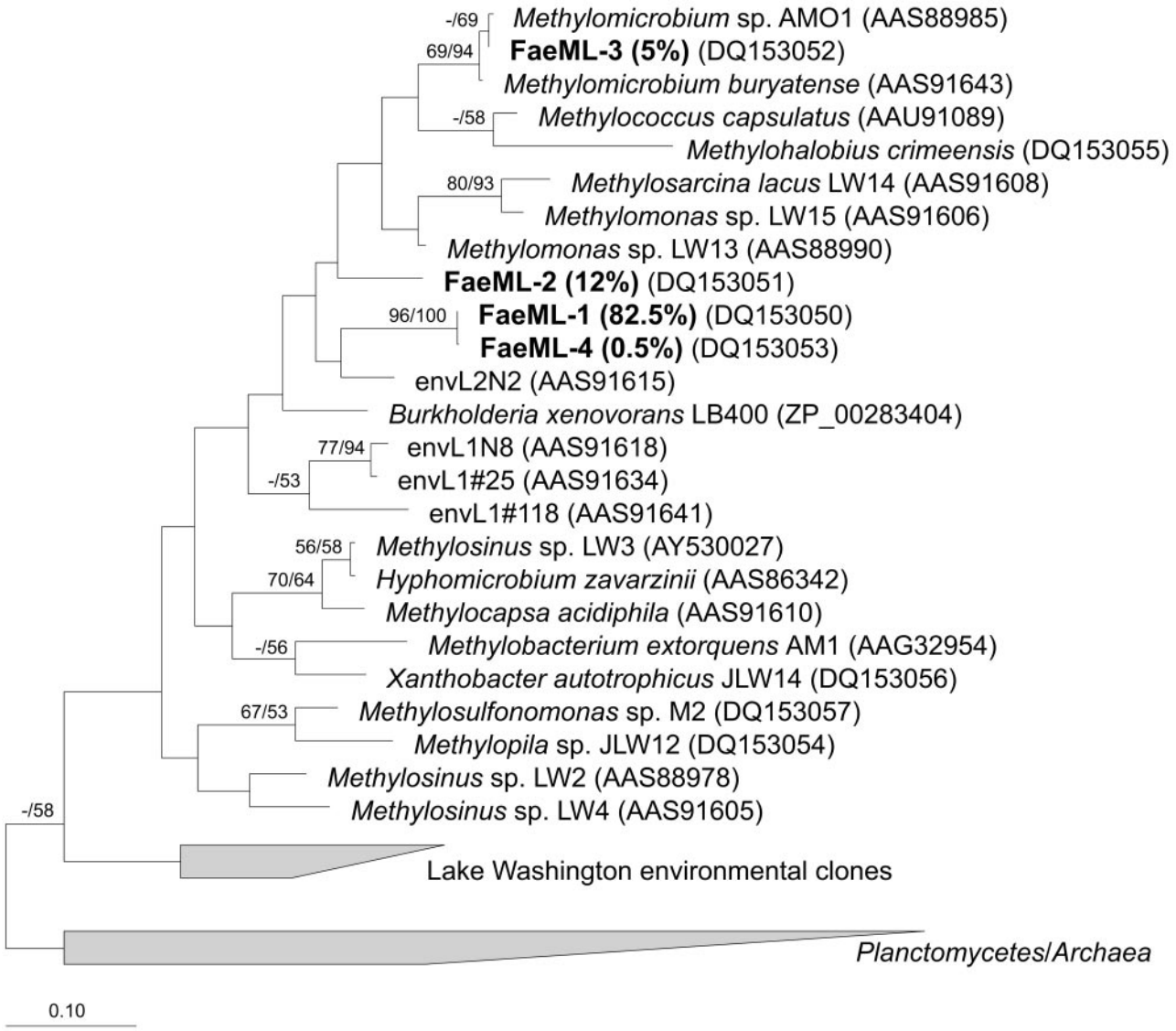

FIG. 1. Phylogenetic tree reflecting relationships of Fae sequences detected in Mono Lake water column. Analyses were performed using neighbor-joining (NJ) and maximum parsimony (MP) methods, using inferred amino acid sequences (92 positions). The scale bar indicates the number of expected amino acid substitutions per site per unit of branch length. Bootstrap values above 50% for NJ/MP analyses are shown above branches. Note that two types of *fae* are found in *Methylosinus* strains (represented here by *Methylosinus* sp. LW2 and *Methylosinus* sp. LW3) that cluster separately on phylogenetic trees (12).

methane oxidation increased at the base of the oxycline as methane concentrations increased and oxygen concentrations decreased (Table 1).

Water samples for DNA extraction were stored in clean sample-rinsed plastic cubitainers at 4°C until filtration through a Sterivex filter cartridge (0.22 μm; Millipore). Excess water was expelled, and the cartridge was filled with 1.8 ml of lysis buffer (22). Total community DNA was extracted from 20 discrete samples from different depths by using a previously described method (9), and these were used as templates to PCR amplify *fae* and *fhcD*, as described previously (12, 13). Nine samples (between the depths of 5 and 15 m) were positive for *fae*, and 13 samples (between the depths of 5 and 20 m) were positive for *fhcD* (Table 1). PCR products were then pooled, cloned into the pCR2.1 vector (Invitrogen), and analyzed, as described below.

A total of 189 plasmids containing *fae* inserts were analyzed based on their restriction fragment length polymorphism (RFLP) patterns, as described before (12). A total of five RFLP patterns were identified. Two to five representatives of each pattern were sequenced, and the sequences were categorized into phylotypes, based on a 94% DNA similarity cutoff value, a value recently suggested for discriminating between microbial species (16, 29), based on extensive comparisons between closely related bacterial strains (16). A total of four unique phylotypes were identified, and these were distributed as follows: phylotype FaeML1, 155 clones (82.5%); phylotype FaeML2, 23 clones (12%); phylotype FaeML3, 10 clones (5%);

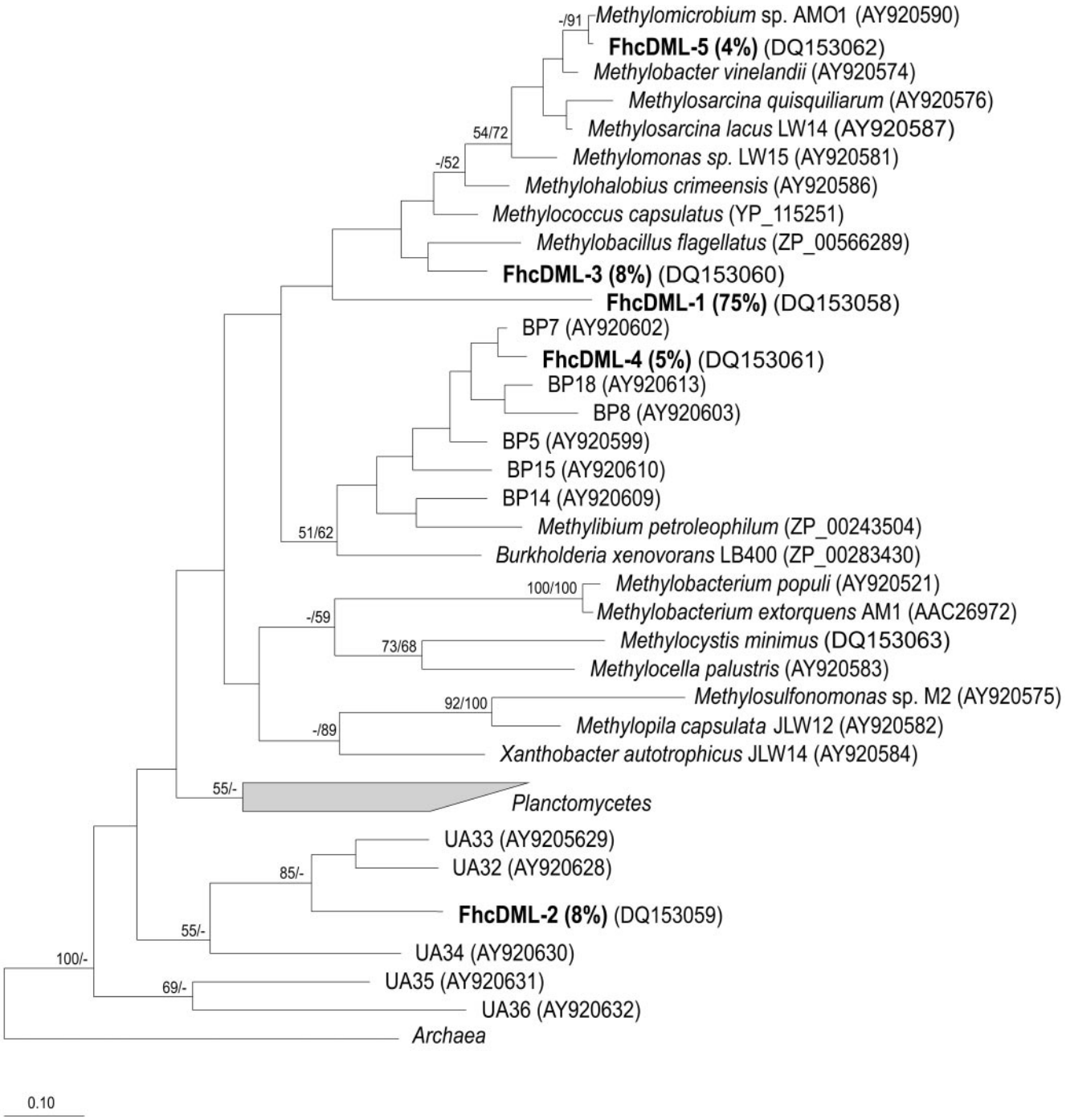

FIG. 2. Phylogenetic tree reflecting relationships of FhcD sequences detected in Mono Lake water column. Analyses were performed using neighbor-joining (NJ) and maximum parsimony (MP) methods, using inferred amino acid sequences (130 positions). The scale bar indicates the number of expected amino acid substitutions per site per unit of branch length. Bootstrap values above 50% for NJ/MP analyses are shown above branches. Note that sequences of *Methylobacillus flagellatus* genes for the H_4MPT-linked reactions cluster with γ-proteobacterial rather than β-proteobacterial sequences (14). Sequences designated with BP and UA represent yet-uncultured β-proteobacteria and unaffiliated bacteria detected in Lake Washington (13), respectively.

and phylotype FaeML4, 1 clone (0.5%). The homologous coverage value (7) for this library was calculated at 0.99, suggesting that the sampling effort was adequate and covered the major phylotypes present in the library. Comparisons with the sequences deposited with GenBank as well as with our proprietary databases showed that only one phylotype, FaeML3, showed over 94% identity at the DNA level with known *fae* sequences, those belonging to *Methylomicrobium* species that are γ-proteobacterial methanotrophs. A representative of each unique phylotype was included in the phylogenetic analyses,

along with the sequences from a variety of cultured bacteria, as well as the sequences previously recovered from Lake Washington by use of the same primer set, as previously described (12). Phylogenetic analyses (Fig. 1) revealed that, while phylotype FaeML3, as expected, tightly clustered with *Methylomicrobium* sequences, the three remaining phylotypes loosely clustered with known sequences belonging to γ-proteobacteria.

A total of 186 plasmids containing *fhcD* inserts were analyzed in a similar fashion, as previously described (13). A total of five RFLP patterns and a total of five phylotypes were identified, based on a 94% cutoff at the DNA level, and these were distributed as follows: phylotype FhcDML1, 139 clones (75%); phylotype FhcDML2, 15 clones (8%); phylotype FhcDML3, 14 clones (8%); phylotype FhcDML4, 10 clones (5%); and phylotype FhcDML5, 8 clones (4%). The homologous coverage value (7) for this library was calculated at 1. Comparisons with the sequences deposited with GenBank as well as with our proprietary databases revealed that only one phylotype, FhcDML5, showed significant identity at the DNA level (90%) with known *fhcD* sequences that belonged to *Methylomicrobium*. The remaining four phylotypes were only distantly related to known *fhcD* sequences (67 to 87% identity at the amino acid level). Phylogenetic analyses (Fig. 2) revealed that, as expected, phylotype FhcDML5 tightly clustered with *Methylomicrobium* sequences, while phylotypes FhcDML1 and FhcDML3 loosely clustered with known γ-proteobacterial sequences, phylotype FhcDML4 clustered with the sequences of uncultured β-proteobacteria, and phylotype FhcDML2 clustered with sequences of unaffiliated uncultured bacteria previously identified in Lake Washington (13).

Overall, our data suggest that only a few species possessing the genes for the H_4MPT-linked C_1 transfer pathway are present in Mono Lake and that of these, only one group, the *Methylomicrobium* group, is identifiable. Sequences highly similar to the 16S rRNA sequences from *Methylomicrobium* strains isolated from soda lakes (11, 18, 28) have recently been detected in Mono Lake (4), pointing toward the ubiquitous nature of these species in environments characterized by high salinity and high alkalinity. The remaining sequences detected in this work, including the most abundant *fae* and *fhcD* phylotypes, only loosely clustered with sequences of known γ-proteobacterial methanotrophs. Based on the abundance of these phylotypes in PCR-amplified libraries, they likely represent species with an ecologically important function, which may be in methane oxidation or in oxidation of other C_1 compounds. Carini et al. have recently reported on the presence of α-proteobacterial 16S rRNA gene sequences, including those closely related to *Methylosinus* sequences (4). However, no α-proteobacterial *fae* or *fhcD* sequences were recovered in this work. This is unlikely to be due to primer bias, as the same primers have readily detected α-proteobacterial *fae* or *fhcD* sequences from Lake Washington (12, 13), or to insufficient sampling in the clone libraries (see above) but is likely due to the low abundance of these sequences in the samples. The low diversity of detected *fae* and *fhcD* sequences in Mono Lake is in contrast with the diversity previously uncovered in a freshwater lake, Lake Washington, where a variety of sequences belonging to α-, β-, and γ-proteobacteria have been identified using the same molecular tools (12, 13). In addition, sequences clustering with planctomycete-related sequences have been identified, as have sequences deeply diverging from the sequences affiliated with known organisms, suggesting the presence of novel phyla with no cultured representatives (12, 13).

The presence of a phylotype in the *fhcD* Mono Lake library (FhcDML2) that is related to this latter group of sequences is intriguing, but nothing is known about the phylogenetic position of the organisms possessing these sequences, their physiological properties, or their role in the environment. Work is under way to address these questions, including metagenomic analysis of the Lake Washington microbial community and specific cell separation using flow cytometry.

In conclusion, we provided evidence that the pathway involved in C_1 transfers mediated by H_4MPT is present in microbial populations inhabiting an extreme environment, a soda lake, but that the diversity of the detected genes is very limited compared to the diversity previously found in a freshwater lake. The observed lower diversity may result from special adaptations required for C_1 microbes to survive in the unique geochemical environment present in Mono Lake. The dominant groups of *fae* and *fhcD* sequences recovered in PCR-based libraries belong to unknown bacterial species likely to be involved in oxidation of C_1 compounds, while about 5% of the sequences belong to the well characterized genus *Methylomicrobium*.

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