Molecular Characterization of the Nonphotosynthetic Partner Bacterium in the Consortium “*Chlorochromatium aggregatum*”

Birgit E. M. Kanzler, Kristina R. Pfannes, Kajetan Vogl, and Jörg Overmann*

Bereich Mikrobiologie, Ludwig-Maximilians-Universität München, Maria-Ward-Strasse 1a,
D-80638 Munich, Germany

Received 21 April 2005/Accepted 22 July 2005

Phototrophic consortia represent valuable model systems for the study of signal transduction and coevolution between different bacteria. The phototrophic consortium “*Chlorochromatium aggregatum*” consists of a colorless central rod-shaped bacterium surrounded by about 20 green-pigmented epibionts. Although the epibiont was identified as a member of the green sulfur bacteria, and recently isolated and characterized in pure culture, the central colorless bacterium has been identified as a member of the β -*Proteobacteria* but so far could not be characterized further. In the present study, “*C. aggregatum*” was enriched chemotactically, and the 16S rRNA gene sequence of the central bacterium was elucidated. Based on the sequence information, fluorescence in situ hybridization probes targeting four different regions of the 16S rRNA were designed and shown to hybridize exclusively to cells of the central bacterium. Phylogenetic analyses of the 1,437-bp-long sequence revealed that the central bacterium of “*C. aggregatum*” represents a so far isolated phylogenetic lineage related to *Rhodospirillum rubrum* spp., *Polaromonas vacuolata*, and *Variovorax paradoxus* within the family *Comamonadaceae*. The majority of relatives of this lineage are not yet cultured and were found in low-temperature aquatic environments or aquatic environments containing xenobiotics or hydrocarbons. In CsCl-bisbenzimidazole equilibrium density gradients, genomic DNA of the central bacterium of “*Chlorochromatium aggregatum*” formed a distinct band which could be detected by quantitative PCR using specific primers. Using this method, the G+C content of the central bacterium was determined to be 55.6 mol%.

During the course of evolution, prokaryotes have entered into numerous symbiotic relationships. So far, the symbioses between bacteria and eukaryotes have mostly been investigated (36). In contrast, interactions between different prokaryotes have received much less attention, such that only the syntrophic associations of anaerobic chemotrophic bacteria with archaea are understood in sufficient detail (42). Microscopic studies have revealed, however, that morphologically highly structured associations of different prokaryotes exist in natural habitats (33, 36). In these so-called consortia, prokaryotes maintain a permanent cell-to-cell contact, and hence their mutual interdependence may be obligatory.

Of the consortia known, only two phototrophic consortia have been cultivated in the laboratory (11, 16). Phototrophic consortia consist of a colorless central rod-shaped bacterium surrounded by 13 to 69 green- or brown-pigmented epibionts (32), and they typically occur in the chemocline of many stratified lakes (7, 8, 15, 17, 19), where they may constitute up to 66% of the total bacterial biomass (15).

Several lines of evidence indicate that a direct communication exists between the two different types of bacteria in phototrophic consortia (11, 18). Intact consortia accumulate scotophobically in the light, at wavelengths which correspond to the absorption maxima of the bacteriochlorophylls present in the epibionts (11). Epibiont cells are nonflagellated, however, whereas the central bacterium is motile by means of a single polar flagellum (18, 38). Consequently, the scotophobic re-

sponse must involve signal exchange between the epibionts and the central bacterium. As a second observation, intact phototrophic consortia take up 2-oxoglutarate, most likely mediated by the central bacterium. This uptake is strictly dependent on the presence of sulfide and light, both utilized by the epibionts (18). Accordingly, the physiological state of the epibiont cells appears to control the 2-oxoglutarate uptake by the central bacterium. Phototrophic consortia thus represent valuable model systems for the study of signal transduction and coevolution between different bacteria.

Using 16S rRNA-based methods, the epibionts of phototrophic consortia have been identified as green sulfur bacteria (12, 45). In the associated state, epibionts grow photoautotrophically like their free-living green sulfur bacterial relatives (17). Recently, the epibiont of the phototrophic consortium “*Chlorochromatium aggregatum*” could be isolated in pure culture, and its physiology was characterized in detail (47). Also, the genome sequence of the epibiont has just been completed (see http://genome.jgi-psf.org/finished_microbes/chlag/chlag.download.html). In contrast, only very little is known of the central bacterium. By fluorescence in situ hybridization (FISH), it could be identified as a member of the β -subclass of the *Proteobacteria* (12). However, its precise phylogenetic position could not be determined because of the notoriously low cell numbers of central bacteria which are present in the available “*Chlorochromatium aggregatum*” cultures.

MATERIALS AND METHODS

Source of organisms. Enrichment cultures of “*Chlorochromatium aggregatum*” were established previously from a sediment sample of the eutrophic Lake Dagow (100 km north of Berlin, Germany) (11). “*Chlorochromatium aggregatum*” consists of a colorless central rod and approximately 20 green-pigmented

* Corresponding author. Mailing address: Bereich Mikrobiologie, Ludwig-Maximilians-Universität München, Maria-Ward-Strasse 1a, D-80638 Munich, Germany. Phone: 49-89-2180-6123. Fax: 49-89-2180-6125. E-mail: j.overmann@lrz.uni-muenchen.de.

epibionts. A recently isolated pure culture of the epibiont strain CaD of "*C. aggregatum*" (47) and cultures of *Rhodocyclus tenuis* DSMZ 109^T, *Ralstonia eutropha* DSMZ 428, *Chlorobium phaeobacteroides* DSMZ 266^T, and *Clostridium acetobutylicum* DSMZ 792 were used for reference.

Media and growth conditions. "*C. aggregatum*" was grown in K4 medium of the following composition (components in grams per liter): KH_2PO_4 , 0.25; NH_4Cl , 0.05; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05; HEPES, 2.38; NaHCO_3 , 0.84. After autoclaving, the medium was cooled under an N_2 - CO_2 atmosphere, and sterile sulfide solution ($\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, 0.12 g in 20 ml), 1 ml of a seven-vitamin solution (39), 1 ml trace element solution SL10 (49), and 0.25 ml lipoic acid solution ($100 \text{ mg} \cdot \text{l}^{-1}$) were added. The pH was adjusted to 7.4, and the medium was dispensed into air-tight, screw-cap bottles. Prior to inoculation with 5% (vol/vol) of an enrichment culture of "*C. aggregatum*," the medium was supplemented with 0.05% (vol/vol) of trace element solution SL12B (35) and 0.5 mM 2-oxoglutaric acid (final concentrations).

Cultures were incubated at 15°C and at $20 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ of a daylight fluorescent tube (Lumilux de Lux, 18 W; Osram, Munich, Germany). Light intensities were monitored using a LiCor LI-250 lightmeter equipped with a model PY38153 pyranometer sensor (Walz, Effeltrich, Germany). During exponential growth, cultures received 0.5 mM neutral sulfide solution (43) and 0.5 mM 2-oxoglutaric acid every 2 days.

Enrichment by chemotaxis. For subsequent molecular analyses, "*C. aggregatum*" was enriched by exploiting the chemotactic behavior of the intact consortia. The original method (11) was modified. All manipulations were carried out in an anaerobic chamber under an atmosphere of 95% N_2 and 5% H_2 . Meplats (100-ml-volume) bottles with 12 bore holes (11) were filled with 20 ml of a densely grown culture of "*C. aggregatum*" containing $2.6 \cdot 10^5$ consortia per ml. Sulfide solution (1 mM) was prepared in sterile filtered (0.1- μm -pore-size Durapore membrane filters; Millipore, Eschborn, Germany) culture supernatant. Flat rectangular capillaries (length, 50 mm; inside diameter, 0.1 by 1.0 mm or 0.1 by 2.0 mm; Vitrocom, New Jersey) or round capillaries (volumes, 5 μl , 10 μl , 20 μl , and 100 μl ; Servoprax, Wesel, Germany; Brand, Wertheim, Germany; or Assistant, Sondheim/Röhn, Germany) were filled by capillary action with the sulfide solution and sealed at one end with plasticine (Münchener Künstler Plastilin, Munich, Germany). Capillaries were then inserted through the holes in the Meplats bottle until their open ends reached the culture liquid and were then fixed with plasticine. Incubations proceeded overnight at 15°C and with an ambient light intensity of $20 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$. Afterwards, the contents of the capillaries were transferred into 100 μl of sterile double-distilled water and centrifuged for 15 min at 13,000 rpm. The cell pellet was resuspended in 10 μl of double-distilled water and stored at -20°C.

PCR. Chemotaxis enrichments were lysed by five consecutive freeze-thaw cycles (each cycle consisting of a 3-min incubation each at 100°C and at -20°C). One microliter of the cell lysate was used for amplification. Amplification reactions for pure cultures received 50 ng of genomic DNA.

Standard PCR conditions (34) were performed with a DNA thermal cycler (GeneAmp PCR system 2400; Applied Biosystems, Foster City, CA), and PCR products were analyzed by standard agarose gel electrophoresis.

For amplifications with primers GC341f or 341f and 907r (30), the cycling conditions described previously were employed (37). For the specific amplification of β -proteobacterial sequences, primers Beta680f (34) and an improved version of primer 13R (1) (5'-TCGCCAAGGCATCCACC-3', *E. coli* positions 23 to 39 of the 23S rRNA) were used. The step-down PCR program comprised 10 cycles with denaturation at 94°C for 30 s, primer annealing at 61°C for 1 min, and elongation at 72°C for 3 min, followed by 25 cycles with the annealing temperature changed to 56°C. Two primers, CRa641f (5'-ACTGCAGATGCTAGAGTA-3') and CRa641r (5'-CGTACTCTAGCATCTGCAGT-3) were designed in the present study and are specific for the 16S rRNA gene sequence of the central bacterium of "*C. aggregatum*." The specific primers were combined either with GC341f or with universal primer 8f or 1492r (27). For amplification with primer pair 8f/CRa641r, the optimized step-down program comprised 10 cycles with denaturation at 94°C for 30 s, primer annealing at 70°C for 30 s, and elongation at 72°C for 2 min, followed by 20 cycles with the annealing temperature changed to 65°C. Cycling conditions for primer pair GC341f/CRa641r were 10 cycles with denaturation at 94°C for 30 s, primer annealing at 58°C for 45 s, and elongation at 72°C for 1 min, followed by 30 cycles with the annealing temperature changed to 53°C. For amplification with the primer pair CRa641f/1492r, the annealing temperature was set to 60°C for 30 s during the first 10 cycles and to 55°C for the subsequent 25 cycles. In this case, elongation proceeded for 1 min at 72°C.

Quantitative PCR. The relative amount of genomic DNA of the central bacterium from "*C. aggregatum*" in CsCl-bis-benzimidazole density gradients was determined by quantitative PCR (iQ iCycler; Bio-Rad, Munich, Germany) em-

ploying the primer pair 341f/CRa641r and SYBR Green Supermix (Bio-Rad) for the detection of double-stranded PCR products. Each reaction received 4 ng of template DNA. For standardization of the values, 10-fold dilutions of genomic DNA from the "*C. aggregatum*" culture (concentration range, 4 pg to 400 ng) were measured in parallel. Cycling conditions included 5 min of denaturation (95°C), 45 s of annealing (67°C, 40 cycles), and 1 min of elongation (72°C). All measurements were done in quadruplicate, and negative controls were included throughout. The relative enrichment factor for genomic DNA of the central bacterium was derived from a comparison of the cycle threshold (C_t) values determined directly for the enrichment culture and the C_t values determined for DNA fractions from the CsCl gradients.

DGGE. 16S rRNA gene fragments amplified were separated by denaturing gradient gel electrophoresis (DGGE) (29, 30) conducted with an Ingeny phorU2 system (Ingeny International BV, Goes, The Netherlands) for 15 min at 200 V, then for 12 h at 180 V at a constant temperature of 60°C. After being stained with SYBRGold (MoBiTec, Göttingen, Germany), the DNA bands were visualized on a UV transilluminator, and DNA fragments of interest were excised with a sterile scalpel. Gel pieces were incubated for 1 h at 65°C in 20 μl of 2 mM Tris-HCl (pH 8.0), the eluted DNA was reamplified, and the amplification products were purified for sequencing using a QiaQuick PCR purification kit (QIAGEN GmbH, Hilden, Germany). Gel images were captured with a digital camera (Spot RT color; Diagnostic Instruments, Inc.) and processed with the Spot RT version 3.1 software.

Cloning. PCR products were cloned through chemical transformation with a TOPO TA cloning kit (version P; Invitrogen, Carlsbad, CA). Plasmids were extracted with a QIAprep spin miniprep kit (Invitrogen), and the presence of inserts was verified by digestion with EcoRI, PvuI, and HaeIII (MBI Fermentas, St. Leon-Rot, Germany).

Sequencing. Sequencing was performed by the dideoxynucleotide method (41) using an AmpliTaq FS Big Dye Terminator cycle sequencing kit according to the protocol of the manufacturer and an ABI Prism 310 genetic analyzer (Applied Biosystems, Weiterstadt, Germany). In addition to the primers described above, oligonucleotides 926f, 1055f, and 1055r (4, 27) were employed in the sequencing reactions. The computer program Lasergene (Seqman II; DNASTAR, Inc., Madison, WI) was used for sequence editing.

Phylogenetic analyses. 16S rRNA sequences were analyzed using the software packages ARB (28) and PHYLIP (Phylogeny Inference Package, version 357c) (9). Sequences of the 50 phylogenetically closest relatives of the central bacterium were retrieved from the GenBank database by using BLAST version 2.0.4 (3) and imported into the ARB database. The Fast Aligner V1.03 tool was used for automatic sequence alignment. The sequence alignment was checked and manually corrected based on secondary-structure information, yielding an alignment of 1,542 informative nucleotide positions.

Phylogenetic trees were constructed using the maximum likelihood and maximum parsimony algorithms within the ARB package and the neighbor-joining and distance-based methods (DNADIST plus FITCH) as implemented in the PHYLIP software package. To identify variable branching points, the phylogenetic trees generated were compared pairwise by employing the COMPARE TOPOLOGY function of the ARB program. In addition, reproducibility of the branching pattern of the maximum likelihood tree was tested by bootstrap analysis, generating a set of 100 resamplings. Those branches, which were observed to differ between the four methods and which had low bootstrap support, were collapsed with deeper branching points to yield multifurcations, using the ARB software (W. Ludwig, personal communication).

FISH. In order to verify the 16S rRNA gene sequence determined for the central rod of "*C. aggregatum*," specific FISH probes were created with the DESIGN PROBES function of the ARB software package. The accessibility of the target sites was checked based on data available for *Escherichia coli* (14). For probes targeting sites with limited accessibility, corresponding helper oligonucleotides (13) were designed. Overall, four specific probes and eight helper oligonucleotides were used (Table 1). Fluorescence in situ hybridization was carried out on black polycarbonate filters, resulting in disintegration of the phototrophic consortia, thereby exposing the central rod (45). Twenty nanograms each of the Cy3-labeled probe and the corresponding helper oligonucleotides at the appropriate hybridization stringency were used (Table 1). Hybridization stringency was tested and optimized by varying the formamide concentrations between 5 and 35%. After counterstaining with 4',6-diamidino-2-phenylindole (DAPI), hybridizations were analyzed by epifluorescence microscopy.

CsCl-bis-benzimidazole density gradient centrifugation. The mol% G+C content of the central bacterium was determined in cesium chloride-bis-benzimidazole equilibrium density gradients (5, 23, 24, 31, 46), using a modified protocol. Ten microliters of bis-benzimidazole stock solution (Hoechst no. 33258; $25 \mu\text{g} \cdot \mu\text{l}^{-1}$) was added to 10 ml of CsCl-TE ($1.3 \text{ g CsCl} \cdot \text{ml}^{-1}$ in Tris

TABLE 1. Fluorescently labeled probes, helper oligonucleotides, and hybridization conditions employed for FISH

Probe or helper ^a	Sequence	Temp (°C)	Formamide (%)
CR-207	5'-CGC GCG AGG CCC TCT-3'	48	20
CR-207-H1	5'-CAG GTC CCC CGC TTT CAT-3'		
CR-207-H2	5'-CTG ATA TCA GCC GCT CCA AT-3'		
CR-442	5'-AAG GCT GTT TCG CTC CGT-3'	45	20
CR-641	5'-TAC TCT AGC ATC TGC AGT-3'	45	20
CR-641-H1	5'-CAC AAA TGC AAT TCC CAG-3'		
CR-641-H2	5'-GTT GAG CCC GGG GAT-3'		
CR-641-H3	5'-TTC ACA TCC GTC TTA CAG-3'		
CR-641-H4	5'-CAT CCC CCT CTG CCG-3'		
CR-1282	5'-CGA CTG ACT TTA TGG-3'	45	5
CR-1282-H1	5'-GGT TGG CTC CCT CTC-3'		
CR-1282-H2	5'-CTG CGA TCC GGA CTA-3'		
Cont-645	5'-TGC CAT ACT CTA GCC TTC-3'	45	20
Cont-645-H1	5'-CAG TCA CAA GCG CAG TT-3'		
Cont-645-H2	5'-CCC AAG TTG AGC CCG-3'		
Cont-645-H3	5'-GGG ATT TCA CGC CTG-3'		
Cont-645-H4	5'-AAT TCC ACC CCC CTC-3'		
Cont-995	5'-CTT CAG GCT CCT GGA CAT-3'	45	20
Cont-995-H1	5'-GTC AAG GGT AGG TAA GGT TTT-3'		
Cont-995-H2	5'-TCG GGC ACA CCC AAA TCT-3'		
Cont-995-H3	5'-CCT GTG TTC CAG TTC CCT T-3'		

^a Numbers indicate 5'-end according to *E. coli* numbering. "CR" denotes probes specific for the central rod-shaped bacterium of "*C. aggregatum*"; "Cont" denotes for probes specific for free-living *β-Proteobacteria*. Helper oligonucleotides are denoted by the suffix "H".

HCl-EDTA [10 mM:1 mM]). The mixture was transferred to a 16- by 76-mm ultracentrifuge Quick-Seal tube (no. 342413; Beckman Coulter), overlaid with 1 ml of DNA extract containing 150 µg DNA from a consortium culture, with the headspace completely filled with paraffin oil, balanced, and sealed. Centrifugation proceeded at $232,000 \times g$ for 48 h at 15°C in a fixed-angle rotor (70.1 Ti; Beckman Coulter). DNA bands were visualized under UV at 365 nm, and 100-µl fractions were collected from below via a 21-gauge needle connected to silicon tubing, by use of a peristaltic pump.

Subsequently, the refractive index of each fraction was determined. Bis-benzimidazole and CsCl were removed using three repeated extractions in CsCl-saturated isopropanol, followed by two wash steps with Tris-HCl in Centricon-50 ultrafiltration units (Millipore, Eschborn, Germany). The DNA contents of all purified fractions were determined fluorimetrically using PicoGreen (Molecular Probes, Eugene, OR). For standardization, genomic DNA of bacterial strains with known GC contents (*Clostridium acetobutylicum*, mol% G+C = 30.9; *Chlorobium phaeobacteroides*, mol% G+C = 49.0; and *Ralstonia eutropha*, mol% G+C = 64.4) was separated in the same gradient, and a standard curve was established correlating the known GC contents to the refractive index.

Nucleotide sequence accession numbers. The almost-full-length 16S rRNA gene sequence of the central bacterium of "*Chlorochromatium aggregatum*" has been deposited in the EMBL database under accession number DQ009030. Partial sequences of the accompanying bacteria are deposited under accession numbers DQ009027 to DQ009029 and DQ009031 to DQ009034.

RESULTS AND DISCUSSION

Improved chemotactic enrichment of intact phototrophic consortia. Based on DAPI counting of standard enrichment cultures of "*Chlorochromatium aggregatum*," cells of the central bacterium reach $\leq 0.071 \pm 0.003\%$ of total cell counts. At such low frequencies, 16S rRNA gene sequences cannot be detected by PCR-DGGE (29, 44). Therefore, the chemotaxis of "*C. aggregatum*" towards sulfide (11) was exploited as a rapid means to selectively enrich intact consortia. In small, flat, rectangular capillaries (0.1 mm by 1.0 mm), "*C. aggregatum*" accumulated within the first 3 mm from the capillary

opening. However, these enrichments also contained a large number of motile chemotrophic bacteria which still amounted to 85% of all cells (similar to the accumulation depicted in Fig. 1B). Experiments with round capillaries of different sizes and volumes did not yield suitable enrichments. In flat capillaries with a larger width (0.1 mm by 2.0 mm), however, "*C. aggregatum*" accumulated in two distinct zones. In addition to the primary enrichment detected near the capillary opening, a second zone of accumulation formed in the center of the capillary, at a distance of approximately 1 cm from the primary accumulation. Direct phase-contrast microscopy of the capillaries revealed that many fewer accompanying bacteria were present in this secondary accumulation (Fig. 1A) than in the accumulation near the capillary end (Fig. 1B).

In order to gather sufficient material for subsequent molecular analyses, chemotactic enrichments from 48 flat rectangular capillaries were collected by breaking off the capillaries in the center (Fig. 1A) and blowing out their contents into an Eppendorf vial using sterile filtered air. Then, the cells were concentrated by centrifugation to a final titer of 10^7 consortia ml⁻¹.

Analysis of the 16S rRNA gene sequence of the central bacterium of "*C. aggregatum*." In a first step, partial 16S rRNA genes were amplified from the chemotactic enrichment of "*Chlorochromatium aggregatum*" using primers GC341f and 907r. Subsequent separation by DGGE revealed the presence of seven different DNA fragments among the amplification products (Fig. 2, bands a through g). All bands were excised, reamplified, and sequenced. In parallel, a 1,400-bp-long DNA fragment was amplified with the *β*-proteobacterial primer pair Beta680f/13R and was also sequenced.

FIG. 1. (A) Phase-contrast photomicrograph of the accumulation of "*Chlorochromatium aggregatum*" (black arrows) formed in the center of the 0.1- by 2-mm rectangular capillary. (B) Photomicrograph of the bacteria accumulated at the opening of the capillary. Besides phototrophic consortia (black arrows), numerous motile contaminants (white arrowheads) are present in this accumulation. Bar, 20 μ m.

FIG. 2. Separation by DGGE of 16S rRNA gene fragments amplified with primers GC341f and 907r from the chemotaxis enrichment of "*Chlorochromatium aggregatum*" and from a pure culture of the epibiont of "*Chlorochromatium aggregatum*." Italic letters denote DNA bands which were excised and sequenced. A negative image of an SYBR Gold-stained gel is shown.

Band b could be identified as the 16S rRNA gene fragment of the epibiont (Fig. 2), which was confirmed by sequence comparison. Of the remaining sequences, three (bands c, d, and e) were affiliated with the β -*Proteobacteria*, two (bands f and g) with the δ -*Proteobacteria*, and one (band a) with the ϵ -*Proteobacteria*. In a previous FISH analysis of the "*C. aggregatum*" enrichment culture, the central bacterium could be identified as a member of the β -*Proteobacteria* (12). In order to investigate whether the 16S rRNA sequence of the central bacterium was present among sequences designated c to e, specific oligonucleotide probes were constructed for each of the sequences and used to analyze the central bacterium by FISH. The sequence of the long DNA fragment amplified with the β -proteobacterial primer pair matched sequence type c and was therefore used to construct a specific probe (Cont-995) for this sequence type.

Probes Cont-995 and Cont-645, targeting sequence type c and type d, respectively, hybridized only to free-living bacteria and not to the central bacterium of "*C. aggregatum*" and hence must originate from accompanying bacteria present in the enrichment. In contrast, probes CR-442 and CR-641, targeting sequence type e, hybridized exclusively to the central bacterium (Fig. 3A to D).

Subsequently, the missing sequence stretches at the beginning and the end of the 16S rRNA gene of the central bacterium were amplified. To this end, PCR primers identical (CRA641r) or complementary (CRA641f; see Materials and Methods) to the specific probe CR-641 were combined with primers 8f and 1492r, respectively. Genomic DNA of two members of the β -*Proteobacteria*, *Rhodocyclus tenuis* DSMZ 109^T and *Ralstonia eutropha* DSMZ 428, was employed to establish highly specific PCR conditions. Sequencing of the resulting amplification products yielded a 650-bp-long sequence from the 5' end and an 850-bp-long sequence from the 3' end of the 16S rRNA gene. Both sequences showed 100%

FIG. 3. Specific detection of the central bacterium of “*Chlorochromatium aggregatum*” by FISH with four different oligonucleotide probes (see Table 1). All bacterial cells were stained with DAPI. (A) FISH with probe CR-442 and DAPI fluorescence. Arrow indicates location of the central bacterium. (B) Same field of view as in panel A but showing Cy3 fluorescence. (C) Overlay of panels A and B. (D) Overlay of DAPI fluorescence and Cy3 fluorescence after FISH using probe CR-641. (E) Overlay of DAPI fluorescence and Cy3 fluorescence after FISH using probe CR-207. (F) Overlay of DAPI fluorescence and Cy3 fluorescence after FISH using probe CR-1282. Bar, 10 μ m.

identity in the overlapping regions to the central fragment (sequence e) obtained by PCR-DGGE.

Finally, the origin of the two 16S rRNA gene sequences was verified by FISH. Two probes were designed for the terminal sequence regions (CR-207 and CR-1282) (Table 1) and were found to hybridize exclusively to cells of the central bacterium

of “*Chlorochromatium aggregatum*” (Fig. 3E and F). Assembling all three 16S rRNA gene sequence fragments of the central bacterium yielded an almost complete 16S rRNA gene sequence of a total length of 1,437 bp.

Phylogenetic classification of the central rod. Based on 16S rRNA gene sequence comparisons, the phylogenetically clos-

FIG. 4. Phylogenetic affiliation of the central bacterium of “*Chlorochromatium aggregatum*.” The consensus tree was constructed using ARB, based on maximum likelihood, maximum parsimony, neighbor-joining, and distance-based phylogenetic analyses (see Materials and Methods). Percentages at nodes indicate bootstrap values out of 100 bootstrap resamplings, as determined for the maximum likelihood tree. Only values above 50% are shown. Uncult., uncultured bacteria detected as environmental clones. Bar indicates 3% fixed point mutations per nucleotide base.

FIG. 5. (A) Separation of genomic DNA of different bacteria present in the enrichment culture of “*Chlorochromatium aggregatum*” using CsCl-bis-benzimidazole gradient density centrifugation. A negative image of DNA bands visualized by fluorescence under UV illumination is shown. (B) DNA concentration (●), refractive index (△), and enrichment factor of the genomic DNA from the central bacterium (◆) along the CsCl gradient. DNA concentrations are presented as moving averages ($n = 3$). Numbers 1 to 4 refer to the respective bands in panel A. (C) DGGE fingerprinting of 16S rRNA gene fragments amplified from the different fractions using eubacterial primers GC341f and 907r. Melting types a to h represent DNA fragments from accompanying bacteria. (D) DGGE fingerprinting of 16S rRNA gene fragments of the central bacterium amplified with primers GC341f and the specific primer CR641r. CR, melting position of the DNA fragment of the central bacterium.

est relatives of the central bacterium are *Rhodoferrax ferrireducens*, *Rhodoferrax antarcticus*, and a variety of not-yet-cultured bacteria. Sequence similarity was always lower than 95%, however (the closest cultured relative *R. ferrireducens* DSMZ15236^T being 94.77% similar). According to our phylogenetic analyses (Fig. 4), the central rod of “*C. aggregatum*” represents a so far isolated phylogenetic lineage, and clusters with the genera *Rhodoferrax* and *Polaromonas* within the family *Comamonadaceae* (βI subgroup [20]). The majority of relatives are not yet cultured and were found in low-temperature aquatic environments or aquatic environments containing pollutants like monochlorobenzene and tetrachloroethene or hydrocarbons (Fig. 4) (2, 6, 10, 22, 26, 48).

GC content and enrichment of genomic DNA of the central bacterium. CsCl-bis-benzimidazole equilibrium density centrifugation was used to determine the mol% G+C content of

the central bacterium and to establish a large-scale purification method for its genomic DNA. Due to the inherently small volumes which can be generated in the chemotaxis enrichments, the DNA had to be extracted directly from conventional laboratory cultures of “*C. aggregatum*” in this case.

Density gradient centrifugation separated the genomic DNA into four distinct bands as visualized by UV illumination (bands 1 through 4) (Fig. 5A). Since the fluorescence of the DNA-bis-benzimidazole complex is proportional to the amount of bis-benzimidazole bound and thus decreases with mol% G+C content (23), genomic DNA with a high mol% G+C content is barely detectable. The gradient was separated into 54 fractions, and the DNA content of each fraction was determined by PicoGreen dye binding (Fig. 5B). This second method confirmed the presence of band 4 and demonstrated its high DNA content, whereas band 2 was barely detectable

with PicoGreen. Each fraction was PCR amplified with eubacterial primers, and the products were separated by DGGE and sequenced. Five major phylotypes could be distinguished (Fig. 5C, melting types a, b, d, g, h). With one exception (sequence type h), these sequences could be assigned to those identified in the chemotaxis enrichment. The genomic DNA of the epibiont of "*C. aggregatum*" (type b) accumulated in the center of the gradient, corresponding to the GC content of 46.7 mol%, as recently determined (47). Band 1 of the CsCl gradient was formed mainly by genomic DNA from the accompanying *Sulfospirillum arsenophilum* (sequence type a). According to our analysis, band 2 contained a mixture of genomic DNA from *S. arsenophilum*, the epibiont and a relative of *Desulfovibrio aerotolerans* (sequence types a, b, and g). In band 3, predominantly *Desulfovibrio aerotolerans* was detected, whereas band 4 also contained DNA from an actinobacterium related to *Celulomonas terrae* (sequence type h).

The genomic DNA of the central bacterium of "*C. aggregatum*" in the density gradients did not reach a concentration sufficient for detection with eubacterial primers. However, amplification with the specific primer pair GC341f/CRA641r yielded amplification products for several fractions of the gradient. DGGE analysis revealed the presence of only one melting type (Fig. 5D), and sequencing confirmed that it was indeed identical to that of the central bacterium of "*C. aggregatum*." The specific amplification protocol was therefore used to quantify the enrichment factor for genomic DNA of the central bacterium in the CsCl gradient by quantitative PCR (Fig. 5B). Compared to the original enrichment culture of "*C. aggregatum*," genomic DNA of the central bacterium was enriched 150-fold in fractions 27 to 29. At an enrichment factor of 150 $\times$ and a frequency of 0.07% in the original culture, the genomic DNA of the central bacterium therefore amounted to approximately 10% of the total DNA in these fractions. Despite this relatively high frequency, the 16S rRNA gene sequence of the central bacterium could not be detected in the complex bacterial communities by PCR-DGGE with eubacterial primers. Our result is in line with data from another study, in which a detection limit of 9% was determined (44).

The distinct banding of the genomic DNA of the central bacterium of "*Chlorochromatium aggregatum*" in CsCl density gradients was used to estimate its GC content, using the median of the refractive indices of all 10 fractions with enrichment factors of ≥ 50 (Fig. 5B). This yielded a mol% G+C content of the central bacterium of "*C. aggregatum*" of 55.6%, which is commensurate with the values determined for other *Comamonadaceae*, spanning a range between 52 and 70% (22, 25, 26, 50). Whereas the most closely related *Rhodospirillum rubrum* species have a GC content of 59.8 to 61.5%, values for *Polaromonas vacuolata* strains are between 52 and 57 mol% and those of *Variovorax paradoxus* strains range between 67 and 69 mol%.

Relevance for future studies. For future enrichment and isolation attempts with the central bacterium of "*Chlorochromatium aggregatum*," it is important to resolve stimulating effects of accompanying bacteria in the enrichment culture. Interestingly, one bacterium which reacted chemotactically toward sulfide was identified to be *Sulfospirillum arsenophilum*. A similar bacterium has been detected as an essential partner in coculture with the green sulfur bacterium *Chloro-*

bium ferrooxidans, where it is thought to provide the green sulfur bacterium with an essential growth factor (21). It therefore appears possible that the accompanying bacteria fulfill similar functions in the "*Chlorochromatium aggregatum*" enrichment culture. Also, sulfur cycling between the *Sulfospirillum* bacteria and consortia may occur in the enrichment cultures, since the phylogenetically related sulfur-reducing *Sulfospirillum deleyianum* DSMZ6946^T is known to grow syntrophically by sulfur cycling with green sulfur bacteria (51). Another interesting finding is the presence of a member of the β -Proteobacteria, *Azonexus fungiphilus*, which is known to require 2-oxoglutarate for growth (40), which may explain the failure to eliminate this bacterium from the enrichment cultures.

So far, only very little is known of the physiology of the central rod-shaped bacterium of phototrophic consortia. Due to the large phylogenetic distance to other known bacteria, however, physiological properties cannot be inferred from its phylogenetic position. Phototrophic consortia exhibit a chemotactic response towards 2-oxoglutarate (11, 19), which is taken up by the cells (18). Recently, the epibiont of "*C. aggregatum*" could be isolated in pure culture and was found to be incapable of using 2-oxoglutarate (47). Taken together, these findings suggest that 2-oxoglutarate is utilized by the central bacterium. The specific oligonucleotide probes developed in the present work now allow the performance of enrichment experiments with different substrates and the selective and sensitive screening for the growth of the central bacterium alone. One question central to the understanding of the bacterial association in phototrophic consortia is whether the association is an obligatory one. The specific oligonucleotide probes now available permit the tracking of the central bacterium in its natural habitat in order to determine whether it occurs in the free-living state. Finally, the CsCl-bis-benzimidazole density gradient centrifugation is suitable for the separation of genomic DNA of the central bacterium from DNA of some of the accompanying bacteria and therefore is relevant for subsequent genome sequencing efforts.

ACKNOWLEDGMENTS

We thank Martina Müller for initial help with the specific PCR amplifications.

This work was supported by the Deutsche Forschungsgemeinschaft (grant Ov20/10-1).

REFERENCES

1. Aakra, A., J. B. Utaker, and I. F. Nes. 1999. RFLP of rRNA genes and sequencing of the 16S–23S rDNA intergenic spacer region of ammonia oxidizing bacteria: a phylogenetic approach. *Int. J. Syst. Bacteriol.* **49**: 123–130.
2. Alfreider, A., C. Vogt, and W. Babel. 2002. Microbial diversity in an *in situ* reactor system treating monochlorobenzene contaminated groundwater as revealed by ribosomal DNA analysis. *Syst. Appl. Microbiol.* **25**:232–240.
3. Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
4. Amann, R. L., W. Ludwig, and K.-H. Schleifer. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**:143–169.
5. Bostock-Smith, C. L., and M. S. Searle. 1999. DNA minor groove recognition by bis-benzimidazole analogues of Hoechst 33258: insights into structure DNA affinity relationships assessed by fluorescence titration measurements. *Nucleic Acids Res.* **27**:1619–1624.
6. Brinkmeyer, R., K. Knittel, J. Jürgens, H. Weyland, R. Amann, and E. Helmke. 2003. Diversity and structure of bacterial communities in arctic versus antarctic pack ice. *Appl. Environ. Microbiol.* **69**:6610–6619.

7. Caldwell, D. E., and J. M. Tiedje. 1974. A morphological study of anaerobic bacteria from the hypolimnia of two Michigan lakes. *Can. J. Microbiol.* **21**:362–376.
8. Croome, R. L., and P. A. Tyler. 1984. Enrichment and nutritional studies on "*Chlorochromatium aggregatum*" in two meromictic lakes in Tasmania. *J. Gen. Microbiol.* **130**:2717–2723.
9. Felsenstein, J. 1989. PHYLIP—phylogenetic interference package (version 3.2). *Cladistics* **5**:164–166.
10. Finneran, K. T., C. V. Johnsen, and D. R. Loveley. 2003. *Rhodoferrax ferrireducens* sp. nov., a psychrotolerant, facultatively anaerobic bacterium that oxidizes acetate with the reduction of Fe(III). *Int. J. Syst. Evol. Microbiol.* **53**:669–673.
11. Fröstl, J. M., and J. Overmann. 1998. Physiology and tactic response of the phototrophic consortium "*Chlorochromatium aggregatum*." *Arch. Microbiol.* **169**:129–135.
12. Fröstl, J. M., and J. Overmann. 2000. Phylogenetic affiliation of the bacteria that constitute phototrophic consortia. *Arch. Microbiol.* **174**:50–58.
13. Fuchs, B. M., F. O. Glöckner, J. Wulf, R. Amann. 2000. Unlabeled helper oligonucleotides increase the in situ accessibility to 16S rRNA of fluorescently labeled oligonucleotide probes. *Appl. Environ. Microbiol.* **66**:3606–3607.
14. Fuchs, B. M., G. Wallner, W. Beisker, I. Schwiippl, W. Ludwig, and R. Amann. 1998. Flow cytometric analysis of the in situ accessibility of *Escherichia coli* 16S rRNA for fluorescently labeled oligonucleotide probes. *Appl. Environ. Microbiol.* **64**:4973–4982.
15. Gasol, J. M., K. Jürgens, R. Massana, J. I. Celadérón-Paz, and C. Pedrós-Alíó. 1995. Mass development of *Daphnia pulex* in a sulfide-rich pond (Lake Ciso). *Arch. Microbiol.* **132**:279–296.
16. Glaeser, J., L. Baneras, H. Rütters, and J. Overmann. 2002. Novel bacteriochlorophyll *a* structures and species-specific variability of pigment composition in green sulfur bacteria. *Arch. Microbiol.* **177**:475–485.
17. Glaeser, J., and J. Overmann. 2003. Characterization and in situ carbon metabolism of phototrophic consortia. *Appl. Environ. Microbiol.* **69**:3739–3750.
18. Glaeser, J., and J. Overmann. 2003. The significance of organic carbon compounds for *in situ* metabolism and chemotaxis of phototrophic consortia. *Environ. Microbiol.* **5**:1053–1063.
19. Glaeser, J., and J. Overmann. 2004. Biogeography, evolution, and diversity of epibionts in phototrophic consortia. *Appl. Environ. Microbiol.* **70**:4821–4830.
20. Glöckner, F. O., E. Zaichikov, N. Belkova, L. Denissova, J. Pernthaler, A. Pernthaler, and R. Amann. 2000. Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of actinobacteria. *Appl. Environ. Microbiol.* **66**:5053–5065.
21. Heising, S., L. Richter, W. Ludwig, and B. Schink. 1999. *Chlorobium ferrooxidans* sp. nov., a phototrophic green sulfur bacterium that oxidizes ferrous iron in coculture with a "*Geospirillum*" sp. strain. *Arch. Microbiol.* **172**:116–124.
22. Hiraishi, A., Y. Hoshino, and T. Satoh. 1991. *Rhodoferrax fermentans* gen. nov., sp. nov., a phototrophic purple nonsulfur bacterium previously referred to as the "*Rhodocyclus gelatinosus*-like" group. *Arch. Microbiol.* **155**:330–336.
23. Holben, W. E., and D. Harris. 1995. DNA-based monitoring of total bacterial community structure in environmental samples. *Mol. Ecol.* **4**:627–631.
24. Holben, W. E., K. P. Feris, A. Kettunen, and J. H. A. Apajalahti. 2004. GC-fractionation enhances microbial community diversity assessment and detection of minority populations of bacteria by denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* **70**:2263–2270.
25. Irgens, R. L., J. J. Gosink, and J. T. Staley. 1996. *Polaromonas vacuolata* gen. nov., sp. nov., a psychrophilic, marine, gas vacuolate bacterium from Antarctica. *Int. J. Syst. Bacteriol.* **46**:822–826.
26. Jung, D. O., L. A. Achenbach, E. A. Karr, S. Takaichi, and M. T. Madigan. 2004. A gas vesiculate planktonic strain of the purple non-sulfur bacterium *Rhodoferrax antarcticus* isolated from Lake Fryxell, Dry Valleys, Antarctica. *Arch. Microbiol.* **182**:236–243.
27. Lane, D. J. 1991. 16S/23S rRNA sequencing, p. 115–175. In E. Stackebrandt and M. Goodfellow (ed.), *Nucleic acid techniques in bacterial systematics*. Wiley, Chichester, United Kingdom.
28. Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettiske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüßmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K.-H. Schleifer. 2004. ARB: a software environment for sequence data. *Nucleic Acids Res.* **32**:1363–1371.
29. Muyzer, G., E. C. De Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**:695–700.
30. Muyzer, G., S. Hottenträger, A. Teske, and C. Waver. 1995. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA—a new molecular approach to analyse the genetic diversity of mixed microbial communities, p. 3.4.4.1–3.4.4.22. In A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (ed.), *Molecular microbial ecology manual*, 2nd ed. Kluwer, Dordrecht, The Netherlands.
31. Nüsslein, K., and J. M. Tiedje. 1998. Characterization of the dominant and rare members of a young Hawaiian soil bacterial community with small-subunit ribosomal DNA amplified from DNA fractionated on the basis of its guanine and cytosine composition. *Appl. Environ. Microbiol.* **64**:1283–1289.
32. Overmann, J. 2001. Green sulfur bacteria, p. 601–623. In G. M. Garrity (ed.), *Bergey's manual of systematic bacteriology*, vol. 1. Williams and Wilkins, Baltimore, Md.
33. Overmann, J. 2001. Phototrophic consortia: a tight cooperation between non-related eubacteria, p. 241–255. In J. Seckbach (ed.), *Symbiosis*. Kluwer, Dordrecht, The Netherlands.
34. Overmann, J., M. J. L. Coolen, and C. Tuschak. 1999. Specific detection of different phylogenetic groups of chemocline bacteria based on PCR and denaturing gradient gel electrophoresis of 16S rRNA gene fragments. *Arch. Microbiol.* **172**:83–94.
35. Overmann, J., U. Fischer, and N. Pfennig. 1992. A new purple sulfur bacterium from saline littoral sediments, *Thiorhodovibrio winogradskyi* gen. nov. and sp. nov. *Arch. Microbiol.* **157**:329–335.
36. Overmann, J., and K. Schubert. 2002. Phototrophic consortia: model systems for symbiotic interrelations between prokaryotes. *Arch. Microbiol.* **177**:201–208.
37. Overmann, J., and C. Tuschak. 1997. Phylogeny and molecular fingerprinting of green sulfur bacteria. *Arch. Microbiol.* **167**:302–309.
38. Overmann, J., C. Tuschak, and J. M. Fröstl. 1998. The ecological niche of the consortium "*Pelochromatium roseum*." *Arch. Microbiol.* **169**:120–128.
39. Pfennig, N. 1978. *Rhodocyclus purpureus* gen. nov., a ring-shaped, vitamin B₁₂-requiring member of the family *Rhodospirillaceae*. *Int. J. Syst. Bacteriol.* **28**:283–288.
40. Reinhold-Hurek, B., and T. Hurek. 2000. Reassessment of the taxonomic structure of the diazotrophic genus *Azoarcus sensu lato* and description of three new genera and new species, *Azovibrio restrictus* gen. nov., sp. nov., *Azospira oryzae* gen. nov., sp. nov., and *Azonexus fungiphilus* gen. nov., sp. nov. *Int. J. Syst. Evol. Microbiol.* **50**:649–659.
41. Sanger, F., G. M. Air, B. G. Barrell, N. L. Brown, A. R. Coulson, C. A. Fiddes, C. A. Hutchison, P. M. Slocumbe, and M. Smith. 1977. Nucleotide sequence of bacteriophage phi X174 DNA. *Nature* **265**:687–695.
42. Schink, B. 1991. Syntrophism among prokaryotes, p. 276–299. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), *The prokaryotes*, 2nd ed. Springer-Verlag, New York, N.Y.
43. Siefert, E., and N. Pfennig. 1984. Convenient method to prepare neutral sulfide solution for cultivation of phototrophic sulfur bacteria. *Arch. Microbiol.* **139**:100–101.
44. Straub, K. L., and B. E. E. Buchholz-Cleven. 1998. Enumeration and detection of anaerobic ferrous iron-oxidizing, nitrate-reducing bacteria from diverse European sediments. *Appl. Environ. Microbiol.* **64**:4846–4856.
45. Tuschak, C., J. Glaeser, and J. Overmann. 1999. Specific detection of green sulfur bacteria by *in situ* hybridization with a fluorescently labeled oligonucleotide probe. *Arch. Microbiol.* **171**:265–272.
46. Vinograd, J., and J. E. Hearst. 1962. Equilibrium sedimentation of macromolecules and viruses in a density gradient. *Fortschr. Chem. Org. Naturst.* **20**:373–422.
47. Vogl, K., J. Glaeser, K. R. Pfannes, G. Wanner, and J. Overmann. Submitted for publication.
48. Watanabe, K., K. Watanabe, W. Kodama, K. Sytupso, and S. Harayama. 2000. Molecular characterization of bacterial populations in petroleum-contaminated groundwater discharged from underground crude oil storage cavities. *Appl. Environ. Microbiol.* **66**:4803–4809.
49. Widdel, F., G. W. Kohring, and F. Mayer. 1983. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. III. Characterization of the filamentous gliding *Desulfonema limicola* gen. nov., sp. nov., and *Desulfonema magnum* sp. nov. *Arch. Microbiol.* **134**:286–294.
50. Willems, A., J. DeLey, M. Gillis, and K. Kersters. 1991. *Comamonadaceae*, a new family encompassing the *Acidovorans* rRNA complex, including *Variovorax paradoxus* gen. nov., comb. nov., for *Alcaligenes paradoxus* (Davis 1969). *Int. J. Syst. Bacteriol.* **41**:445–450.
51. Wolfe, R. S., and N. Pfennig. 1977. Reduction of sulfur by *Spirillum* 5175 and syntrophism with *Chlorobium*. *Appl. Environ. Microbiol.* **33**:427–433.