

Thiocystis chemoclinalis sp. nov. and *Thiocystis cadagnonensis* sp. nov., motile purple sulfur bacteria isolated from the chemocline of a meromictic lake

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Two isolates, designated CadH11^T and Cad448^T, representing uncultured purple sulfur bacterial populations H and 448, respectively, in the chemocline of Lake Cadagno, a crenogenic meromictic lake in Switzerland, were obtained using enrichment and isolation conditions that resembled those used for cultured members of the genus *Thiocystis*. Phenotypic, genotypic and phylogenetic analyses of these isolates confirmed their assignment to the genus *Thiocystis*. However, 16S rRNA gene sequence similarities of 98.2% between CadH11^T and Cad448^T, and similarities of 97.7 and 98.5%, respectively, with their closest cultured relative *Thiocystis gelatinosa* DSM 215^T, as well as differences in DNA G + C content and carbon source utilization suggested that the isolates belonged to two distinct species. DNA–DNA hybridization of CadH11^T and Cad448^T with *T. gelatinosa* DSM 215^T showed relatedness values of 46.4 and 60.8%, respectively; the relatedness value between CadH11^T and Cad448^T was 59.2%. Based on this evidence, strains CadH11^T and Cad448^T represent two novel species within the genus *Thiocystis*, for which the names *Thiocystis chemoclinalis* sp. nov. and *Thiocystis cadagnonensis* sp. nov. are proposed, respectively. The type strains of *T. chemoclinalis* sp. nov. and *T. cadagnonensis* sp. nov. are CadH11^T (=JCM 15112^T =KCTC 5954^T) and Cad448^T (=JCM 15111^T =KCTC 15001^T), respectively.

Molecular tools have been used for many years to study the diversity and population dynamics of bacteria inhabiting the chemocline of Lake Cadagno, a crenogenic meromictic lake located 1923 m above sea level in the Piora Valley in the southern Alps of Switzerland (46° 33' N 8° 43' E) [see Tonolla *et al.* (2004) for review]. Comparative analysis of sequences in a 16S rRNA gene clone library suggested a relatively limited diversity of phototrophic sulfur bacteria in the chemocline of Lake Cadagno with two clones containing 16S rRNA gene fragments resembling those of *Chromatium okenii* and *Lamprocystis purpurea*. In addition, sequences of three groups of purple sulfur bacteria related to *L. purpurea* and *Lamprocystis roseopersicina* (Tonolla

et al., 1999) and two groups clustering with *Thiocystis minor* and *Thiocystis gelatinosa* were retrieved. In contrast to species of the genus *Lamprocystis*, bacteria affiliated with the genus *Thiocystis*, referred to as populations H and 448, were found in small numbers in only two years of a decade-long monitoring study (Tonolla *et al.*, 1999, 2005). Unlike species of the genus *Lamprocystis*, these populations did not form aggregates and were not associated with sulfate-reducing bacteria related to *Desulfocapsa thiozymogenes*.

Samples from the chemocline of Lake Cadagno were taken from the layer of maximum turbidity corresponding to the highest bacterial density at a depth of 12.7 m on August 28, 2001, and manipulated as described previously (Peduzzi *et al.*, 2003). They served as concentrated inocula, i.e. as a natural enrichment, for liquid and deep agar dilutions (1%, v/v) prepared by the Hungate technique. Small-celled phototrophic sulfur bacteria were enriched and cultured in medium containing (l⁻¹) 0.25 g KH₂PO₄, 0.34 g NH₄Cl,

Abbreviations: BS, bootstrap support; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour joining.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CadH11^T and Cad448^T are AM086641 and AM086640, respectively.

0.5 g MgSO₄ · 7H₂O, 0.25 g CaCl₂ · 2H₂O, 0.34 g KCl, 1.5 g NaHCO₃, 0.5 ml trace element solution SL10 and 0.02 mg vitamin B₁₂ (Eichler & Pfennig, 1988). The medium was prepared in a 2 l bottle with an N₂/CO₂ (80% : 20%) gas phase according to Widdel & Bak (1992), supplemented with 2 mM acetate, reduced with 0.3 g Na₂S · 9H₂O l⁻¹ (1.10 mM) and adjusted to a pH of ~7.2. Enrichments and purification procedures were conducted as described previously (Peduzzi *et al.*, 2003) according to Pfennig (1978). Two isolates, designated Cad448^T and CadH11^T, were obtained and considered pure after repeated colony transfers from subsequent agar-shake dilution series when all cells hybridized with one specific probe, S453H or S448. The probes were developed specifically for purple sulfur bacteria from Lake Cadagno related to the genus *Thiocystis* (Tonolla *et al.*, 2005). 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. All cultures were incubated at room temperature (20–23 °C). Dilution series of these cultures were subjected to a photoperiod (6 h light/6 h dark) using low light intensities generated with an incandescent 40 W bulb resulting in 10 μmol photons m⁻² s⁻¹.

Nucleic acids were extracted from pure cultures using the MagNA Pure LC automated extractor and DNA isolation extraction kit (Roche Molecular Biochemicals). Nearly complete 16S rRNA gene fragments were amplified using primers EUB7f (5'-AGAGTTTGATCNTGGCTCAG) and EUB1392r (5'-ACGGGCGGTGTGTAC), purified as described previously (Tonolla *et al.*, 1999) and sequenced with an ABI PRISM Ready Reaction dye deoxy terminator cycle sequencing kit and an ABI Prism 310 automated sequencer (Applied Biosystems). The sequences were then aligned with related sequences from GenBank/EMBL (Altschul *et al.*, 1997; Pearson & Lipman, 1988) using Sequencher 4.2.2 (Gene Codes), CLUSTAL_X and MacClade 4.05 (Maddison & Maddison, 1999; Thompson *et al.*, 1997). The length of all compared sequences was 1393 bp. Phylogenetic analyses included maximum-parsimony (MP), neighbour joining (NJ) and maximum-likelihood (ML) methods using nucleic acid sequences in PAUP*4.0b10 (Swofford, 2002). Confidence in tree topologies was gauged using bootstrap support (BS) resampling methods in PAUP; only values over 70% were included in the tree (Hillis & Bull, 1993). Additionally, Bayesian methods were used in MRBAYES version 3.0 (Huelsenbeck & Ronquist, 2001) and a 95% majority rule consensus tree was generated in PAUP.

The tree topology was very similar for each of the phylogenetic methods employed. A representative ML tree with BS values for ML, NJ and MP analyses and probability values for Bayesian analysis shows that isolates CadH11^T and Cad448^T both clustered with representative strains of the genus *Thiocystis* within the *Gammaproteobacteria* with high BS (Fig. 1). The 16S rRNA gene sequences of isolates CadH11^T and Cad448^T were identical to those of clones 13 (AJ831744) and 130 (AJ831743), respectively. Clones 13 and 130 were used previously as representatives of the

uncultured populations H and 448, respectively (Tonolla *et al.*, 2005). The nearest cultured relative of isolates CadH11^T and Cad448^T was *T. gelatinosa* DSM 215^T, with sequence similarities of 97.7 and 98.5%, respectively (Fig. 1). 16S rRNA sequence similarity between the isolates was 98.2%. These results demonstrated that both isolates were members of the genus *Thiocystis* but differed from each other and from other members of the genus (Stackebrandt & Ebers, 2006).

DNA–DNA hybridization analyses with the most closely related species, *T. gelatinosa* DSM 215^T, showed relatedness values of 46.4 and 60.8% with strains Cad448^T and CadH11^T, respectively; the DNA–DNA relatedness value between Cad448^T and CadH11^T was 59.2%. DNA–DNA hybridization analyses with *Thiocystis violascens* DSM 198^T showed relatedness values of 34.3 and 45.3% with strains Cad448^T and CadH11^T, respectively, and 53.2% with *T. gelatinosa* DSM 215^T. For these analyses, cells were disrupted using a French pressure cell (Thermo Spectronic) and DNA was isolated and subsequently purified using the hydroxy-apatite chromatography method as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) with modifications described by Huß *et al.* (1983), using a Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in situ* temperature probe (Varian). The values obtained for strains Cad448^T and CadH11^T and their closest cultured relative *T. gelatinosa* DSM 215^T were below 70%, the threshold value recommended for the delineation of bacterial species, thus, supported the description of the strains as representatives of novel species within the genus *Thiocystis*.

The genomic DNA G + C contents of strains CadH11^T and Cad448^T, determined by HPLC analysis according to the method described by Mesbah *et al.* (1989), were 68.1 and 69.5 mol%, respectively (Table 1). These values were far higher than those generally found within the genus *Thiocystis* (61–64 mol%; Imhoff, 2005). The DNA G + C contents of members of the genus *Thiocystis* were all determined by buoyant density in CsCl (Mandel *et al.*, 1971) and, thus, variations of 2–3 mol% might be attributed to the different methodologies used. However, these values are too small to explain the difference in DNA G + C content between strains CadH11^T and Cad448^T and type strains of the genus *Thiocystis*. In addition, the most closely related species, *T. gelatinosa* DSM 215^T (DNA G + C content 61.3 mol%) and *T. minor* DSM 178^T (62.2 mol%), differed significantly from the isolates in other characteristics, which would support the classification of both isolates as members of novel species within the family *Chromatiaceae* (Imhoff, 2005).

Cells of isolates CadH11^T and Cad448^T were Gram-reaction-negative, flagellated and motile, similar to all species of the genus *Thiocystis* used in this study (Table 1). Bright-field microscopy revealed the presence of sulfur

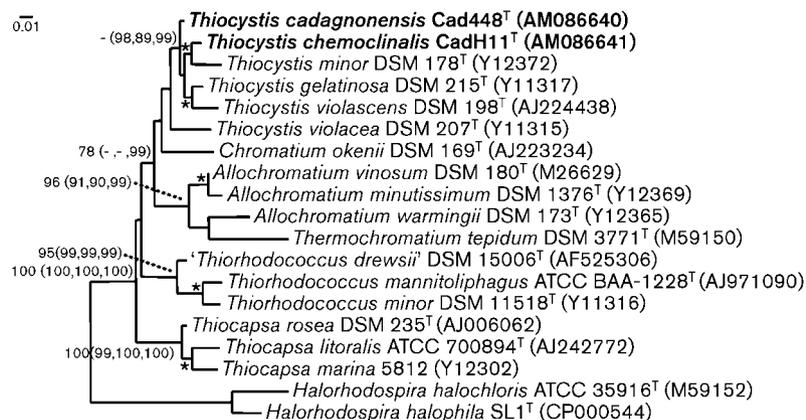


Fig. 1. Maximum-likelihood tree topology based on 16S rRNA gene sequences of strains Cad448^T and CadH11^T and other closely related strains of the family *Chromatiaceae* created using PAUP*4.0b10 and a GTR+I+G model of sequence evolution (Swofford, 2002). Numbers reflect BS values >70% generated in PAUP; numbers in parentheses reflect BS values from NJ and MP analyses in PAUP and Bayesian posterior probabilities created using MRBAYES version 3.0 (Huelsenbeck & Ronquist 2001), respectively; -, values <70%. Asterisks indicate that the node had high BS values or posterior probabilities in all four types of phylogenetic analyses performed. *Halorhodospira halochloris* ATCC 35916^T and *Halorhodospira halophila* SL1^T were used as an outgroup. The length of the compared sequences was 1393 bp. Bar, 0.01 substitutions per nucleotide position.

Table 1. Differential characteristics of strains CadH11^T and Cad448^T and species of the genus *Thiocystis*

Taxa: 1, CadH11^T; 2, Cad448^T; 3, *T. violacea*; 4, *T. violascens*; 5, *T. minor*; 6, *T. gelatinosa*. Data for taxa 3–6 are from Imhoff (2005) and Zaar *et al.* (2003), except for butyrate and lactate assimilation data for *T. violascens* DSM 198^T (this study). Data for *T. violacea* are from the type strain DSM 207^T except for glucose and glycerol assimilation. Motility by flagella, storage of sulfur, chemolithotrophic growth and utilization of sulfide and elemental sulfur as photosynthetic electron donors were positive for all taxa. Succinate was not utilized in the presence of sulfide and bicarbonate by any taxa. Pyruvate and acetate were photoassimilated by all taxa. +, Positive; -, negative; +/-, variable depending on the strain; NR, not reported.

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 |
|---|-------------|-------------|---------------|----------------|---------------|------------|
| Shape | Oval/sphere | Oval/rod | Sphere | Rod | Sphere/rod | Sphere |
| Size (µm) | 2.3–3.6 | 2.3–4.7 | 2.5–3.5 | 2.0 × 2.5–6.0 | 2.0 × 2.5–6.0 | 3 |
| Colour of cell suspension | Purple-red | Purple-red | Purple-violet | Purple-violet | Purple-red | Purple-red |
| Carotenoid group | Okenone | Okenone | Rhodopinal | Rhodopinal | Okenone | Okenone |
| DNA G+C content (mol%) | 68.1 (HPLC) | 69.5 (HPLC) | 63.1 (Bd) | 61.8–64.3 (Bd) | 62.2 (Bd) | 61.3 (Bd) |
| pH optimum | 7.2 | 7.2 | 7.3 | 7.0–7.3 | 7.0–7.3 | 7.0–7.3 |
| Temperature optimum (°C) | 20 | 20 | 25–35 | 25–35 | 30 | 30 |
| Salt requirement (% w/v) | None | None | None | None | None | 0–1 |
| Use of thiosulfate as a photosynthetic electron donor | + | + | + | + | + | - |
| Assimilation of (in the presence of sulfide and bicarbonate): | | | | | | |
| Formate | - | - | - | + | - | NR |
| Propionate | - | - | + | + | - | - |
| Butyrate | - | - | + | - | - | NR |
| Lactate | - | + | - | - | NR | - |
| Fumarate | - | - | + | - | - | - |
| Malate | - | - | NR | - | - | NR |
| Fructose | + | - | + | - | - | - |
| Glucose | + | + | +/- | - | + | - |
| Ethanol | + | + | - | - | - | - |
| Propanol | - | - | + | - | - | - |
| Glycerol | - | - | +/- | - | - | - |

globules that were randomly distributed in the cells and a slime capsule was not observed. The colour of the cell suspensions was purple–red, similar to cultures of *T. minor* DSM 178^T and *T. gelatinosa* DSM 215^T but different from cultures of *T. violascens* DSM 198^T and *Thiocystis violacea* DSM 207^T, which were purple–violet (Table 1). These differences in colour were also demonstrated by *in vivo* absorption spectra of pigments in cell suspensions, which were determined using the sucrose method and a UV/Vis Spectrometer Lambda 2S (Perkin–Elmer) (Peduzzi *et al.*, 2003). Spectra for both isolates displayed absorption maxima around 590 nm and 830 nm and a low peak at 800 nm, indicating the presence of bacteriochlorophyll *a*, and an additional maximum at 525 nm suggesting the presence of the carotenoid okenone, which has also been found in *T. minor* DSM 178^T and *T. gelatinosa* DSM 215^T but not in *T. violascens* DSM 198^T or *T. violacea* DSM 207^T (Table 1). Absorption peaks at 830 nm rather than 860 nm with an 800 nm peak as a shoulder of the 830 nm peak are typical for purple bacteria containing bacteriochlorophyll *a* and okenone as major carotenoid pigments. However, with other carotenoids, this second peak is more distant in the close infrared region around 860 nm and, thus, is completely separate from the 800 nm peak.

Both strains differed from each other and other *Thiocystis* species based on morphological and physiological criteria. Cells of isolate CadH11^T were spherical to oval with a width of 2.3–3.6 µm, whereas those of Cad448^T were oval to rod-shaped with a width of 2.3–4.7 µm (Fig. 2; Table 1). Differences in morphologies were confirmed by observing populations 448 and H in their natural environment, the chemocline of Lake Cadagno, detected by fluorescent *in situ* hybridization with specific probes, during long-term studies and observations in the field (Tonolla *et al.*, 2005) (Fig. 2). In liquid media, both isolates grew as single motile cells. CadH11^T, however, lost motility under continued laboratory maintenance and cells formed small aggregates of up to 5–10 cells. However, neither Cad448^T nor CadH11^T, which are representatives of populations 448 and H, respectively, were found to aggregate in their natural environment.

Further physiological characterization focused on utilization of different combinations of electron donors and acceptors that were added aseptically from sterile stock solutions (5 mM final concentration) of formate, acetate, pyruvate, propionate, butyrate, lactate, fumarate, succinate, malate, fructose, glucose, ethanol, propanol and glycerol. Headspace gas was 80 % N₂ and 20 % CO₂. Stock solutions of inorganic sulfur compounds, thiosulfate, sulfide and elemental sulfur, were prepared according to Janssen *et al.* (1996). Final concentrations in culture were 10 mM thiosulfate, 2 mM sulfide and 20–30 mg sulfur ml⁻¹ (Finster *et al.*, 1998). Chemolithoautotrophic growth of both strains was tested as described by Kämpf & Pfennig (1980) in uniformly inoculated deep agar-shake cultures.

Similar to other *Thiocystis* species, both strains grew photolithoautotrophically under anaerobic conditions with

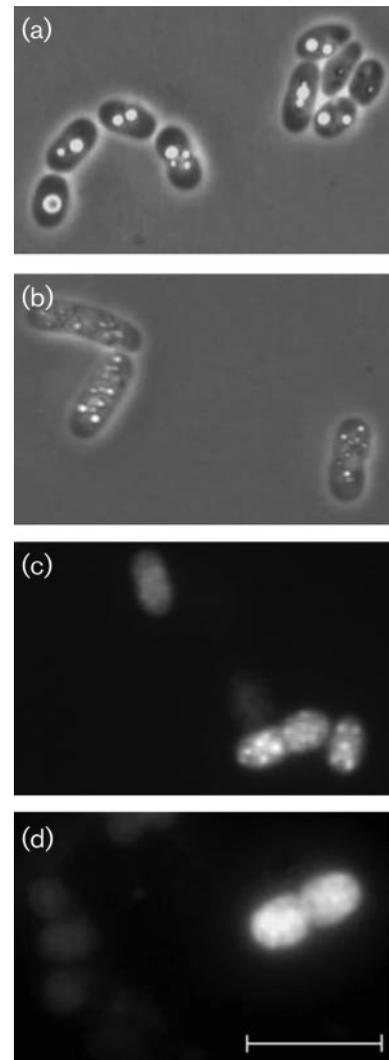


Fig. 2. Phase-contrast micrographs of isolates CadH11^T (a) and Cad448^T (b), and epifluorescence micrographs of the corresponding uncultured populations H (c) and 448 (d) in the chemocline of Lake Cadagno after *in situ* hybridization with Cy3-labelled probes S453H and S448, respectively. Bar, 10 µm.

sulfide, thiosulfate and elemental sulfur as electron donors (Table 1). *T. gelatinosa* DSM 215^T, the closest cultured relative of strains CadH11^T and Cad448^T, however, did not grow with thiosulfate. In the presence of sulfide and bicarbonate, photoassimilation of acetate was observed (Table 1). Doubling times in the presence of sulfide (0.6 mM) and acetate (1 mM) for strains CadH11^T and Cad448^T were 90 h and 66 h, respectively. Chemolithoautotrophic growth was observed with both hydrogen sulfide (0.02 %) and thiosulfate (0.07 %) or with sulfide alone (0.07 %) in the dark and under oxic atmospheric conditions with air as headspace and in a micro-oxic atmosphere with 5 % O₂, 10 % CO₂ and 85 % N₂ in the headspace as suggested by Kämpf & Pfennig (1980) in deep agar-shake cultures. A marked increase in growth was observed in the presence of sulfide (0.07 %) alone compared

to sulfide (0.02 %) plus thiosulfate (0.07 %) in the medium. Like all members of the genus *Thiocystis*, both isolates were able to use simple organic compounds such as pyruvate and acetate. However, CadH11^T and Cad448^T were more versatile than other species of the genus *Thiocystis*, particularly the most closely related okenone-containing species. In contrast to all other strains, growth of strains CadH11^T and Cad448^T was promoted by ethanol assimilation. No fatty acids were assimilated. Moreover, CadH11^T also grew by assimilating fructose and Cad448^T was able to assimilate lactate (Table 1).

The high versatility of these isolates, the differences in oxygen tolerance displayed in chemolithoautotrophic growth and their capacity to use diverse organic compounds to promote their growth might be prerequisites allowing them to thrive and compete successfully within a well-developed and dynamic anaerobic phototrophic community inhabiting the chemocline of Lake Cadagno, where seven different populations of purple sulfur bacteria and two different populations of green sulfur bacteria have been observed to date (Tonolla *et al.*, 2005). Based on comparisons of 16S rRNA genes sequences and basic morphological and physiological properties, both isolates were clearly affiliated with the genus *Thiocystis*. Based on the large differences in the DNA G+C contents of these isolates compared to other species, the results of DNA–DNA hybridization analyses and differences in specific physiological traits of the isolates, strains CadH11^T and Cad448^T, isolated from freshwater stratified sulfidic environments, represent two novel species of the genus *Thiocystis*, for which the names *Thiocystis chemoclinalis* sp. nov. and *Thiocystis cadagnonensis* sp. nov. are proposed, respectively.

Description of *Thiocystis chemoclinalis* sp. nov.

Thiocystis chemoclinalis (che.mo.cli.na'lis. N.L. fem. adj. *chemoclinalis* pertaining to chemocline, found to inhabit environments with steep physico-chemical gradients, isolated from the chemocline of a meromictic freshwater lake).

Cells are spherical to ovoid (~2.3–3.6 µm in diameter) and occur as singularly but can form small aggregates of up to 5–10 cells. Rod-shaped cells may be observed at the end of the stationary phase. Cells are Gram-negative, motile by means of flagella and divide by binary fission. The colour of cell suspensions is purple–red. Photosynthetic pigments, including bacteriochlorophyll *a* and carotenoids of the okenone group, are present. Phototrophic growth occurs under anoxic conditions. In the presence of reduced sulfur compounds (sulfide, thiosulfate), globules of elemental sulfur are formed and appear to be randomly distributed within the cells. Growth also occurs on elemental sulfur as sole electron source. Acetate, pyruvate, glucose, fructose and ethanol are photo-assimilated in the presence of sulfide and bicarbonate. Chemolithotrophic growth is observed in the presence of oxygen in the dark with sulfide and thiosulfate. Growth occurs at 5–25 °C (optimum 20 °C) and pH 6.6–7.5 (optimum pH 7.2). Salt is not required for growth.

The type strain, CadH11^T (=JCM 15112^T =KCTC 5954^T), was isolated from the boundary layer (chemocline) between the oxic upper layer and the anoxic sulfidic layer along steep physico-chemical gradients of Lake Cadagno, a meromictic alpine lake in the southern Swiss Alps at 1923 m above sea level (46° 33' N 8° 43' E). The genomic DNA G+C content of the type strain is 68.1 mol%.

Description of *Thiocystis cadagnonensis* sp. nov.

Thiocystis cadagnonensis (ca.da.gno.nen'sis. N.L. fem. adj. *cadagnonensis* pertaining to Lake Cadagno).

Cells are ovoid to rod-shaped (~2.3–4.7 µm in diameter) and occur as single cells. Rod-shaped cells predominate in pure culture. In their natural environment, ovoid cells are also observed. Cells are Gram-negative, motile by means of flagella and divide by binary fission. The colour of cell suspensions is purple–red. Photosynthetic pigments, including bacteriochlorophyll *a* and carotenoids of the okenone group, are present. Phototrophic growth occurs under anoxic conditions. In the presence of reduced sulfur compounds (sulfide, thiosulfate), globules of elemental sulfur are formed and appear to be randomly distributed within the cells. Growth also occurs on elemental sulfur as sole electron source. Acetate, pyruvate, lactate, glucose and ethanol are photo-assimilated and promote growth in the presence of sulfide and bicarbonate. Chemolithotrophic growth occurs under oxic and micro-oxic conditions in the dark with sulfide and thiosulfate. Growth occurs at 5–25 °C (optimum 20 °C) and pH 6.8–7.5 (optimum pH 7.2). Salt is not required for growth.

The type strain, Cad448^T (=JCM 15111^T =KCTC 15001^T), was isolated from the boundary layer (chemocline) between the oxic upper layer and the anoxic sulfidic layer of Lake Cadagno, a meromictic alpine lake in the southern Swiss Alps at 1923 m above sea level (46° 33' N 8° 43' E). The genomic DNA G+C content of the type strain is 69.5 mol%.

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