Isolation and characterization of aggregate-forming sulfate-reducing and purple sulfur bacteria from the chemocline of meromictic Lake Cadagno, Switzerland

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Abstract

In situ hybridization with specific oligonucleotide probes was used to monitor enrichment cultures of yet uncultured populations of sulfate-reducing and small-celled purple sulfur bacteria found to associate into aggregates in the chemocline of meromictic Lake Cadagno, Switzerland, and to select potential isolates. Enrichment and isolation conditions resembled those of their nearest cultured relatives, the sulfate-reducing bacterium *Desulfocapsa thiozymogenes* and small-celled purple sulfur bacteria belonging to the genus *Lamprocystis*, respectively. Based on comparative 16S rRNA analysis and physiological characterization, isolate Cad626 was found to resemble *D. thiozymogenes* although it differed from the type strain by its ability to grow on lactate and pyruvate. Like *D. thiozymogenes*, isolate Cad626 was able to disproportionate inorganic sulfur compounds (sulfur, thiosulfate, sulfite) and to grow, although growth on sulfur required a sulfide scavenger (FeOOH). Isolate Cad16 represented small-celled purple sulfur bacteria that belonged to a previously detected, but uncultured population designated F and was related to *Lamprocystis purpurea* as evidenced by comparative 16S rRNA analysis and the presence of bacteriochlorophyll *a* and the carotenoid okenone. Mixed cultures of isolates Cad626 and Cad16 resulted in their association in aggregates similar to those observed in the chemocline of Lake Cadagno. Concomitant growth enhancement of both isolates in mixed culture suggested synergistic interactions that presumably resemble a source–sink relationship for sulfide between the sulfate-reducing bacterium growing by sulfur disproportionation and the purple sulfur bacteria acting as biotic scavenger.
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1. Introduction

Permanently stratified lakes such as the meromictic Lake Cadagno, Switzerland, represent optimal model systems for the study of aquatic microorganisms since defined and stable vertical gradients of environmental conditions such as light intensity and quality, oxygen availability, or the presence of sulfide support the development of diverse species of microorganisms adapted to defined ecological niches [1]. Lake Cadagno is characterized by a permanent stratification stabilized by density differences of salt-rich water constantly supplied by subaquatic springs to the monimolimnion and of electrolyte-poor surface water feeding the mixolimnion [2]. A permanent chemocline generally found at a depth between 11 and 13 m and characterized by high concentrations of sulfate and steep gradients of sulfide [3,4] supports the growth of elevated numbers of bacteria (up to 10^7 cells ml^{-1}) indicating that a bacterial community making use of these gradients is present [5,6]. Depending on the season as much as 35–45% of the total microbial community is associated in aggregates consisting of small-celled purple sulfur bacteria (15–35% of the total microbial community) [7,8] and sulfate-reducing bacteria (13–18% of the total microbial community) [8]. Molecular methods identified four major populations of purple sulfur bacteria in these aggregates forming a tight cluster with the genus *Lamprocystis*, i.e. *L. purpurea*, *L. roseopersicina*, and two yet uncultured

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populations D and F [6]. All four populations form associations with sulfate-reducing bacteria related to *Desulfocapsa thiozymogenes* [9]. The latter account for up to 72% of all sulfate-reducing bacteria and almost completely represent those belonging to the family Desulfovibrionaceae [8]. The association between small-celled purple sulfur bacteria and these sulfate-reducing bacteria is not obligate since non-associated cells of bacteria related to *D. thiozymogenes* were frequently found in winter and early summer when limiting light conditions caused by snow and ice cover had reduced the abundance of small-celled phototrophic sulfur bacteria to below 25% of the values found in late summer [8]. Nonetheless, the association suggests an ecological advantage to both groups of organisms under appropriate environmental conditions.

Since the bacterial partners of the association in the chemocline of Lake Cadagno have not been obtained in pure culture yet, specific traits of their closest cultured relatives have been used previously to speculate about their potential interactions in the aggregate [7–9] even though it was acknowledged that phylogenetic relationships do not necessarily reflect metabolic similarities [10]. *D. thiozymogenes* DSM7269, for example, can grow by disproportionation of thiosulfate and sulfite to sulfate and sulfide [11]. It also disproportionates elemental sulfur, though growth was only observed in the presence of a sulfide scavenger such as amorphous ferric hydroxide [11], similar to conditions found for *Desulfocapsa sulfoexigens* DSM10523 and *Desulfobulbus propionicus* DSM2032 [12,13]. This generally results in the formation of sulfate along with iron sulfides [13,14] and thus removes free sulfide from the culture [11]. The small-celled phototrophic sulfur bacteria *L. purpurea* and *L. roseopersicina* both photo-oxidize sulfide to sulfur and further to sulfate [15,16]. Small-celled phototrophic sulfur bacteria in the chemocline of Lake Cadagno might therefore act as sulfide scavengers creating a sink for sulfide produced by sulfur disproportionation of the sulfate-reducing bacteria in the association. The consumption of sulfide by small-celled sulfur phototrophic bacteria might therefore enhance the activity of bacteria related to *D. thiozymogenes* while these would provide a continuous supply of electron donors for the small-celled phototrophic sulfur bacteria. Thus, principally the association would benefit both small-celled sulfur phototrophic bacteria as well as the bacteria related to *D. thiozymogenes*.

Such speculations, however, can only be confirmed with detailed pure culture studies with both partners of this association. The aim of this study was therefore to isolate both small-celled sulfur phototrophic bacteria and the bacteria related to *D. thiozymogenes* found in the chemocline of Lake Cadagno, to confirm their metabolic similarity with their closest cultured relatives, to show aggregate formation and association of both organisms in vitro and to demonstrate beneficial effects of mixed culture on growth performance of both organisms.

2. Materials and methods

2.1. Enrichment and isolation

Samples from the chemocline of Lake Cadagno were taken with a Friedinger-type bottle (Zuellig AG, Rheineck, Switzerland) at the maximum of turbidity corresponding to the highest bacterial density in October 1999. Samples were used to completely fill 0.5-l screw-cap glass bottles that were subsequently stored in the dark at 4°C for a week. Aggregates of sulfate-reducing and small-celled phototrophic sulfur bacteria, macroscopically identified by the characteristic purple-red color of the phototrophic sulfur bacteria, that accumulated at the neck of the bottle and under the screw-cap were then collected with a previously gassed syringe (N_2) and served as concentrated inoculum for liquid and deep agar dilutions (1% v/v) prepared by the Hungate technique [17,18]. Media for both sulfate-reducing and purple sulfur bacteria were prepared in a 2-l bottle with a N_2/CO_2 (80%/20%) gas phase according to Widdel and Bak [18].

Sulfate-reducing bacteria related to *D. thiozymogenes* were enriched and cultivated in a bicarbonate-buffered (30 ml l^{-1} of 1 M of $NaHCO_3$ in water solution), sulfide-reduced (1 ml l^{-1} of a 1 M Na_2S in water solution) and sulfate-free basal medium that also contained (l^{-1}) 0.5 g of KH_2PO_4, 0.3 g of NH_4Cl, 0.5 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 1 ml of non-chelated trace element mixture, 1 ml of selenite–tungstate solution, 1 ml of vitamin mixture, 1 ml of vitamin B_{12} solution, and 1 ml of thiamine solution [18]. Before inoculation, different combinations of electron donors and acceptors were aseptically added from sterile stock solutions (final concentration): ethanol (5 mM) and sulfate (20 mM); propanol (5 mM) and sulfate (20 mM); lactate (5 mM) and sulfate (20 mM); and thiosulfate (10 mM) and acetate (1 mM) as used for the isolation of *D. thiozymogenes* DSM7269 [11]. Headspace gas was 80% N_2 and 20% CO_2.

Small-celled phototrophic sulfur bacteria were enriched and cultured in medium containing (l^{-1}) 0.25 g KH_2PO_4, 0.34 g NH_4Cl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.25 g $CaCl_2 \cdot 2H_2O$, 0.34 g KCl, 1.5 g $NaHCO_3$, 0.5 ml trace element solution SL_{10}, and 0.02 mg vitamin B_{12} [15]. The medium was reduced with 0.3 g l^{-1} $Na_2S \cdot 9H_2O$ (1.10 mM final concentration) and adjusted to a pH around 7.2. Acetate (2 mM) was added to pure cultures of phototrophic bacteria.

All cultures were incubated at room temperature (20–23°C). Sulfate-reducing bacteria were incubated in the dark, while purple sulfur bacteria were subjected to a photoperiod (6 h light/6 h dark) with low light intensities generated with an incandescent 40-W bulb placed at a distance of 60 cm from the cultures [15]. Enrichments of sulfate-reducing bacteria in liquid culture were periodically checked for growth by microscopy and for sulfide formation or iron sulfide precipitation, when FeOOH was

present. Small-celled phototrophic sulfur bacteria were initially enriched exploiting the tendency of gas-vacuolated species to accumulate in the upper part of the culture bottle [15,19]. Several transfers with cells taken from the surface of the culture vessel [20] thus preceded the purification steps in agar-shake dilutions series [17]. Enrichments in liquid media as well as single colonies from deep agar dilutions were always resuspended in 5 ml liquid medium before inoculation into a new agar-shake series.

2.2. Identification and characterization

Enrichments and single colonies were analyzed for target organisms by in situ hybridization in a top-to-bottom approach. Sulfate-reducing bacteria were initially monitored using Cy3-labeled probes SRB385 [21] and SRB385Db [22] targeting members of the families Desulfovibrionaceae and Desulfobacteriaceae, respectively. Cells hybridizing with probe SRB385 were further analyzed with probe SRB441 targeting yet uncultured free-living sulfate-reducing bacteria with no identified cultured relative and combined probes DSC213 and DSC441 targeting sulfate-reducing bacteria related to *D. thiozymogenes* [9]. Small-celled phototrophic sulfur bacteria were analyzed with Cy3-labeled probes Apur453 targeting *L. purpurea* DSM4197, Laro453 targeting *L. roseopersicina* DSM229, and S453D and S453F, both targeting yet uncultured populations of phototrophic sulfur bacteria [6]. In situ hybridization was performed on aliquots (3 μl) of fresh cultures spotted onto gelatin-coated slides [23] as described previously [6,9]. A strain was considered pure when all cells hybridized with one specific probe. Purity of sulfate-reducing strains was also tested using the medium described above supplemented with 0.25% (w/v) yeast extract, 5 mM pyruvate, 5 mM glucose and 5 mM fumarate [11].

Pure cultures that hybridized to probes DSC213 and DSC441 (targeting sulfate-reducing bacteria related to *D. thiozymogenes*) or to probe S453F (targeting yet uncultured phototrophic sulfur bacteria) were initially identified by comparative 16S rRNA sequence analysis. Nucleic acids were extracted from pure cultures using the MagNA Pure LC automated extractor (Roche Molecular Biochemicals, Indianapolis, IN, USA) and the DNA isolation extraction kit produced by the same manufacturer. 16S rDNA was amplified and purified as described previously [6] and sequenced with an ABI Prism Ready Reaction dye deoxy terminator cycle sequencing kit and an ABI Prism 310 automated sequencer (Perkin-Elmer). Sequences were aligned with a subset of bacterial 16S rDNA sequences obtained from the Ribosomal Database Project (RDP) [24] using the CLUSTAL W service at EBI [25]. Sequences from the 16S rRNA gene clone library of Lake Cadagno [6,9,26] as well as sequences of other purple sulfur and sulfate-reducing bacteria were included in the phylogenetic analysis. Phylogenetic relationships were estimated using the Phylogeny Inference Package (PHYLIP version 3.573c). Kimura two-parameter evolutionary distances were calculated using the DNADIST program and phylogenetic trees were derived using the FITCH program with random order input of sequences and the global rearrangement option [27]. The sequences obtained were deposited in the EMBL/GenBank databases with accession numbers AJ511274 (Cad16) and AJ511275 (Cad626), respectively.

The sulfate-reducing bacterium, isolate Cad626, was further characterized with respect to its ability to grow with different electron donors and acceptors [11]. *D. thiozymogenes* DSM7269, purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), was analyzed concomitantly. Stock solutions (300 mM) of amorphous ferric hydroxide (FeOOH) solution were prepared as described by Lovely and Philips [28] and diluted to a final concentration of 30 mM in cultures [11,12]. Stock solutions of inorganic sulfur compounds, i.e. $Na_2S_2O_3$, Na_2SO_3 and flowers of sulfur, were prepared according to Janssen et al. [11]. Final concentrations in culture were 10 mM for both thiosulfate and sulfite, and 20–30 mg S ml^{-1} culture medium [12].

The small-celled phototrophic sulfur bacterium, isolate Cad16, was further characterized based on morphological criteria and pigment analysis. The absorption spectra of living cells were measured with a UV/Vis Spectrometer Lambda 2S (Perkin-Elmer) in a 1-cm cuvette following the procedure described by Pfennig [29]. Before measurements, cell suspensions were treated in an ultrasonic bath to reduce scattering [15]. Chemolithoautotrophic growth of strain Cad16 was tested as described by Kämpf and Pfennig [30]. Characteristics of isolate Cad16 were compared to those published for its closest cultured relatives *L. purpurea* DSM4197 [15] and strain LcCad1 [15], and *L. roseopersicina* DSM229 [16,20].

2.3. Mixed culture study

Studies on interactions between isolates Cad626 and Cad16 were performed in basal medium used for sulfate-reducing enrichments supplemented with 1 mM acetate and flowers of sulfur in excess (20–30 mg ml^{-1}) as the sole energy source for isolate Cad626. Headspace gas was 80% N_2 and 20% CO_2, and the incubation temperature 20°C. Experiments were carried out in duplicate at low light intensities and a 6-h light/dark photoperiod for 60 days. Growth of the sulfate-reducing bacterium, isolate Cad626, was analyzed in pure culture, with or without FeOOH as scavenger, and in mixed culture with the purple sulfur bacterium, isolate Cad16. Growth of isolate Cad16 was monitored in pure culture and in mixed culture with isolate Cad626. Both isolates were inoculated at an initial density of approx. 5×10^5 cells ml^{-1}. Rough estimates of
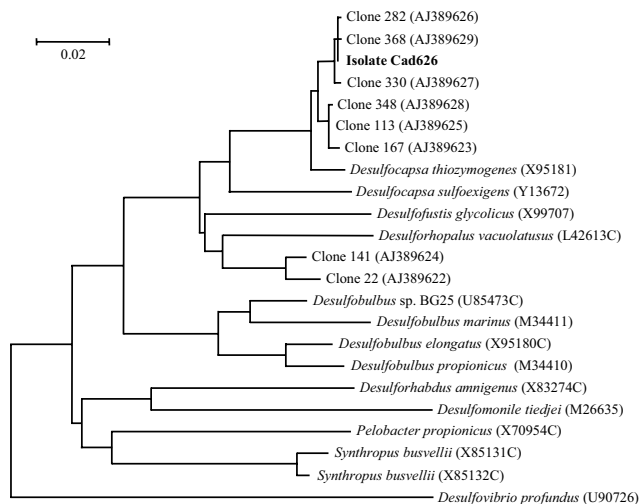
Fig. 1. Neighbor-joining tree showing the relationship of isolate Cad626 to clones from the 16S rRNA gene clone library of Lake Cadagno, to *Desulfocapsa* species and other related sulfate-reducing bacteria. The distance scale indicates the expected number of changes per sequence position.

the abundance of both organisms by OD measurements at 440 nm and 650 nm, respectively, were followed by microscopic enumeration after DAPI staining and in situ hybridization with oligonucleotide probes DSC213 and DSC441 or probe S453F.

3. Results

3.1. Isolation and characterization of the sulfate-reducing bacterium, isolate Cad626

After about 10–12 weeks of incubation, sulfide and iron sulfide formation was detected in enrichments with ethanol, propanol, lactate and thiosulfate as electron donors and acceptor, respectively. Cells hybridizing to probes DSC213 and DSC441, however, were only detected in enrichments with either 1 mM acetate and 10 mM thiosulfate or 10 mM lactate and 20 mM sulfate. From these enrichments and several subsequent series of agar-shake dilutions with 10 mM lactate and 20 mM sulfate, isolate Cad626 was finally obtained in pure culture with all cells hybridizing to probes DSC213 and DSC441.

Comparative sequence analysis of the 16S rRNA gene confirmed the hybridization data placing isolate Cad626 into the δ-subdivision of Proteobacteria with 99.9% and 99.7% similarity, respectively, to clones 282 (AJ389626) and 368 (AJ389629) previously retrieved from the chemocline of Lake Cadagno, and 97.8% similarity to *D. thiozymogenes* DSM7269, the closest cultured relative and the type strain of the genus (Fig. 1).

Cells of isolate Cad626 were Gram-negative, motile rods with a width of 0.4–0.5 μm and a length of 1.0–2.2 μm (Fig. 2). During exponential growth, single rods were
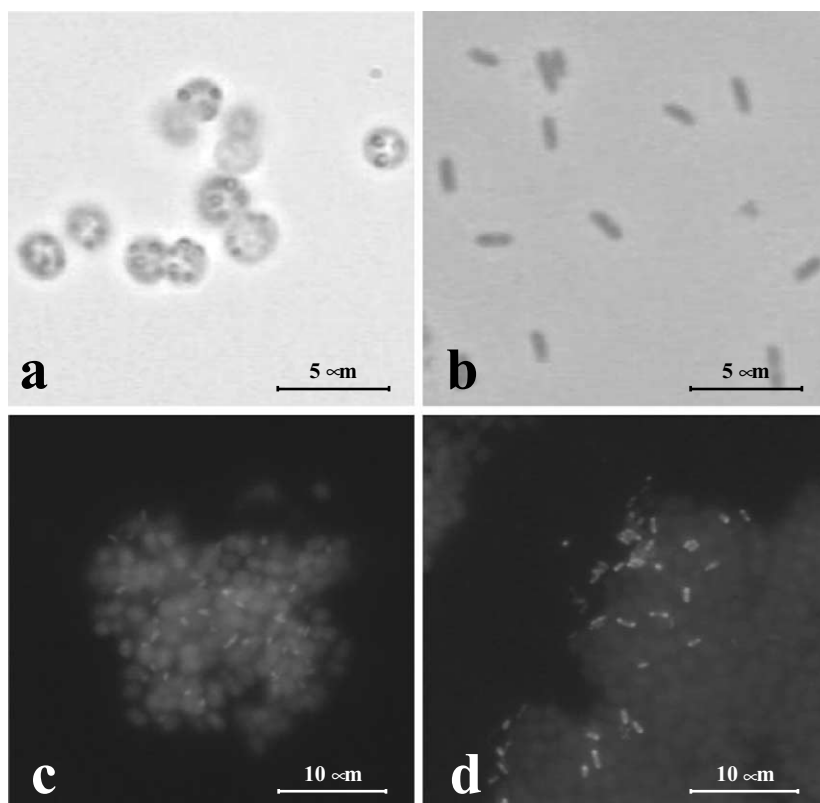

Fig. 2. Phase contrast micrographs of isolates Cad16 (a) and Cad626 (b), as well as epifluorescence micrographs of aggregates formed in the chemocline of Lake Cadagno (c) or by mixed cultures of isolates Cad16 and Cad626 (d) after in situ hybridization with probes DSC213 and DSC441.

Table 1
Comparison of selected metabolic characteristics of isolate Cad626 and its closest cultured relatives

	Isolate Cad626	*Desulfocapsa thiozymogenes* (DSM 7269)	*Desulfocapsa thiozymogenes* (strain Bra2)[a]	*Desulfocapsa sulfoexigens* (DSM 10523)[a]	*Desulfobulbus propionicus* (DSM 2032)[a]	*Desulfobulbus elongatus* (DSM 2908)[a]	*Desulfobulbus marinus* (DSM 2058)[a]	*Desulforhopalus vacuolatus* (DSM 9700)[a]
Sulfate reduction with								
Ethanol	+	+	+	−	+	+	+	+
Propanol	+	+	+	−	+	+	+	+
Butanol	+	+	+	−	n.a.	n.a.	n.a.	−
Lactate	**+**	−	−	−	+	+	+	+
Propionate	−	−	−	−	+	+	+	+
Pyruvate	**+**	−	−	−	+	+	+	(+)[c]
Acetate	−	−	−	−	−	−	−	−
Disproportionation of								
Sulfur								
+FeOOH	+	+	+	+	+	−	−	−
−FeOOH	(+)[b]	(+)[b]	(+)[b]	(+)[b]	n.a.	−	−	n.a.
Thiosulfate								
+FeOOH	+	+	+	+	+	n.a.	n.a.	−
−FeOOH	+	+	+	+	+	n.a.	n.a.	n.a.
Sulfite								
+FeOOH	+	+	+	+	n.a.	n.a.	n.a.	n.a.
−FeOOH	+	+	+	+	n.a.	n.a.	n.a.	n.a.

n.a., not available.
[a] Data from Janssen et al. [11].
[b] No increase in cells number but disproportionation.
[c] No sulfide detection.

evenly distributed in the culture medium, but developed into elongated cells that formed small aggregates during stationary phase. Growth was inhibited at temperatures higher than 25°C. Cells grew by sulfate reduction (20 mM) and oxidation of butanol, ethanol, lactate, propanol and pyruvate (each 10 mM), but not of acetate, glucose, propionate, and fumarate (each 10 mM) (Table 1). In the absence of sulfate, no fermentative growth was observed on butanol, ethanol, lactate, propanol, propionate or pyruvate.

Isolate Cad626 could grow by thiosulfate and sulfite disproportionation. A scavenger for sulfide was not necessary, although its presence (i.e. amorphous FeOOH) reduced the lag phase and resulted in higher growth yields. Sulfur disproportionation was indicated in the absence of FeOOH through the production of sulfide and sulfate was not accompanied by an increase in cell number. In the presence of FeOOH, isolate Cad626 grew by disproportionation of sulfur. The metabolic characteristics of isolate Cad626 generally resembled those of *D. thiozymogenes* DSM7269 although it differed from the type strain by its ability to grow on lactate and pyruvate (Table 1).

3.2. Isolation and characterization of the phototrophic sulfur bacterium, isolate Cad16

After about 4–6 weeks of incubation at low light intensity, growth of phototrophic sulfur bacteria was macroscopically detected in enrichments. Strain Cad16 was isolated from enrichments in liquid media with 0.83 mM sulfide as electron donor and 2 mM acetate as organic carbon source after several series of agar-shake dilutions. All cells hybridized to probe S453F.

Comparative sequence analysis of the 16S rRNA gene confirmed the hybridization data placing isolate Cad16 in the γ-subdivision of Proteobacteria with 100% sequence similarity to clone 371 (AJ006061) representing population F of small-celled purple sulfur bacteria in the chemocline of Lake Cadagno (Fig. 3). The highest sequence similarities to cultured relatives were found for *L. purpurea* (95.3%) and *L. roseopersicina* (95.4%).

Cells of isolate Cad16 were non-motile, spherical to oval cells of 1.4 μm to 2.4 μm wide (Fig. 2; Table 2). In liquid
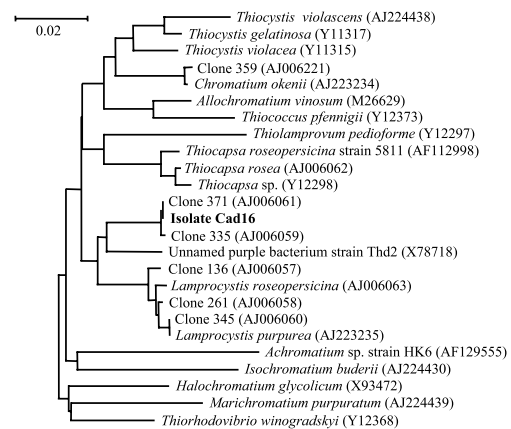

Fig. 3. Neighbor-joining tree showing the relationship of isolate Cad16 to clones from the 16S rRNA gene clone library of Lake Cadagno, to *Lamprocystis* species and other related phototrophic sulfur bacteria. The distance scale indicates the expected number of changes per sequence position.

Table 2
Comparison of some characteristics of isolate Cad16, isolated from chemocline samples of Lake Cadagno as a representative of purple sulfur bacteria belonging to population F, with those of its closest cultured relatives

	Population F (strain Cad16)	*Lamprocystis purpurea* (strain LcCad1)	*Lamprocystis purpurea* (DSM4197^T)	*Lamprocystis roseopersicina* (DSM229)
Shape	Spherical to oval	Spherical to oval	Spherical to oval	Spherical
Size (μm)	1.4–2.4	3.3–3.8×3.5–4.5	1.9–2.3×2.0–3.2	3.0–3.5
Aggregate formation	+	+	+	+
Gas vacuoles	+	+	+	+
Sulfur storage	+	+	+	n.d.
Slime capsule	+	+	+	−
Color of cell suspension	purple-red	purple-red	purple-red	purple
Motility	−	−	−	+
Carotenoid group	okenone	okenone	okenone	lycopenal, lycopenol
Chemolithotrophic growth	+	+	+	−

n.d., not determined.

media isolate Cad16 developed as single cells as well as in irregular aggregates of variable size with up to about 100 cells. Cells of isolate Cad16 stained Gram-negative, contained gas vacuoles and had a slime capsule. Bright field microscopy revealed the presence of sulfur globules in the cells. The color of cell suspensions was purple-red. In vivo absorption spectra of cell suspensions displayed absorption maxima at 833 nm, 582 nm and 374 nm indicating the presence of bacteriochlorophyll *a* and one at 528 nm suggesting the presence of the carotenoid okenone (Fig. 4).

Photolithoautotrophic growth of isolate Cad16 under anaerobic conditions occurred with hydrogen sulfide, and elemental sulfur as electron donors. Globules of sulfur were stored inside the cells as intermediary oxidation products. In the presence of carbon dioxide and sulfide, photoassimilation of acetate was observed. Chemolithoautotrophic growth was observed with hydrogen sulfide or thiosulfate under micro-oxic conditions in the dark. These characteristics correspond to those published for *L. purpurea* DSM4197 and strain LcCad1 (Table 2).

3.3. Mixed culture study

Mixed cultures of the sulfate-reducing bacterium, isolate Cad626, and the purple sulfur bacterium, isolate Cad16, generally resulted in higher cell numbers for both organisms than obtained in pure culture under the same conditions. Highest numbers were obtained for isolate Cad626 in pure culture in the presence of FeOOH as sulfide scavenger with cell numbers increasing about 155-fold during the incubation period of 60 days. No growth of isolate Cad626 was observed in the absence of FeOOH (Fig. 5). Without FeOOH, but in the presence of isolate Cad16, however, isolate Cad626 grew significantly with number of cells increasing about 47-fold during the incubation period of 60 days. Isolate Cad16 grew in pure culture though at lower cell density than in mixed culture with isolate Cad626 (Fig. 6). During the incubation, sulfide was not detectable in mixed cultures and in pure cultures with FeOOH. In pure as well as in mixed cultures, cells of the small-celled purple sulfur bacterium Cad16 developed aggregates during the incubation period (Fig. 2). In contrast to the appearance of these aggregates in the chemocline of Lake Cadagno, however, they seemed to be less dense (Fig. 2). In mixed cultures, the sulfate-reducing bacterium Cad626 in part associated with these aggregates but was also observed with no direct contact to cells of Cad16. With about half of the cells of isolate Cad626 being non-
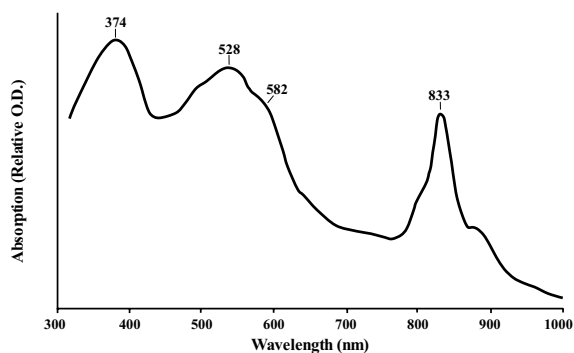

Fig. 4. Absorption spectrum of living cells of isolate Cad16. The presence of bacteriochlorophyll *a* is indicated by absorption peaks at 833 nm, 582 nm and 374 nm, that of the carotenoid okenone by the absorption peak at 528 nm.
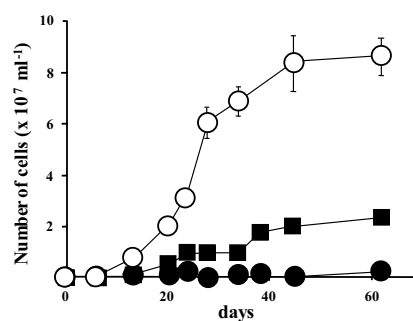

Fig. 5. Growth of the sulfate-reducing bacterium, isolate Cad626, in pure culture, with (○) or without (●) FeOOH as scavenger, or in mixed culture with isolate Cad16 (■), a small-celled purple sulfur bacterium representing population F.
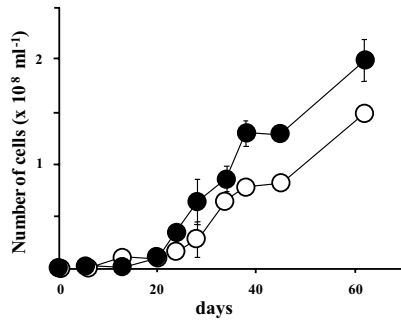
Fig. 6. Growth of isolate Cad16, a small-celled purple sulfur bacterium representing population F in pure culture (○) and in mixed culture with the sulfate-reducing isolate Cad626 (●).

associated, their proportion was higher compared to observations in the chemocline of Lake Cadagno. Isolate Cad16 grew faster than the sulfate-reducing bacterium Cad626 which resulted in large proportional shifts from about 1 at the beginning of the incubation to 0.12 after 60 days. The latter proportion approximated the value generally found in the chemocline of Lake Cadagno in June (0.18).

4. Discussion

A variety of methods can be used for the isolation and cultivation of physiologically and genetically different bacteria from environmental samples. The reliance on culture techniques alone, however, bears the risk of retrieving the most easily culturable bacteria from the natural community only, and not the most frequently occurring ones [31,32]. Our isolation attempt on yet uncultured populations of sulfate-reducing and small-celled purple sulfur bacteria from the chemocline of Lake Cadagno took advantage of results of previous studies that provided background data on the usefulness of molecular tools, i.e. specific probes detecting the dominant bacterial populations in the chemocline [6–9]. Using these tools, we successfully followed the strategy of others to monitor enrichment cultures [22,33,34] with the aim of isolating bacteria with a high ecological significance, i.e. a numerical abundance with up to 50% of the total number of bacteria and a potential interaction since they were found to occur mainly associated in aggregates in the chemocline of Lake Cadagno [8].

Although phylogenetic relationships do not necessarily reflect physiological relationships [10,35,36], enrichment and isolation conditions that resembled those for the nearest cultured relatives of our target organisms, the sulfate-reducing bacterium D. thiozymogenes DSM7269 and small-celled purple sulfur bacteria belonging to the genus Lamprocystis, respectively, were successfully used to retrieve isolate Cad626 resembling D. thiozymogenes and isolate Cad16 representing population F related to L. purpurea. The limited number of physiological traits of the

isolates analyzed in this study resembled those of D. thiozymogenes and L. purpurea, respectively, that had been used in previous studies to speculate about the potential nature of the association [7–9]. The availability of pure cultures of both organisms now opened up the opportunity to study potential interactions by comparing growth of a mixed culture with that of the respective pure cultures using the same experimental conditions and inoculation densities [37,38].

In mixed culture, cells of both organisms grew and assembled in aggregates similar to those observed in the chemocline of Lake Cadagno. The cells of isolate Cad626, however, seemed to be more attached to the surface of aggregates of purple sulfur bacteria rather than deeply inserted into the aggregate as encountered in the chemocline [7,9]. The association is not obligatory since about half of the cells of the sulfate-reducing bacterium remained non-associated, a situation similarly encountered in the chemocline of Lake Cadagno during winter and spring when purple sulfur bacteria were significantly reduced numerically due to limited light conditions [8]. In the presence of large numbers of purple sulfur bacteria in the chemocline during summer and fall, however, most of the Desulfocapsa-like sulfate-reducing bacteria were associated [8]. Although the association is not highly structured as described for phototrophic consortia [39], it seems to be a highly specific synergistic relationship between sulfate-reducing bacteria related to the genus Desulfocapsa and four distinct groups of purple sulfur bacteria of the genus Lamprocystis [7,8].

In addition to sulfate reduction, isolate Cad626 was able to grow by disproportionation of inorganic sulfur compounds similar to D. thiozymogenes DSM7269 [11]. Disproportionation of sulfur to sulfate and sulfide and growth required the presence of a sulfide scavenger (FeOOH) similar to observations with D. thiozymogenes DSM7269 [11], Desulfocapsa sulfoexigens DSM10523 [12] and Desulfobulbus propionicus DSM2032 [13]. Without a sulfide scavenger, isolate Cad626 could not grow. FeOOH, however, could be replaced by the purple sulfur bacterium, isolate Cad16. In mixed culture, isolates Cad626 and Cad16 displayed a synergistic relationship since both benefited from the presence of the other organism showing increased growth compared to pure cultures. These results and the close spatial proximity of both organisms in aggregates suggest a physiological interaction presumably resembling a source–sink relationship for sulfide between the sulfate-reducing bacterium growing by sulfur disproportionation and the purple sulfur bacteria acting as biotic scavenger.

Sulfate reduction or disproportionation of isolate Cad626 in association with aggregates of isolate Cad16 might also overcome sulfide limitations of these small-celled phototrophic sulfur bacteria during periods of intensive photo-oxidation in the upper part of the layer where the highest light intensities are encountered [7,40,41]. The latter assumption is probably more pro-

nounced under high light intensity conditions and might explain the closer association between both partners during summer and fall than during winter and spring when low light conditions prevail [7,8]. Under artificial conditions in the laboratory with excess of sulfur, the additional sulfide source might only marginally increase its availability for the purple sulfur bacterium and thus explain the only small effect on growth of isolate Cad16. The source–sink relationship for sulfide between both organisms might be a reasonable explanation for their association and the growth increase under laboratory conditions, however, it still remains speculative since no data on sulfur transformations are yet available.

Under natural conditions in the chemocline, several additional facets of potential interactions in aggregates must be considered since both organisms are metabolically highly versatile and interactions may not be limited only to sulfur compounds. The metabolic properties of phototrophic sulfur bacteria, for example, are different in the presence or absence of light [42–44] and depend on the position of the organisms in the bacterial plume [45,46]. In the absence of light, interactions with sulfate-reducing bacteria might lose their synergistic character if storage polymers such as glycogen or polyhydroxyalkanoates are oxidized with the concomitant reduction of sulfur stored intracellularly [43]. Aggregation, however, could also confer better a resilience of both associated organisms to environmental stresses such as the presence of oxygen [47–50] that could occur in zones with overlapping oxygenic and anoxygenic photosynthesis [44] or reduced sulfide availability in the upper part of the bacterial layer [51]. Thus, further studies on the interaction between isolates Cad626 and Cad16 need to address the effects of varying environmental conditions on growth and aggregate formation of both organisms. In addition, the remaining three populations of uncultured small-celled purple sulfur bacteria must be incorporated into these studies that should also include an attempt to imitate and maintain the environmental conditions found in the upper part of the chemocline of Lake Cadagno.

Acknowledgements

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