RESEARCH ARTICLE

High genetic similarity between two geographically distinct strains of the sulfur-oxidizing symbiont '*Candidatus* Thiobios zoothamnicoli'

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Abstract

The giant marine ciliate *Zoothamnium niveum* (*Ciliophora*, *Oligohymenophora*) is obligatorily covered by a monolayer of putative chemoautotrophic sulfur-oxidizing (thiotrophic) bacteria. For *Z. niveum* specimens from the Caribbean Sea it has been demonstrated that this ectosymbiotic population consists of only a single pleomorphic phylotype described as *Candidatus* Thiobios zoothamnicoli. The goal of our study was to identify and phylogenetically analyse the ectosymbiont(s) of a recently discovered *Z. niveum* population from the Mediterranean Sea, and to compare marker genes encoding key enzymes of the carbon and sulfur metabolism between the two symbiont populations. We identified a single bacterial phylotype representing the ectosymbiont of *Z. niveum* from the Mediterranean population showing 99.7% 16S rRNA gene (99.2% intergenic spacer region) similarity to the Caribbean *Z. niveum* ectosymbiont. Genes encoding enzymes typical for an inorganic carbon metabolism [ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO)] and for sulfur metabolism (5′-adenylylsulfate reductase, dissimilatory sulfite reductase) were detected in both symbiotic populations. The very high amino acid sequence identity (97–100%) and the high nucleic acid sequence identity (90–98%) of these marker enzymes in two geographically distant symbiont populations suggests that the association of *Z. niveum* with *Cand*. Thiobios zoothamnicoli is very specific as well as temporally and spatially stable.

Introduction

Symbioses with chemoautotrophic, sulfur-oxidizing (thiotrophic) Bacteria are widespread among invertebrate and protist hosts and have been reported from diverse marine, shallow-water to deep-sea habitats (Fisher, 1996; Polz *et al*., 2000; Bright & Giere, 2005). A putative thiotrophic symbiosis hosted by the sessile, colonial, giant ciliate *Zoothamnium niveum* (*Ciliophora*, *Oligohymenophora*) has been reported from subtropical, tropical, and temperate shallow subtidal areas (Bauer-Nebelsick *et al*., 1996a, b; Ott *et al*., 1998; Rinke *et al*., 2006, 2007) (Fig. 1). The ciliate builds a colonial formation that consists of a central stalk with alternate branches bearing three different cell types: the microzooids are feeding types, the macrozooids are dispersal types, capable of leaving the colony to form an entire new colony after settlement, and the terminal zooids are responsible for asexual reproduction by longitudinal fission (Bauer-Nebelsick *et al*., 1996a, b). *Zoothamnium niveum* is obligatorily covered by a dense coat of ectosymbiotic bacteria (Bauer-Nebelsick *et al*., 1996a; Clamp & Williams, 2006; Rinke *et al*., 2006), whereby the microorganisms establish a monolayer composed of two morphotypes. Stalk, branches, terminal zooids and macrozooids bear rod-shaped symbionts, whereas microzooids are covered by rods on the aboral side as well as by coccoid rods on the oral side. A series of intermediate shapes between these two bacterial morphotypes on the microzooids was noted (Bauer-Nebelsick *et al*., 1996b). Phylogenetic 16S rRNA gene analyses and FISH of specimens from the Belize Barrier Reef (Caribbean Sea) revealed a single pleomorphic phylotype named *Candidatus* Thiobios zoothamnicoli, which clustered with thiotrophic

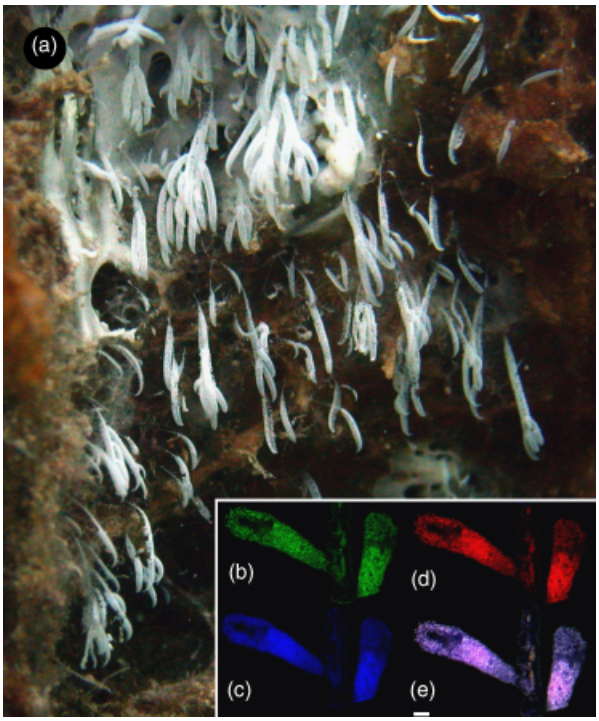

Fig. 1. (a) Several colonies of the giant ciliate *Zoothamnium niveum* populate a hydrogen sulfide-releasing mini vent on a vertical mangrove peat wall in the main channel of the island Twin Cays, Belize in the Caribbean Sea. The white color of the symbiont is due to a symbiotic monolayer of the *Gammaproteobacteria Candidatus* Thiobios zoothamnicoli which covers almost the entire host colony. (b–e) FISH micrographs of the Mediterranean *Z. niveum* symbiosis. Two *Z. niveum* microzooids attached to a branch and covered with ectosymbionts are visualized using a hierarchical probe set: (b) EUBmix probes in Fluos (green); (b) probe GAM42a in Cy5 (blue); (d) probe ZNS196 in Cy3 (red); (e) overlay. Scale bar = 10 μm.

free-living and symbiotic *Gammaproteobacteria* (Rinke *et al.*, 2006). Based on the hydrogen sulfide-rich habitat of the symbiosis (Ott *et al.*, 1998) and the white color of the symbiont (Bauer-Nebelsick *et al.*, 1996a), which was attributed to membrane-bound vesicles containing elemental sulfur, a chemoautotrophic sulfur-oxidizing nature of the ectosymbiotic bacteria was suggested (Bauer-Nebelsick *et al.*, 1996a). This assumption is supported by recent cultivation experiments which revealed that the symbiosis is not able to survive without sulfide (Rinke *et al.*, 2007). However, further studies that would support these putative physiological capabilities of the symbiont, such as screening for genes involved in carbon fixation or sulfur oxidation metabolic pathways, have not been undertaken so far.

The reductive pentose phosphate cycle (Calvin–Benson cycle) represents the CO_2 fixation pathway in almost all aerobic autotrophic bacteria (Watson & Tabita, 1997). One of the key enzymes involved in the Calvin–Benson cycle is ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39), which catalyzes the assimilation of CO_2 to organic carbon. The enzyme exists in two main forms. Form I consists of large and small subunits (L_nS_n, typically L_8S_8), and form II contains only large subunits (L_n) (Kellogg & Juliano, 1997); however, only the large subunits are responsible for carbon fixation (Miziorko & Lorimer, 1983). The genes coding for the large subunits of form I and form II are *cbbL* and *cbbM*, respectively.

Thiotrophic bacteria require a reduced sulfur source, usually hydrogen sulfide or thiosulfate, as electron donor and oxygen, or in some cases, nitrate as electron acceptor. The electrons derived from sulfur oxidation are used for energy transformation via the respiratory chain and for carbon dioxide fixation. However, a common mechanism for bacterial sulfur oxidation does not exist (Friedrich *et al.*, 2005). An enzyme system present in some but not all photo and chemotrophic microorganisms that oxidize reduced sulfur species was found to be encoded in the *dsr* gene cluster, which was originally identified in the phototrophic sulfur-oxidizing bacterium *Allochromatium vinosum* (Pott & Dahl, 1998; Dahl *et al.*, 2005). The *dsrAB* gene duo encode the α and β subunits of a dissimilatory sulfite reductase (dsr; EC 1.8.99.3), a characteristic enzyme used by sulfate-/sulfite-reducing microorganisms for generating energy via anaerobic respiration (Zverlov *et al.*, 2005; Stahl *et al.*, 2007; Loy *et al.*, 2008a) and which is operating in the reverse direction in sulfur-oxidizing bacteria. Molecular and (meta)genomic studies indicate that *dsrAB* genes are widespread among sulfur oxidizers of the phyla *Proteobacteria* (the classes *Alpha-*, *Beta-*, and *Gammaproteobacteria*) and *Chlorobi* (green sulfur bacteria) (Dahl *et al.*, 1999; Sabehi *et al.*, 2005).

In the sulfur metabolism of sulfur-oxidizing Bacteria and Archaea studied so far the main intermediate in the oxidation of reduced sulfur compounds to sulfate is the highly reactive sulfite. Two main pathways of sulfite oxidation are known: the direct oxidation to sulfate, or the indirect oxidation catalyzed by the enzymes adenosine-5′-phosphosulfate (APS) reductase (EC 1.8.99.2) and ATP sulfurylase (EC 2.7.7.4) with APS as an intermediate. APS reductase, consisting of two subunits (α and β) encoded by the genes *apsA* and *apsB*, respectively, is also found in sulfate reducers catalyzing the two-electron reduction of APS to sulfite and AMP, and was therefore proposed as a phylogenetic marker for bacteria involved in oxidative and reductive sulfur metabolism (Hipp *et al.*, 1997). Lateral gene transfer was, however, reported for *apsA* of sulfate-reducing as well as sulfur-oxidizing bacteria (Friedrich, 2002; Meyer & Kuever, 2007a, b).

The main objectives of this study were to identify and phylogenetically analyse the ectosymbiont(s) of a recently discovered *Z. niveum* population from the Mediterranean Sea (Rinke *et al.*, 2007), and to compare the genetic makeup (with respect to three key enzymes involved in autotrophic

carbon fixation, RuBisCO, and in sulfur metabolism, dsr and APS reductase) between the Mediterranean symbiont(s) and a recently described symbiont of *Z. niveum* from the Caribbean Sea (Rinke *et al.*, 2006). A single bacterial phylotype could be indentified representing the ectosymbiont of *Z. niveum* from the Mediterranean population showing 99.7% 16S rRNA gene similarity to the Caribbean *Z. niveum* ectosymbiont. We detected genes encoding enzymes typical for inorganic carbon (RuBisCO – *cbbL*) and sulfur metabolism (*dsrAB*, *apsA*) in both symbiotic populations. Comparing two geographically distant symbiont populations we found very high amino acid sequence identity (97–100%) of these marker enzymes, suggesting that the association of *Z. niveum* with *Cand.* Thiobios zoothamnicoli is very specific and highly stable over temporal and spatial scales. However, a universal free-living population of the symbiont serving as reinfection source cannot be ruled out completely.

Materials and methods

Specimen collection and preparation

Colonies of *Z. niveum* (Hemprich & Ehrenberg, 1831) were collected by scuba diving in the bay of Calvi (42°34′N, 8°43′E) in Corsica, France (Mediterranean Sea) on decaying *Posidonia oceanica* leaves at a depth of 13.8 m in 2005 (site 3, Rinke *et al.*, 2007). Colonies of the Caribbean *Z. niveum* population were sampled in 2006 at Cuda Cut (16°83′N, 88°10′W) at the island Twin Cays belonging to the Belize Barrier Reef. Specimens were cut from a mangrove peat wall, with a vertical elongation of about 15 m, at 1–3 m depth and combined during scuba diving. Immediately after collection, colonies from the respective sampling site were randomly picked and fixed in 99% ethanol for molecular biology experiments or in 50% Bouin's solution for FISH and subsequent confocal laser scanning microscopy.

Gene amplification, cloning and sequencing

From each sampling site (Caribbean and Mediterranean Sea) 20 colonies were randomly chosen, pooled and used for PCR-mediated amplification of gene fragments of interest. To avoid contamination with other bacteria occurring on the lower parts of large colonies (Rinke *et al.*, 2006), the basal third of each colony was cut off. Subsequently, samples were transferred to phosphate-buffered saline (PBS; 0.1 M, pH 7.4), followed by two short pulses of sonication for 10 s each (HD 2070 sonicator, Bandelin, Berlin, Germany), to mechanically disintegrate the large colonies. For each PCR, 1 μL of these PBS solutions containing a mixture of host tissue and symbionts was directly used as a template without prior DNA extraction, except for the PCR amplification of the *cbbL*, *cbbM*, and *apsA* genes of the Caribbean samples, for which DNA was isolated as described by Groot *et al.* (2005). PCR reactions amplifying the 16S rRNA genes and the intergenic spacer region (ISR) between the 16S and the 23S rRNA genes were performed with a standard PCR cycling program using an annealing temperature of 50 and 54 °C, respectively. PCR reactions targeting *cbbL* and *cbbM* genes were carried out according to Elsaied & Naganuma (2001), those targeting *apsA* genes according to Blazejak *et al.* (2006), and those targeting *dsrAB* genes according to Kjeldsen *et al.* (2007). All primers used in this study are shown in Table 1. Positive controls applied were: *Ralstonia metallidurans* DNA extract for *cbbL*; *Polaromonas naphthalenivorans* DNA extract for *cbbM*; *Riftia pachyptila* trophosome extract for *cbbM* and *apsA*. Each assay contained a negative control, in which no DNA was added to the reaction mixture.

PCR products were separated by electrophoresis using 1.5% agarose gels, which were stained with ethidium bromide and visualized by UV transillumination. Amplification products of the desired size (*c.* 1500 bp for the 16S rRNA gene, 1200 bp for ISR, 800 bp for *cbbL*, 400 bp for *apsA*,

Table 1. PCR primers used in this study

Primer	Target gene	Sequence (5′–3′)*	References
616V	16S rRNA gene (most Bacteria)	AGA GTT TGA TYM TGG CTC	Juretschko *et al.* (1998)
1492R	16S rRNA gene (most Bacteria and Archaea)	GGY TAC CTT GTT ACG ACT T	Loy *et al.* (2005)
1100F	16S rRNA gene (most Bacteria)	CAA CGA GCG CAA CCC T	Weisburg *et al.* (1991)
1035R	23S rRNA gene (most Bacteria)	TTC GCT CGC CRC TAC	Ludwig *et al.* (1992)
cbbL 595F	*cbbL* (form IA RuBisCO)	GAC TTC ACC AAA GAC GAC GA	Elsaied & Naganuma (2001)
cbbL 1405R	*cbbL* (form IA RuBisCO)	TCG AAC TTG ATT TCT TTC CA	Elsaied & Naganuma (2001)
cbbM 663F	*cbbM* (form II RuBisCO)	ATC ATC AAR CCS AAR CTS GGC CTG CGT CCC	Elsaied & Naganuma (2001)
cbbM 1063R	*cbbM* (form II RuBisCO)	MGA GG TGA CSG CRC CGT GRC CRG CMC GRTG	Elsaied & Naganuma (2001)
apsA 1F	*ApsA*	TGG CAG ATC ATG ATY MAY GG	J. Kuever (unpublished data)
apsA 4R	*ApsA*	GCG CCA ACY GGR CCR TA	J. Kuever (unpublished data)
rDSR1Fc	*dsrAB* of some sulfur oxidizers	ATG GGN TAY TGG AAR G	Loy *et al.* (2008b)
rDSR4Ra	*dsrAB* of some sulfur oxidizers	CC RAA RCA IGC NCC RCA	Loy *et al.* (2008b)

*Sequences are indicated in IUPAC nomenclature; base analogon inosine (I).

and 1900 bp for *dsrAB*) were extracted from the gel and used for cloning reactions with the TopoTA cloning kit (Invitrogen Life Technologies, Lofer, Austria), except for cloning of the *dsrAB*, which was carried out using the TopoXL cloning kit (Invitrogen Life Technologies).

Clones of *cbbL* and *aprA* genes were analysed with a restriction fragment length polymorphism (RFLP) applying a combination of the restriction enzymes AluI and MspI at 0.67 U μL^{-1} each (Fermentas GmbH, St Leon-Rot, Germany), whereas *dsrAB* clones were analysed applying MspI only. The enzyme sample mix was incubated for 90 min at 37 °C and subsequently visualized using 3% agarose gels, which were thereafter stained with ethidium bromide and visualized by UV transillumination.

Nucleotide sequences of cloned DNA fragments were determined on an ABI 3130 XL genetic analyser using the BigDye Terminator kit v3.1 (ABI, Vienna, Austria).

Sequence analysis and phylogenetic reconstruction

Maintenance and phylogenetic analysis of the obtained sequences were conducted using the ARB program package (Ludwig *et al.*, 2004). New sequences were added to the corresponding 16S rRNA gene, ISR, RuBisCO, *apsA* and *dsrAB* nucleotide databases. Automatic alignment was performed with the fast aligner of the ARB package and subsequently corrected by visual inspection. The *cbbL*, *apsA*, and *dsrAB* sequences were translated to amino acids. The phylogenetic relationships of 16S rRNA gene and ISR nucleic acid sequences were inferred applying the following methods: neighbor-joining (Jukes–Cantor correction), maximum parsimony (Phylip DNAPARS 3.573, with and without 100 resamplings), maximum likelihood (AxML), and TREEPUZZLE 5.0. The results of all applied treeing methods were combined in a consensus tree. Phylogenetic calculations of RuBisCO, APS reductase, and Dsr were applied with the deduced amino acid sequences using the following treeing methods: neighbor-joining (using the PAM matrix for amino acid substitution), maximum parsimony (Phylip PROTPARS 3.573, with and without 100 resamplings), and maximum likelihood (PROTML). APS reductase and dsr amino acid sequence phylogenies were additionally inferred using TREEPUZZLE 5.0. Obtained results were combined in a consensus tree.

For 16S rRNA gene phylogeny, an outgroup of 34 sequences covering the main phyla of the domain Bacteria was used. A filter considering only those alignment positions that were conserved in at least 50% of all *Gammaproteobacteria* sequences was applied, and only sequences with a length of more than 1400 nucleotides were used, resulting in 1288 positions for calculations. Phylogenetic analyses of RuBisCO were performed without a conservation filter based on an alignment of 271 amino acids, reflecting the sequence length obtained in this study. An outgroup consisting of five RuBisCO form II sequences was used. Amino acid sequences shorter than that indicated above or with only partial overlaps to the sequence region used for calculation were individually added to the different trees using the parsimony interactive tool of ARB. The phylogeny of APS reductase was calculated from partial sequences consisting of 130 amino acids. Amino acid sequences shorter than indicated above were added to the different trees using the parsimony interactive tool of ARB. Dsr trees were calculated based on sequences > 592 amino acids and using a filter that excludes sequence regions affected by insertions and deletions (indels), leaving a total of 551 amino acid positions for phylogenetic analyses. ISR sequences were screened for tRNA genes with the online software tool ARAGORN (Laslett & Canback, 2004). The retrieved nucleotide sequences have been deposited in the public databases GenBank/EMBL/DDBJ under accession numbers EU439003 (16S rRNA gene and ISR); EU439000, EU439002 (*cbbL*); EU439004, EU439001 (*apsA*); and EU817177, EU817178, EU817179 (*dsrAB*).

FISH

For FISH, specimens from the bay of Calvi (Mediterranean Sea) were fixed with 50% Bouin's solution (EMS, Hatfield, PA) for 15 min, rinsed in PBS three times for 5 min each, dehydrated in 30% and 50% ethanol for 5 min each, and stored in 70% ethanol until further treatment up to a year later. Specific probes for Bacteria (EUB338, EUB338II, EUB338III; Amann *et al.*, 1990; Daims *et al.*, 1999), *Gammaproteobacteria* (GAM42a; Manz *et al.*, 1992), *Betaproteobacteria* (BET42a; Manz *et al.*, 1992) as well as two *Cand.* Thiobios zoothamnicoli-specific probes ZNS196 (5′-CTG ATA GTG ACC GAA GTC T-3′) and ZNS1439 (5′-GAG CGC CCA TTA TTA AGC TA-3′) (Rinke *et al.*, 2006) were labeled on their 5′ end with the fluorescent dyes Cy3, Cy5, or fluorescein (Fluos) (Thermo Hybaid, Ulm, Germany). Probe NON338 (Wallner *et al.*, 1993) complementary to EUB338 was applied as negative hybridization control. Detailed information on the oligonucleotide probes used in this study is available at probeBase (Loy *et al.*, 2007). FISH on whole-mounted *Z. niveum* colonies was performed as described previously (Rinke *et al.*, 2006).

Results and discussion

16S rRNA gene phylogeny and FISH

For the Calvi (Mediterranean Sea) ciliate sample, five 16S rRNA genes and four ISR clones were randomly chosen and sequenced completely. The 16S rRNA gene sequences were all 1453 nucleotides in length and of identical composition.

Table 2. Sequences obtained from the two geographically distinct *Candidatus* Thiobios zoothamnicoli strains from Twin Cays (Caribbean Sea) and Calvi (Mediterranean Sea)

Accession no.	Gene (encoded enzyme)	Clones screened	Clones sequenced	Length (NA/AA)	Strain	References
AJ879933	16S rRNA, ISR, partial 23S rRNA gene	7	2	2956	Twin Cays	Rinke *et al*. (2006)
EU439003	16S rRNA, ISR	25, 25	5, 4	1869	Calvi	This study
EU439002	*cbbL* (form IA RuBisCO)	26	6	812/271	Twin Cays	This study
EU439000	*cbbL* (form IA RuBisCO)	6	6	812/271	Calvi	This study
EU817178	*dsrAB* (dissimilatory sulfite reductase) *TZdsrAB1*	14	5	1908/602	Twin Cays	This study
EU817179	*dsrAB* (dissimilatory sulfite reductase) *TZdsrAB2*	16	5	1908/602	Twin Cays	This study
EU817177	*dsrAB* (dissimilatory sulfite reductase)	2	2	1908/602	Calvi	This study
EU439001	*apsA* (APS reductase)	28	10	389/130	Twin Cays	This study
EU439004	*apsA* (APS reductase)	9	5	389/130	Calvi	This study

Clones were screened with RFLP.

The ISR sequences were also identical with each other (416 nucleotides in length) and contained two tRNA genes (isoleucine and alanine; Fig. 3). The ISR could be unambiguously attributed to the 16S rRNA gene due to the sequence identity of 372 overlapping nucleotides.

The 16S rRNA gene sequence analysis revealed that the symbiont of *Z. niveum* from Calvi shows 99.7% similarity to *Cand*. Thiobios zoothamnicoli, the ectosymbiont of *Z. niveum* colonies from the Caribbean Sea (island Twin Cays, Belize) (Rinke *et al*., 2006). According to Stackebrandt & Ebers (2006) a 16S rRNA gene sequence similarity below a threshold range of 98.7–99% is recommended for the unambiguous differentiation of two species. However, a 16S rRNA gene sequence similarity of over 99%, as found in this study, can still mask high genome heterogeneity (Thompson *et al*., 2005). It is thus not possible to decide unambiguously whether the two symbiont populations belong to the same species. Tentatively, we will refer thereafter to the 16S rRNA gene sequence obtained in this study from Calvi, France, as 'strain Calvi', and to the sequence from Twin Cays, Belize (Rinke *et al*., 2006) as 'strain Twin Cays' (Table 2). All treeing methods used to resolve the phylogenetic relationships of the obtained 16S rRNA gene sequence to other *Gammaproteobacteria* in our dataset provided highly congruent results. The Mediterranean *Z. niveum* symbiont formed a monophyletic group together with *Cand*. Thiobios zoothamnicoli from the Caribbean Sea, the free-living sulfur-oxidizing bacterium ODIII6 (Kuever *et al*., 2002), a symbiont (named 'scaly snail endosymbiont') of a recently discovered hydrothermal vent gastropod (family *Peltospiridae*) from the Indian Ocean (Goffredi *et al*., 2004) and three uncultured marine *Gammaproteobacteria* retrieved from presumably sulfide-rich habitats (Fig. 2). Furthermore, all treeing methods supported the monophyly of a symbiont-containing group comprising the gammaproteobacterial ectosymbiont of the nematode *Laxus oneistus* of the subfamily Stilbonematinae (Polz *et al*., 1994), the endosymbiont of the nematode *Astomonema* sp. (Musat *et al*., 2007), and endosymbionts of the oligochaete subfamily Phallodrilinae (Dubilier *et al*., 1999).

The obtained ISR sequences from strain Calvi showed a sequence similarity of 99.2% to strain Twin Cays. In previous studies the ISR has been a powerful tool for comparing bacterial strains and populations (Otsuka *et al*., 1999; Boyer *et al*., 2001), because ISR are highly variable due to frequent DNA recombination and mutation events (Gürtler, 1999). The ISR sequence similarity between strain Calvi and strain Twin Cays is surprisingly high, showing a variation of only five-nucleotide position, three occurring within tRNA coding regions (Fig. 3). Nevertheless, the available ISR sequences unambiguously distinguish the two *Cand*. Thiobios zoothamnicoli strains.

The very high sequence similarity of the 16S rRNA gene and the ISR points to a relatively recent separation of the Caribbean and the Mediterranean *Cand*. Thiobios zoothamnicoli populations in geological times. Using molecular clocks of bacteria, assuming that bacterial 16S rRNA gene sequences diverge at a substitution rate of 1–2% per 50 million years (Ochman & Wilson, 1987; Moran *et al*., 1993), the diversity observed between strain Calvi and strain Twin Cays of 0.36% results in an estimated separation of the two lineages 9–18 million years ago. Since the Atlantic Ocean was already established 65 million years ago, the *Z. niveum* symbiosis must have found ways of bridging far distances of what is nowadays over 9200 km (5700 miles) to achieve such a wide distribution.

Macrozooids, which are covered by a monolayer of symbionts (Bauer-Nebelsick *et al*., 1996a), leave the colony as swarmers and survive a maximum of 24 h as motile stages, in which they cover a maximum distance of 400 m before settling (Ott & Bright, 2004). Therefore it is more likely that drifting sulfide sources, such as mangrove tree trunks or other massive macrophyte debris, serve as vessels to explore new distant habitats. Hydrogen sulfide, generated

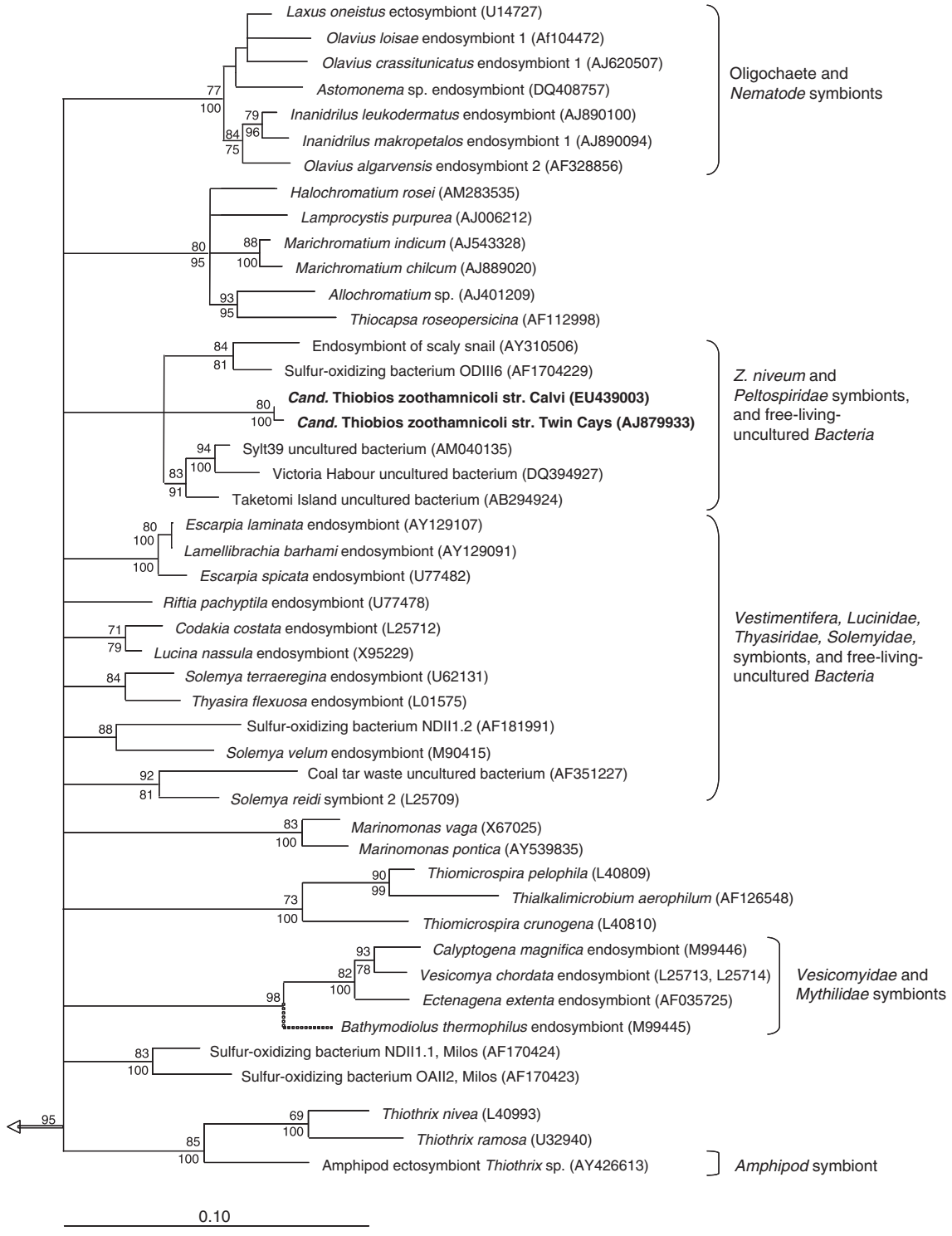

Fig. 2. 16S rRNA gene phylogenetic consensus tree based on a TREEPUZZLE tree (with the HKY correction/uniform model of substitution) showing the relationships of the *Candidatus* Thiobios zoothamnicoli strain Calvi' and chemoautotrophic symbiotic and free-living *Gammaproteobacteria*. For symbiotic bacteria the respective host organisms are indicated. TREEPUZZLE support values (%) are depicted above the respective branches; maximum parsimony bootstrap values (%) are shown below the branches. Only TREEPUZZLE supports values > 70% and parsimony bootstrap values > 75% are displayed. GenBank accession numbers are given in parentheses. The arrow points to the outgroup, and the bar represents 10% estimated evolutionary distance.

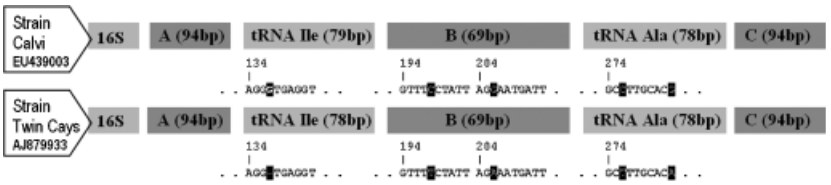

Fig. 3. Comparison of the ISR adjacent to the 16S rRNA gene of strain Calvi and strain Twin Cays. The ISR sequence consists of three potential noncoding regions (a–c) and two segments encoding RNAs for isoleucine (Ile) and alanine (Ala). The length of each region is given in basepairs (bp). The sequence variations between both strains are shown according to the region of appearance and numbered starting with base 1 of the ISR.

by a diverse community of microorganisms using sulfate as an oxidizing agent, is known to be a major product of anaerobic degradation of cellulose in marine systems (Leschine, 1995).

The Mediterranean Sea dried up for the last time 5–6 million years ago (Hsü, 1983), and thus it is most likely that the *Z. niveum* symbiosis (re)colonized this habitat from the Atlantic Ocean. Furthermore, the land connection between North and South America started developing around 5 million years ago, which makes it possible that the *Z. niveum* symbiosis also swept out into the Pacific Ocean.

Alternatively, one cannot rule out the possibility that the symbiont has its own additional way of distribution by co-occurring in a motile, free-living population in the water column interacting with the sessile symbiotic population, as suggested for the thiotrophic symbionts of *Rimicaris exoculata*, a hot vent shrimp from the Mid-Atlantic Ridge (Polz & Cavanaugh, 1995). However, considering the scattered distribution of sulfide sources in the *Z. niveum* habitat (Ott *et al*., 1998) and results showing that the swarmers actively find and colonize new sulfide minivents (Pöhn, 2002) within a few 100 m day^{-1} (Ott & Bright, 2004), it seems rather unlikely that a free-living population of the bacterial symbiont could keep up with this speed of distribution.

FISH of whole-mounted *Z. niveum* colonies strain Calvi using *Cand*. Thiobios zoothamnicoli-specific probes ZNS196 and ZNS1439 under stringent conditions (35% formamide in the hybridization buffer) demonstrated that both ectosymbiotic morphotypes, the rods as well as the coccoid rods, hybridized with the symbiont-specific probes, the probe for *Gammaproteobacteria*, and the probe for *Bacteria* (Fig. 1b–e), and therefore belong to one phylotype. These results are consistent with those reported for the Caribbean *Z. niveum* population from the mangrove island Twin Cays, Belize (Rinke *et al*., 2006).

cbbL, *apsA* and *dsrAB* gene diversity

From the Caribbean *Z. niveum* sample, 26 clones of the amplified *cbbL* gene, encoding the large subunit of RuBisCO, were analysed with RFLP, resulting in identical patterns. Thereafter six clones were randomly chosen, sequenced and found to be identical. From the Mediterranean sample, six *cbbL* clones were retrieved, which were fully sequenced and also found to be identical. The obtained *cbbL* gene sequences of both symbionts (Caribbean and Mediterranean) were 812 nucleotides in length (Table 2) and showed 99% nucleic acid sequence similarity. The deduced amino acid sequences (271 amino acids) were 100% identical. The closest relative of both symbiont RuBisCo sequences was the RuBisCO of an uncultured bacterium, retrieved from suboxic mobile muds from French Guyana (V. Madrid, pers. commun.), showing 97% amino acid identity (Fig. 4). All amino acid identities to other RuBisCO sequences in public databases were below 88.5%. Although multiple PCR assays under various conditions were performed, form II RuBisCO, encoded by *cbbM*, was not detected in the *Z. niveum* samples. All applied phylogenetic treeing methods placed the RuBisCO sequences of both *Z. niveum* strains in a monophyletic group together with thiotrophic symbiotic (*Inanidrillus leukodermatus* and *Inanidrillus makropealos* endosymbiont) and free-living (*A. vinosum*) *Gammaproteobacteria*, as well as with an uncultured bacterium from suboxic mobile mud and *Cyanobacteria* of the *Prochlorococcus* and *Synechoccus* spp. clade (PS clade), all bearing form 1A RuBisCO (Fig. 4).

Amplified *apsA* sequences, encoding the APS reductase α subunit, were cloned and the RFLP analyses of 28 clones from the Caribbean sample resulted in two distinct, equally distributed patterns. Thereafter five clones of each pattern were randomly chosen and sequenced, revealing identical sequences, showing only three nucleotide sequence differences at the primer binding sites, most likely due to the use of degenerate primers applying PCR. From the Mediterranean sample, five clones were sequenced and found to be identical. The *apsA* genes of both symbionts were 389 nucleotides in length (Table 2) and shared 95.7% nucleic acid sequence identity. The deduced amino acid sequences (130 amino acids) were 98.4% identical. Phylogenetic analysis of *apsA* amino acid sequences of strain Twin Cays and strain Calvi revealed the highest amino acid sequence identity to *Thiobacillus denitrificans apsA2* (95.1/95.1%), *A. vinosum* strain DSM 180 (93.4/92.6%), and to *Candidatus* Vesicomyosocius okutanii (93.4/91.8%), a thiotrophic intracellular symbionts of the deep-sea clam *Calyptogena okutanii* (Fig. 5). All treeing methods applied placed the *apsA* gene of

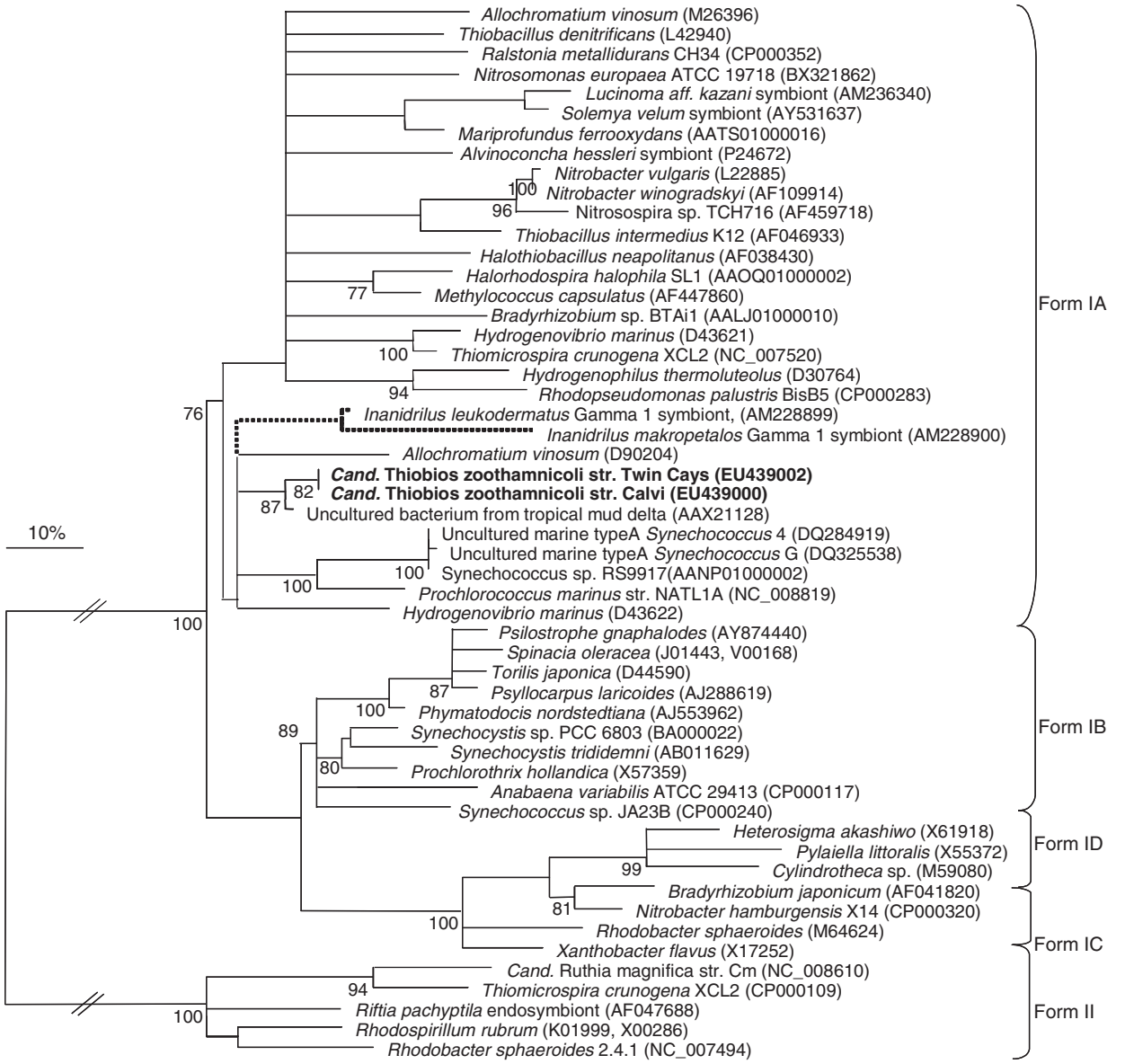

Fig. 4. Maximum likelihood (PROTEINML)-based consensus tree displaying the phylogenetic positions of the sequences encoding for the large subunit of RuBisCO retrieved from the Caribbean and the Mediterranean strain of *Candidatus* Thiobios zoothamnicoli. For the symbiotic bacteria the respective host organisms are indicated or the taxonomic status 'Candidatus' is provided if available. The four different forms (A–D) of RuBisCO form I and RuBisCO form II are marked. Partial sequences added to the initial tree by applying parsimony insertion are marked with dashed lines. Maximum parsimony bootstrap values (in%) are shown below the branches. Only TREEPUZZLE support values $> 70\%$ and parsimony bootstrap values $> 75\%$ are displayed. GenBank accession numbers are given in parentheses. The bar represents 10% estimated evolutionary distance.

the two *Z. niveum* ectosymbiont strains in a well-supported group together with sulfur-oxidizing symbiotics (*Inanidrilus exumae* endosymbiont, *Cand.* Vesicomyosocius okutanii, *Candidatus* Ruthia magnifica, *Idas* sp. endosymbiont, *Bathymodiolus brevior* endosymbiont) and free-living (*A. vinosum*) *Gammaproteobacteria*, with *T. denitrificans*, a sulfur-oxidizing *Betaproteobacteria*, and with an unclassified environmental clone S8CL5 from metalliferous peat soils. This group clusters within the *apsA* lineage I, which was proposed to contain the 'authentic' *apsA* gene loci not affected by lateral gene transfer (Meyer & Kuever, 2007b). A second group of symbiotic and free-living sulfur-oxidizing bacteria was found to possess *apsA* genes showing close affiliation to sulfate-reducing bacteria. These sulfur-oxidizing *Gamma-* and *Betaproteobacteria* were recently placed into *apsA* lineage II, where acquisition of apsA genes was hypothesized to be due to lateral gene transfer from

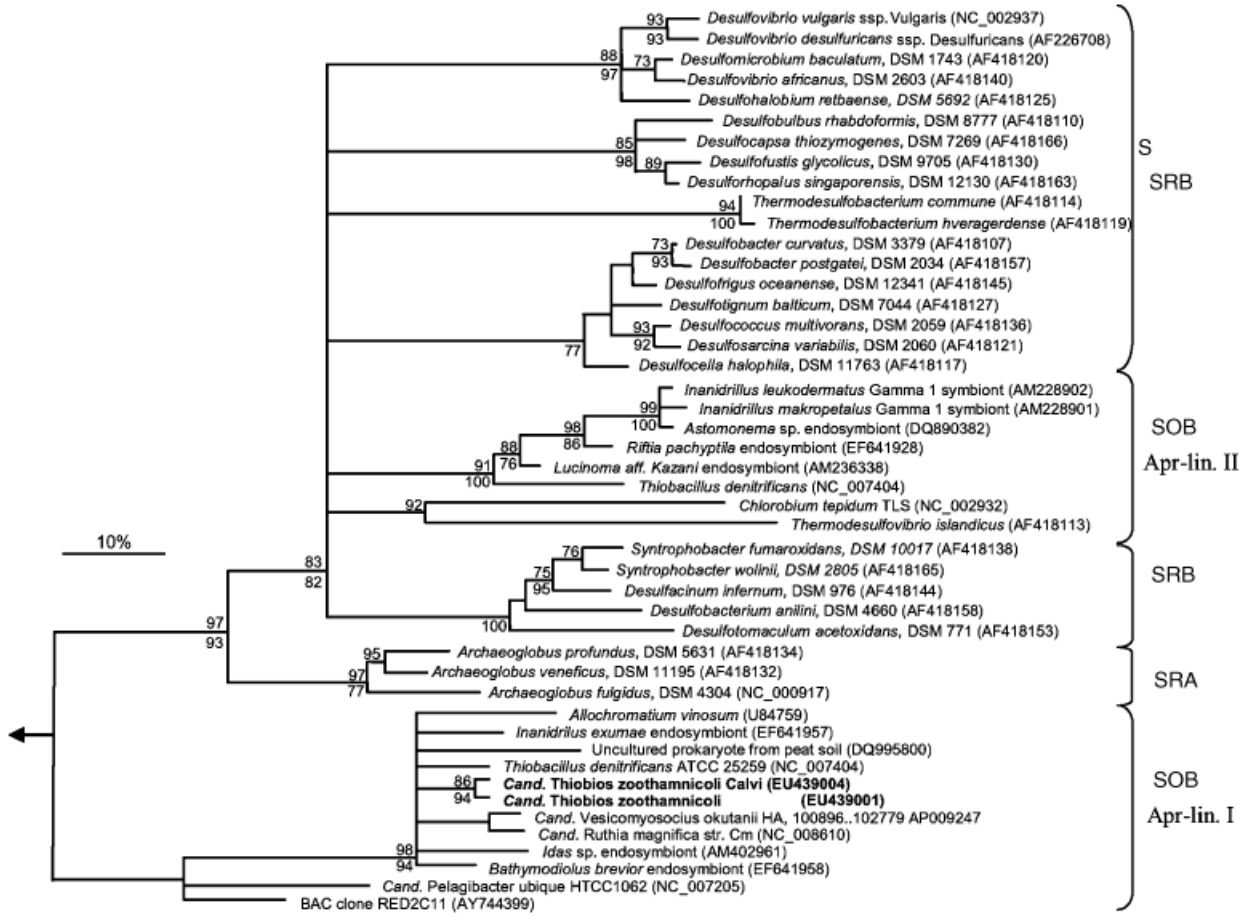

Fig. 5. Maximum likelihood (PROTEINML)-based consensus tree displaying the phylogenetic positions of the sequences encoding for APS reductase retrieved from the Caribbean and the Mediterranean strain of *Candidatus* Thiobios zoothamnicoli. For the symbiotic bacteria the respective host organisms are indicated or the taxonomic status 'Candidatus' is provided if available. Phylogenetic groups of sulfate-reducing Bacteria (SRB), sulfur-oxidizing Bacteria (SOB), and sulfate-reducing Archaea (SRA) are marked. TREEPUZZLE support values (in%) are depicted above the respective branches; maximum parsimony bootstrap values (in%) are shown below the branches. Only TREEPUZZLE support values $> 70\%$ and parsimony bootstrap values $> 75\%$ are displayed. GenBank accession numbers are given in parentheses. The arrow points to the outgroup, and the bar represents 10% estimated evolutionary distance.

sulfate-reducing bacteria (Meyer & Kuever, 2007b). Despite these lateral gene transfer events, *apsA* phylogeny is still instrumental in distinguishing between bacteria involved in the oxidative vs. the reductive sulfur metabolism.

For *dsrAB*, encoding dsr α and β subunit, two evenly abundant distinct RFLP patterns were obtained by screening 30 clones from the Caribbean symbiont. For each pattern, five clones were randomly chosen, sequenced and two distinct clone sequences *TZdsrAB1* and *TZdsrAB2* were obtained. From the Mediterranean symbiont, two clones were retrieved, sequenced and found to be identical. The *dsrA* genes from both *Z. niveum* symbionts were 1080 nucleotides (360 amino acids) in length, separated by a spacer region of 97 nucleotides from the *dsrB* gene of 731 nucleotides (242 amino acids) length, resulting in a total of 1908 nucleotides (602 amino acids) for *dsrAB* (Table 2). The Caribbean sequences *TZdsrAB1/TZdsrAB2* showed 90.3% nucleic acid similarity and the deduced amino acid sequences 97.7% identity to each other, and 98.2/90.1% nucleic acid similarity as well as 99.4/97.1% amino acid sequence identity to the Mediterranean clone. Phylogenetic analysis revealed the sulfur-oxidizing *Gammaproteobacteria A. vinosum* as most similar relative, with 82.2–83.3% DsrAB amino acid identity to strain Twin Cays and strain Calvi. All applied phylogenetic treeing methods grouped the dsr sequences of the two *Z. niveum* ectosymbiont strains next to *A. vinosum* within a well-supported cluster of sulfur-oxidizing *Gammaproteobacteria* (Fig. 6). The latter were clearly separated from sulfur-oxidizing *Alpha-* and *Betaproteobacteria*, and *Chlorobi*, as well as from sulfate/sulfite-reducing microorganisms in all treeing methods applied (Fig. 6). In contrast to sulfate-/sulfite-reducing microorganisms (Zverlov *et al.*, 2005), there is

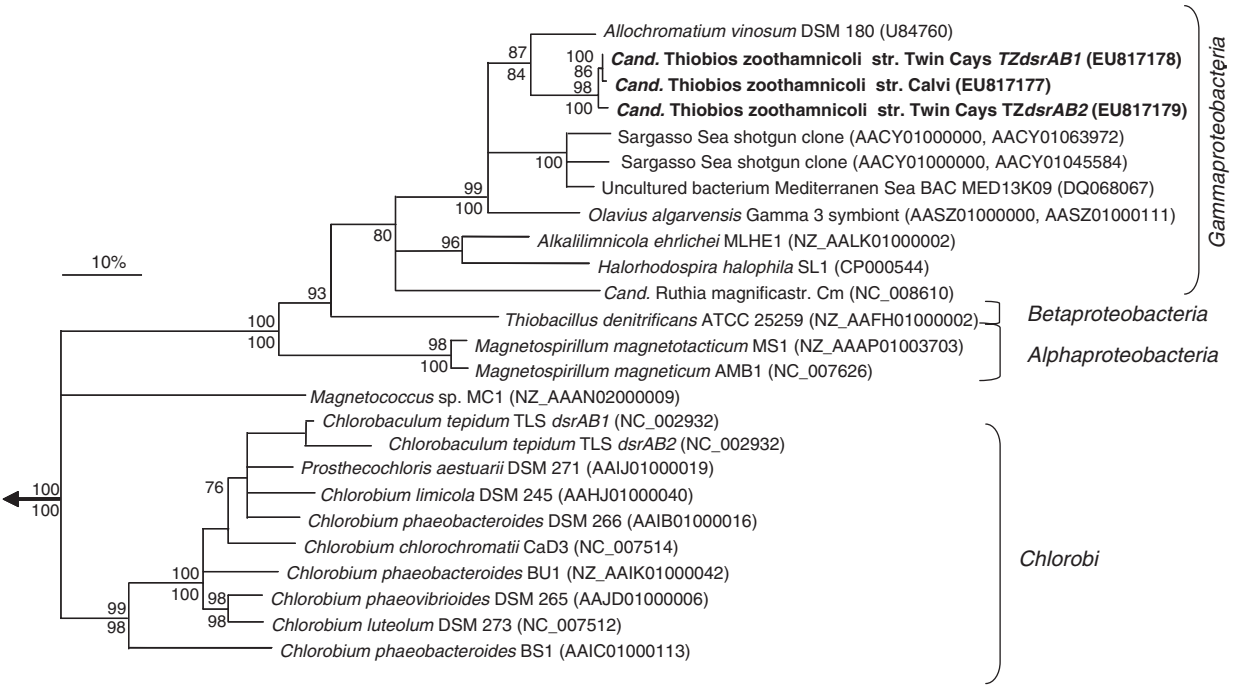

Fig. 6. Maximum likelihood (PROTEINML)-based consensus tree displaying dsr sequences, of sulfur-oxidizing bacteria belonging to *Gamma-*, *Beta-*, and *Alphaproteobacteria*, and *Chlorobi* (green sulfur bacteria). For the symbiotic bacteria the respective host organisms are indicated or the taxonomic status 'Candidatus' is provided if available. TREEPUZZLE support values (in%) are depicted above the respective branches; maximum parsimony bootstrap values (in%) are shown below the branches. Only TREEPUZZLE support values $> 70\%$ and parsimony bootstrap values $> 75\%$ are displayed. GenBank accession numbers are given in parentheses. The bar represents 10% estimated evolutionary distance.

currently no hint that the distribution of *dsrAB* from sulfur oxidizers was affected by LGT (lateral gene transfer) among major bacterial taxa (phyla/classes). However, only a limited number of *dsrAB* sequences from cultivated sulfur oxidizers are available (Loy *et al.*, 2008). Interestingly, two distinct *dsrAB* sequences were obtained from the Twin Cays sample. These two *dsrAB* types showed only moderate nucleic acid similarity (90.3%), but high amino acid identity (97.7%) to each other. So far only two microorganisms are known to harbor two *dsrAB* copies in their genomes. The two *dsrAB* sequences from the photoautotrophic sulfur-oxidizer *Chlorobaculum tepidum* show 95.5% nucleic acid similarity (94.8% amino acid identity). However, one copy contains an authentic frame shift within *dsrB*, suggesting that this copy is nonfunctional. The sulfite-reducing archaeon *Pyrobaculum aerophilum* possesses two *dsrAB* copies with only 74% nucleic acid similarity (70.6% amino acid identity). Nevertheless, whether both *dsrAB* sequences retrieved from the Caribbean sample derive from the same organism or from two closely related microorganisms remains to be determined. However, an organism's ability to cope with varying environmental conditions might be extended by the presence of multiple copies of a functional gene in one strain, considering that the different gene versions may code for functional enzymes with slightly different characteristics (Tchawa Yimga *et al.*, 2003).

Our results show that the colonial ciliate *Z. niveum* is obligatorily covered by the single polymorphic bacterial phylotype *Cand.* Thiobios zoothamnicoli, whereby symbionts sampled in the Caribbean and the Mediterranean Sea show high 16S rRNA gene similarity, suggesting a differentiation on the strain level. Detection of sulfide was reported from both sampled *Z. niveum* habitats (Ott *et al.*, 1998; Rinke *et al.*, 2007), and *in vivo* experiments revealed that the *Z. niveum* symbiosis requires sulfide for its survival (Ott & Bright, 2004; Rinke *et al.*, 2007). Consistent with these observations the detection of highly similar RuBisCO, APS reductase, and dsr-encoding genes in two samples from different oceanic sites provides the first molecular evidence that *Cand.* Thiobios zoothamnicoli is indeed a chemoautotrophic sulfur-oxidizing (thiotrophic) *Gammaproteobacteria*. Observations of membrane-bound vesicles, most likely containing elemental sulfur, by transmission electron microscopy (Bauer-Nebelsick *et al.*, 1996a), the white color of the bacteria, an attribute to intracellular stored sulfur, and our confirmation of dsr-encoding genes support the hypothesis that *Cand.* Thiobios zoothamnicoli generates intracellularly stored sulfur vesicles as essential intermediates in the sulfur metabolism, as reported for *A. vinosum* (Pott & Dahl, 1998).

Because a variety of thiotrophic symbionts are known to form sulfur deposits, dsr could very well be one of the mayor

pathways of sulfur-oxidizing symbiotic bacteria from shallow water to the deep sea. This concept is supported by available metagenome data, showing *dsrAB* genes for the *Olavius algarvensis* and the *Calyptogena magnifica* symbiont (*Cand.* Ruthia magnifica; Fig. 6).

In general, microbial symbioses with eukaryotic hosts are characterized by the physiological capabilities of the symbionts. Thus this study is a crucial step in illuminating the thiotrophic metabolism of two geographically separated *Cand*. Thiobios zoothamnicoli populations.

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