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Communities of green sulfur bacteria in marine and saline habitats analyzed by gene sequences of 16S rRNA and Fenna-Matthews-Olson protein

Summary. Communities of green sulfur bacteria were studied in selected marine and saline habitats on the basis of gene sequences of 16S rRNA and the Fenna-Matthews-Olson (FMO) protein. The availability of group-specific primers for both 16S rDNA and the *fmoA* gene, which is unique to green sulfur bacteria, has, for the first time, made it possible to analyze environmental communities of these bacteria by culture-independent methods using two independent genetic markers. Sequence results obtained with *fmoA* genes and with 16S rDNA were largely congruent to each other. All of the 16S rDNA and *fmoA* sequences from habitats of the Baltic Sea, the Mediterranean Sea, Sippewissett Salt Marsh (Massachusetts, USA), and Bad Water (Death Valley, California, USA) were found within salt-dependent phylogenetic lines of green sulfur bacteria established by pure culture studies. This strongly supports the existence of phylogenetic lineages of green sulfur bacteria specifically adapted to marine and saline environments and the exclusive occurrence of these bacteria in marine and saline habitats. The great majority of clone sequences belonged to different clusters of the *Prosthecochloris* genus and probably represent different species. Evidence for the occurrence of two new species of *Prosthecochloris* was also obtained. Different habitats were dominated by representatives from the *Prosthecochloris* group and different clusters or species of this genus were found either exclusively or as the clearly dominant green sulfur bacterium at different habitats. [**Int Microbiol** 2006; 9(4):259-266]

Key words: *Prosthecochloris* · Chlorobiaceae · environmental diversity · Fenna-Matthews-Olson protein

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Introduction

Green sulfur bacteria are anoxygenic phototrophic bacteria, obligate phototrophic and strictly anaerobic. Although widely distributed, green sulfur bacteria are restricted to anoxic habitats exposed to light; thus, they are found in brackish waters, marine lagoons, hypersaline habitats, and freshwater pools [4,11,17, 18,31,33]. Due to their adaptation to low light intensities and high sulfide concentrations [2], these bacteria prevail in the deeper anoxic layers of lagoons and lakes, in the Black Sea [27,34], as well as within the top millimeters of sediments with an oxic/anoxic boundary. A limited variety of green sulfur bacteria have been isolated from marine habitats [11,33,34] and in most cases were identified as belonging to *Prosthecochloris aestuarii*. This limited genetic variation of cultured green sulfur bacteria known from marine habitats could be due to the selectivity of their growth conditions and may represent only a fraction of the environmental communities. Therefore, a genetic approach—one that was not biased by the limitations imposed by specific culture conditions—was used in the present study to analyze environmental communities of green sulfur bacteria.

One of the properties responsible for the specific low-light adaptation of green sulfur bacteria is the presence of characteristic light-harvesting structures, the chlorosomes, which are attached to the cytoplasmic membrane [6,29,30]. The Fenna-Matthews-Olson protein is part of this unique photosynthetic apparatus in green sulfur bacteria. The water-soluble protein bacteriochlorophyll *a* (Bchl *a*) mediates energy transfer between the chlorosomes and the reaction center in the cytoplasmic membrane. It is unique to green sulfur bacteria and is not even present in *Chloroflexus aurantiacus* [3]. *fmoA* codes for the monomer of the FMO protein, which binds Bchl *a* and is associated in a trimeric structure [9]. Its unique occurrence in green sulfur bacteria makes *fmoA* an appropriate target for specifically analyzing environmental communities of these bacteria.

Phylogenetic relationships of green sulfur bacteria have been investigated based on pure-culture studies that examined 16S rRNA [1,10,22,26] and *fmo*A [1] genes. These studies of pure culture isolates demonstrated different phylogenetic lines for strains derived from marine and saline habitats and from freshwater habitats. However, evidence from environmental studies for this distinction is so far lacking. Since cultivation approaches generally are believed to detect only a small fraction of the natural population, it would be interesting to instead prove this relationship by genetic analyses with environmental samples. In this study, several marine and one hypersaline habitat were selected and the compositions of communities of green sulfur bacteria were analyzed using specific primers for the 16S rRNA and FMO genes [1].

Materials and methods

Environmental samples. DNA was obtained from environmental samples derived from different geographic locations on two continents and from different habitats, including brackish and true marine coastal habitats as well as saline inland waters. These habitats were represented by sediments from the German Baltic Sea shore near Laboe, Stakendorf, and Stein (samples Laboe-3, Stak-2 and Stein-1), by sediment and water samples from the saline lake Malo Jezero, which is connected to the Adriatic Sea (Island of Mljet, Croatia [7]; sample Cviic-2), by sediments from Great Sippewissett Salt Marsh (MA, USA; samples Sip-4 and SSM-3), and from the salt pool Bad Water (Death Valley, CA, USA; sample BW-4).

The sediments from the Baltic Sea (salinity ca. 1.9 %) showed a pink layer within the top 2 mm, but there were no signs indicating the development of green sulfur bacteria. From previous studies in which agar dilution series with Pfennig's medium were used to count viable numbers of green and purple sulfur bacteria (JFI, unpublished), it was known that green sulfur bacteria were present in these sediments, in numbers about three orders of magnitude below those of purple sulfur bacteria, i.e., at 10^3–10^4 cells/cm^3.

Well-established layered communities of phototrophic bacteria have been described in sediments from Sippewissett Salt Marsh [21]. However, at the time of sampling for this study, a distinct green layer below that of purple bacteria was not observed. Samples were taken from the top 5 mm of the sediment, including the thin pink layer of purple sulfur bacteria. The salinity approximated that of sea water, about 3.5%.

The sample of Malo Jezero originated from a Winogradsky column set up by Vlaho Cvii? in 1951 with mud and water from this lake; the column was reestablished in 1973 by JFI [7,13,14] and has been maintained since then in the author's laboratory. At the time of sampling, the red water in the column had a salinity of ca. 3.8% [7]. Although these conditions are considered to be quite close to those of the natural situation, the relative proportions of individual strains of the community may have changed over time. Nonetheless, the green sulfur bacteria identified from the column play a significant role in Malo Jezero under appropriate environmental conditions. The sample from Bad Water (a hypersaline pool at the depth of Death Valley) was taken from the top of the highly reduced and actively sulfate-reducing sediment.

Extraction of genomic DNA and PCR amplification. DNA was extracted by using the CTAB method [35]: 1 g sediment was mixed with 2.7 ml DNA extraction buffer [100 mM Tris/HCl, pH 8.0; 100 mM EDTA, pH 8.0; 1500 mM NaCl; 1% *N*-cetyl-*N*,*N*,*N*-trimethylammoniumbromide (CTAB)] and 20 µl proteinase K solution (10 mg/ml) and incubated for 30 min at 37ºC. Three hundred µl of 20% SDS solution was added; the sample was mixed and then incubated for 2 h at 65ºC with additional mixing every 15–20 min. After centrifugation (10 min, 6000 ×*g*, 20ºC), the pellet was washed twice with 0.9 ml DNA extraction buffer and 0.1 ml SDS solution, mixed thoroughly for 10 s, and centrifuged. The pellet was discarded, and the three solutions were combined and mixed with the same volume of chloroform/isoamylalcohol (24:1). After a brief centrifugation, the aqueous phase was separated from the organic one, mixed with 0.6 vol% isopropanol, and centrifuged (20 min, 16,000 ×*g*, 20ºC). The supernatant was discarded; the DNA pellet was washed with 500 µl ice-cold ethanol (70%), and centrifuged again (10 min, 16,000 ×*g*, 20ºC). The DNA contained in the pellet was solved in 1 ml water and then purified with the QIAquick PCR Purification Kit (QIAgen, Hilden, Germany) according to the manufacturer's instructions.

PCR amplifications. Reactions were carried out in a total volume of 25 µl using ReadyToGo PCR beads (Amersham-Pharmacia, Freiburg), 10 pmol of each primer (10 pmol per ml), and DNA (10–100 ng). To decrease primer specificity, the annealing temperatures for PCR amplification of fragments from environmental samples were lower than those used for pure-culture studies. Optimum reproduction was achieved by individually adapting the PCR conditions to each environmental sample.

Specific primers, well-established for all pure cultures of green sulfur bacteria (F-Start-fmo and R-889-fmo [1]), were used for amplification of *fmoA* gene sequences. After an initial denaturation step of 2 min at 94ºC, PCR was carried out in a Progene-Cycler (Thermo-Dux, Wertheim, Germany). The amplification of *fmoA* from environmental samples consisted of the following cycles: 30 s denaturation at 94ºC, 30 s elongation at 72ºC, and 40 s annealing. The number of cycles and the annealing temperature varied depending on the sample (25–35 cycles, 40–45ºC). The final cycle consisted of 1 min at 42ºC and 5 min at 72ºC. A combination of primer F-99-GSB, specifically designed for green sulfur bacteria [1], and the eubacterial R-1388 primer (see below) were used to amplify 16S rRNA gene sequences. PCR cycles were as described above but consisted of 25–30 cycles and an annealing temperature varying from 48–60ºC (depending on the sample). The amplification products were checked on agarose gels (1% wt/vol, Biozym, Hess; Oldendorf, Germany) stained with ethidium bromide (0.5 mg/l). Both 16S rDNA and *fmoA* fragments could be amplified from the majority of the samples. Due to unknown biases with the mixed populations of the environmental samples, in those from Bad Water and Stein only 16S rDNA could be amplified and in those from Stakendorf only *fmoA*.

Cloning. After amplification of DNA from environmental samples, the different DNA molecules were separated by cloning using the Topo TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, USA) according to the

manufacturer's manual. This procedure consists of ligation of the DNA into the vector pCR4-TOPO (included in the kit) and transformation in competent cells of *Escherichia coli* TOP10 One Shot (included in the kit). From each environmental sample, 100 clones were screened for the presence of the correct insert. Vectors were screened for environmental DNA of the suspected length by PCR and using the primer pair M13F/M13R (see below) with binding sites in the vector pCR4-TOPO surrounding the insert. PCR amplification started with a denaturation step of 2 min at 94ºC using a ProgeneCycler (Thermo-Dux, Wertheim, Germany) followed by 25 cycles under constant conditions (30 s denaturation at 94ºC, 45 s elongation at 72ºC, and 40 s annealing at 55ºC). The final cycle consisted of 1 min at 42ºC and 5 min at 72ºC.

Sequencing. 16S rDNA and *fmoA* amplificates were purified with spin columns from the QIAquick PCR Purification Kit (QIAgen, Hilden, Germany) according to the manufacturer's manual. Sequences were obtained by cycle sequencing with the ABI Prism Sequencing Kit (Perkin-Elmer, Weiterstadt, Germany) and the chain-termination reaction [28] using a capillary sequencer (Perkin-Elmer, Weiterstadt, Germany). Cycle sequencing reactions were carried out at an annealing temperature of 50ºC and according to the manufacturer's manual. For sequencing, the F-99-GSB primer specific for green sulfur bacteria [1] and the eubacterial R-519 5′-ATTACCGCGGCTGCTGG-3′ and F-519 5′-CCAGCAGCCGCGGTAAT-3′ [19] as well as the eubacterial R-908 5′-GTCAATTCCTTTGAGTTT-3′, F-908 5′-AAACTCAAAGGAATTGAC-3′ and R-1388 5′-CGGGCGGTGTGTACAAGG-3′ (constructed using the ARB program by J. Süling, IFM-GEOMAR Kiel) were used. Sequences of *fmoA* were analyzed using the specifically designed primers F-Start-fmo and R-889-fmo [1]. For sequencing of the cloned environmental DNA, the primers M13F 5′-GTAAAACGACGGCCAG-3′ and M13R 5′-CAGGAAACAGCTATGAC-3′ were used. Putatively positive PCR products were partially sequenced (more than 400 nucleotides of 16S rDNA with primer R-519 in direction of the primer binding site F-99-GSB, including the V3 zone; more than 450 nucleotides of *fmoA* with primer M13F in direction of the insert). All sequences were compared to those in the EMBL prokaryote library using the Fasta3 search program [23, 24]; those which could be identified as parts of the *fmoA* gene or the 16S rDNA of green sulfur bacteria were aligned and manually checked for base pair differences. Clone sequences with less than 1% base pair difference within each sample were considered to belong to a so-called microheterogeneity group (MHG) with variations most likely due to natural variation among closely related strains. One representative sequence of each MHG was selected and completely sequenced (ca. 1240 nt total length for 16S rDNA and 875 nt length for *fmoA* fragments). These complete sequences were subsequently aligned together with sequences from pure cultures, and phylogenetic trees were constructed for 16S rDNA and *fmoA* sequences (Figs. 1, 2).

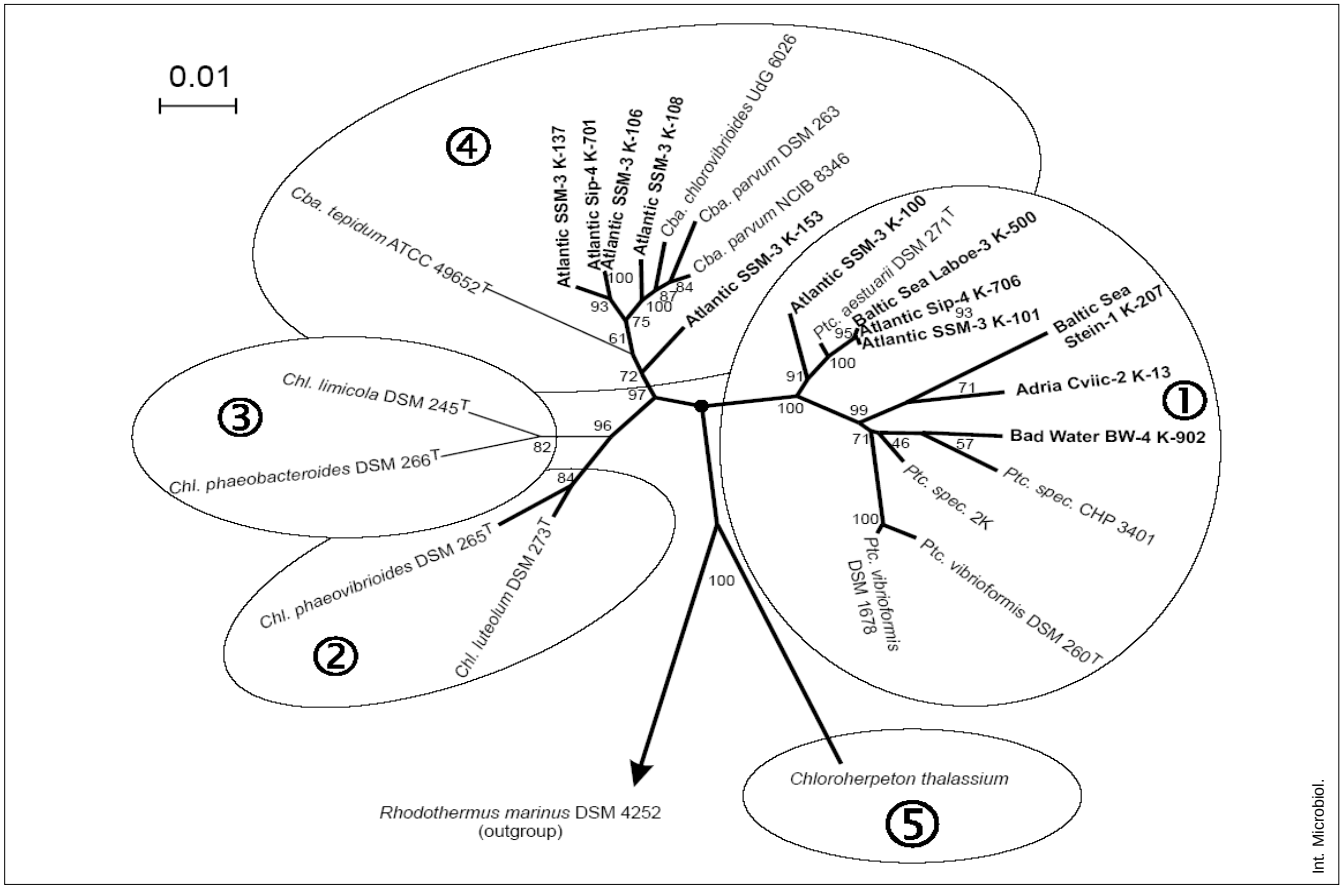

Fig. 1. Phylogenetic tree based on 16S rDNA sequences. The tree topology was based on ca. 1240 nt. Sequences derived from this work (Table 1) were obtained with the primer pairs F-99-GSB/R-1369 and F-99-GSB/R-1388. Tree and branch lengths were calculated by distance methods using DNADIST and FITCH from the PHYLIP program package. Bootstrap values are proportional. Bold lines connect sequences from salt-dependent strains or sequences obtained from saline habitats. Names from environmental sequences are in bold letters. Numbers 1 to 4 mark the groups of green sulfur bacteria according to Alexander et al. [1].

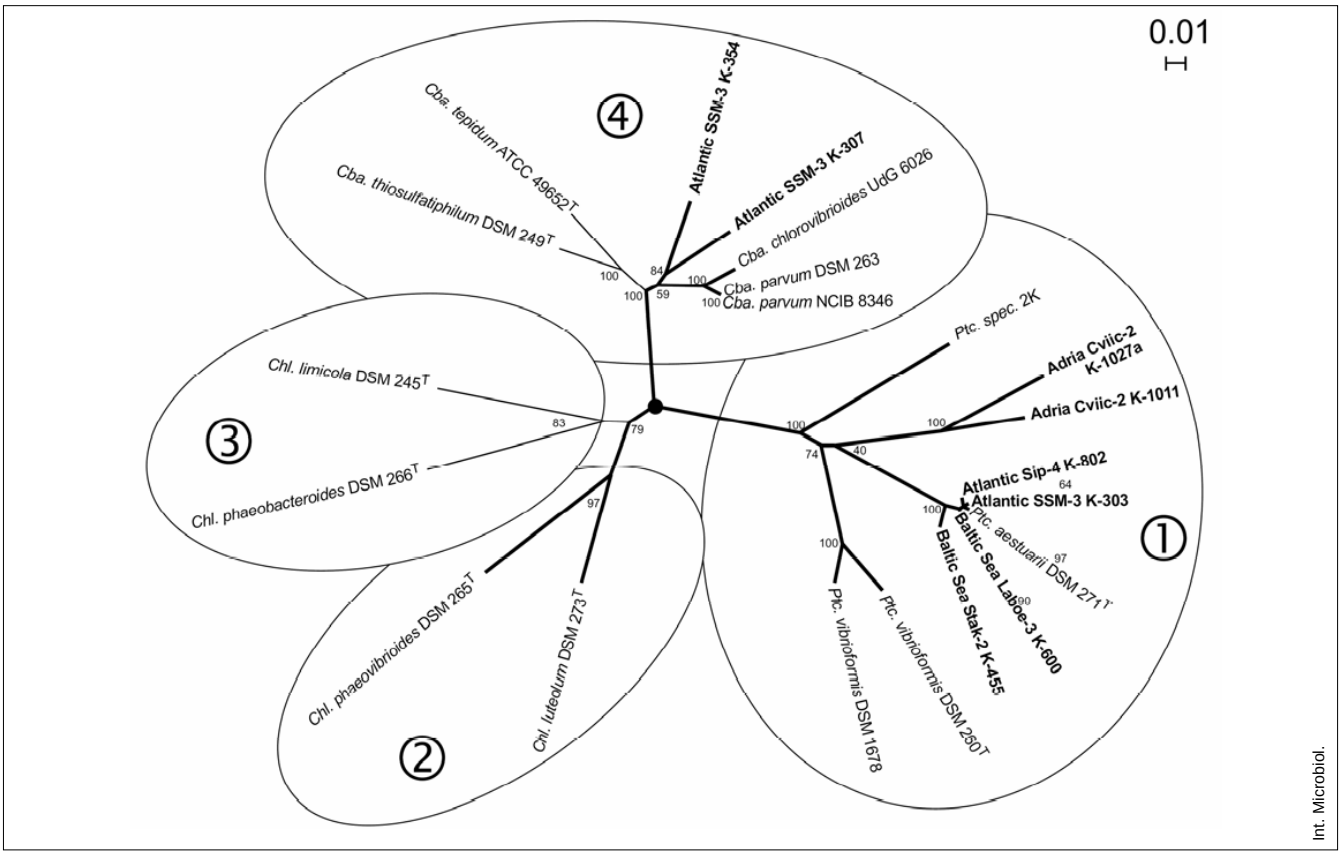

Fig. 2. Phylogenetic tree of the Fenna-Matthews-Olson *fmoA* gene. The tree topology was based on sequences of ca. 875 nt. Sequences derived from this work (Table 2) were obtained with the primer pair F-Start-fmo/R-889-fmo. Tree and branch lengths were calculated by distance methods using DNADIST and FITCH from the PHYLIP program package. Bootstrap values are proportional. Bold lines connect sequences from salt-dependent strains or sequences obtained from saline habitats. Names from environmental sequences are in bold letters.

Phylogeny of 16S rDNA and *fmoA*. For phylogenetic evaluation, 16S rDNA and *fmoA* nucleotide sequences were aligned using the ClustalW program package [32] with subsequent manual editing. Nucleotide distances were calculated from the data set according to the algorithm of Jukes and Cantor [16] by using DNADIST from the PHYLIP program package [8]. Phylogenetic trees were inferred from the distance data with global rearrangements using FITCH from the same program package. Bootstrap analysis of nucleotide sequences (100 resamplings) were done with SEQBOOT from the PHYLIP program package. CONSENSE was used to generate consensus trees. All environmental sequences were compared to a backbone of sequences from established pure cultures of green sulfur bacteria (Table 1).

Results

The clone sequences of green sulfur bacteria from brackish, marine, and hypersaline habitats (more than 370 16S rDNA sequences and more than 130 *fmoA* sequences) all belonged to the branches defined as marine and salt-dependent green sulfur bacteria, according to pure culture studies by Alexander et al. [1]. The majority of sequences from all habitats belonged to group 1 (according to [1]), which represents the genus *Prosthecochloris*. Sequences belonging to *Chlorobium* (groups 2 and 3 according to [1]) were not retrieved and only a few clone sequences were identified as belonging to the salt-dependent branch of group 4 (the genus *Chlorobaculum*) of green sulfur bacteria [1]. Results were verified for the 16S rDNA and *fmoA* sequences (Table 2, Figs. 1, 2).

Genetic variation of environmental 16S rDNA sequences. For comparison of the clone sequences, all sequences from the same habitat were arranged in MHGs, in which sequences with > 99% sequence similarity were combined (see Materials and methods). For the purpose of convenience, representative sequences from each MHG were considered (Table 2). In all samples, a restricted genetic variation of sequences and the clear dominance or exclusive presence of a single MHG was observed. As expected according to our postulation of strict separation between phylogenetic lines of green sulfur bacteria from salty environments and those adapted to freshwater environments, all sequences clustered with branches established with salt-

Table 1. Pure cultures of green sulfur bacteria included in this study

Species	Strain	EMBL accession number 16S rDNA	EMBL accession number *fmo*A	Salt required[c]	Group of GSB
Ptc. vibrioformis	DSM 1678	[b]AJ290833	[b]AJ391163	+	1
Ptc. vibrioformis	DSM 260[T]	[a]M62791	[b]AJ391145	+	1
Ptc. aestuarii	DSM 271[T]	[a]Y07837	[b]AJ391151	+	1
Ptc. spec.	CHP 3401	[a]AJ291826		+	1
Ptc. spec.	2K	[b]AJ290835	[b]AJ290823	+	1
Chl. phaeovibrioides	DSM 265[T]	[b]AJ290829	[b]AJ391160	+	2
Chl. luteolum	DSM 273[T]	[a]Y08107	[b]AJ391152	+	2
Chl. limicola	DSM 245[T]	[a]Y10113	[b]AJ391153	–	3
Chl. phaeobacteroides	DSM 266[T]	[a]Y08104	[b]AJ391148	–	3
Cba. chlorovibrioides	UdG 6026	[a]Y10649		+	4
Cba. parvum	DSM 263	[a]Y10647	[b]AJ391147	+	4
Cba. parvum	NCIB 8346	[b]AJ290830	[b]AJ391161	+	4
Cba. thiosulfatiphilum	DSM 249[T]	[a]Y08102	[b]AJ391143	–	4
Cba. tepidum	ATCC 49652[T]	[a]M58468	[a]L13700	–	4
Chp. thalassium		[a]AF170103		+	5

[a]Sequences were obtained from the EMBL database. [b]Alexander et al. [1]. [c] + Minimum of 1% NaCl is required for growth; – no or low salt composition (<1% NaCl) is required.

dependent pure cultures [1]. The dominant sequences were always from green sulfur bacteria of the *Prosthecochloris* cluster (group 1 according to [1]), which represents bacteria from marine and hypersaline environments, with *Prosthecochloris aestuarii* as a well-known representative (Tables 1, 2).

The greatest diversity of green sulfur bacteria was found in samples from the Sippewissett Salt Marsh. The majority of the sequences recovered from the Sippewissett sediments (Sip-4 and SSM-3) belonged to phylogenetic group 1 of green sulfur bacteria (97% of sequences from clone Sip-4 K-706, 66% of those from SSM-3 K-101, and 1% of those from SSM-3 K-100). All three sequences, i.e., MHGs, were highly similar to that of the type strain of *Prosthecochloris aestuarii* DSM 271[T], with sequence similarities of 99.4% for Sip-

Table 2. Type and abundance of microheterogeneity groups (MHGs) obtained from environmental sequences

Sample[1]	Type sequence of MHG	EMBL accession number 16S rDNA	EMBL accession number *fmo*A	Abundance of MHG sequence[2]	Group of GSB
Adria Cviic-2	K-13	AJ428420		100 (34)	1
Adria Cviic-2	K-1011		AJ428448	95 (20)	1
Adria Cviic-2	K-1027a		AJ428449	5 (20)	1
Atlantic Sip-4	K-706	AJ428431		97 (87)	1
Atlantic Sip-4	K-701	AJ428430		3 (87)	4
Atlantic Sip-4	K-802		AJ428446	100 (29)	1
Atlantic SSM-3	K-100	AJ428422		1 (92)	1
Atlantic SSM-3	K-101	AJ428423		66 (92)	1
Atlantic SSM-3	K-106	AJ428424		17 (92)	4
Atlantic SSM-3	K-108	AJ428425		12 (92)	4
Atlantic SSM-3	K-137	AJ428426		2 (92)	4
Atlantic SSM-3	K-153	AJ428427		1 (92)	4
Atlantic SSM-3	K-303		AJ428435	88 (17)	1
Atlantic SSM-3	K-307		AJ428436	6 (17)	4
Atlantic SSM-3	K-354		AJ428439	6 (17)	4
Bad Water BW-4	K-902	AJ428433		100 (77)	1
Baltic Sea Laboe-3	K-500	AJ428429		100 (84)	1
Baltic Sea Laboe-3	K-600		AJ428442	100 (67)	1
Baltic Sea Stak-2	K-455		AJ428441	100 (4)	1
Baltic Sea Stein-1	K-207	AJ428428		100 (3)	1

[1]All environmental sequences were obtained during this study and have been deposited with the EMBL database. [2]The numbers give the relative abundance of the MHG as percent of the total sequences per sample and gene (total number of clone sequences analyzed per sample and gene are given in parentheses). Microheterogeneity groups represent sequences from one habitat with more than 99% sequence identity.

4 K-706 and SSM-3 K-101 and 98.2% for SSM-3 K-100. In addition, in both sediment samples, sequences from the salt-dependent branch of group 4 of the green sulfur bacteria (genus *Chlorobaculum*, [1,15]) were recovered in small numbers. These sequences revealed *Chlorobaculum parvum* as a close phylogenetic relative available in pure culture (Fig. 1). The similarity of all clone sequences of group 4 to the sequence of *Chlorobaculum parvum* NCIB 8346 ranged from 96.2% (clone SSM-3K-153) to 98.8% (SSM-3K-106). None of the environmental sequences clustered with freshwater lineages of green sulfur bacteria, including the freshwater cluster of group 4 with *Chlorobaculum tepidum* ATCC 49652^T as representative and groups 2 and 3 (*Chlorobium limicola* and relatives).

All sequences retrieved from habitats of the Baltic Sea (Laboe-3 K-500 and Stein-1 K-207), Malo Jezero (Cviic-2 K-13), and Bad Water (BW-4 K-902) belonged to group 1 of green sulfur bacteria. From these, only Laboe-3 K-500 was highly similar to *Prosthecochloris aestuarii* DSM 271^T and fitted the cluster of sequences from Sippewissett Salt Marsh. Sequences from Stein and Malo Jezero formed a separate new cluster within group 1 of green sulfur bacteria, with similarities of 96–97% to other sequences of this group. They may represent a so far uncultured new species. The sequence derived from Bad Water (BW-4 K-902), which was the only saline, non-marine habitat of this study, had a similarity of 97.8% to *Prosthecochloris* sp. CHP 3401. This clone sequence and the culture of CHP 3401 [34] were obtained from hypersaline inland lakes. They formed a third cluster within the *Prosthecochloris* group of green sulfur bacteria that may represent another new species of this genus.

Genetic variation of environmental *fmoA* sequences. The results from *fmoA* sequences were congruent with those from the 16S rDNA sequences. Sequences belonging to groups 1 and 4 of green sulfur bacteria were retrieved (Fig. 2). As was the case for their 16S rDNA counterparts, the dominant *fmoA* sequences from Sippewissett Salt Marsh (Sip-4 K-802, SSM-3 K-303) fitted very well into the cluster of the *fmoA* sequence of the type strain of *Prosthecochloris aestuarii* DSM 271^T (>99% sequence similarity). This also held true for the *fmoA* sequences from the Baltic Sea at Laboe (Laboe-3 K-600) and Stakendorf (Stak-2 K-455).

Two *fmoA* sequences (98.7% similar to each other) were retrieved from Malo Jezero (relative abundance: 95% Cviic-2 K-1011 and 5% Cviic-2 K-1027a). As was found for the single 16S rDNA sequence from this sample, both *fmoA* sequences were distant to other *fmoA* sequences from pure cultures and environmental samples. The sequence similarity to *Prosthecochloris aestuarii* DSM 271^T was as low as 93.2%. Two *fmoA* sequences belonging to group 4 of green sulfur bacteria (*Chlorobaculum parvum* and relatives) were retrieved from sediments of Sippewissett Salt Marsh (SSM-3 K-354 and SSM-3 K-307) and both represented minor numbers of clones from this sample. They were 98.4% similar to each other and less than 97% similar to other members of this group (e.g., *Chlorobaculum parvum*).

Discussion

In order to test the hypothesis that special marine phylogenetic lines of green sulfur bacteria exist and develop in marine and saline habitats, samples were selected from a few representative habitats and clone libraries of selectively amplified genes of green sulfur bacteria were analyzed. In anticipation of possible experimental biases of the PCR reactions with environmental samples, two independent primer pairs well-established in pure culture studies with green sulfur bacteria were used, i.e., those for the 16S rRNA and *fmoA* genes [1]. Detailed pure culture studies demonstrated a largely congruent phylogeny of 16S rRNA and *fmoA* genes of green sulfur bacteria and supported a clear separation into different phylogenetic groups [1]. Although 16S rDNA is universal, it allows the use of group-specific PCR primers for green sulfur bacteria. The *fmoA* gene is unique to green sulfur bacteria and is thus an appropriate tool to specifically investigate this group by using primers with specificity for this gene [1].

The results were quite remarkable. First, only sequences that fit into the marine phylogenetic lines established by pure culture studies were obtained. Thus, the origin and salt relationships of pure cultures apparently correctly reflect the situation in the environment. Second, it was verified that true marine and saline phylogenetic lines of green sulfur bacteria exist which are specifically adapted to these kinds of habitats. Among more than 500 sequences, not a single one related to freshwater forms of green sulfur bacteria was found.

Evidence for new green sulfur bacteria at the genus level was not obtained. Despite the fact that low-stringency conditions were used for PCR amplification and that high numbers of clone sequences (up to 92 clone sequences for 16S rDNA from sample SSM-3 and up to 67 *fmoA* sequences from sample Laboe-3) were obtained, all sequences fit into currently recognized genera of green sulfur bacteria [15]. However, evidence from sequence data demonstrated the presence and dominance of at least two new species of the major phylogenetic branch of marine green sulfur bacteria, both from 16S rDNA (Stein-1 K-207, Cviic-2 K-13 and BW-4 K-902) and from *fmoA* (Cviic-2 K-1027a and Cviic-2 K1011) sequences

in the habitats of the Baltic Sea at Stein, the marine lake Malo Jezero at Mljet (Croatia), and Bad Water (Death Valley).

The clear dominance of representatives of the true marine group 1 of green sulfur bacteria (the genus *Prosthecochloris* [1,15]) was indicated in all investigated habitats and with both genes. This is agreement with data from culture studies, which yielded *Prosthecochloris aestuarii* in enrichments and isolation attempts from many marine habitats [5,12–15,18, 19,25]. It also supports the postulation of separate phylogenetic lines of freshwater and marine green sulfur bacteria and distinct populations in the two types of habitats [1,14]. However, the phylogenetic diversity of marine green sulfur bacteria belonging to *Prosthecochloris* apparently is much higher than known from pure culture studies. Available sequence information allows at least four different clusters within this group to be recognized. Representatives of three of these were found to be dominant in the different habitats investigated during this study. The fourth one, with strains of *P. vibrioformis*, was not found during this study but is known from pure cultures.

The first cluster, including *Prosthecochloris aestuarii* DSM 271^T, was represented by the great majority of the environmental sequences. It was dominant in clone libraries of the marine sediments from Sippewissett Salt Marsh and the Baltic Sea at Laboe and Stakendorf. Interestingly, the dominant sequence and a second minor one in sample SSM-3 (according to 16S rDNA sequences) were both located in this cluster. Also, in the sample from Malo Jezero, both the dominant sequence and a second minor component (according to *fmoA* clone sequences) were representatives of this cluster.

The second cluster of sequences was represented by *Prosthecochloris* sp. CHP 3401 (possibly also *Prosthecochloris* sp. strain 2K) and the Bad Water sequences (Fig. 1). *Prosthecochloris aestuarii* CHP 3401 was isolated from a continental salt lake, Salada de Chiprana, Spain [34]. This bacterium is clearly distinct from the type strain of *Prosthecochloris aestuarii* (96.6% sequence similarity of 16S rDNA). The close relationship of the dominant 16S rDNA sequence from Bad Water (BW-4 K-902) to strain CHP 3401 (97.8 similarity) suggests that this cluster represents green sulfur bacteria from inland salt-water lakes and that these bacteria apparently separated during evolution from those green sulfur bacteria living in marine habitats.

The third cluster comprises 16S rDNA sequences from the Baltic Sea at Stein and from Malo Jezero (Stein-1 K-207 and Cviic-2 K-13); these had a sequence similarity of 97.0% to each other (Fig. 1). In the *fmoA* tree (Fig. 2), the sequences of Cviic-2 K-1027a and K-1011 had a topological corresponding position. The corresponding bacterial strains form a new, so far not known cluster within group 1 of green sulfur bacteria and possible represent a new species.

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Comunidades de bacterias verdes del azufre en hábitats marinos o salinos analizados mediante las secuencias de genes rRNA 16S y de la proteína Fenna-Matthews-Olson

Resumen. Se han estudiado las comunidades de bacterias verdes del azufre en hábitats marinos o salinos seleccionadas a partir de las secuencias de genes del rRNA 16S y de la proteína Fenna-Matthews-Olson (FMO). La disponibilidad de cebadores específicos de grupo para el rDNA 16S y para el gen *fmoA*, que es exclusivo de las bacterias verdes del azufre, ha permitido por primera vez analizar las comunidades naturales de estas bacterias por métodos que no requieren cultivo usando dos marcadores genéticos independientes. Los resultados de la secuencia obtenidos con los genes *fmoA* y con rDNA 16S concordaban entre sí. Todas las secuencias de rDNA 16S y *fmoA* procedentes de hábitats del mar Báltico, del Mediterráneo, de las salinas de Sippewissett (Massachusetts, EE UU) y de Bad Water (Death Valley, California, EE UU) se encuentran en las líneas filogenéticas dependientes de sal de las bacterias verdes del azufre establecidas mediante estudios de cultivo puro. Esta constatación respalda la existencia de linajes filogenéticos de bacterias verdes del azufre adaptadas específicamente a medios marinos o salinos y a su distribución exclusiva en tales ambientes. La gran mayoría de secuencias clónicas pertenece a agrupaciones genéticas (*clusters*) del género *Prosthecochloris* y probablemente representan especies diferentes. Se han conseguido pruebas de la presencia de dos nuevas especies de *Prosthecochloris*. En diversos hábitats dominaban representantes del grupo *Prosthecochloris* y varias agrupaciones genéticas o especies de ese género eran exclusivos de esos hábitats o bien eran las bacterias verdes del azufre claramente dominantes en ellos. [**Int Microbiol** 2006; 9(4):259-266]

Palabras clave: *Prosthecochloris* · Chlorobiaceae · diversidad ambiental · proteína Fenna-Matthews-Olson

Comunidades de bactérias verdes do enxofre em habitats marítimos ou salinos analisados mediante as seqüências de genes rRNA 16S e da proteína Fenna-Matthews-Olson

Resumo. Foram estudadas as comunidades de bactérias verdes do enxofre em habitats marítimos ou salinos selecionadas a partir das seqüências de genes do rRNA 16S e da proteína Fenna-Matthews-Olson (FMO). A disponibilidade de iniciadores específicos de grupo para o rDNA 16S e para o gene *fmoA*, que é exclusivo das bactérias verdes do enxofre, permitiu pela primeira vez analisar as comunidades naturais destas bactérias por métodos que não requerem cultivo usando dois marcadores genéticos independentes. Os resultados das seqüências obtidos com os genes *fmoA* e com rDNA 16S concordavam entre si. Todas as seqüências de rDNA 16S e *fmoA* procedentes de habitats do mar Báltico, do Mediterrâneo, das salinas de Sippewissett (Massachusetts, EUA) e de Bad Banheiro (Death Valley, a Califórnia, EUA) se encontram nas linhas filogenéticas dependentes de sal das bactérias verdes do enxofre estabelecidas mediante estudos de cultivo puro. Esta constatação respalda a existência de linhagens filogenéticos de bactérias verdes do enxofre adaptadas especificamente a meios marítimos ou salinos e a sua distribuição exclusiva em tais ambientes. A grande maioria de seqüências clônicas pertencem a agrupamentos genéticos (*clusters*) do gênero *Prosthecochloris* que provavelmente representam espécies diferentes. Foram alcançadas provas da presença de duas novas espécies de *Prosthecochloris*. Em diversos habitats dominavam representantes do grupo *Prosthecochloris* e vários agrupamentos genéticos ou espécies desse gênero eram exclusivos desses habitats ou eram as bactérias verdes do enxofre claramente dominantes neles. [**Int Microbiol** 2006; 9(4):259-266]

Palavras chave: *Prosthecochloris* · Chlorobiaceae · diversidade ambiental · proteina Fenna-Matthews-Olson