Clonothrix fusca Roze 1896, a Filamentous, Sheathed, Methanotrophic γ -Proteobacterium^{∇†}

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Crenothrix polyspora Cohn 1870 and *Clonothrix fusca* Roze 1896 are two filamentous, sheathed microorganisms exhibiting complex morphological differentiation, whose phylogeny and physiology have been obscure for a long time due to the inability to cultivate them. Very recently, DNA sequencing data from uncultured *C. polyspora*-enriched material have suggested that *Crenothrix* is a methane-oxidizing γ -proteobacterium (39). In contrast, the possible ecological function of *C. fusca*, originally considered a developmental stage of *C. polyspora*, is unknown. In this study, temporal succession of two filamentous, sheathed microorganisms resembling Cohn's *Crenothrix* and Roze's *Clonothrix* was observed by analyzing the microbial community of an artesian well by optical microscopy. Combined culture-based and culture-independent approaches enabled us to assign *C. fusca* to a novel subgroup of methane-oxidizing γ -proteobacteria distinct from that of *C. polyspora*. This assignment was supported by (i) methane uptake and assimilation experiments, (ii) ultrastructural data showing the presence in *C. fusca* cytoplasm of an elaborate membrane system resembling that of methanotrophic γ -proteobacteria, and (iii) sequencing data demonstrating the presence in its genome of a methanol dehydrogenase α subunit-encoding gene (*mxhF*) and a conventional particulate methane mono-oxygenase α subunit-encoding gene (*pmoA*) that is different from the unusual *pmoA* (*u-pmoA*) of *C. polyspora*.

Sheathed bacteria are a group of microorganisms found widely in nature in slowly running water, often attached to submerged surfaces. They are characterized by a filamentous arrangement of cells enclosed within a tube of extracellular material referred to as a sheath. Under a phase-contrast microscope, sheaths are usually transparent and resemble microscopic plastic tubes, generally but not always containing cells. The sheaths of *Leptothrix* sp., *Crenothrix* sp., and *Clonothrix* sp. are variously encrusted or impregnated with iron or manganese oxides and appear from yellow to dark brown. Due to the fact that many sheathed bacteria have never been obtained in pure cultures so far, the taxonomy of this heterogeneous group of microorganisms reflects a deterministic model more than their actual phylogeny (14).

The taxonomy of the two morphologically similar species *Crenothrix polyspora* Cohn 1870 (5) and *Clonothrix fusca* Roze 1896 (29) is paradigmatic. Although these microorganisms became well known for blocking wells in various European countries and the United States (12, 16, 21, 25, 32), their phylogenies and physiologies have for a long time remained unknown because, in spite of excellent seminal studies addressing their

morphologies, life cycles, and ecologies (5, 12, 16, 21, 25, 26, 30, 31, 38, 45), they have not been cultivated to date (36).

Ultrastructural analysis demonstrated that *C. polyspora* was prokaryotic, with a gram-negative cell wall surrounded by several layers of sheath material (42). Distinctive features of this microorganism were the presence of large hexagonal bodies, rod-shaped fibrillar elements, polyphosphate granules and, more interestingly, an elaborate membrane system in the form of lamellar stacks resembling that of methanotrophic bacteria. On the basis of these results, it was suggested that *C. polyspora* might be a methane oxidizer (42). Indeed, the habitat of this microorganism is represented by stagnant and running waters containing low concentrations of organic matter and Fe^{2+} and traces of methane. In a recent study, this hypothesis was supported by DNA sequencing data from uncultured *C. polyspora*-enriched material demonstrating that Cohn's *Crenothrix* is a possible methane-oxidizing member of the γ -proteobacteria with a very unusual particulate methane mono-oxygenase (pMMO) gene (*u-pmoA*) closely allied to genes encoding subunits of ammonia mono-oxygenase of the β -proteobacteria (*amoA*) (39).

In this study, temporal succession of *C. polyspora* and *C. fusca* was observed by analyzing the microbial community of an artesian well by optical microscopy. By taking advantage of the luxuriant growth of *C. fusca* in this environment, the problem of defining the taxonomy of this microorganism, originally considered a developmental stage of *C. polyspora* (16, 21, 25), was addressed.

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TABLE 1. Oligonucleotide primers used in this study

Primer	Target gene	Sequence ^a	PCR annealing temp (°C)	Position ^b	Reference
16SE20-42-F	16S rRNA	5'-TGGCTCAGATTGAACGCTGGCGG-3'	55	20-42	This study
16SEB20-42-F	16S rRNA	5'-TGGCTCAGGAYGAACGCTGGCGG-3'	58	20-42	This study
16SEB683-R	16S rRNA	5'-CTACGCATTTACCCGCTACAC-3'	58	683-703	This study
16SEB786-F	16S rRNA	5'-GATTAGATACCCTGGTAGTCCACG-3'	58	786-809	This study
16SEB1488-R	16S rRNA	5'-TACCTTGTTACGACTTACC-3'	58	1488-1507	This study
Com1-F	16S rRNA	5'-CAGCAGCCGCGGTAATAC-3'	50	519-536	18
Com2-R	16S rRNA	5'-CCGTCAATTCCTTTGAGTTT-3'	50	908-927	18
un-pmoAfl	u-pmoA	5'-GGGGACTGGGACTTCTGGCTCGAC-3'	55	1-24	This study
un-pmoAr	u-pmoA	5'-GGCGGAGATCATGGTGCTGTGACC-3'	55	493-516	This study
pmofl	pmoA	5'-GGGGGAACCTCTGGGGITGGAC-3'	55	329-350	4
pmor	pmoA	5'-GGGGGRCIACGTCITTACCGAA-3'	55	636-657	4
mxafF	mxaf	5'-CTTGAACGAAACCATGCG-3'	55	875-893	This study
mxafR	mxaf	5'-CCCTGGTTGTGATAACCC-3'	55	1251-1268	This study

^a Y = C or T; R = A or G.

^b Numbering is relative to the *Escherichia coli* 16S rRNA gene, *Crenothrix polyspora* u-pmoA, *Methylosinus trichosporium* pmoA, or *Methylococcus capsulatus* Bath mxaf and in a 5'- to -3' orientation relative to the coding strand.

MATERIALS AND METHODS

Sampling and growth media. Samples were taken monthly from the filters of an artesian well water pumping system in a farm in Salento (Apulia, Italy) over a period of 1 year (November 2005 to October 2006). The filters were found to be obstructed by a thick population of filamentous, sheathed bacteria with whitish-beige to yellowish-brown color.

Flavobacterium sp. strains AWR-3 and AWR-4 were isolated on ATCC media 1103 and 1503 (ATCC, Manassas, VA) after incubation at 28°C for 72 h. *Bacillus* sp. strain AWR-5 was isolated on ATCC medium 3 after incubation at 28°C for 24 h. The enrichment media for *C. fusca* were formulated using DSMZ medium 569 (<http://www.dsmz.de/>) that was diluted 1:5 (569-0.2X) as a base. The composition of 569-0.2X is 0.1 g liter⁻¹ KNO₃, 0.02 g liter⁻¹ MgSO₄ · 7H₂O, 0.002 g liter⁻¹ CaCl₂ · 2H₂O, 0.023 g liter⁻¹ Na₂HPO₄, 0.007 g liter⁻¹ NaH₂PO₄, 0.1 mg liter⁻¹ FeSO₄ · 7H₂O, 0.5 µg liter⁻¹ CuSO₄ · 5H₂O, 1.0 µg liter⁻¹ H₃BO₃, 1.0 µg liter⁻¹ MnSO₄ · 5H₂O, 7 µg liter⁻¹ ZnSO₄ · 7H₂O, 1 µg liter⁻¹ MoO₃. The medium was adjusted to pH 5.0. Samples were incubated under a methane (0.1 to 10%)/air atmosphere at 10°C in serum bottles with aluminum-paper covers placed in closed glass desiccators. Alternatively, 569-0.2X was supplemented with 0.5% methanol or 0.15% formaldehyde or 0.25% methanol and 0.075% formaldehyde and incubation was carried out in an air atmosphere at the same temperature. The optimal concentrations of methanol and formaldehyde in the media were determined in preliminary experiments testing a range between 0.05 and 1%. Selection in the presence of ampicillin (10 µg ml⁻¹) was performed by incubating samples in 596-0.2X containing 0.5% methanol in an air atmosphere at 10°C. Solid 596-0.2X media containing 0.5% methanol or 0.15% formaldehyde were prepared by adding 0.8% agarose.

Analytical procedures. The physical-chemical parameters (temperature; pH; specific conductance; hardness; alkalinity; ammonia, nitrite, chloride, calcium, magnesium, and iron concentrations; salinity; and oxygen consumption) of the artesian well water were assayed by standard procedures (<http://www.epa.gov/waterscience/methods/>).

To evaluate methane uptake, almost-pure cultures of *C. fusca* (about 100 mg, dry weight) grown in 100 ml of 596-0.2X containing 0.15% formaldehyde were centrifuged, washed with 596-0.2X without added carbon sources, gently resuspended in 100 ml of the same medium, and incubated at 10°C in flasks under a methane (0.45%)/air atmosphere (headspace of 150 ml) at 10°C for 0 to 72 h. Flasks without bacteria were used as a control. Methane consumption was measured in headspace samples by using a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector and a Chrompack PLOT fused-silica capillary column (length, 50 m; inside diameter, 0.53 mm; film thickness, 10 µm) coated with Al₂O₃/KCl, according to standard methods (43).

Assimilation of [¹⁴C]methane into cellular compounds. Almost-pure *C. fusca* cultures (about 100 mg, dry weight) were incubated in 100 ml of 569-0.2X in the presence of 100 µCi [¹⁴C]methane (1 mCi mmol⁻¹) (American Radiolabeled Chemicals, Inc.) in a methane (about 1.5%)/air atmosphere (headspace of 150 ml) at 10°C. Incubation was stopped after 0, 24, and 72 h. Cells were collected by centrifugation, washed with 0.9% NaCl, resuspended in phosphate buffer (pH 7.4) and then homogenized in a glass Potter-Elvehjem homogenizer with a Teflon pestle attached by rubber tubing to a constant-speed stirring motor (19).

For protein analysis, the homogenate was subjected to two cycles of freezing in liquid nitrogen and thawing, incubated in the presence of 3% sodium dodecyl sulfate for 30 min at 37°C, and then centrifuged at 10,000 × g for 10 min at 4°C. Supernatant was recovered and proteins were purified as described previously (44). The protein concentration was assayed according to Lowry's procedure (22). Total lipids were extracted according to the modified Bligh and Dyer method (2) as reported previously (46). Labeled neutral or polar lipids, were separated by thin-layer chromatography and stained with iodine vapors as described previously (33). Regions of thin-layer chromatography corresponding to the different classes of lipids were scraped with a scalpel and analyzed for radioactivity. The radioactivity in each fraction was measured on a scintillation analyzer (Packard Tri-carb 1900TR) using a scintillation cocktail for aqueous and nonaqueous samples (PerkinElmer). As a control, heat-killed *C. fusca* cells were used.

DNA procedures. To achieve maximum extraction of DNA from the total microbial community obstructing the filter or from enriched microbial fractions, samples (10 mg, dry weight) were subjected to two cycles of freezing in liquid nitrogen and thawing at 55°C. Homogenates were suspended in 1.7 ml SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris-Cl [pH 7.5]), processed with the Potter-Elvehjem system, and incubated in the presence of 0.5 mg/ml proteinase K and 1% sodium dodecyl sulfate for 2 h at 55°C. Genomic DNA was then purified as previously described (41).

Primers and PCR conditions are listed in Table 1. All PCRs were performed as follows: initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 45 s, annealing for 30 s at the indicated temperature, and extension at 72°C for 1 to 2 min, depending on the amplicon length. They were carried out in a Perkin-Elmer Cetus DNA thermal cycler 2400.

To perform PCR single-strand conformational polymorphism (PCR-SSCP) experiments, PCR products were purified by using a High Pure PCR product purification kit (Boehringer Mannheim), denatured, and resolved on 10% polyacrylamide gel (acrylamide/*N,N*-methylenebisacrylamide, 49:1) in 0.8× TBE (72 mM Tris-borate, 1.6 mM EDTA) containing 5% glycerol (35). Bands identified after silver staining were excised with razorblades and single-strand DNAs were eluted from the gel by using a Qiaex II DNA purification kit (QIAGEN). The resulting DNAs were reamplified, cloned into pGEM-T Easy vector by using a TA cloning kit (Promega), and finally sequenced using primers M13F and M13R as a service of MWG Biotech.

For restriction fragment length polymorphism (RFLP) analysis, the PCR products generated by 16S-specific primers Com1-F and Com2-R were cloned into pGEM-T Easy vector to generate a library. Two hundred clones were screened by RFLP analysis with either EcoRI or HinfI on 6% polyacrylamide gel (35). The different clones were then grouped on the basis of their restriction profiles, and for each group, five representative clones were sequenced.

DNA similarity searches were carried out using BLAST at NCBI (<http://www.ncbi.nlm.nih.gov/>). Sequence alignments were performed with ClustalW at EBI (<http://www.ebi.ac.uk/>). Phylogenetic analyses were conducted using neighbor-joining with the MEGA 3.1 program (17). Bootstrap analysis (34) was used to estimate the reliability of phylogenetic reconstructions (1,000 replicates).

FIG. 1. Optical microscopy and culture-independent analysis of the microbial community obstructing the filter. (A to C) Gram stains of samples collected in December 2005, January 2006, and March 2006. The arrows indicate filamentous microorganisms resembling *C. polyspora* or *C. fusca*. The white bars represent 10 μm . (D to F) PCR-SSCP analysis of the microbial community. (D) PCR-SSCP profile of total sample (lanes 1 and 2) and filter-passing (lane 3) or filter-retained (lane 4) fractions with 16S rRNA gene-specific primers. The sample was collected in November 2005. (E and F) PCR-SSCP profile of filter-retained fractions with 16S rRNA gene-specific or *pmoA*-specific primers. The samples were collected in the period from November 2005 to March 2006 (lanes 1 to 5). In panels D to F, the arrows indicate specific bands (a, a', b, b', c, c', d, d', e, and e') that were excised from the gel, eluted, and subjected to nucleotide sequence analysis. The lines indicate bands that were not characterized at the molecular level.

Light, fluorescence, and electron microscopy. For light and fluorescence microscopy, a Leica DMLB microscope and a Nikon Optiphot-2 fluorescence microscope were used, respectively. Fluorescence staining was performed with diamidino-2-phenylindole (DAPI) or the lipophilic dye FM4-64 (Molecular Probes, Invitrogen).

For transmission electron microscopy, samples were fixed with 2% glutaraldehyde and 1% formaldehyde in 0.04 M piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) buffer at pH 7 for 2 h at room temperature. The samples were rinsed in 0.08 M PIPES buffer and twice in 0.08 M Na-cacodylate buffer and postfixed in 1% OsO_4 in 0.08 M Na-cacodylate buffer, pH 6.7, overnight at 4°C. Following dehydration in a step gradient of ethanol with three exchanges of anhydrous ethanol and one exchange of propylene oxide at 4°C, the samples were slowly infiltrated with Epon 912 resins at 4°C, transferred to polypropylene dishes and incubated at 70°C for 24 h. Thin sections were stained with 3% uranyl acetate in 50% methanol for 15 min and in Reynold's lead citrate for 10 min and, finally, observed with a Leo 912AB electron microscope.

Nucleotide sequence accession numbers. Nucleotide sequences determined in this study were deposited at GenBank under accession numbers DQ984190, partial 16S rRNA gene from *C. fusca* AW-b; DQ984191, partial 16S rRNA gene from *Crenothrix* sp. AW-a; DQ984192, partial *pmoA* gene from *C. fusca* AW-b; DQ984193, partial *u-pmoA* gene from *Crenothrix* sp. AW-a; DQ984194, partial

pmoA gene from uncultured bacterium cAW-d; DQ984195, partial *pmoA* gene from uncultured bacterium cAW-e; DQ984196, partial *pmoA* gene from uncultured bacterium cAW-f; EF530351, partial *mxhF* gene from *C. fusca* AW-b; and EF530352, partial *mxhF* gene from uncultured bacterium cAW-h. The nucleotide sequences of partial or almost entire 16S rRNA, *pmoA*, and *mxhF* genes are reported in Tables S2 to S4 of the supplemental material.

RESULTS

Detection of *C. polyspora* and *C. fusca* by optical microscopy.

The microbial community obstructing a filter of an artesian well water pumping system in a farm in Salento (Apulia, Italy) was studied over a period of 1 year (November 2005 to October 2006). The succession of two filamentous, sheathed microorganisms was observed by analyzing the microbial community by optical microscopy during this time. Tentative identification of these two microorganisms as *C. polyspora* and *C. fusca* was performed by macro- and micromorphological criteria accord-

TABLE 2. Similarities between 16S rRNA gene sequences from the artesian well and the most similar database sequences

Clone or isolate	Closest relative(s)	GenBank accession no.	Similarity(ies) (%)
AWS-a/a	<i>Crenothrix polyspora</i> clone 6	DQ295898.1	97.69
AWS-b/b	<i>Methylobacter tundripaludum</i> SV96, <i>Methylobacter psychrophilus</i> Z-0021	AJ414655.1, AF152597.1	94.82, 94.23
AWR-1	Uncultured bacterium clone AAE6, <i>Niastella jeongjuensis</i>	AY692239.1, DQ244076.1	97.04, 95.76
AWR-2	Uncultured organism clone D129, <i>Methylophilus methylotrophus</i>	AY707601.1, AB193724.1	99.76, 98.78
AWR-3	<i>Flavobacterium</i> sp. strain EP235, <i>Flavobacterium succinicans</i>	AF493641.1, AM230493.1	100.00, 99.46
AWR-4	<i>Flavobacterium</i> sp. strain Tibet-S721, <i>Flavobacterium columnare</i>	DQ177492.1, AY747592.1	100.00, 99.73
AWR-5	<i>Bacillus cereus</i>	DQ234855.1	100.00

ing to Kolk (16) and Bergey's Manual (14), the physiological properties of these microorganisms being largely unknown.

C. polyspora exhibited its typical habitus, consisting of long, unbranched, sheathed clusters of cell filaments with brownish coloration at the cluster's basal area. The filaments were up to 1 cm long, about 2 to 4.0 μm wide, did not taper to the tip, often appeared attached to a mass substrate, and contained disk-shaped to cylindrical cells that reacted negatively to the Gram stain and exhibited variation in size (Fig. 1A). These cells were frequently divided by longitudinal or oblique septa leading to formation of microgonidia (small spherical cells, possibly spores) at the filament tips (Fig. 1A), a pattern typical of these kinds of bacteria. *C. fusca* formed filaments of about the same width and length as *C. polyspora*, attached or free, surrounded by a more or less distinct sheath that may be encrusted (possibly with iron or manganese oxides), giving a yellowish-brown color. At variance with those of *C. polyspora*, *C. fusca* filaments had the tendency to taper to the tip and contained gram-negative rod-shaped cells divided by cross-septation with considerable variation in size, up to 20 μm in length (Fig. 1A to C). Microgonidia were never observed, whereas massive "acropetal" production of macrogonidia (large spherical cells, possibly spores) was frequently detected at the tips of longer filaments as carefully described by Kolk (16) (Fig. 1C).

In our samples, *C. polyspora* was largely predominant during the first month (November 2005) (data not shown) and coexisted with *C. fusca* during the next three months (December 2005 to February 2006) (Fig. 1A and B), and then it almost disappeared, and *C. fusca* became prevalent (March to May 2006) (Fig. 1C). During the period from June to October 2006, the abundance of both microorganisms progressively decreased. In September to October 2006, luxuriant growth of *C. polyspora* causing severe obstruction of the filter was observed (data not shown). The available data of the standard water quality parameters suggested a possible correlation between the *C. polyspora* overgrowth and an increase of iron, calcium, magnesium, ammonia, and nitrite levels in the water, with a moderate decrease of pH values (see Table S1 in the supplemental material). At a depth of 54 m, where sampling was carried out, the water temperature, 18.5°C, was very close to the annual average temperature, slightly under 20°C.

Culture-independent analysis of the microbial community. The microbial community obstructing the filter was then analyzed by culture-independent methods. For this purpose, the DNA of the microorganisms was amplified using the bacterium-specific primers Com1-F and Com2-R (Table 1). These primers targeted a 409-nucleotide-long (in *Escherichia coli*) central region of the prokaryotic small-subunit rRNA gene (16S rRNA

gene) and allowed high taxonomic resolution, often at the species level (18). The November 2005 sample was analyzed first. The SSCP profile of the amplified 16S rRNA gene pool revealed a relatively low degree of complexity of the microbial community, with a few species being predominant (Fig. 1D, lanes 1 and 2). The profile was further simplified when the starting sample was first passed through a 100- μm pore size fine-meshed sieve to retain the filamentous mass (Fig. 1D, lane 4) and eliminate microorganisms that were not arranged in long filaments (Fig. 1D, lane 3). The SSCP profile of the enriched sample was dominated by the presence of two major bands (a and a') (Fig. 1D, lane 4) that were excised from the gel, eluted, cloned, and subjected to nucleotide sequence analysis. To avoid any bias due to the reamplification step, only PCR products showing the same SSCP profile as the original bands were further processed. The sequence corresponding to bands a/a' exhibited the highest level of similarity (97.69% identity) with sequences published by Stoecker and coworkers (39) and attributed to *C. polyspora* Cohn (Table 2), suggesting that the filamentous microorganism in our sample might belong to the same genus as Cohn's *Crenothrix*.

Due to the inability to recover the other SSCP bands efficiently, the identities of the other microorganisms that did not form long filaments (Fig. 1D, lane 3) were determined by a different approach. The PCR products generated by the bacterium-specific primers targeting the total DNA from the filterable fraction were cloned to construct a library. Two hundred clones gave different RFLP profiles, and five clones representative of each profile were subjected to sequence analysis (Table 2). This approach led us to identify two microorganisms, AWR-3 and AWR-4, belonging to the genus *Flavobacterium*, closely allied to the species *F. succinicans* and *F. columnare*, respectively; an unknown microorganism, AWR-1, grouping with *Niastella jeongjuensis* of the *Bacteroidetes/Chlorobi* group; a β -proteobacterium, AWR-2, close to *Methylophilus methylotrophus*, a methylotrophic β -proteobacterium; and a *Bacillus* sp. strain isolate, AWR-5, close to *B. cereus*. *Flavobacterium* sp. strains AWR-3 and AWR-4 and *Bacillus* sp. strain AWR-5 were obtained in pure cultures.

The other samples, from December 2005 to March 2006, were then analyzed by PCR-SSCP. To recover the filamentous mass and to eliminate microorganisms that were not arranged in long filaments, the samples were filtered as above. The SSCP profiles of the amplified 16S rRNA gene pool showed a succession of the microbial community (Fig. 1E). During the last month (March 2006), the fingerprinting corresponding to *Crenothrix* (Fig. 1D, lane 4, and Fig. 1E, lane 1) disappeared, and two major bands (b and b') became predominant (Fig. 1E, lane 5). These bands were subjected to nucleotide sequence analysis

as described above. The results of this analysis suggested that the filamentous microorganism that was prevalent in this sample (March 2006), tentatively identified as *C. fusca* on the basis of its micromorphology (Fig. 1A to C), was a member of the type I methanotrophic γ -proteobacteria distinct from *Crenothrix* and from any other known member of this group of prokaryotes. Among the cultivated species, the psychrophilic methanotrophs *Methylobacter tundripaludum* SV96 and *Methylobacter psychrophilus* Z-0021 (3) exhibited the highest similarities, with sequence identities of 94.82% and 94.23%, respectively (Table 2). The levels of similarity were too low to assign our microorganism to any known genus of the methanotrophs.

To substantiate with further molecular data the idea that Roze's *Clonothrix* might be a type I methanotroph, we looked for the presence of the particulate methane mono-oxygenase (pMMO) α subunit-encoding gene *pmoA* in the filter-retained fraction of our samples by using PCR-SSCP (Fig. 1F). MMO is responsible for the initial transformation of methane into methanol. MMO exists in two forms, a cytoplasmic, soluble form and the membrane-bound form pMMO. Soluble MMO is found in only a subset of methanotrophs, whereas pMMO is a marker of most methanotrophs (7, 20). Using the primer pair *pmof1*/*pmor*, designed to amplify evolutionarily conserved regions of *pmoA* (4), we observed specific SSCP bands in all samples, from November 2005 to March 2006 (Fig. 1F, lanes 1 to 5). As seen with the 16S rRNA genes, the SSCP profile of the amplified *pmoA* sequences demonstrated a succession, with four major bands predominating in the November 2005 sample (d, d', e, and e') and two different bands (c and c') predominating in the March 2006 sample. We deduced that these two bands might correspond to the two filaments encoded by the putative *C. fusca pmoA* gene, while the four bands in the sample of November 2005 might correspond to *pmoA* sequences of contaminating methanotrophs. Bands c and c' were excised from the gel and subjected to nucleotide sequence analysis. The results confirmed that c and c' represented the two cDNA strands of an unknown *pmoA* gene. Among characterized *pmoA* genes, that of *Methylosarcina fibrata* exhibited the highest similarity (91.59% identity at the level of deduced amino acid sequence). The other bands were similarly characterized. d and d' and e and e' corresponded, respectively, to the two strands of two *pmoA* genes from bacteria phylogenetically close to *Methylomicrobium buryatense* 5B (94.44% identity at the level of deduced amino acid sequence) and to *Methylomonas methanica* S1 (96.30% identity at the level of deduced amino acid sequence).

Enrichment media for *C. fusca*. The results of the 16S rRNA gene and *pmoA* sequence analyses prompted us to hypothesize that Roze's *Clonothrix* might be a methane oxidizer physiologically close to the psychrophilic type I methanotrophs. On the basis of this hypothesis, we attempted to enrich the starting sample (January 2006) in *C. fusca* by a three-step procedure. The three steps were (i) enrichment of the starting sample in a diluted medium for type I methanotrophs (596-0.2X) without added carbon sources, under a methane (10%)/air atmosphere at 10°C for 2 weeks in the dark; (ii) further selection in the presence of ampicillin in 596-0.2X medium containing methanol, in an air atmosphere for 24 h at the same temperature; and (iii) incubation in 596-0.2X containing methanol, formaldehyde, or both, in an air atmosphere at 10°C for 2 weeks in the

dark. Methanol and, in some cases, formaldehyde, formate, and methylamines may be utilized as C₁ carbon (and energy) sources by methanotrophic microorganisms (3). However, as formaldehyde is a highly reactive molecule and a powerful poison for living systems (9), the optimal concentrations of formaldehyde and methanol (that generates formaldehyde) in the media were determined in preliminary experiments testing a range between 0.05 and 1%. The ampicillin treatment was effective in eliminating the rapid-growing nonfilamentous microorganisms in the starting sample.

In the media containing methanol, formaldehyde, or both, *C. fusca* was able to grow at extremely low rates, as deduced from the elongation and thickening of single inoculated filaments and from the increase in dry weight. In the medium containing formaldehyde, growth rates of about 0.01 h⁻¹ were estimated. In the media with either methanol or methanol and formaldehyde, growth rates could not be evaluated by dry weight, because rapid-growing bacteria had a selective advantage and outgrew *C. fusca*. In contrast, an almost pure culture of *C. fusca* could be obtained in 596-0.2X containing 0.15% formaldehyde, as determined by Gram staining (see Fig. S1A to D in the supplemental material), plating on complex organic media (nutrient agar) to exclude the presence of heterotrophic contaminants, and PCR-SSCP profiles (see Fig. S2 in the supplemental material). In the medium with formaldehyde, *C. fusca* filaments nucleating at the base and sporulating at the tip (see Fig. S1A to D in the supplemental material) were observed, in addition to spores and younger, short, thinner filaments often lacking a distinct sheath and exhibiting false-branching (data not shown), such as were carefully described by Kolk (16). The filaments were also observed by fluorescence microscopy after being stained with the membrane-labeling dye FM4-64 (see Fig. S1E in the supplemental material) or with DAPI (see Fig. S1F in the supplemental material). The DAPI staining results were suggestive of the presence of multiple nucleoids within each larger cell.

However, *C. fusca* did not grow on 596-0.2X containing agar with methane, methanol, or formaldehyde. The inability to grow on agar is a common feature of several methanotrophs (3). *C. fusca* also failed to grow in the medium 596-0.2X containing 0.5% glucose. This result was consistent with the taxonomic position of this microorganism. Indeed, all type I methanotrophs that have been isolated to date are able to utilize only reduced C₁ substrates (11, 13). In all tested media, *Crenothrix* was progressively lost.

Methane uptake and assimilation of [¹⁴C]methane into cellular compounds. To obtain direct evidence of the ability of *C. fusca* to utilize methane, uptake and assimilation studies were carried out, utilizing cultures enriched in formaldehyde medium. After removing formaldehyde, methane uptake was evaluated in 596-0.2X medium under a methane (0.45%)/air atmosphere at 10°C. The measurements, made at various intervals (0, 24, 48, and 72 h) with triplicate samples, demonstrated that methane consumption occurred as a function of time (Fig. 2A). A decrease of about 27% in methane concentration was observed during the first 24 h, when methane uptake was maximal (about 5.4 $\mu\text{mol g}^{-1}$ [dry weight] h⁻¹).

The assimilation of [¹⁴C]methane into cellular compounds was then analyzed. In these experiments, *C. fusca* cultures were incubated in 596-0.2X under a [¹⁴C]methane (about 1.5%)/air

FIG. 2. Methane uptake by *C. fusca* cultures and assimilation of [¹⁴C]methane or [¹⁴C]methanol into cellular compounds. (A) The methane uptake was estimated in triplicate samples. (B) ¹⁴C assimilation from [¹⁴C]methane into indicated lipids. The values are expressed as nmol g⁻¹ (dry weight). (C) ¹⁴C assimilation from [¹⁴C]methane into proteins. The values are expressed as nmol g⁻¹ (protein). In all graphs, the values represent means with standard deviations (bars).

atmosphere. ¹⁴C distribution into total lipids, phospholipids, neutral lipids (Fig. 2B), or proteins (Fig. 2C) was evaluated in triplicate samples at different incubation times (0, 24, and 72 h). The results demonstrated that incorporation of ¹⁴C in all macromolecules occurred as a function of time, and it was maximal during the first 24 h, consistent with the methane uptake data (Fig. 2A).

Ultrastructural analysis of *C. fusca* Roze. Transmission electron microscopy analysis of *C. fusca* from enrichment medium containing methane demonstrated multilayered sheaths surrounding cell filaments consisting of both granular and fibrillar material (Fig. 3A to D). The architecture of the cell wall was typical of that of gram-negative bacteria (Fig. 3B to D). The vegetative cells (Fig. 3A) exhibited a bacillar shape and were, in our microphotographs, about 1.0 μ m wide and 2.5 μ m long (without sheaths), although larger elements could be observed. They contained an elaborate membrane system in the cytoplasm, in the form of lamellar stacks arranged perpendicularly to the main cell axis and lining the periphery of the cytoplasm. At higher magnifications, the membrane system consisted of closely packed, swollen sacks (Fig. 3B and C). This membrane system resembles that of type I methanotrophs, and seems to be different from that of *C. polyspora*, which is characterized by flattened sacks (39, 42). Electron-dense storage granules resembling polyphosphate granules and large electron-transparent organelles delimited by membranes and resembling gas vesicles were also found (Fig. 3C). The role of these organelles is obscure; their close association with the lamellar stacks arranged perpendicularly to the membrane is noteworthy (Fig. 3C). Indeed, gas vesicles were also observed in *M. psychrophilus*, the psychrophilic methanotroph closely allied to *C. fusca* (27). Occasionally, slightly smaller cells arranged in unsheathed filaments with the above-mentioned features were observed (Fig. 3D to F). This finding may correspond to contaminant methanotrophs (for instance, to the uncultured bacterium cAW-f) (Fig. 4B and C, and see Fig. S2 in the supplemental material) or to younger, short, thinner filaments of *C. fusca* lacking a distinct sheath.

Phylogeny of *C. fusca* Roze. Using *C. fusca* cultures in formaldehyde medium as a source of DNA, almost the entire 16S

rRNA-encoding gene was amplified by using the proteobacteria-specific primers 16SEB20-42-F and 16SEB1488-R (Table 1) and was cloned into a multicopy plasmid. Three of these clones were subjected to nucleotide sequencing that gave identical results. Phylogenetic analysis demonstrated the taxonomic position of our isolate in a subgroup of type I methanotrophs including *C. polyspora* and the psychrophilic methanotrophs *M. tundripaludum* and *M. psychrophilus* (Fig. 4A). Almost the entire 16S rRNA gene sequence of the *Crenothrix* sp. which was predominant during the first month (November 2005) was then determined by using the sample fraction enriched in filaments as a source of DNA (Fig. 1D, lane 4). The results confirmed the taxonomic position of our *Crenothrix* in a clade including the other *C. polyspora* Cohn clone sequences from Stoecker and coworkers' study (39) (Fig. 4A). However, if we consider the phylogenetic distances, we may hypothesize that our and Stoecker and coworkers' microorganisms may belong to the same genus but to different species.

The DNA from *C. fusca* cultures in formaldehyde medium was then used to amplify *pmoA* by using the above-mentioned primer pair (4). We could observe a specific PCR product of the expected size that was subjected to direct DNA sequencing or to DNA cloning and sequencing. Both approaches gave identical sequences, corresponding to those of the c and c' PCR-SSCP bands (Fig. 1F). Phylogenetic analysis confirmed the presence in *C. fusca* of a *pmoA* gene typical of type I methanotrophs (Fig. 4B). The phylogenetic analysis also included the *pmoA* sequences from bands d/d' (uncultured bacterium cAW-d) and e/e' (uncultured bacterium cAW-e) of Fig. 1F.

Stoecker and coworkers' study (39) described the presence in *C. polyspora* Cohn-enriched material of a very unusual *pMMO* gene (*u-pmoA*). Thus, we looked for the presence of this gene in *C. fusca* cultures by PCR using a *u-pmoA*-specific primer pair. However, we did not obtain any specific PCR product. In contrast, we were able to amplify *u-pmoA*-specific DNA sequences when the starting sample (January 2006) was used as the DNA template. *u-pmoA*-specific PCR products were cloned and subjected to nucleotide sequencing. This analysis confirmed the presence of the *u-pmoA* gene in the unenriched sample, exhibiting

FIG. 3. Transmission electron microscopy of *C. fusca* filaments. (A to C) Longitudinal sections of *C. fusca* filaments at different magnifications. The bars represent 1 μm in panel A and 0.5 μm in panels B and C. Note in panel B (i) the thick, multilayered sheath; (ii) the gram-negative type of cell wall; and (iii) the membrane system in the cytoplasm, consisting of closely packed, swollen sacks arranged perpendicularly to the main axis of the cell. In the filament shown in panel C, the lamellar stacks are arranged perpendicularly to the membrane of a large, electron-transparent organelle associated with electron-dense granules. (D) Cross section of *C. fusca* filaments. The bar represents 0.5 μm . Note the circular shape and thickness of the sheath. (E to G) Longitudinal sections at different magnifications of unsheathed microorganisms arranged in chains. The bars represent 2 μm in panel E, 0.5 μm in panel F, and 0.2 μm in panel G.

high similarity (95.23% identity at the level of the nucleotide sequence; 97.14% identity at the level of the deduced amino acid sequence) with that of *C. polyspora* (Fig. 4B).

The presence of *mxoF*, a marker gene of both methanotrophs and methylotrophs, encoding the α subunit of the methanol dehydrogenase (24), was also investigated in *C. fusca* cultures. The primer pair *mxoFf* and *mxoFr* (Table 1) amplified a specific PCR product that was subjected to sequencing. Phylogenetic analysis demonstrated that the *C. fusca* methanol dehydrogenase was typical of that of type I methanotrophs (Fig. 4C).

DISCUSSION

In this study, we provide evidence, on the basis of physiological, ultrastructural, and molecular data (Fig. 2 to 4), that *C.*

fusca Roze is a filamentous, sheathed, methane-oxidizing γ -proteobacterium distinct from *C. polyspora* Cohn. Both of these microorganisms were found in oligotrophic groundwater environments, often growing attached to the same mass substrate (Fig. 1). Such a biocenosis was described at the beginning of the past century in a number of studies carefully addressing the morphologies, life cycles, and ecologies of the two microorganisms (16, 21, 25). In other studies, the succession of *C. fusca* and *C. polyspora* was reported. In the summers of 1934, 1935, and 1936, *C. fusca* appeared in the water supplied from the house tap in a section of Brooklyn in sufficiently large dark brown clumps of filaments to be visible to the eye. In December of 1936, both *C. fusca* and *C. polyspora* appeared in great quantity during a period of unseasonably warm weather

(16). Although our data suggest a possible correlation between several physical-chemical water parameters and *C. polyspora* overgrowth (see Table S1 in the supplemental material), understanding the physiologies of these two microorganisms will help to define the role of environmental factors in this microbial succession.

The 16S rRNA gene sequence-based phylogenetic analysis places both *C. fusca* and *C. polyspora* within a subgroup of psychrophilic type I methanotrophs also including *M. tundripaludum* and *M. psychrophilus* (Fig. 4A). In addition to molecular phylogeny, an additional element that both these psychrophilic methanotrophs and *C. fusca* share is the presence of large electron-transparent organelles delimited by membranes and resembling gas vesicles (Fig. 3C).

The results of phylogenetic analysis based on PmoA/AmoA sequences are not entirely consistent with this view. Indeed, the putative *C. fusca* PmoA sequence is more similar to those of *Methylosarcina* spp. than to those of the above-mentioned psychrophilic methanotrophs (Fig. 4B). This result confirms that the molecular evolution of this enzyme is very complex and does not simply reflect the 16S rRNA phylogeny. The very unusual u-PmoA of *C. polyspora* (39), the two divergent families of PmoA proteins (PmoA1 and PmoA2) coexisting in several type II methanotrophs (40), and the AmoA of *Nitrosococcus oceanus* that is much more similar to PmoA of the methane oxidizers than to the other AmoAs of ammonia-oxidizing bacteria (1) well exemplify this concept (Fig. 4B).

C. fusca filaments were able to grow at extremely low rates in a diluted medium for type I methanotrophs (596-0.2X) using methanol or formaldehyde as the sole carbon source (see Fig. S1 in the supplemental material). Although in this study we did not try to propagate *C. fusca* from macrogonidia (spores), and we did not estimate the actual growth rates of *C. fusca* in its natural environment, our data suggest that this may be a slow-growing microorganism adapted to oligotrophic aquatic environments. This belief is supported by the inability of *C. fusca* to grow in 596-0.2X-glucose, undiluted 569, or complex organic media.

In the diluted medium for methanotrophs, *C. polyspora* failed to grow and was progressively lost. We do not know the reason why this occurred, but it may be relevant to note that this microorganism, in contrast to *C. fusca*, has a very unusual PmoA (u-PmoA), phylogenetically much more closely allied to subunits of the ammonia mono-oxygenase of the β -proteobacteria (AmoA) than to the PmoA of the γ -proteobacterial methanotrophs (39) (Fig. 4B). The substrate specificity of u-PmoA for ammonia, as well as the requirement of ammonia for the growth of *C. polyspora*, is currently unknown.

The variations in vegetative cell size, ability to sporulate, filamentous habit, and presence of encrusted sheaths are specific traits of these two microorganisms. It is reasonable to assume that these traits represent successful strategies to counteract the disadvantage of being slow growing. Regulation of cell size, filamentous habit, and presence of sheaths may be effective against predation by bacterivorous protozoans (6, 8, 10, 15, 23, 28, 37). In movie S1 in the supplemental material, we show how these concepts might apply to the environment where *C. fusca* was isolated during our study, which is characterized by strong protozoan grazing pressure.

In conclusion, after more than a century, the mystery of

Cohn's *Crenothrix* and Roze's *Clonothrix* begins to be solved with the unraveling of surprising aspects that improve our knowledge of the phylogeny of filamentous, sheathed bacteria, encouraging the study of these microorganisms as a model for differentiation in prokaryotes.

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REFERENCES

1. Bedard, C., and R. Knowles. 1989. Physiology, biochemistry, and specific inhibitors of CH₄, NH₄⁺, and CO oxidation by methanotrophs and nitrifiers. *Microbiol. Rev.* **53**:68–84.
2. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**:911–917.
3. Bowman, J. 20 January 2000, posting date. The methanotrophs—the families *Methylococcaceae* and *Methylocystaceae*. In M. Dworkin et al. (ed.), *The prokaryotes: an evolving electronic resource for the microbiological community*, 3rd ed., release 3.1. Springer, New York, NY. <http://link.springer-ny.com/link/service/books/10125/>.
4. Cheng, Y. S., J. L. Halsey, K. A. Fode, C. C. Remsen, and M. L. Collins. 1999. Detection of methanotrophs in groundwater by PCR. *Appl. Environ. Microbiol.* **65**:648–651.
5. Cohn, F. 1870. Über den Brunnenfaden (*Crenothrix polyspora*) mit Bemerkungen fiber die mikroskopische Analyse des Brunnenwassers. *Beitr. Biol. Pflanz.* **1**:108–131.
6. Corno, G., and K. Jürgens. 2006. Direct and indirect effects of protist predation on population size structure of a bacterial strain with high phenotypic plasticity. *Appl. Environ. Microbiol.* **72**:78–86.
7. Dedys, S. N., W. Liesack, V. N. Khmelenina, N. E. Suzina, Y. A. Trotsenko, J. D. Semrau, A. M. Bares, N. S. Panikov, and J. M. Tiedje. 2000. *Methylocella palustris* gen. nov., sp. nov., a new methane-oxidizing acidophilic bacterium from peat bogs, representing a novel subtype of serine-pathway methanotrophs. *Int. J. Syst. Evol. Microbiol.* **50**:955–969.
8. Gonzalez, J. M., E. B. Sherr, and B. F. Sherr. 1990. Size-selective grazing on bacteria by natural assemblages of estuarine flagellates and ciliates. *Appl. Environ. Microbiol.* **56**:583–589.
9. Grafstrom, R. C., A. J. Fornace, Jr., H. Autrup, J. F. Lechner, and C. C. Harris. 1983. Formaldehyde damage to DNA and inhibition of DNA repair in human bronchial cells. *Science* **220**:216–218.
10. Hahn, M. W., E. R. Moore, and M. G. Höffle. 1999. Bacterial filament formation, a defense mechanism against flagellate grazing, is growth rate controlled in bacteria of different phyla. *Appl. Environ. Microbiol.* **65**:25–35.
11. Hanson, R. S., and T. E. Hanson. 1996. Methanotrophic bacteria. *Microbiol. Rev.* **60**:439–471.
12. Harder, E. 1919. Iron-depositing bacteria and their geologic relations. U.S. Geological Survey Professional Paper 113. U.S. Geological Survey, Washington, DC.
13. Higgins, I. J., D. J. Best, R. C. Hammond, and D. Scott. 1981. Methane-oxidizing microorganisms. *Microbiol. Rev.* **45**:556–590.
14. Holt, J. G., P. Hirsch, and E. G. Mulder. 1989. Sheathed bacteria, p. 1994–2009. In J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, 1st ed., vol. 3. Williams & Wilkins, Baltimore, MD.
15. Jürgens, K., J. Pernthaler, S. Schalla, and R. Amann. 1999. Morphological and compositional changes in a planktonic bacterial community in response to enhanced protozoan grazing. *Appl. Environ. Microbiol.* **65**:1241–1250.
16. Kolk, L. A. 1938. A comparison of the filamentous iron organisms, *Clonothrix fusca* Roze and *Crenothrix polyspora* Cohn. *Am. J. Bot.* **25**:11–17.
17. Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform.* **5**:150–163.
18. Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace. 1985. Determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc. Natl. Acad. Sci. USA* **82**:6955–6959.
19. Levine, M., and F. C. Miller. 1996. An *Eikenella corrodens* toxin detected by plaque toxin-neutralizing monoclonal antibodies. *Infect. Immun.* **64**:1672–1678.
20. Lieberman, R. L., and A. C. Rosenzweig. 2004. Biological methane oxidation: regulation, biochemistry, and active site structure of particulate methane monooxygenase. *Crit. Rev. Biochem. Mol. Biol.* **39**:147–164.
21. Lieske, R. 1922. Bakterien und Strahlenpilze, p. 48–52. In K. Linsbauer (ed.), *Handbuch der Pflanzenanatomie*, vol. 6. Gebrüder Borntraeger, Berlin, Germany.

22. Lowry, O. H., N. J. Roseborough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
23. Matz, C., and K. Jürgens. 2003. Interaction of nutrient limitation and protozoan grazing determines the phenotypic structure of a bacterial community. *Microb. Ecol.* **45**:384–398.
24. McDonald, I. R., and J. C. Murrell. 1997. The methanol dehydrogenase structural gene *mxhF* and its use as a functional gene probe for methanotrophs and methylotrophs. *Appl. Environ. Microbiol.* **63**:3218–3224.
25. Molisch, H. 1910. Die Eisenbakterien. Gustav Fischer, Jena, Germany.
26. Nauman, E. 1921. Untersuchungen über die Eisenorganismen Schwedens. *Kungl. Sv. Vetenskapsakad. Handl.* **62**:1–68.
27. Omelchenko, M. V., L. V. Vasileva, G. A. Zavarzin, N. D. Saveleva, A. M. Lysenko, L. L. Mityushina, V. N. Khmelenina, and Y. A. Trotsenko. 1996. A novel psychrophilic methanotroph of the genus *Methylobacter*. *Microbiologiya* **65**:339–343.
28. Pernthaler, J., E. Zollner, F. Warnecke, and K. Jürgens. 2004. Bloom of filamentous bacteria in a mesotrophic lake: identity and potential controlling mechanism. *Appl. Environ. Microbiol.* **70**:6272–6281.
29. Roze, E. 1896. Le *Clonothrix*, un nouveau type generique de Cyanophycees. *J. Bot.* **10**:325–330.
30. Rullman, W. 1907. Photogramme von *Crenothrix polyspora* Cohn. *Centralbl. Bakteriol. II Abt.* **20**:97–99.
31. Rullman, W. 1912. Über Eisenbakterien. *Centralbl. Bakteriol. II Abt.* **33**:277–289.
32. Ruttner, F. 1906. Die Mikroflora der Prager Wasserleitung. *Arch. Natwiss. Landesdurchforsch. Boehmens* **13**:6.
33. Safonova, E. F., A. A. Nazarova, V. F. Selemeney, T. A. Brezhneva, and A. I. Slivkin. 2002. Selecting optimum parameters for the TLC separation of phospholipids. *Pharm. Chem. J.* **36**:206–208.
34. Salemi, M., and A.-M. Vandamme. 2003. The phylogenetic handbook: a practical approach to DNA and protein phylogeny. Cambridge University Press, Cambridge, United Kingdom.
35. Sambrook, J., and D. W. Russell. 2001. Molecular cloning. a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
36. Schweisfurth, R. 1974. *Crenothrix polyspora* Cohn als Indikator für eine organische Belastung von Grundwasser, p. 129–144. In P. Müller (ed.), *Verhandlungen der Gesellschaft für Ökologie*, Erlangen, 1974. Dr. W. Junk B. V., The Hague, Holland.
37. Simek, K., and T. H. Chrzanowski. 1992. Direct and indirect evidence of size-selective grazing on pelagic bacteria by freshwater nanoflagellates. *Appl. Environ. Microbiol.* **58**:3715–3720.
38. Smith, E. G. 1903. Growths in water mains. *Proc. Amer. Water Works Assoc.* **1903**:524–536.
39. Stoecker, K., B. Bendinger, B. Schoning, P. H. Nielsen, J. L. Nielsen, C. Baranyi, E. R. Toenshoff, H. Daims, and M. Wagner. 2006. Cohn's *Crenothrix* is a filamentous methane oxidizer with an unusual methane monooxygenase. *Proc. Natl. Acad. Sci. USA* **103**:2363–2367.
40. Tchawa Yim, M., P. F. Dunfield, P. Ricke, J. Heyer, and W. Liesack. 2003. Wide distribution of a novel *pmoA*-like gene copy among type II methanotrophs, and its expression in *Methylocystis* strain SC2. *Appl. Environ. Microbiol.* **69**:5593–5602.
41. Vigliotta, G., S. M. Tredici, F. Damiano, M. R. Montinaro, R. Pulimeno, R. di Summa, D. R. Massardo, G. V. Gnoni, and P. Alifano. 2005. Natural merodiploidy involving duplicated *rpoB* alleles affects secondary metabolism in a producer actinomycete. *Mol. Microbiol.* **55**:396–412.
42. Volker, H., R. Schweisfurth, and P. Hirsch. 1977. Morphology and ultrastructure of *Crenothrix polyspora* Cohn. *J. Bacteriol.* **131**:306–313.
43. Watanabe, T., K. Kato, N. Matsumoto, and T. Maeda. 2006. Development of a precise method for the quantitative analysis of hydrocarbons using post-column reaction capillary gas chromatography with flame ionization detection. *Chromatography* **27**:49–55.
44. Wessel, D., and U. I. Flugge. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* **138**:141–143.
45. Wolfe, R. S. 1960. Observations and studies of *Crenothrix polyspora*. *J. Am. Water Works Assoc.* **52**:915–918.
46. Zelles, L., Q. Y. Bai, T. Beck, and F. Beese. 1992. Signature fatty acid profiles of phospholipids and lipo-polysaccharids in differently cultivated soils. *Soil Biol. Biochem.* **24**:317–323.