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Phylogenetic biodiversity and *in situ* detection (whole cell hybridization) of the microbial flora from Lake Cadagno

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Riassunto: Il lago di Cadagno è un lago alpino situato ad un'altitudine di 1923 m nella val Piora, posta a sud delle Alpi svizzere (46°33' N, 8°43' E). Il lago ha una superficie di 26 x 10^5 m^2 ed una profondità massima di 21 m. L'infiltrazione di acqua percolata in rocce di dolomia saccaroide ricca di gesso ed immessa nel lago attraverso sorgenti sottolacustri determina uno stato di meromissi crenogenica. Il lago è caratterizzato dunque da una elevata salinità del monimolimnio e dalla presenza di un chemoclinio ad una profondità di 9-14 m (Peduzzi et al. 1998). Le analisi iniziali centrate sulla distribuzione verticale e temporale delle popolazioni di zolfobatteri del lago Cadagno sono state effettuate grazie all'utilizzo di fattori discriminanti quali la morfologia cellulare e l'autofluorescenza. Studi seguenti si sono focalizzati sull'analisi dei gruppi filogenetici maggiori quali le suddivisioni α, β, γ e δ del dominio *Bacteria* utilizzando le tecniche di ibridazione cellulare *in situ* combinata con l'analisi morfologica.

Alfine di descrivere la natura delle popolazioni batteriche osservate microscopicamente nel chemoclinio e nel monimolimnio sottostante, abbiamo amplificato (attraverso PCR utilizzando primers del dominio *Bacteria*) e clonato i geni 16S rDNA dei batteri del monimolimnio del lago Cadagno (campione di acqua prelevato a 17 m di profondità).

Gli inserti clonati sono stati riamplificati per confermare la presenza dell'inserto globale di 1.4 kb, che è stato detettato in 276 cloni e parzialmente sequenziato.

La distribuzione delle diverse popolazioni di zolfobatteri fototrofi nel chemoclinio è stata analizzata utilizzando 5 sonde diverse sviluppate specificatamente. La sonda oligonucleotidica **Cmok453** diretta contro l'ARN ribosomale 16S è stata progettata per la detezione specifica di *Chromatium okenii* DSM 169 come pure la sequenza dell'ARN ribosomale 16S inserita nel clone 359. La sonda **Apur453** effettua la detezione di *Amoebobacter purpureus* DSM 4197 e la sequenza nel clone 345. La sonda **Laro453** è specifica per *Lamprocystis roseopersicina* DSM 229 e la sequenza nel clone136. Le sonde **S453D** e **S453F** ibridizzano con l'ARN ribosomale 16S di batteri con sequenza uguale a quella presente rispettivamente nel clone 261 e nei cloni 335, 371.

Abstract: Lake Cadagno is an alpine lake situated 1923 m above sea level in the Piora valley in the south of Switzerland (46°33' N, 8°43' E). The lake has a surface area of 26 x 10^5 m^2 and a maximum depth of 21 m. Due to the infiltration of water through dolomite rich in gypsum, Lake Cadagno is a meromictic lake characterized by a high salinity of the monimolimnion and a permanent chemocline in a depth between 9 and 14 m (Peduzzi et al. 1998). Initial analyses on the spatial and temporal distribution of different populations of sulfur bacteria of the meromictic lake Cadagno where performed by means of distinctive parameters, namely cell morphology and autofluorescence. Further studies dealt with the analysis of major phylogenetic groups of bacteria, i.e. the α, β, γ and δ subdivisions of Proteobacteria, by *in situ* hybridization and a concomitant characterization of these populations by morphological criteria.

To resolve the nature of the bacterial populations observed by microscopy in the chemocline and in the underlying monimolimnion, we amplified (by PCR with universal primers for the domain *Bacteria*) and cloned the 16S rDNA genes of bacteria from the monimolimnion of the Lake Cadagno (water sample taken at 17 meters depth).

The cloned inserts were reamplified to confirm the presence of the 1.4 kb full-length insert, which was detected in 276 clones, and partially sequenced.

The distribution of the different populations of phototrophic sulfur bacteria in the chemocline could be analysed by the utilization of 5 probes which we had specifically developped.

16S rRNA-targeted oligonucleotide probe **Cmok453** was designed to detect *Chromatium okenii* DSM 169 as well as our clone 359. Probe **Apur453** targeted *Amoebobacter purpureus* DSM 4197, and also the sequence of our clone 345. Probe **Laro453** targeted *Lamprocystis roseopersicina* DSM 229, as well as the sequence of clone 136. Probes **S453D** and **S453F** targeted the 16S rRNA of bacteria harboring the sequence of clone 261 and those of clones 335 and 371, respectively.

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ANALYSIS OF BACTERIOPLANKTON BY DIRECT MICROSCOPICAL OBSERVATION

The Lake Cadagno was well stratified during the whole period of analysis (1993-1998), and only small spacial and temporal variations in the depth profiles of the different physico-chemical parameters were observed. The temperature profiles, the conductivity values (with over 300 µS cm^{-1} below a depth of 16 m), the changes in redox conditions (from 346 mV to 68 mV), the rapid decrease of oxygen (concentrations dropping below detection limit at a depth of approx. 10 m) and the concomitant increase in sulfide concentrations (from 10 to 100 mg l^{-1} below 11 m) with depth indicated the formation of a condensed chemocline in a depth between 10 and 14 m. In this layer the maximum turbidity was also found. The increase in turbidity in the chemocline was correlated to an increase in bacterial numbers determined after DAPI-staining. In mixolimnion between 5.9 (± 2.0) x 10^5 and 28.1 (± 3.7) x 10^5 cells ml^{-1} were found, while in the chemocline numbers increased to 1.3 (± 0.3) x 10^6 and 7.4 (± 1.6) x 10^6 cells ml^{-1}. In the monimolimnion the bacterial counts ranged between 2.2 (± 0.6) x 10^6 and 11.0 (± 1.7) x 10^6 cells ml^{-1}.

The analysis of Acridine Orange and DAPI-stained bacterial cells revealead very different morphologies especially at the upper sulfide border in the chemocline. Both total DNA staining methods demonstrated the presence of cells with volumes ranging from 0.1 to up to 70 µm³. Due to their distinct morphologies and their intensive autofluorescence, a major portion of the largest bacteria was identified as phototrophic sulfur bacteria, i.e. as *Chromatium okenii* or as *Amoebobacter purpureus* resembling morphotypes. These bacteria occurred in highest densities in a small layer of 1 to 2 meters at the upper sulfide border where light intensities during the day ranged from 0.1 to 4 µE m^{-2} sec^{-1} depending on the weather conditions. The *Cm. okenii* morphotype reached concentrations of up to 2.0 (± 0.5) x 10^5 cells ml^{-1} in July corresponding to approx. 3% of the total number of bacteria at this depth.

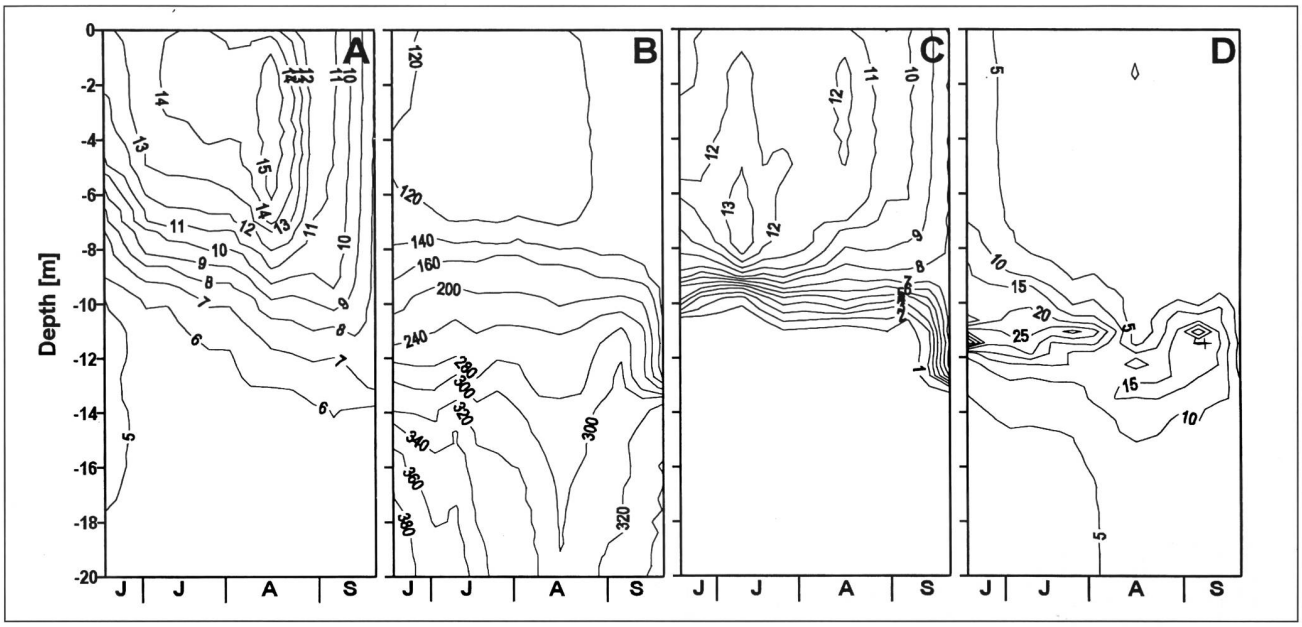

Fig. 1 - Physico-chemical characteristics of Lake Cadagno from June 8 to September 30, 1993: A: temperature (°C); B: Conductivity (µS cm^{-1}); C: oxygen (mg l^{-1}); D: turbidity (FTU).

Cells resembling *A. purpureus* showed a broader vertical distribution than *Cm. okenii* and relatively high cell concentrations not only in the chemocline, but also in the lower part of the monimolimnion. They were generally present in higher numbers than *Cm. okenii* reaching a maximum cell density in the chemocline of 9.0 (± 2.0) x 10^5 cells ml^{-1} which corresponded to approx. 30 % of the total number of bacteria. The average cell volumes were 54 (± 14) and 3 (± 0.8) µm³ $cell^{-1}$ for *Cm. okenii* and *A. purpureus*, respectively. The biovolumes of 2.7 ± (3.0) and of 0.5 ± (0.4) x 10^3 mm^3m^{-3} for *Cm. okenii* and *A. purpureus*, respectively, accounted for 40 and 9% of the total bacterial biovolume.

In addition to the phototrophic sulfur bacteria *Cm. okenii* and *A. purpureus*, two other morphotypes that contained gas-vacuoles were distinguished in the chemocline and in the monimolimnion from other bacteria by using morphological criteria. The first morphotype, named morphotype R, was a rod with round ends (0.4-0.5 µm wide and 1-4 µm long) similar to Procaryote T5 described by Caldwell & Tiedje (1975). The distribution of this morphotype correlated to the profiles of conductivity and hydrogen sulfide. The highest cell densities of this morphotype with 44.0 (± 0.6) x 10^6 cells ml^{-1} were obtained in the monimolimnion at a depth of 19 m in June 1993 and decreased slightly towards the end of the year. The second morphotype had an ovoid shape and was similar to morphotype H1 discovered in the lakes Blake Mere, Kettle Mere and Crose Mere (CLARK & WALSBY, 1978). The highest cell densities of this morphotype of 6.8 (± 3.0) x 10^3 cells ml^{-1}, representing 0.1% of total bacteria, were observed in August 1993 at a depth of 13.3 m depth.

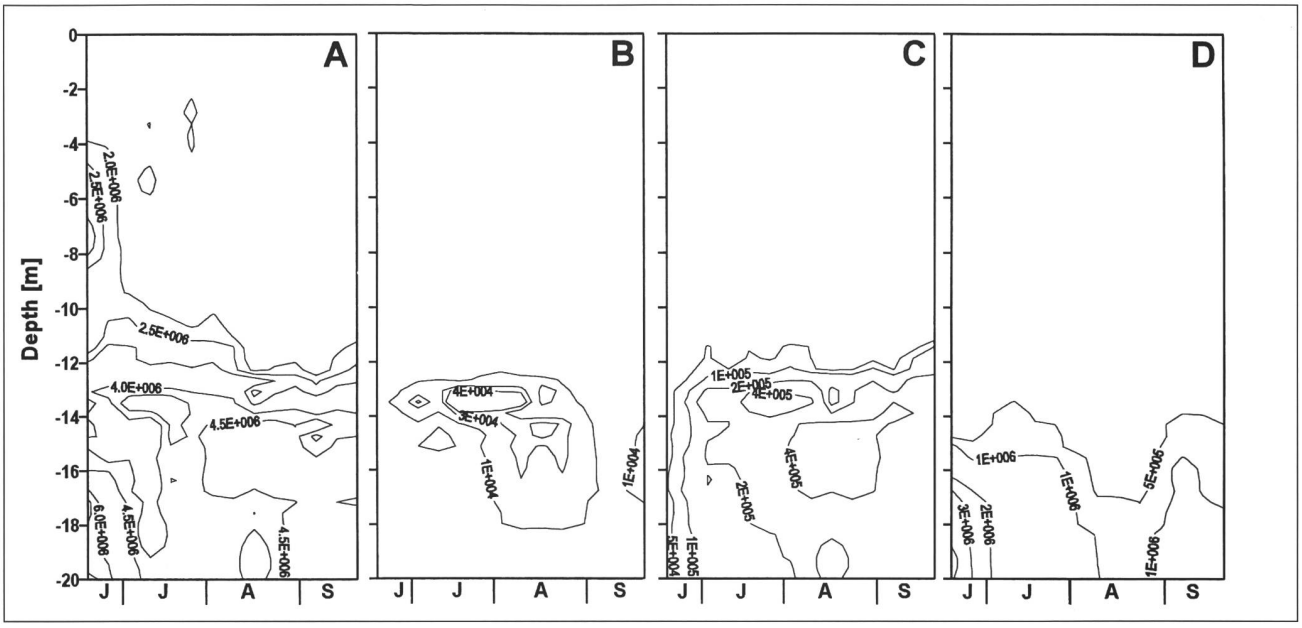

Fig. 2 - Vertical distribution of bacteria in Lake Cadagno from June 8 to September 30, 1993: A: total DAPI-stained bacteria (cells ml^{-1}); B: morphotypes resembling *Chromatium okenii* (cells ml^{-1}); C: morphotypes resembling *Amoebobacter purpureus* (cells ml^{-1}); D: morphotype R like cells (cells ml^{-1}).

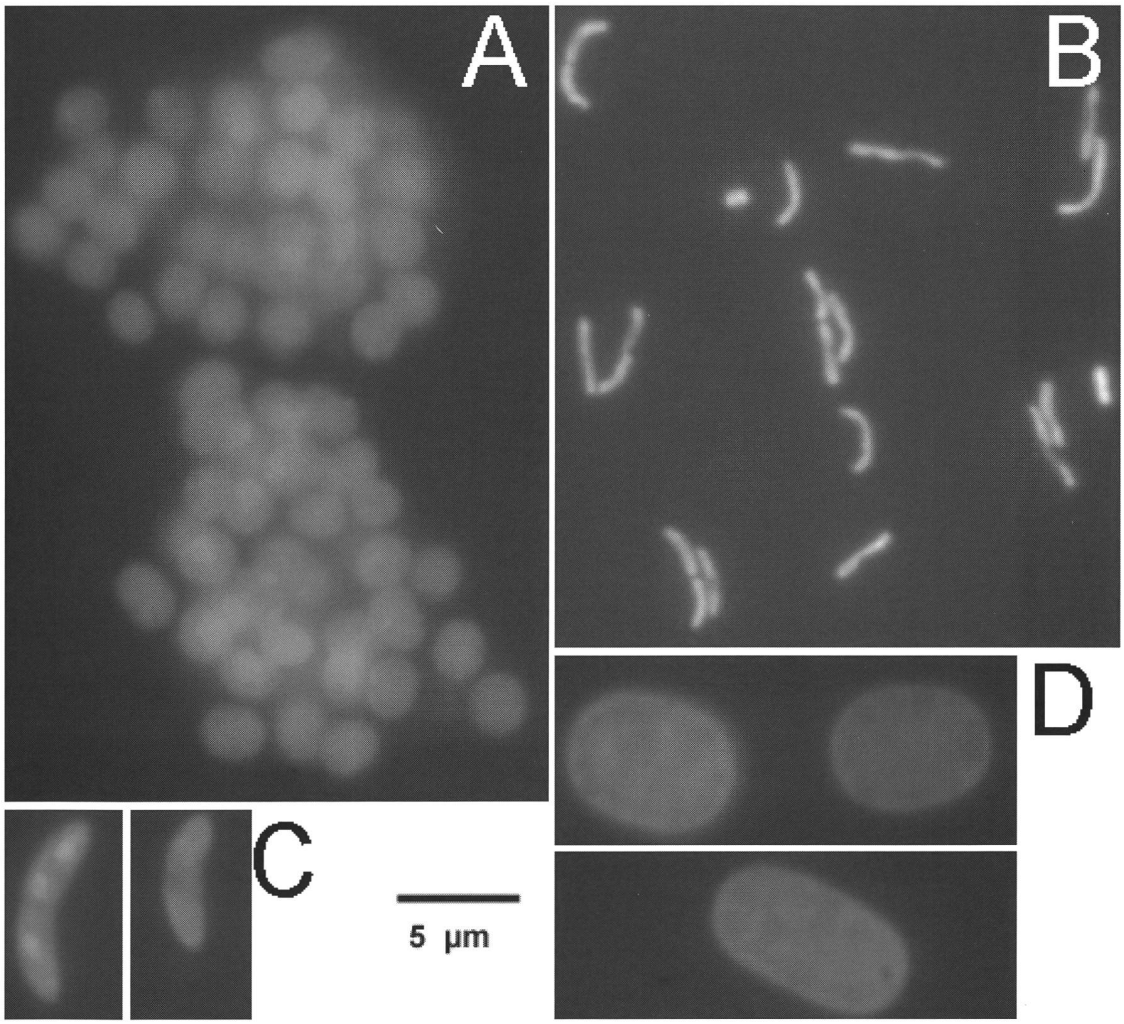

Fig. 3 - Fluorescence microscopy after acridine orange stain showing bacterial morphotypes resembling (A) *Amoebobacter purpureus*, (B) morphotype R, (C) morphotype H1-like and (D) *Chromatium okenii*.

ANALYSIS OF BACTERIOPLANKTON BY *IN SITU* HYBRIDIZATION

From October 1994, the *in situ* hybridization technique was applied to study water samples. Autofluorescence of phototrophic sulfur bacteria did not interfer with probe-conferred signals when the high quality filter system HQ-Cy3 (HQ535/50, Q565LP, HQ610/50, F41 AHF Analysentechnik, Tübingen Germany) was used.

Between 1.1 ± (0.6) x 10^4 and 1.8 ± (0.5) x 10^6 cells ml^{-1} were detectable in the chemocline by *in situ* hybridization with probe Eub338, targeting all members of the domain *Bacteria*. These cell numbers corresponded to percentages between 38 and 90% of the DAPI-stained cells. In the anoxic monimolimnion up to 70% of the DAPI-stained bacteria were detected with the same probe. With probe nonEub338, which should not bind to any rRNA, less than 1% of the total number of DAPI-stained bacteria were detectable, indicating a low non-specific binding of the probe to cell components.

Averaged over the whole chemocline, 49 (± 18)% of the DAPI-stained bacteria hybridized to probe Gam42a, targeting bacteria of the γ subdivision of Proteobacteria, whereas cells hybridizing to probes Alf1b, Bet42a, and SRB385, targeting bacteria of the α, β, and δ subdivisions of Proteobacteria respectively, accounted for 23 (± 3), 15 (± 7), and 16 (± 4)% of the DAPI-stained bacteria. In the monimolimnion, bacteria hybridizing to probe Alf1b were the most abundant organisms with 32 (± 9)% of the DAPI total count.

Cells hybridizing to probes Alf1b, Bet42a, Gam42a, and SRB385 were further differentiated into classes of morphotypes. Bacteria detected with probes Bet42a and SRB385 showed a higher number of different morphotypes than bacteria detected with probes Alf1b and Gam42a throughout the whole water column, although the latter were on average more abundant. Probes Bet42a and SRB385 generally detected the broadest range of morphotypes from small coccoid cells, with a volume of 0.1 μm^3, to large elongated cells, with a volume of up to 7 μm^3, whereas probe Gam42a generally detected large morphotypes that resembled phototrophic sulfur bacteria of the genera *Chromatium* and *Amoebobacter*.

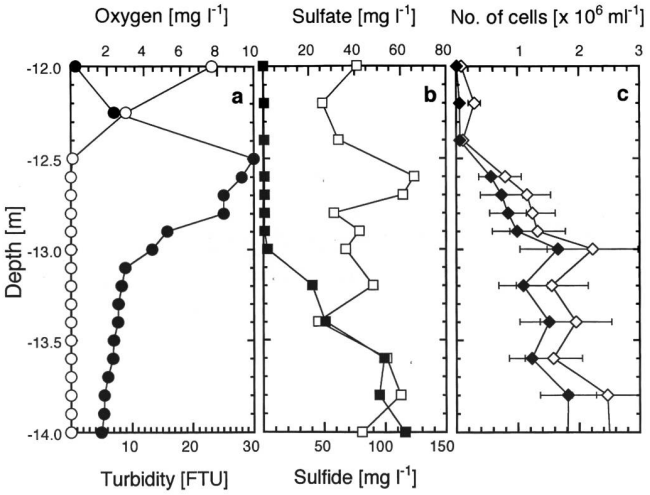

Fig. 4 - Vertical distribution of physico-chemical parameters and bacteria in the chemocline of Lake Cadagno at a depth between 12 m and 14 m (October 13, 1994);

a, oxygen (○), and turbidity (●) determined with a Hydropolytester HPT-C profiler;

b, sulfate (□), and sulfide (■);

c, numbers of DAPI-stained bacteria (◇), and cells detectable after *in situ* hybridization with probe Eub338 (◆).

Sulfate, sulfide and numbers of bacteria were determined in water samples obtained with a pneumatic multi-syringe sampler.

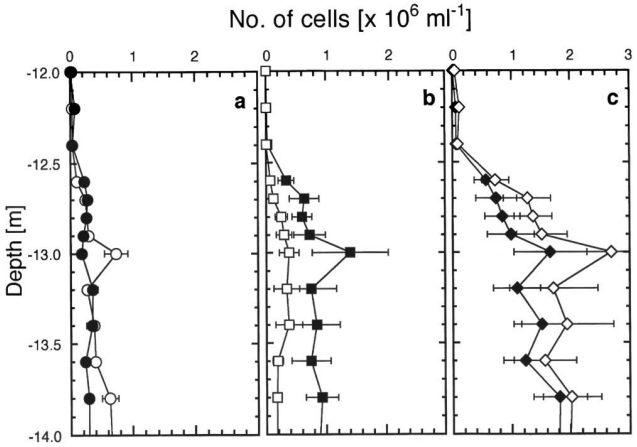

Fig. 5 - Vertical distribution of bacterial populations in the chemocline of Lake Cadagno at a depth between 12 m and 14 m (October 13, 1994);

a, cells detectable after *in situ* hybridization with probe Alf1b (○), and Bet42a (●);

b, cells detectable after *in situ* hybridization with probe Gam42a (■), and SRB385 (□);

c, cells detectable after *in situ* hybridization with probe Eub338 (◆), and the sum of cells detectable after *in situ* hybridization with probes Alf1b, Bet42a, Gam42a, and SRB385 (◇).

Probe	Morphotype[1]	Area (μm²)	Length (μm)	Width (μm)	Volume (μm³)	Cell description[2]
Alf1b	A1	1.1	1.6	0.9	0.7	Large elongated
	A2	0.9	1.6	0.7	0.5	Large elongated
	A3	2.8	3.8	0.7	1	Large elongated
	A4	4	4.6	0.7	1.2	Large elongated
	A5	4.1	5.6	0.7	1.4	Large elongated
Bet42a	B1	11.5	4.9	1.7	7.4	Large elongated
	B2	5.6	3.8	1.8	6.6	Large elongated
	B3	5.9	6	1	3.1	Large elongated
	B4	4	4.5	1.7	6.4	Large elongated
	B5	3.5	4.2	1.1	2.4	Large elongated
	B6	1.4	2.9	0.8	1	Large elongated
	B7	0.7	1.2	0.9	0.5	Large coccoid
	B8	0.8	1.6	0.7	0.4	Large elongated
	B9	1.1	1.6	0.7	0.4	Large elongated
	B10	1	2	0.4	0.2	Large elongated
	B11	0.3	0.7	0.6	0.1	Small coccoid
	B12	0.3	0.7	0.6	0.1	Small coccoid
	B13	0.7	1.6	0.7	0.4	Large elongated
	B14	0.3	0.7	0.6	0.1	Small coccoid
Gam42a	G1	25.6	7	4.6	81.1	Large coccoid
	G2	3.7	2.4	2	5.8	Large coccoid
	G3	3.9	2.3	2.2	5.6	Large coccoid
	G4	3.3	2.3	1.9	4.4	Large coccoid
	G5	2.5	2.5	1.2	2	Large elongated
	G6	0.3	0.8	0.5	0.1	Small coccoid
SRB385	S1	1.9	2.2	1.2	1.8	Large coccoid
	S2	4.4	3.3	1.3	3	Large elongated
	S3	2	3.7	0.7	1	Large elongated
	S4	0.8	1.3	1	0.7	Large coccoid
	S4	1.2	1.5	1.1	1	Large coccoid
	S5	0.3	0.9	0.4	0.1	Small elongated
	S6	0.7	1.9	0.5	0.2	Large elongated
	S7	0.9	1.8	0.7	0.5	Large elongated
	S8	0.6	1.3	0.6	0.2	Large elongated
	S9	0.9	1.6	0.8	0.5	Large elongated
	S10	1.1	1.3	1.1	0.8	Large coccoid
	S11	0.3	0.7	0.6	0.1	Small coccoid

[1] morphotype description in Figure 6

[2] according to the morphologic criteria described by Ramsing *et al.* (1996)

Tab. 1 - Morphotype description of bacteria detected by *in situ* hybridization with probes Alf1b, Bet42a, Gam42a, and SRB385.

SEQUENCES ANALYSIS, PROBES DESIGN AND ANALYSIS OF PHOTOTROPHIC SULFUR BACTERIA

To resolve the nature of the bacterial populations observed by microscopy in the anoxic layer of the meromictic Lake Cadagno, we isolated the genes encoding the small subunit ribosomal RNA from a water sample taken at 17 meters depth, using PCR with universal primers for the domain *Bacteria* followed by cloning and sequencing of the obtained fragments.

The cloned inserts were reamplified to confirm the presence of the 1.4 kb full-length insert, which was detected in 276 clones over a total of 293 putative transformants.

To identify unique clone types, ARDRA (amplified rDNA restriction analysis) was performed on all of the 276 reamplified inserts, and the profiles obtained were grouped in similar clusters using the GelCompare software. The analysis identified 69 distinct groups of restriction profiles.

Some chosen clones were further analyzed by sequencing (ABI PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit and ABI Prism 310 automated sequencer -Perkin-Elmer, Rotkreutz, Switzerland).

16S rRNA-targeted oligonucleotide probes were designed, and their specificity with reference to available 16S rRNA sequences, was checked with the ARB program (STRUNK & LUDWIG 1996) and in the EMBL/GenBank databases using FASTA (PEARSON & LIPMAN 1988).

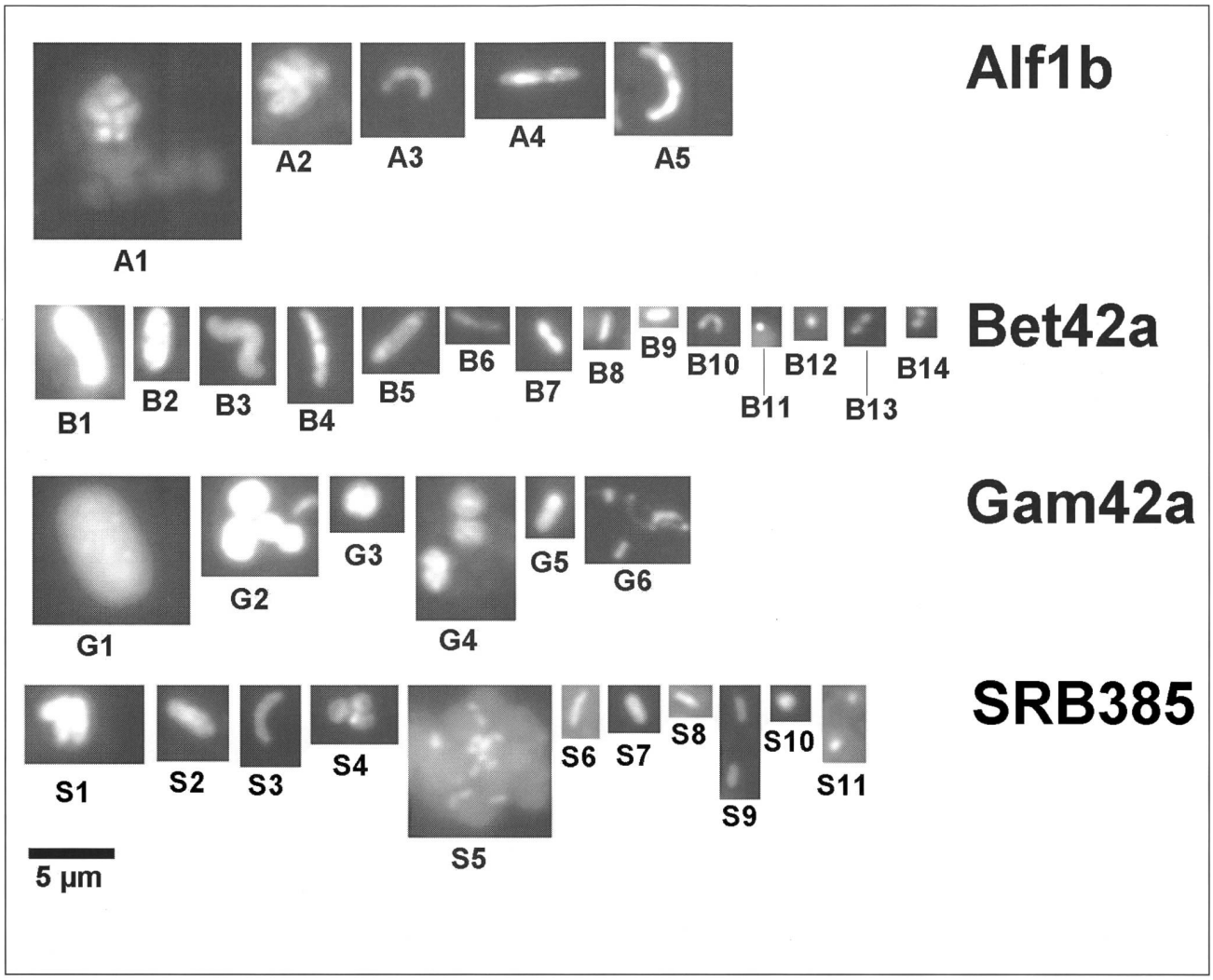

Fig. 6 - Typical morphotypes of bacteria hybridizing to Cy3-labeled oligonucleotide probes Alf1b, Bet42a, Gam42a and SRB385.

Probes Cmok453 (5'AGC CGA TGG GTA TTA ACC ACC AGG TT, pos. 453-478 according to the *E. coli* numbering of Brosius et al. 1981) and Apur453 (5'TCG CCC AGG GTA TTA TCC CAA ACG AC, pos. 453-479) were designed to detect *Cm. okenii* type strain DSM169 and *A. purpureus* type strain DSM4197, respectively.

Probe Apur453 hybridized to only a minor portion of the *A. purpureus* population identified by autofluorescence and morphological criteria in the Lake Cadagno. Even though *in situ* hybridization with probe Gam42a demonstrated the permeability to the probe of all the *Amoebobacter*-like cells in our samples, only approx. 20% (2.2 ± 0.8 x 10^5 cells ml^{-1}) could be detected with this specific probe Apur453. This result showed that the type strain *A. purpureus* DSM 4197 did not represent the major population of *Amoebobacter*-like cells in the chemocline of Lake Cadagno and that at least two different populations might be present.

A further characterization of these populations was thus based on *in situ* hybridization with probes designed after the analysis of the 16S rDNA gene sequences of the library and of the phototrophic sulfur bacteria *Lamprocystis roseopersicina* DSM229 and *Amoebobacter roseus* DSM235. All these probes were targeting 16S rRNA at the position 453 to 478, according to the *E. coli* numbering, which is a highly variable region revealing at least five differences between sequences of the phototrophic sulfur bacteria investigated.

Probe Apur453 targeted *A. purpureus* DSM 4197, but also the rRNA of bacteria harboring the sequence of our clone 345. Probe Laro453 targeted *L. roseopersicina* DSM229 as well as the sequence of clone 136, while probe S453D targeted the 16S rRNA of bacteria harboring the sequence of clone 261, and probe S453F this of clones 335 and 371.

The specificity of the probes was checked with pure cultures of phototrophic sulfur bacteria as well as with those of bacteria from other phyla.

In situ hybridization with probes Apur453, Laro453, S453D, and S453F, targeting a phylogenetically and morphologically very tight cluster of different small-celled phototrophic sulfur bacteria, resulted in the detection of distinct populations in the chemocline of Lake Cadagno. Probe Apur453 detected up to 7 x 10^5 cells ml^{-1}, whereas probes Laro453, S453D, and S453F detected 1 x 10^5, 7 x

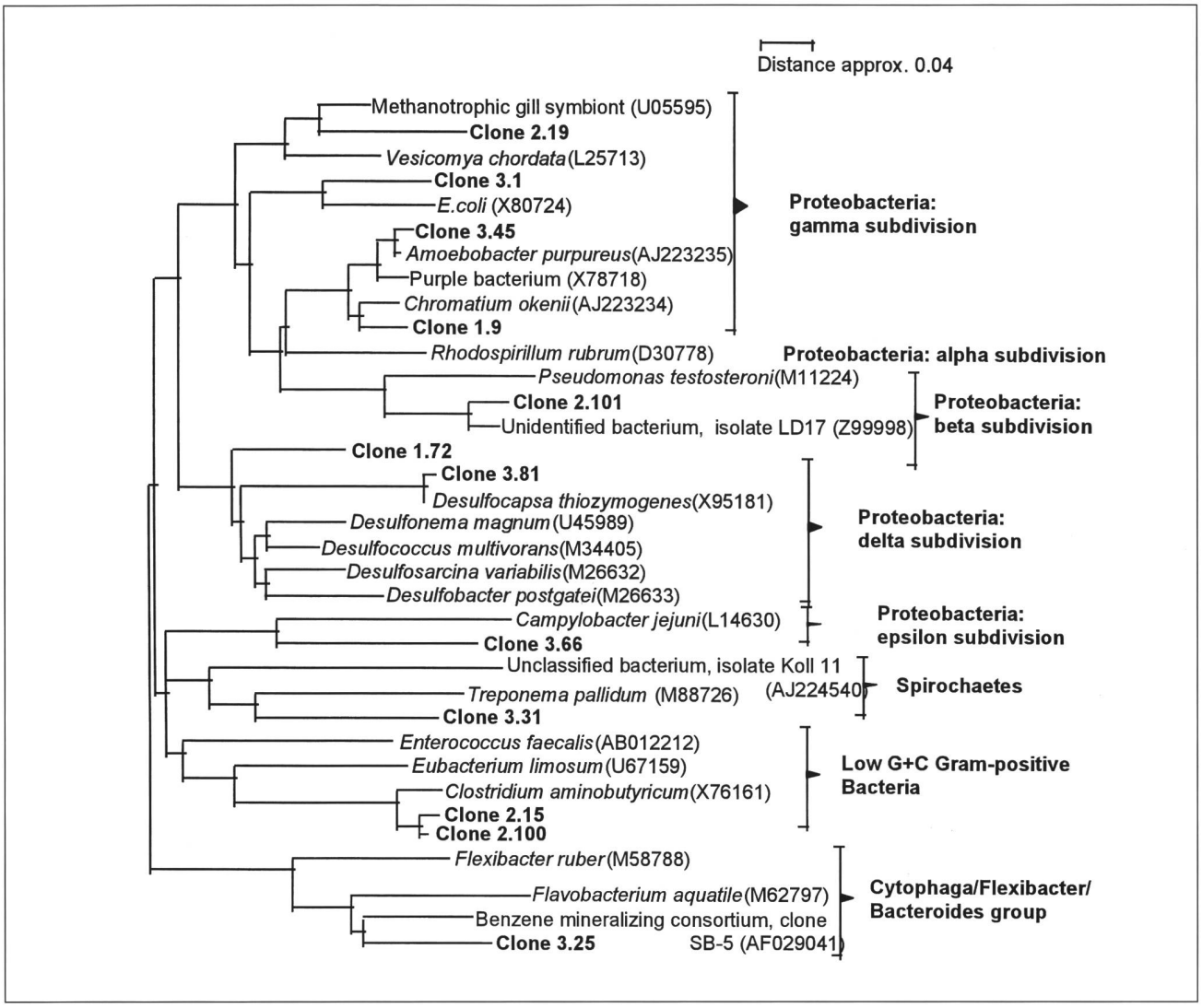

Fig. 7 - Phylogenetic tree showing the relationship of the clones partially sequenced to other members of the domain *Bacteria*. 334 nucleotide positions were included in the analysis (Jukes-Cantor distance and Neighbor-joining method). Between brackets the RDP accession numbers of each organism retrieved from this database are indicated. The scale bar indicates 4 substitutions per 100 nucleotide positions (Demarta A. et al., 1998)

10^5, and 4 x 10^5 cells ml^{-1} at their maximum occurrence, respectively.

The analysis revealed that the *Amoebobacter*-like populations showed a broader distribution than *Cm. okenii,* with high cell densities in the lower part of the chemocline which might be due to their high sulfide tolerance.

Populations of small-celled phototrophic sulfur bacteria not only differed with respect to total population sizes but also differed with respect to population profiles in depth. While populations detected with probe S453D only revealed a distinct maximum occurrence at depth of 12.5 m (sampling of the 3rd of October 1997), populations detected with probe S453E showed a second maximum occurrence at a depth of 13.1 m, and those detected with probe S453F showed an evenly high distribution at a depth of between 12 and 13.1 m.

Between 96 and 100% of autofluorescent cells were detected through the whole chemocline when a combination of all probes was used.

The type strain of *A.purpureus* DSM4197 did thus not represent the major population of *Amoebobacter*-like cells in the chemocline of Lake Cadagno and different populations were present. This result confirmed earlier studies reporting some size differences between an isolate obtained from Lake Cadagno (LcCAD1) and the type strain, which was isolated from Lake Schleinsee in Germany,.were already reported (EICHLER & PFENNIG, 1988).

Further studies on the populations of small-celled phototrophic sulfur bacteria in the chemocline of Lake Cadagno will be focused on the analysis of temporal and spatial distributions of specific populations in relation to the environmental factors such as light, electron donors, oxygen and carbon sources.

The 16S rDNA sequences determined have been depostited in the EMBL/GenBank databases under accession no. AJ223234, AJ223235, AJ006221, and AJ006057 to AJ006063.

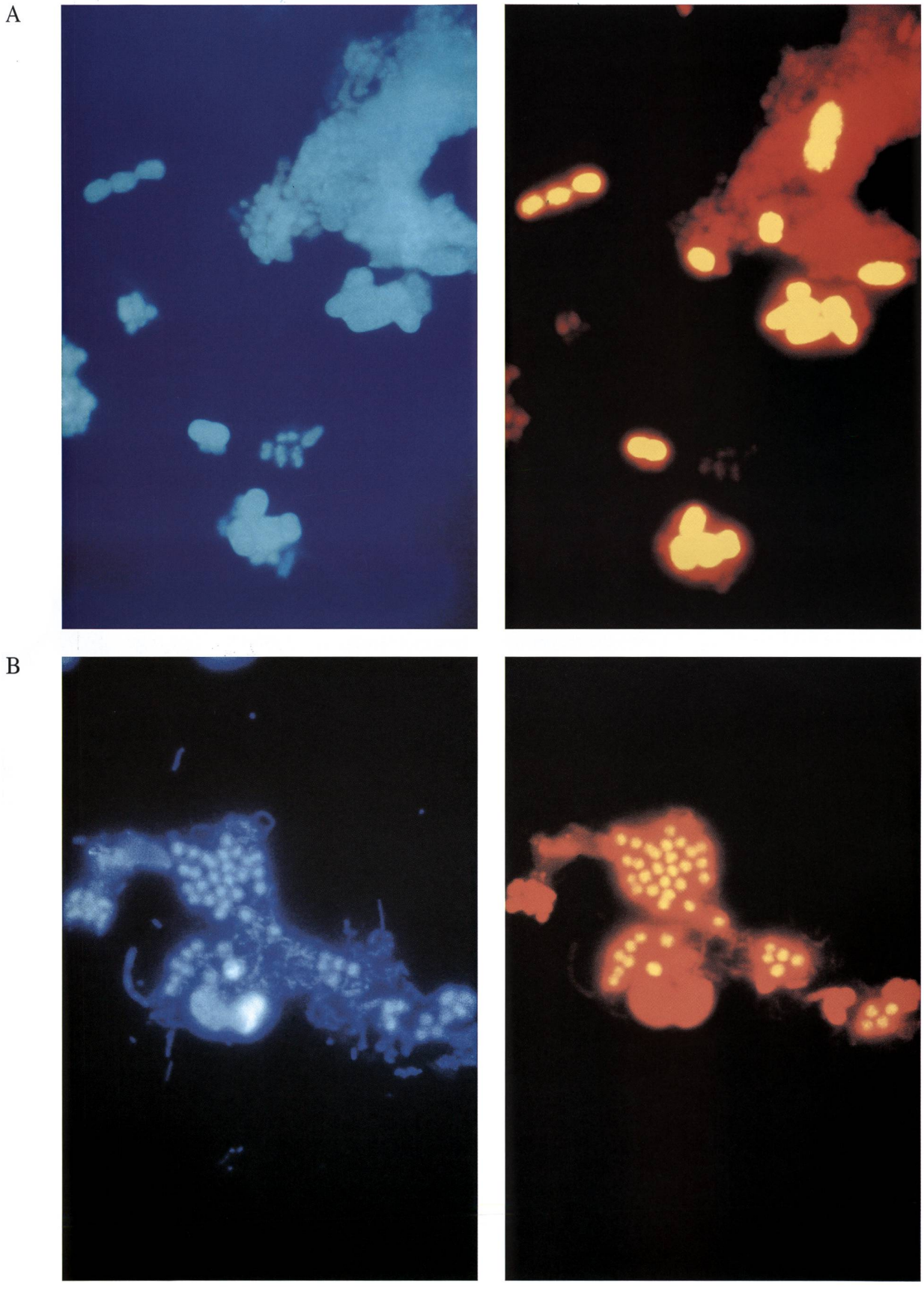

Fig. 8 - Total bacterial stains with DAPI (**left**) and *in situ* detection of phototrophic sulfur bacteria with Cy3-labeled probes (**right**). **Panel A**: Probe Cmok453 targeting *Cm. okenii*. **Panel B**: Probe Apur453 targeting *A. purpureus*.
Bar represents 10 µm.

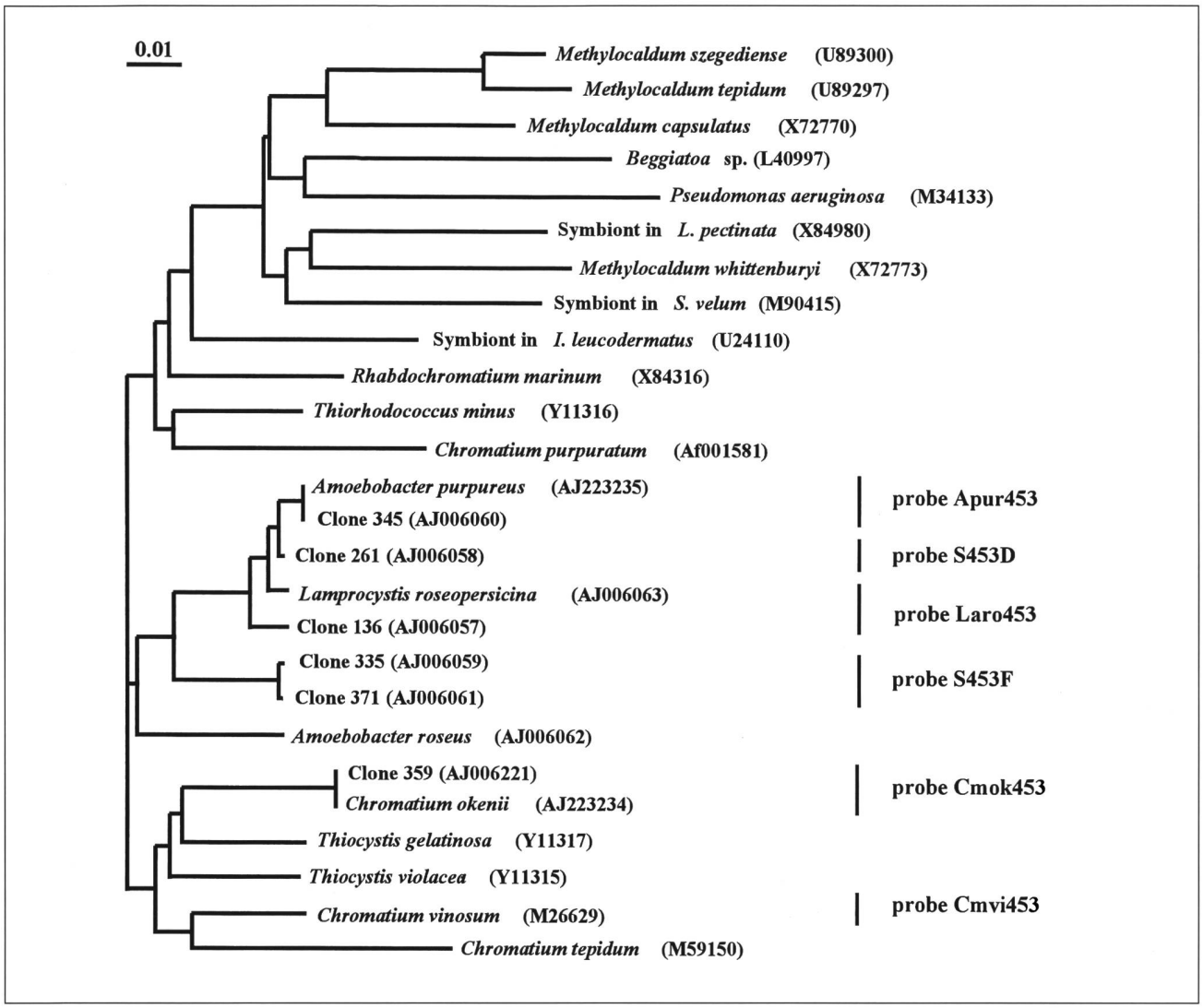

Fig. 9 - Neighbor-Joining tree based on the aligned sequences of selected clones from the 16S rRNA gene library of the chemocline of Lake Cadagno and of selected bacteria searched from the EMBL/GenBank databases using FASTA through the GCG package. The distance scale indicates the expected number of changes per sequence position. Bars and probe designations indicate target groups of phototrophic sulfur bacteria for specific oligonucleotide probes (Tonolla M. et al. 1999).

DISTRIBUTION OF SULFATE-REDUCING BACTERIA IN THE CHEMOCLINE.

In situ hybridization with probe SRB385 targeting bacteria of the d-subdivision of Proteobacteria (presumably mainly sulfate-reducing bacteria) showed the presence of sulfate-reducing bacteria which number went together with some environmental parameters. The maximum of bacteria detected with probe SRB385 of $3.8 \pm 1.7 \times 10^5$ cells ml^{-1} was obtained in the chemocline, where also the phototrophic sulfur bacteria reached their maxima. At the same depth a minimum concentration of sulfate and increasing concentrations of sulfide where present.

Sulfate-reducing bacteria and sulfur bacteria such as purple and green sulfur bacteria have been shown to co-occur in the same habitat (CAUMETTE *et al.* 1994, OVERMANN *et al.* 1996, ØVREÅS *et al.* 1997). It was therefore speculated that sulfate-reducing bacteria may complement sulfur- and sulfide-oxidizing bacteria ecologically (MUYZER *et al.* 1995). Our results on the association of both sulfate-reducing and phototrophic sulfur bacteria in the chemocline of Lake Cadagno add some more evidence to this speculation thought the character of a possible interaction, competitive or commensalistic, remains uncertain. A deeper analysis of these co-occurence as been undertaken by the use of new and specific 16S rRNA targeting probes.

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