Chloroflexus aggregans sp. nov., a Filamentous Phototrophic Bacterium Which Forms Dense Cell Aggregates by Active Gliding Movement

SATOSHI HANADA,^{1*} AKIRA HIRAISHI,² KEIZO SHIMADA,¹ AND KATSUMI MATSUURA¹

Department of Biology, Tokyo Metropolitan University, Hachioji, Tokyo 192-03,¹ and Laboratory of Environmental Biotechnology, Konishi Co., Ltd., Sumida, Tokyo 130,² Japan

Two strains of thermophilic photosynthetic bacteria, designated MD-66^T (T = type strain) and YI-9, were isolated from bacterial mats in two separate hot springs in Japan. These new isolates were phenotypically similar to *Chloroflexus aurantiacus* in some respects. They were thermophilic filamentous photosynthetic bacteria that grew well at 55°C either anaerobically as photoheterotrophs or aerobically as chemoheterotrophs. They exhibited gliding motility, produced bacteriochlorophylls *a* and *c*, contained chlorosomes, and required thiamine and folic acid as growth factors. However, isolates MD-66^T and YI-9 had the ability to rapidly form mat-like dense aggregates of filaments, an ability which has not been observed in any *C. aurantiacus* strain. Carbon source utilization tests revealed that unlike *C. aurantiacus*, the new isolates did not utilize acetate, citrate, ethanol, or glycylglycine. An analysis of the carotenoid components revealed that isolates MD-66^T and YI-9 contained mainly γ -carotene and OH- γ -carotene glucoside fatty acid esters. These isolates also contained only trace amounts of β -carotene, which is a major carotenoid component (28.4% of the total carotenoids) in *C. aurantiacus*. The results of DNA hybridization studies suggested that the new strains were genetically distinct from *C. aurantiacus* (levels of similarity, 9 to 18%), and 16S rRNA sequence comparisons showed that strain MD-66^T was related to *C. aurantiacus* at a similarity level of 92.8%. On the basis of our data, we propose that a new *Chloroflexus* species should be created for our new isolates; the name of this new species is *Chloroflexus aggregans*, and the type strain is strain MD-66 (= DSM 9485).

The first filamentous anoxygenic phototrophic bacteria were discovered by Pierson and Castenholz in thermophilic environments (25). These organisms were described as members of a new genus and species, *Chloroflexus aurantiacus*, and strain J-10-fl, which was isolated from a Japanese hot spring, was designated the type strain of this species (26). Following this description, Gorlenko and coworkers found members of two apparently related mesophilic filamentous phototrophic genera in freshwater habitats, the genera *Chloronema* (4) and "*Oscillochloris*" (9). These organisms exhibited gliding motility and contained pigment-bearing vesicles called "chlorosomes" (32). The group containing the phototrophic bacteria includes representatives of another genus, the genus *Heliothrix*, as described by Pierson et al. (29). Members of the genus *Heliothrix* have not been isolated in pure culture yet, but they are known to lack chlorosomes. The results of a 5S rRNA sequence analysis showed that *Heliothrix oregonensis* is phylogenetically related to *Chloroflexus aurantiacus*.

A phylogenetic analysis based on 16S rRNA sequence data revealed that *Chloroflexus aurantiacus* is not closely related to any other phototrophic bacteria and forms a remarkably deep branch on the common line of eubacterial descent (23). Consequently, the filamentous phototrophs are now placed in a group called the "multicellular filamentous green bacteria" (24, 36) or the "filamentous anoxygenic phototrophs" (27).

Chloroflexus strains and their relatives have been detected and observed in hot springs, freshwater lakes, and marine environments (1–3, 7, 8, 16, 19, 30, 33, 34). However, most strains have not been isolated in axenic culture, and molecular

phylogenetic data are not available for any organisms except *Chloroflexus aurantiacus*, *Heliothrix oregonensis*, and the recently described isolate *Oscillochloris trichoides* DG-6 (16, 26, 29).

We isolated several strains of thermophilic filamentous phototrophic bacteria from various hot springs in Japan by using an improved isolation method (11). Most of these new isolates were assigned to *C. aurantiacus* on the basis of morphological, physiological, and chemotaxonomic characteristics and DNA-DNA hybridization data. However, two of the isolates, designated MD-66^T (T = type strain) and YI-9, clearly differed from *Chloroflexus aurantiacus* in that they formed mat-like dense aggregates. The results of a preliminary DNA-DNA hybridization assay suggested that there is a distinct genetic distance between these two isolates and *Chloroflexus aurantiacus*.

In this study, we characterized these aggregate-forming strains by determining their morphological, physiological, and genetic properties. We found that the new strains represent a distinct species of the genus *Chloroflexus*, and we propose the name *Chloroflexus aggregans* sp. nov. for this taxon.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strain MD-66^T (= DSM 9485) was isolated from bacterial mats in an outflow (57°C, pH 7.0) of the Okukinu Meotobuchi hot spring in Tochigi Prefecture, Japan. This spring contains sodium chloride (18 mM) and sodium bicarbonate (3 mM) as its major salts and trace amounts of sulfide, and the bacterial mat consisted of a dark green layer of filamentous and unicellular cyanobacteria covered with bright yellow streamers (length, approximately 1 cm) that were composed of filamentous green bacteria. Strain YI-9 (= DSM 9486) was isolated from the Yufuin hot spring (67°C, pH 8.2) in Oh-ita Prefecture, Japan. These strains were isolated out by using PE medium supplemented with 1.5% agar. This medium contained (per liter) 0.5 g of sodium glutamate, 0.5 g of sodium succinate, 0.5 g of sodium acetate, 0.5 g of yeast extract (Difco), 0.5 g of Casamino Acids (Difco), 0.5 g of Na₂S₂O₃ · 5H₂O, 0.38 g of KH₂PO₄, 0.39 g of K₂HPO₄, 0.5 g of (NH₄)₂SO₄, 1 ml of a vitamin mixture, and 5 ml of a basal salt solution. The pH of the medium was adjusted to 7.5 with NaOH. The vitamin mixture contained (per 100 ml) 100 mg of

* Corresponding author. Mailing address: Department of Biology, Tokyo Metropolitan University, 1-1 Minamiohsawa, Hachioji, Tokyo 192-03, Japan. Phone: (81) 426 77 2582. Fax: (81) 426 77 2559. Electronic mail address: shanada@yamabuki.comp.metro-u.ac.jp.

nicotinic acid, 100 mg of thiamine hydrochloride, 5 mg of biotin, 50 mg of *p*-aminobenzoic acid, 1 mg of vitamin B₁₂, 50 mg of calcium pantothenate, 50 mg of pyridoxine hydrochloride, and 50 mg of folic acid. The basal salt solution contained (per liter) 1.11 g of FeSO₄ · 7H₂O, 24.65 g of MgSO₄ · 7H₂O, 2.94 g of CaCl₂ · 2H₂O, 23.4 g of NaCl, 111 mg of MnSO₄ · 4H₂O, 28.8 mg of ZnSO₄ · 7H₂O, 29.2 mg of Co(NO₃)₂ · 6H₂O, 25.2 mg of CuSO₄ · 5H₂O, 24.2 mg of Na₂MoO₄ · 2H₂O, 31.0 mg of H₃BO₃, and 4.53 g of trisodium EDTA. Enrichment cultures were established by using the same medium and were incubated in screw-cap tubes or bottles at 55°C under incandescent light (approximately 30 W/m²) (11).

Electron microscopy. Phototrophically grown cells were harvested and embedded in 2% agar (27). The agar was cut into 1-mm³ cubes and fixed for 4 h at room temperature with 2.5% glutaraldehyde in Kellenberger buffer (pH 6.8) supplemented with 0.05% ruthenium red (15, 18). The cubes were washed and postfixed with 1% OsO₄ in buffer containing 0.05% ruthenium red at 4°C for 6 h. They were then washed again and placed in 0.5% uranyl acetate in water for 1 h at room temperature. After washing and dehydration, the cubes were embedded in Spurr embedding medium (17). Thin sections were stained with 5% uranyl acetate for 20 min at 45°C and with 0.002% lead citrate for 1 min at room temperature (37). Micrographs were taken with a JEOL model JEM-1010 electron microscope operating at 80 kV.

Physiological tests. For nutritional experiments we used the media used for *Chloroflexus aurantiacus*, as previously described by Madigan et al. (21), with some modifications. The basic medium contained (per liter) 0.38 g of KH₂PO₄, 0.39 g of K₂HPO₄, 0.5 g of Na₂S₂O₃ · 5H₂O, 0.5 g of (NH₄)₂SO₄, 1 ml of the vitamin mixture, and 5 ml of the basal salt solution (see above). The final pH of the medium was adjusted to 7.5. For each nutritional test performed under aerobic conditions, we used 5 ml of autoclaved basic medium containing an organic carbon source at a final concentration of 0.25% (wt/vol) in a 30-ml L-shaped tube closed with a silicon sponge stopper; the tubes were incubated under aerobic conditions in the dark at 55°C. For each nutritional test performed under anaerobic conditions, we used a 30-ml screw-cap tube filled with basic medium supplemented with a filter-sterilized sulfide-bicarbonate solution (Na₂S · 9H₂O at a final concentration of 0.05% [wt/vol], NaHCO₃ at a concentration of 0.03% [wt/vol]) and an organic carbon source at a concentration of 0.25% (wt/vol); the tubes were incubated in the light or in the dark at 55°C. All organic carbon sources were neutralized and sterilized before they were added. Vitamin requirements were determined by using eight vitamin combinations, each of which lacked one of eight vitamins (nicotinic acid, thiamine hydrochloride, biotin, *p*-aminobenzoic acid, vitamin B₁₂, calcium pantothenate, pyridoxine hydrochloride, folic acid). An organic medium containing 2% vitamin-free Casamino Acids (Difco) was used in this test, and the final results were determined after three serial transfers.

Spectroscopy and pigment analyses. Cells were collected from 2-day cultures in PE medium and then were washed and disrupted by sonication at 140 W for 3 min in MOPS-potassium-magnesium buffer (0.01 M MOPS [morpholinepropanesulfonic acid]-NaOH, 0.1 M KCl, 0.001 M MgCl₂; pH 7.0). The absorption spectra of ultrasonically disrupted cells in buffer were determined with a Shimadzu model UV-160 spectrophotometer. Pigments were extracted with chloroform-methanol (3:1, vol/vol) and were analyzed by reverse-phase high-performance liquid chromatography (HPLC) by using methanol as the solvent (35).

Genetic properties. Genomic DNA was purified by the method of Marmur (22). The guanine-plus-cytosine (G+C) content was determined by performing HPLC of nuclease P1 hydrolysates of genomic DNA (13). DNA-DNA hybridization was performed by the quantitative dot blot hybridization method with photobiotin labeling and colorimetric detection (5, 13). 16S rRNA-specific DNA was amplified by PCR and was sequenced directly by the cycle sequencing method modified for automated fluorescence detection (12, 14).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of *Chloroflexus aggregans* MD-66^T and *Chloroflexus aurantiacus* J-10-fl^T determined in this study have been deposited in the DDBJ, EMBL, and GenBank data libraries under accession numbers D32255 and D38365, respectively. The GenBank and EMBL accession numbers for the 16S rRNA sequences used for comparison in the evolutionary distance matrix analysis are as follows: *Bacteroides fragilis*, M11656; *Chlorobium limicola*, M31769; *Chlorobium tepidum*, M58468; *Clostridium ramosum*, M23731; *Escherichia coli*, M24828; *Helio bacterium chlorum*, M11212; *Herpetosiphon aurantiacus*, M34117; *Methanohalophilus mahii*, M59133; *Paracoccus denitrificans*, X69159; *Rhodobacter capsulatus*, M34129; *Thermomicrobium roseum*, M34115; and *Thermotoga maritima*, M21774.

RESULTS

Morphology. The cells of the cluster-forming strains formed unbranched multicellular filaments of indefinite length. The diameters of full-grown filaments ranged from 1.0 to 1.5 μm (Fig. 1). Septa were observed with a phase-contrast microscope, and the lengths of the individual cells in the filaments ranged from 2 to 6 μm. Granular inclusions were found commonly.

FIG. 1. Phase-contrast photomicrograph of strain MD-66^T cells showing septa in filaments. Cells in the stationary growth phase were grown phototrophically at 55°C and 30 W/m² in PE medium. Bar = 10 μm.

Electron microscopic observations of ultrathin sections (Fig. 2a) showed that the cells of strain MD-66^T were 0.5 to 0.6 μm wide. Septa were distinguished, and the lengths of individual cells ranged from 2 to 5 μm. Vesicular structures, presumably chlorosomes, were observed along the cytoplasmic membrane in the cytoplasm. No sheaths were observed (Fig. 2b).

Photosynthetic pigments. The cells of strains MD-66^T and YI-9 were green or greenish brown when the organisms were grown phototrophically in PE medium. Ultrasonically disrupted cells in buffer had absorption maxima at 464, 740, 803, and 868 nm, and a shoulder at around 510 nm (Fig. 3), which indicates that carotenoids and bacteriochlorophylls *a* and *c* were present. This spectrum resembles that of *Chloroflexus aurantiacus*. An HPLC analysis revealed that the carotenoid components of the new isolates were mainly γ-carotene and OH-γ-carotene glycoside fatty acid esters and that there was a trace amount of β-carotene (data not shown). When cultures were grown aerobically in 5-ml portions of PE medium in 30-ml L-shaped tubes shaken vigorously in the dark, the cells of the new isolates were reddish brown or orange because of the production of carotenoids and small amounts of bacteriochlorophylls even in the early log phase. The concentration of bacteriochlorophyll *c* was approximately 0.5 nM when the optical density at 620 nm of an aerobic culture of one of the new isolates was 0.05. Under parallel conditions cultures of *Chloroflexus aurantiacus* J-10-fl^T were light pink in the early log phase because of the presence of small amounts of carotenoids and no bacteriochlorophyll (no bacteriochlorophyll *c* was detected when the optical density at 620 nm of the culture was 0.05), as described previously by Fuller and Redlinger (6).

Active cell aggregation. When strain MD-66^T cells were grown with illumination, they formed mat-like dense aggregates that appeared to be "green balls" (Fig. 4). Aggregation was observed within 20 to 30 min each time that the cells were dispersed by shaking. The most rapid aggregation occurred at temperatures around 55°C, which was the optimum temperature for growth of this organism. Since the aggregation that occurred was dependent on illumination or the oxygen supply, it could be considered active aggregation. This aggregation was probably due to the gliding motility of the filaments, which was

FIG. 2. Electron micrographs of ultrathin sections of strain MD-66^T cells poststained with uranyl acetate and lead citrate. (a) Longitudinal section of a filament. S, septum. Bar = 200 nm. (b) Cross section showing chlorosomes inside the cytoplasmic membrane (arrows). Bar = 100 nm.

FIG. 3. Absorption spectrum obtained with strain MD-66^T cells grown under anaerobic conditions in the light in PE medium. The cells were ultrasonically disrupted in MOPS-sodium-magnesium buffer.

observed on the surface of a glass slide under a microscope, and the maximum rate was 1 to 3 $\mu\text{m/s}$ at 55°C. Details about the motility of *Chloroflexus aggregans* and the formation of mat-like dense aggregates by this organism will be described elsewhere.

Physiological and other characteristics. Strains MD-66^T and YI-9 were able to grow either anaerobically as photoheterotrophs or aerobically as chemoheterotrophs, like *Chloroflexus aurantiacus*. These strains grew at pH values ranging from 7.0 to 9.0. The optimum temperature for growth was between 50 and 60°C. Both strains required thiamine and folic acid as growth factors. Table 1 shows the carbon sources utilized by these strains. Under anaerobic, light conditions, both strains utilized a wide variety of organic substrates. The highest growth rates and greatest cell yields were obtained in media containing yeast extract or Casamino Acids as the carbon source. In contrast, strains MD-66^T and YI-9 did not utilize most simple organic compounds as carbon sources under aerobic, dark conditions. Only yeast extract and Casamino Acids supported growth under aerobic conditions. Under anaerobic conditions in the dark, none of the substrates tested supported growth. Photoautotrophic growth with sulfide or thiosulfate as the electron donor was not observed with either strain.

Genetic properties. The DNA base compositions of the new isolates as determined by HPLC were 56.7 to 57.0 mol% G+C. These values are 2 to 3% higher than the values reported previously for *Chloroflexus aurantiacus* strains (26). The previously published values, however, were determined by the buoyant density method. Therefore, we determined the G+C base

FIG. 4. Mat-like dense aggregates of strain MD-66^T cells growing in the bottom of a 200-ml screw-cap bottle containing PE medium under phototrophic conditions. Bar = 2 cm.

TABLE 1. Utilization of organic compounds by *Chloroflexus aggregans* strains under various conditions^a

Carbon source	Utilization by:					
	Strain MD-66 ^T			Strain YI-9		
	Anaerobic conditions		Aerobic conditions	Anaerobic conditions		Aerobic conditions
	Light	Dark		Light	Dark	
Glutamate	+	—	—	+	—	—
Aspartate	+	—	—	+	—	—
Glycylglycine	—	—	—	—	—	—
Acetate	± ^b	—	—	—	—	—
Pyruvate	—	—	—	—	—	—
Lactate	+	—	—	+	—	—
Succinate	+	—	—	+	—	—
Malate	+	—	—	+	—	—
Butyrate	+	—	—	—	—	—
Citrate	± ^b	—	—	± ^b	—	—
Glucose	+	± ^c	—	+	± ^c	—
Fructose	± ^b	—	—	± ^b	—	—
Mannitol	± ^b	—	—	± ^b	—	—
Methanol	± ^c	—	—	± ^c	—	—
Ethanol	± ^b	—	—	—	—	—
Glycerol	± ^b	—	—	—	—	—
Yeast extract	++	—	++	++	—	++
Casamino Acids	++	—	+	++	—	+
HCO ₃ [—]	—	—	—	—	—	—

^a Relative growth after 3 days of incubation in vitamin-containing medium (see Materials and Methods) was assessed by determining whether the cell yield was comparable to that obtained with yeast extract (++) or was significantly less than that obtained with yeast extract (+). —, carbon source did not support growth.

^b Faint growth was observed.

^c Initial growth occurred, but sustained growth was not observed.

ratios of two strains of *Chloroflexus aurantiacus* (J-10-fl^T and OK-70-fl) by HPLC. The values which we obtained for *Chloroflexus aurantiacus* J-10-fl^T and OK-70-fl (56.9 and 57.1 mol%, respectively) were similar to the values obtained for the new isolates.

The results of DNA-DNA hybridization studies indicated that the two new strains were closely related to each other and could be clearly distinguished from *Chloroflexus aurantiacus* (Table 2). The relative level of homology between MD-66^T and YI-9 was approximately 80%, while the levels of homology between the new strains and *Chloroflexus aurantiacus* strains were approximately 9 to 18%.

The sequences of 16S rRNA genes enzymatically amplified from strain MD-66^T and *Chloroflexus aurantiacus* J-10-fl^T were determined by direct automated sequencing. The sequences which we examined corresponded to positions 28 to 1,491 in the *Escherichia coli* sequence and represented 95% of the entire 16S rRNA gene. The 16S rRNA sequence of

Chloroflexus aurantiacus J-10-fl^T has been reported previously by Oyaizu et al. (23), but there were some undetermined regions in the data of these authors. Since in phylogenetic analyses of closely related species such a lack of information could easily lead to errors in classification, we decided to determine the sequence of *Chloroflexus aurantiacus* J-10-fl^T in this study.

The level of sequence similarity between strain MD-66^T and *Chloroflexus aurantiacus* was 92.8%. Table 3 shows the evolutionary distances among representative members of bacteria as determined by the neighbor-joining method (31). A phylogenetic tree was reconstructed on the basis of the distance matrix data (Fig. 5). Strain MD-66^T formed a cluster with representatives of the green nonsulfur bacteria, a phylogenetic group defined by Woese (38) whose closest relative is *Chloroflexus aurantiacus*.

DISCUSSION

Our new isolates, strains MD-66^T and YI-9, are thermophilic, filamentous, phototrophic bacteria which grow well at 55°C either anaerobically as photoheterotrophs or aerobically as chemoheterotrophs and inhabit freshwater hot springs. These organisms exhibit gliding motility, produce bacteriochlorophylls *a* and *c*, contain chlorosomes, and require thiamine and folic acid as growth factors. In these respects, the new strains are similar to *Chloroflexus aurantiacus* (20, 26).

However, our data revealed marked phenotypic differences between the new strains and *Chloroflexus aurantiacus*. The most distinctive property of the new organisms is their ability to rapidly form mat-like dense cell aggregates. Whenever aggregated cells were dispersed by shaking, dense aggregates formed again within 20 to 30 min, with the energy required supplied by photosynthesis or respiration. *Chloroflexus aurantiacus* OK-70-fl tends to aggregate in tubes containing liquid medium under anaerobic conditions, but this aggregate formation is not rapid, unlike that observed with the new isolates. The rapid aggregation which we observed seemed to be linked to the fast gliding rate of the cells, which was determined on the surface of a glass slide with a microscope; the maximum gliding rate was 1 to 3 µm/s at 55°C. The gliding rate of the new isolates is approximately 100 times higher than the gliding rates of *Chloroflexus aurantiacus* strains (the gliding rates of strain J-10-fl^T filaments on 1.5% agar were 0.01 to 0.04 µm/s) and 10 times higher than the gliding rates of *Heliothrix oregonensis* F-1, which also forms clumps rapidly and has gliding rates of 0.1 to 0.4 µm/s (26, 28).

Morphologically, the new isolates form unbranched multicellular filaments of indefinite length, like *Chloroflexus aurantiacus*. However, the cells of the new isolates are significantly wider than the cells of the type strain of *Chloroflexus aurantiacus* (J-10-fl) and slightly wider than the cells of strain OK-70-fl. The carbon source utilization tests revealed that unlike *Chloroflexus aurantiacus* (21), the new isolates do not utilize acetate, citrate, ethanol, or glycylglycine. Our analysis of carotenoid components revealed that the new isolates contain mainly γ-carotene and its derivatives, as well as trace amounts of β-carotene, while β-carotene is a major carotenoid component (28.4% of the total carotenoids) in *Chloroflexus aurantiacus* (10). In addition, the quinone compositions of the new isolates and *Chloroflexus aurantiacus* differed, as the new isolates produced a small but significant amount of menaquinone 4 in addition to the predominant homolog, menaquinone 10 (11).

The G+C contents of the DNAs of the new isolates are 56.7 to 57.0 mol%, values which are similar to the values obtained for *Chloroflexus aurantiacus* J-10-fl^T and OK-70-fl (56.9 and

TABLE 2. Levels of DNA homology between *Chloroflexus aggregans* and *Chloroflexus aurantiacus* strains

Strain	% DNA homology with genomic DNA from:		
	Strain J-10-fl ^T	Strain MD-66 ^T	Strain YI-9
<i>Chloroflexus aurantiacus</i> J-10-fl ^T	100	10	16
<i>Chloroflexus aurantiacus</i> OK-70-fl	98	14	18
<i>Chloroflexus aggregans</i> MD-66 ^T	10	100	103
<i>Chloroflexus aggregans</i> YI-9	9	73	100

TABLE 3. Evolutionary distance matrix for a collection of bacterial 16S rRNA sequences, including the sequence of *Chloroflexus aggregans* obtained by the neighbor-joining method^a

Species	Evolutionary distance ^b												
	<i>Chloroflexus</i> <i>aggregans</i> MD-66 ^T	<i>Chloroflexus</i> <i>aurantiacus</i>	<i>Herpetosiphon</i> <i>aurantiacus</i>	<i>Thermomicrobium</i> <i>roseum</i>	<i>Clostridium</i> <i>ramosum</i>	<i>Heliobacterium</i> <i>chlorum</i>	<i>Chlorobium</i> <i>limicola</i>	<i>Chlorobium</i> <i>tepidum</i>	<i>Bacteroides</i> <i>fragilis</i>	<i>Rhodobacter</i> <i>capsulatus</i>	<i>Paracoccus</i> <i>denitrificans</i>	<i>Escherichia</i> <i>coli</i>	<i>Thermotoga</i> <i>maritima</i>
<i>Chloroflexus aurantiacus</i>	0.0489												
<i>Herpetosiphon aurantiacus</i>	0.1776	0.1677											
<i>Thermomicrobium roseum</i>	0.1986	0.1956	0.2006										
<i>Clostridium ramosum</i>	0.2515	0.2375	0.2285	0.2246									
<i>Heliobacterium chlorum</i>	0.2285	0.2186	0.2056	0.1946	0.1567								
<i>Chlorobium limicola</i>	0.2405	0.2345	0.2285	0.2285	0.2126	0.2026							
<i>Chlorobium tepidum</i>	0.2395	0.2305	0.2236	0.2295	0.2086	0.2006	0.0240						
<i>Bacteroides fragilis</i>	0.2764	0.2675	0.2705	0.2655	0.2375	0.2365	0.2036	0.2056					
<i>Rhodobacter capsulatus</i>	0.2525	0.2405	0.2345	0.2246	0.1996	0.1876	0.1946	0.1926	0.2295				
<i>Paracoccus denitrificans</i>	0.2455	0.2395	0.2305	0.2206	0.1976	0.1796	0.1836	0.1836	0.2335	0.0399			
<i>Escherichia coli</i>	0.2345	0.2285	0.2345	0.2146	0.1836	0.1717	0.1896	0.1846	0.2365	0.1806	0.1697		
<i>Thermotoga maritima</i>	0.2206	0.2146	0.2375	0.1786	0.2086	0.1886	0.2066	0.2166	0.2315	0.2086	0.2056	0.1986	
<i>Methanohalophilus mahii</i>	0.3743	0.3663	0.3772	0.3543	0.3713	0.3483	0.3623	0.3643	0.3683	0.3323	0.3323	0.3633	0.3134

^a See reference 31.^b Every site with a gap in any sequence was excluded from the analysis. The value for sequences that were different at every site was 1; all values were normalized to this value.

57.1 mol%, respectively). However, the levels of genomic DNA hybridization between the new isolates and *Chloroflexus aurantiacus* are low (range, 9 to 18%), which suggests that these organisms are not conspecific. In view of the phenotypic and genotypic data presented above, it is reasonable to conclude that our isolates should be classified as members of a new species of filamentous green bacteria. The remaining problem is the taxonomic relationship of the new isolates to *Chloroflexus aurantiacus* at the generic level. 16S rRNA gene sequence comparisons showed that the level of sequence similarity between strain MD-66^T and *Chloroflexus aurantiacus* is 92.8%, which seems to be low enough to warrant placement in different genera from a phylogenetic viewpoint. However, we believe that at this time it is appropriate to classify the new isolates as members of a new *Chloroflexus* species, because

there are only a few phenotypic properties that could be used for generic separation.

On the basis of our phenotypic, genotypic, and phylogenetic data, we classify the new strains as members of a new *Chloroflexus* species, for which we propose the name *Chloroflexus aggregans* sp. nov.

Description of *Chloroflexus aggregans* sp. nov. *Chloroflexus aggregans* (ag'gre.gans. L. v. *aggregare*, to flock or band together; L. pres. part. *aggregans*, assembling, aggregating). Cells form unbranched multicellular filaments of indefinite length. The filaments are clearly septate. The diameters of cells range from 1.0 to 1.5 µm. Cells have no flagella but are motile by gliding. Gram negative. Cultures grow by forming mat-like aggregates in liquid that have the appearance of green balls. Cell aggregates reform rapidly whenever a culture is shaken to produce a uniform suspension under growth conditions. Photosynthetic cultures are green to greenish brown. Bacteriochlorophylls *a* and *c* are present in phototrophically grown cells (under anaerobic conditions) and to a certain extent in chemotrophically grown cells under aerobic conditions. Chlorosomes are present. The major carotenoid components are γ-carotene and OH-γ-carotene glucoside fatty acid esters; there is also a trace amount of β-carotene.

Thermophilic facultative phototrophs that grow optimally at 55°C. Aerobic growth in the dark occurs at full atmospheric oxygen tension. Thiamine and folic acid are required as growth factors. Good carbon sources are yeast extract and Casamino Acids. Simple organic compounds such as intermediates of the citric acid cycle, glutamate, and aspartate are utilized as sole carbon sources only under phototrophic conditions. The major quinone is menaquinone 10. A trace amount of menaquinone 4 is also present.

Habitat: bacterial mats with cyanobacteria in freshwater of hot springs (50 to 67°C).

The G+C content of the DNA is 56.7 to 57.0 mol% (as determined by the HPLC method).

The type strain is strain MD-66 (= DSM 9485).

FIG. 5. Phylogenetic position of *Chloroflexus aggregans* MD-66^T among eubacteria. The tree was constructed from the evolutionary distances shown in Table 3. Bar = 10 nucleotide substitutions per 100 nucleotides in 16S ribosomal DNA. An archaeobacterial sequence (the *Methanohalophilus mahii* sequence) (Table 3) was used to root the tree.

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