Ecological diversity and low light adaptation of green sulfur bacteria

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"…we live in the Age of Bacteria as it was in the beginning, is now and ever shall be, until the world ends".

Stephen J. Gould, palaeontologist, in: *The Full House: The spread of Excellence from Plato to Darwin* (NewYork, Three Rivers Press, 1996).

see also: http://brembs.net/gould.html

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ACCEPTED MANUSCRIPTS

CHAPTER 3: Jörg Overmann and **Ann K. Manske**. **2006.** Anoxygenic phototrophic bacteria in the Black Sea chemocline. In: L.N. Neretin (ed.) Past and Present Water Column Anoxia. 523-541. (NATO Science Series)

CHAPTER 4: **Ann K. Manske**, Jens Glaeser1, Marcel M. M. Kuypers and Jörg Overmann. **2005.** Physiology and phylogeny of green sulfur bacteria forming a monospecific phototrophic assemblage at 100 m depth in the Black Sea. Appl. Environ. Microbiol. **71**:8049-8060.

CHAPTER 5: J. Thomas Beatty, Jörg Overmann, Michael T. Lince, **Ann K. Manske**, Andrew S. Lang, Robert E. Blankenship, Cindy L. Van Dover, Tracey A. Martinson, and F. Gerald Plumley. **2005.** An obligately photosynthetic bacterial anaerobe from a deep-sea hydrothermal vent. PNAS **102**:9306-9310.

UNPUBLISHED MANUSCRIPTS

Chapter 6: **Ann K. Manske**, Uta Henßge, Jens Glaeser and Jörg Overmann. Subfossil DNA sequences of green sulfur bacteria as indicators for past water column anoxia in the Black Sea. In preparation

Chapter 7: **Ann K. Manske**, Hanno Teeling, L. Jesús Garcia-Gil and Jörg Overmann. Biodiversity and Phylogeny of the family *Chlorobiaceae* based on comparison of multiple genetic regions. In preparation

Statement regarding the contribution of Ann Manske to the publications listed in this dissertation

Chapter 3: Ann Manske contributed sections 7., parts of sections 2., 4. and 5., and the three figures.

Chapter 4: Ann Manske maintained the cultures of green sulfur bacteria, performed the analysis of photosynthetic pigments, the extraction of DNA, PCR, denaturing gradient gel electrophoresis, sequencing and phylogenetic analyses, and the experiments testing the light dependence of photosynthesis. She wrote the first draft of the manuscript and created Figures 2-6.

Chapter 5: Ann Manske carried out the enrichment, isolation and maintenance of strain GSB1, the subsequent growth experiments, PCR amplifications and sequence analyses of 16S rRNA genes and the survival experiments. She created Fig. 4 and contributed some paragraphs of the text.

Chapter 6: Ann Manske performed the DNA extractions of the sediments, PCR, denaturing gradient gel electrophoresis, part of the cloning and sequencing, constructed phylogenetic trees and enriched green sulfur bacteria. She created all figures and wrote the first draft of the manuscript.

Chapter 7: Ann Manske carried out the amplification of 16S rRNA genes, cloning and PCR of the ITS region, the primer design and PCR for group 1 σ^{70}-type sigma factors, analysis of bchG gene sequences, sequencing, phylogenetic analysis, part of the rarefaction analysis. She prepared all figures and wrote the first draft of the manuscript.

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ABSTRACT

This study focuses on one brown-colored representative of the green sulfur bacteria, *Chlorobium* sp. BS-1, that survives by means of anoxygenic photosynthesis even at very low light intensities. This unusual representative of the green sulfur bacteria lives in the chemocline of the Black Sea, which is located at 80 to 120 m depth, offering only 0.0005 % (winter) to 0.002 % (summer) of surface irradiance (0.00075 to 0.0022 µmol Quanta m^{-2} s^{-1}). The Black Sea represents the world's biggest anoxic water body, and it is permanently stratified. An overview of the habitat Black Sea and the research on phyotosynthetic bacteria in the Black Sea chemocline gives the article *Anoxygenic phototrophic bacteria in the Black Sea chemocline* (pages 53 ff.)

In the second article, *Physiology and phylogeny of green sulfur bacteria forming a monospecific phototrophic assemblage at 100 m depth in the Black Sea* (pages 75 ff.), it is shown that *Chl.* sp. BS-1 represents a novel phylotype in the marine cluster of green sulfur bacteria and is the only detectable phylotype of green sulfur bacteria in the Black Sea chemocline. It was shown that *Chl.* sp. BS-1 is the first organism known to date which fixes $^{14}CO_2$ under laboratory conditions even at light intensities as low as 0.015 µmol Quanta m^{-2} s^{-1} which is much lower than the light intensity in any typical habitat of green sulfur bacteria. Therefore it is the best adapted species to extremely low light intensities documented. The major adaptive mechanism to extremely low light intensities might be a significant change in the secondary homologs of the main photosynthetic pigment, bacteriochlorophyll *e* (BChl *e*). The dominance of farnesyl esters and the presence of four unusual geranyl ester homologs of BChl *e* were revealed by HPLC analysis in cells shifted from 3 to 0.1 µmol Quanta m^{-2} s^{-1}. Together with *in situ* light measurements and in situ BChl *e* concentrations, the growth experiments allowed for the calculation of doubling times and significance of the photosynthetic activity for the carbon and sulfur cycle in the Black Sea chemocline. With doubling times of a minimum of 3.1 years (for summer light intensity) and the contribution of only 0.002 - 0.01 % to total sulfide oxidation in the chemocline *Chl.* sp. BS-1 represents the slowest growing population of green sulfur bacteria known to date and does not contribute significantly to the carbon or sulfur cycle.

The article *Subfossil DNA sequences of green sulfur bacteria as indicators for past water column anoxia in the Black Sea* (page 119 ff.) gives insight into the history of the strain *Chl.* sp. BS-1 and the green sulfur bacteria in the Black Sea during the last few thousand years. The Black Sea today is considered as closest contemporary analogue to past sulfidic oceans and its biogeography over several thousand years is well documented in its stratified sedimentary record. Since the 16S rRNA gene sequence of *Chl.* sp. BS-1 might be a useful indicator for past photic zone anoxia, the

presence of its fingerprints in past periods of the Black Sea was investigated. 16S rRNA gene sequences of green sulfur bacteria from samples of Black Sea sediments up to 7 m below seafloor were amplified and sequenced. Nine green sulfur bacterial 16S rRNA gene sequences were identified. Surprisingly, not only green sulfur bacterial fingerprints were found but also closely related species clustering at the basis of the green sulfur bacterial subtree, together with not yet cultured species detected all over the world. The new cluster was called "deep-branching green sulfur bacteria" though it was not possible to enrich live organisms with medium for photosynthetic green sulfur bacteria. The chemocline strain *Chl.* sp. BS-1, found in Units III, II and I (>9000 years b.p. until today), and two other sequences, found in sediments of Unit IIb (between 8200 yr. b.p. and 5000 yr.b.p) only, were the only two sequences clustering with the marine green sulfur bacteria. The other green sulfur bacterial sequences clustered with freshwater or low-salt adapted species, indicating an allochthonous introduction of these sequences to the Black Sea sediments. A long-distance transport of green sulfur bacteria even through oxygenated water is possible since the group has protective mechanisms agains oxygen intoxication.

In the article *An obligately photosynthetic bacterial anaerobe from a deep-sea hydrothermal vent* (pages 105 ff.) a strain is presented which was enriched from beneath a deep sea hydrothermal vent, *Chlorobium bathyomarinum* GSB1. It was shown to resist more than 16 days of oxygenated medium. Since this strain was isolated only from the vicinity of a vent and was shown to depend on photosynthesis, it might survive photosynthetically by exclusively using geothermal radiation. It might be the first green sulfur bacterium which is adapted to light intensities even lower than in the Black Sea chemocline.

A lot of newly obtained sequences during this study evoked the necessity of a phylogenetic analysis. The article *Biodiversity and Phylogeny of the family* Chlorobiaceae *based on comparison of multiple genetic regions* (pages 145 ff.) discusses the phylogenetic system of the green sulfur bacteria, which is to date not satisfactory with respect to resolution of the group. A new system is presented which is based on green sulfur bacterial sequences of isolated strains and environmental sequences from the NCBI database (www.ncbi.nlm.nih.gov). The 16S rRNA gene phylogenetic tree revealed the need for a revised phylogenetic system of green sulfur bacteria and showed a close correlation of ecology, i.e. the habitat of the respective species, and phylogeny. Consequently, functional genes might reflect the adaptation to different habitats better than 16S rRNA gene sequences. Three different markers were used for an alternative phylogenetic analysis: ITS (16S-23S rRNA intergenic spacer region), *bchG*, and sigma factor A, respectively. Only sigma factor A showed a significantly higher resolution of the phylogenetic system of green

sulfur bacteria and should be considered as more powerful tool than the16S rRNA gene to classify green sulfur bacteria.

CHAPTER 1

INTRODUCTION

GREEN SULFUR BACTERIA (*CHLOROBIACEAE*)

Green sulfur bacteria (*Chlorobiaceae*) represent a monophyletic group of anoxygenic phototrophic bacteria. They are immotile and stain gram-negative. Typical habitats of green sulfur bacteria comprise stagnant lakes, ponds, and microbial mats in hot springs or shallow sandy sediments (van Gemerden and Mas 1995), as well as euxinic saltwater habitats such as the Black Sea (Overmann et al. 1992). Anoxygenic phototrophic bacteria typically occur in the chemocline, where light reaches sulfidic water layers. Among the anoxygenic phototrophic bacteria, green sulfur bacteria are especially well adapted to low-light habitats due to their large photosynthetic antennae, their lower maintenance energy requirements and higher sulfide tolerance (Overmann and Garcia-Pichel 2000).

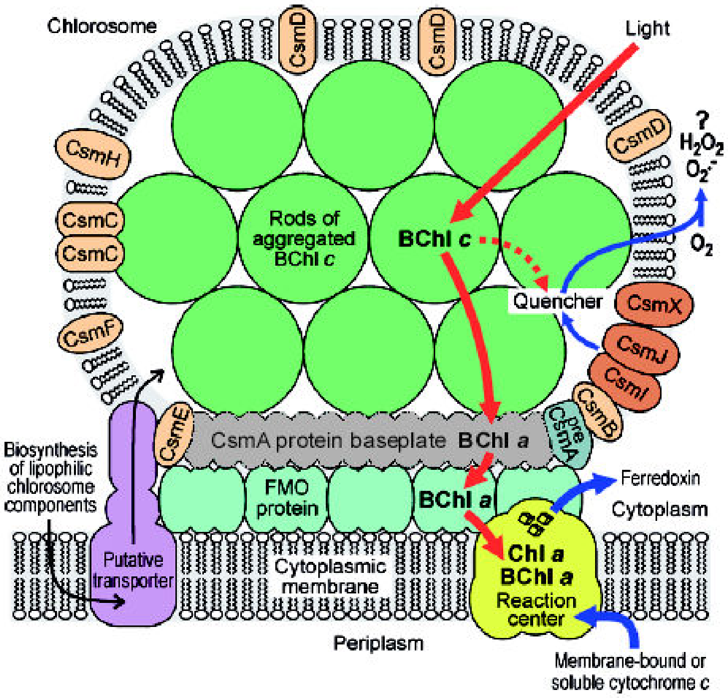

Fig.1. Simplified model of the photosynthetic apparatus in *Chlorobium tepidum*. Carotenoids (not shown) are associated with all complexes containing bacteriochlorophylls, except the Fenna-Matthews-Olson (FMO) protein. The excitation and electron transfer under oxic conditions is depicted with arrows (Grey – excitation transfer, dark Grey – electron transfer; see text for details) (Frigaard and Bryant 2004). BChl – bacteriochlorophyll, Csm – chlorosome protein, Chl – chlorophyll.

Green sulfur bacteria contain pigment carrying organelles, the chlorosomes. These organelles are attached to the inner cytoplasmic membrane and harbor the antenna pigments of the photosynthetic apparatus. The reaction center pigments, however, are located in the plasma membrane (Fig. 1) (Frigaard and Bryant 2004). The photosynthetic reaction center of green sulfur bacteria is similar to the reaction center in photosystem 1 of cyanobacteria and plants (Frigaard 1997).

Green sulfur bacteria contain bacteriochlorophyll (BChl) *c*, *d*, or *e* as their main light harvesting pigment (Imhoff 1995) and therefore are either green (BChl *c* or BChl *d*) or brown (BChl *e*) colored. The long wavelengths absorption maxima of these pigments are between 715 and 755 nm (Schlegel 1992). Bacteriochlorophylls *c*, *d*, and *e* are derived from chlorin. Each of the bacteriochlorophylls *c*, *d*, or *e* consists of porphyrin ring systems that bear up to five different combinations of substituents and are esterified to farnesol or other long-chain alcohols (Fig. 2, Table 1). These different homologs exhibit similar absorption spectra in solution (Borrego and Garcia-Gil 1995, Otte et al. 1993), but differ in regard to their stereochemistry and their solubility (Glaeser et al. 2002). *Chlorobiaceae* also have small amounts of bacteriochlorophyll *a* (BChl *a*), absorbing at 810 nm. in contrast to the bacteriochlorophylls *c*, *d*, and *e*, bacteriochlorophyll *a* is synthesized from bacteriochlorin (Frigaard 1997). The esterification of the chlorine macrocycle in the biosynthesis of BChl *a* is catalyzed by bacteriochlorophyll *a* synthase (Fig. 2 and Table 1) (Addlesee et al. 2000, Frigaard et al. 2002). This enzyme belongs to the UbiA enzyme family of polyprenyltransferases and is encoded by the *bchG* gene. Most of the bacteriochlorophyll *a* is bound to the Fenna-Matthews-Olson protein (FMO) (Fig. 1). The FMO protein itself is an additional antenna complex (Frigaard 1997), which mediates energy transfer between the chlorosomes and the reaction center. It is a characteristic and unique feature of the green sulfur bacteria and is not present in *Chloroflexus* (Blankenship et al. 1995). The monomers of FMO bind bacteriochlorophyll *a* and they are associated in a trimeric structure (Fenna et al. 1974).

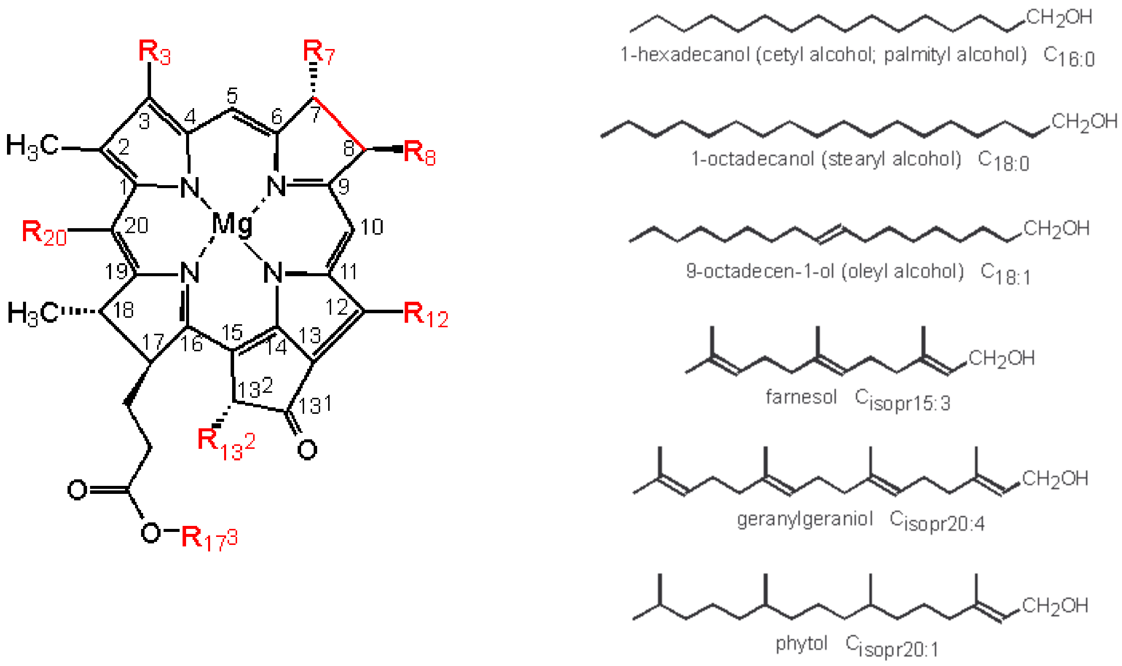

Fig. 2. Basic structure of naturally occurring chlorophylls (cf. Table 1) and esterifying alcohols.

Table 1. Substituents in chlorophylls of green sulfur bacteria (cf. Fig. 2)

Compound	**R$_3$**	**R$_7$**	**R$_8$**	**7,8-bond**	**R$_{12}$**	**R$_{13}^2$**	**R$_{20}$**	**Main R$_{17}^3$**
Chl *a*	-CH=CH$_2$	M	E	double	M	-CO-O-CH$_3$	H	P
BChl *a*	-CO-CH$_3$	M	E	single	M	-CO-O-CH$_3$	H	P, (GG)
BChl *c*	-CHOH-CH$_3$	M	E, Pr, I	double	M,E	H	M	F
BChl *d*	-CHOH-CH$_3$	M	E,Pr,I,N	double	M,E	H	H	F
BChl *e*	-CHOH-CH$_3$	-CHO	E,Pr,I,N	double	M,E	H	M	D, F, Han,Hen, OD, PD, TD

Abbreviations: BChl – bacteriochlorophyll, Chl – chlorophyll, D – Dodecanyl, E – ethyl, F – Farnesyl, GG – geranylgeranyl, H – Hydrogen, Han – Hexadecanyl, Hen – Hexadecenyl, I – Isobutyl (2-methylpropyl), M – methyl, N – neopentyl (2,2-dimethylpropyl), OD – Octadecenyl, P – phytyl, PD – Pentadecenyl, Pr – Propyl, TD - Tetradecenyl. (From Scheer 1991, BChl *e* data supplemented after Glaeser et al. 2002)

The bacteriochlorophyll content– and the type of the main bacteriochlorophyll (BChl *c*, *d*, or *e*) – are directly correlated to the habitat of the strains and the prevailing light intensities *in situ*. The bacteriochlorophyll content is higher in low-light adapted species and lower in high light adapted species (Glaeser et al. 2002).

Brown colored members of the green sulfur bacteria dominate in extreme low-light environments (Montesinos et al. 1983, Overmann et al. 1992, 1998), and BChl *e* contains higher relative amounts of secondary homologs than the other bacteriochlorophylls (Francke and Amesz 1997). In brown colored green sulfur bacteria light-dependent differences in the composition of bacteriochlorophyll homologs were shown (Glaeser et al. 2002). The alkyl side chain of porphyrin ring III is directly involved in the aggregation of bacteriochlorophyll molecules (van Rossum et al. 2001). A high degree of alkylation therefore leads to a red shift of the Q_Y absorption maximum by 7-11 nm (Borrego et al. 1999). It has been hypothesized that this facilitates the channeling of excitation energy towards the reaction center, thereby causing an increase in energy transfer efficiency of the chlorosomes (Borrego et al. 1999). Within the aggregates, bacteriochlorophylls seem to be arranged in a bilayer with the alcohol tails of bacteriochlorophylls in the inner layer oriented towards the central cavity of the rod (Steensgaard et al. 2000). Because of spatial confinements, the structure of esterifying alcohols may therefore influence the stability of the rod structure and the function of the chlorosome as a whole (Glaeser et al. 2002). The variation in length of these side chains therefore represents a physiological adaptation mechanism of green sulfur bacteria to varying light intensities (Glaeser et al 2002).

Green sulfur bacteria are considered as obligately anaerobic bacteria. However, they possess protection mechanisms to protect themselves from oxidation. In *Chlorobaculum tepidum* ATCC 49652^T (previous name: *Chlorobium tepidum*) under oxic conditions, the quencher in the chlorosome prevents excitation transfer from BChl *c* to the reaction center and consequently prevents photosynthetic electron transfer (Fig. 1) (Frigaard and Bryant 2004). The quencher is activated by oxidation, with the electrons likely being delivered directly or indirectly to O_2. Inactivation of the quencher occurs when it is re-reduced by the chlorosome proteins CsmI and CsmJ (Fig. 1). Thus, it is presumed that green sulfur bacteria can protect themselves from oxygen stress and would survive exposure to oxygen for a certain period of time.

Green sulfur bacteria also contain carotenoids as accessory pigments, absorbing light in the range of 400-550 nm. Chlorobactene is the main carotenoid in green colored green sulfur bacteria, whereas isorenieratene is the main carotenoid in brown colored *Chlorobiaceae* (Fig. 3) (Frigaard and Bryant 2004).

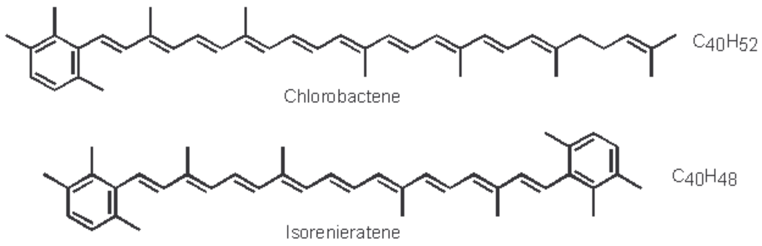

Fig. 3. Carotenoids of green sulfur bacteria.

Latest studies showed that the chlorosomes are associated in a lamellar structure. In brown colored green sulfur bacteria, these lamellar structures are associated with carotenoids which drive lamellar assembly by means of hydrophobic interactions (Psencik et al. 2006). These aggregates may be responsible for the low-light adaption of brown colored species.

Green sulfur bacteria are significant for the understanding of oxic/anoxic habitats in three aspects. First, like other phototrophic sulfur bacteria, anoxyphototrophs are important contributors to the carbon and sulfur cycles (Pfennig 1989, van Gemerden and Beeftink 1983). They may substantially alter the carbon and sulfur cycles by reoxidizing a major portion of the biogenic sulfide to sulfur and further to sulfate (Pfennig 1989, Pierson and Castenholz 1995). At the same time, they recycle electrons, and thus feed additional organic carbon into the pelagic carbon cycle (Overmann 1997). Green sulfur bacteria typically fix CO_2 via the reversed tricarboxylic acid cycle. This is in contrast to the purple bacteria, which use the ribulose bisphosphate cycle. Furthermore, *Chlorobiaceae* are excellent nitrogen-fixers and thereby contribute to the fertility of the habitat (Madigan 1988, Pfennig 1989). In order to be able to understand the role of green sulfur bacteria in anoxic oceans, however, the regulation of photosynthetic activity by environmental factors like light, sulfide and temperature first needs to be determined for those types that are typical for euxinic water bodies. Second, green sulfur bacteria represent an outstanding model systems for the study of low-light adaptation and of mechanisms of adaptation towards extreme energy-limited habitats in general. Third, green sulfur bacteria have been used as indicator organisms for past photic zone anoxia when reconstructing oceanic paleoenvironments (Koopmans et al. 1996). Green sulfur bacteria are superb objects of research for this purpose since their photosynthetic pigments are well preserved in anoxic sediments and since the origin even of pigment derivatives can be verified by their distinct carbon isotopic signatures (Kohnen et al. 1992). Still, a better knowledge of the physiology of green sulfur bacteria typical for the marine oxic/anoxic pelagial is needed to correctly interprete fossil green sulfur bacterial biomarkers and to get a detailed reconstruction of environmental conditions in ancient oceans. The green sulfur bacteria recovered from the Black Sea chemocline represent a particularly well suited model system for the investigation of these basic scientific questions, as will be outlined below.

THE BLACK SEA IS AN EXTREME HABITAT FOR GREEN SULFUR BACTERIA

The Black Sea is a semi-enclosed sea that is located between 40°55' and 46°32' N and between 27°27' and 41°42' E in the valley between the Caucasus Mountains and the Pontian Mountains. It is supplied with high density saltwater through the Bosphorus Strait, and with low density freshwater mainly from the big rivers Danube, Dnepr, Dnestr and Don (Fig. 7). The total area of the Black Sea is about $423 \cdot 10^3$ km^2 and the total volume is $537 \cdot 10^3$ km^2. The bottom of the Black Sea is subdivided into a shallow continental shelf, which is located in the Northwestern part of the basin, the continental slope and the basin apron, and the abyssal (euxine) plain with a maximum depth of 2272 m (Sorokin 2002).

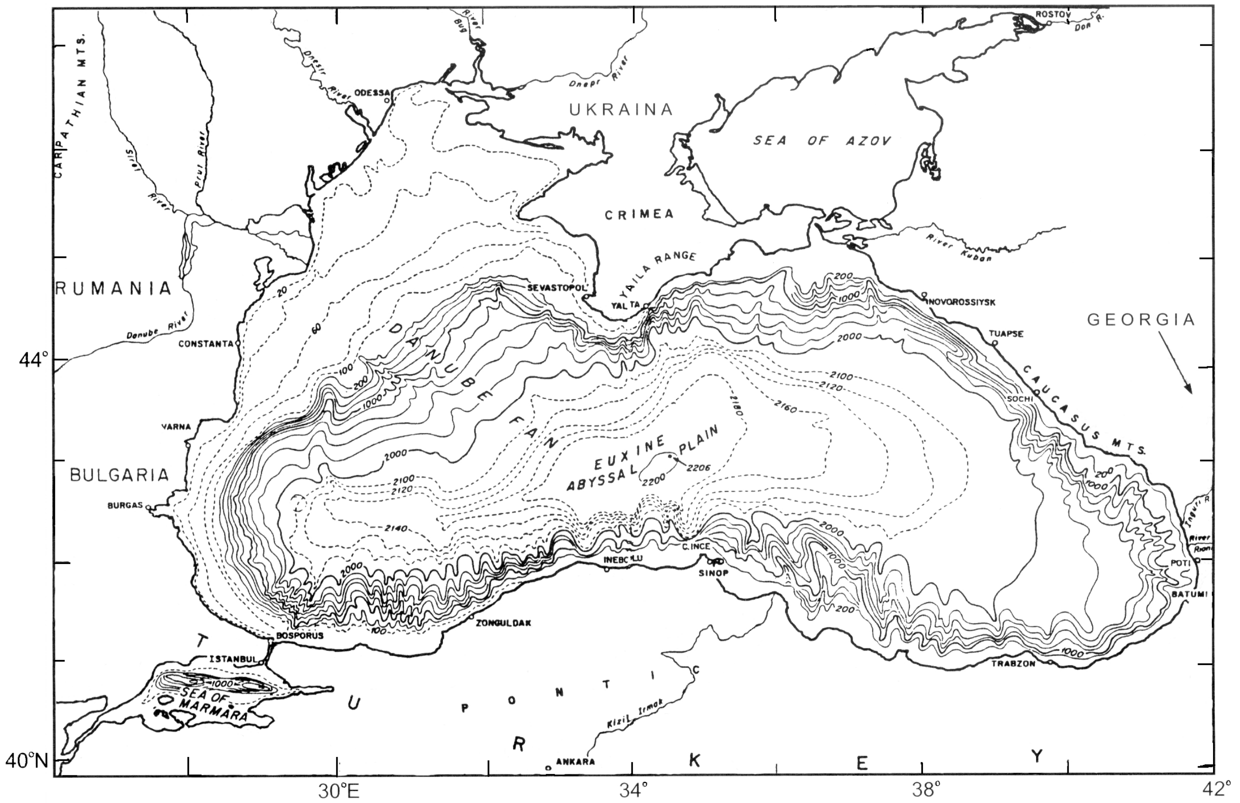

Fig. 7. Physiographic map of the Black Sea. (Sorokin 2002, modified)

Due to its geographical properties, a frequent mixing of the water column does not occur. The result is a permanent stratification of the Black Sea water column (Fig. 8 A, B). Today, 87-92% of the water body of the Black Sea is permanently anoxic (Fig. 8 B) (Codispoti et al. 1991, Konovalov et al. 2001, Sorokin 2002). Hence, the Black Sea basin currently contains the largest volume of anoxic water on the planet. Its permanently stratified water column comprises a ~60 m thick oxic top layer (upper mixed layer UML, and oxycline layer OXCL), a ~40 m thick suboxic intermediate zone devoid of sulfide and oxygen (suboxic zone, SOZ, and redox transition zone, RZ), and a ~2000 m deep sulfidic bottom zone (AZ, Fig. 8 B) (Murray et al. 1989, Sorokin 2002). The redox transition zone is also defined as chemocline, which is today located at a depth of ~60 to 200 m, varying over time and depending on sampling location (Canfield et al. 1996). Several evidences were found which pointed to the existence of a population of green sulfur bacteria in the Black Sea chemocline, for example autofluorescent cells (Bird and Karl 1991) or traces of bacteriochlorophyll *e* (Repeta et al. 1989), derived from the chemocline. Whereas the environmental factors correspond to those in other oxic/anoxic habitats (Fig. 8 B), the Black Sea chemocline is characterized by a very extreme light climate. The chemocline is positioned much deeper in the water column than in other environments of phototrophic sulfur bacteria described so far. Typically, accumulations of phototrophic sulfur bacteria have been observed between 2 and 20 m, rarely up to 30 m depth (van Gemerden and Mas 1995). In such environments, values for the light transmission to populations of phototrophic sulfur bacteria range from 0.015 to 10 % (Parkin and Brock 1980, van Gemerden and Mas 1995). Due to the lower position of the chemocline, the *in situ* light intensities in the Black Sea were expected to be significantly lower and consequently represent the limiting factor for the growth of green sulfur bacteria (Repeta et al. 1989, Repeta and Simpson 1991). Light intensities in the Black Sea chemocline of 0.003 µEinstein m^{-2} s^{-1} (max. in June 0.01 µEinstein m^{-2} s^{-1}) were calculated based on irrandiance measurements at Odessa. Light attenuation was determined from Chl *a* concentrations in the Black Sea, the chlorophyll-specific attenuation coefficient, and the light transmission reported by Repeta et al. (1989) (Overmann et al. 1992). The calculated light intensity, which is available for photosynthesis in the Black Sea chemocline, corresponds to only 0.0005 % of the surface irradiance.

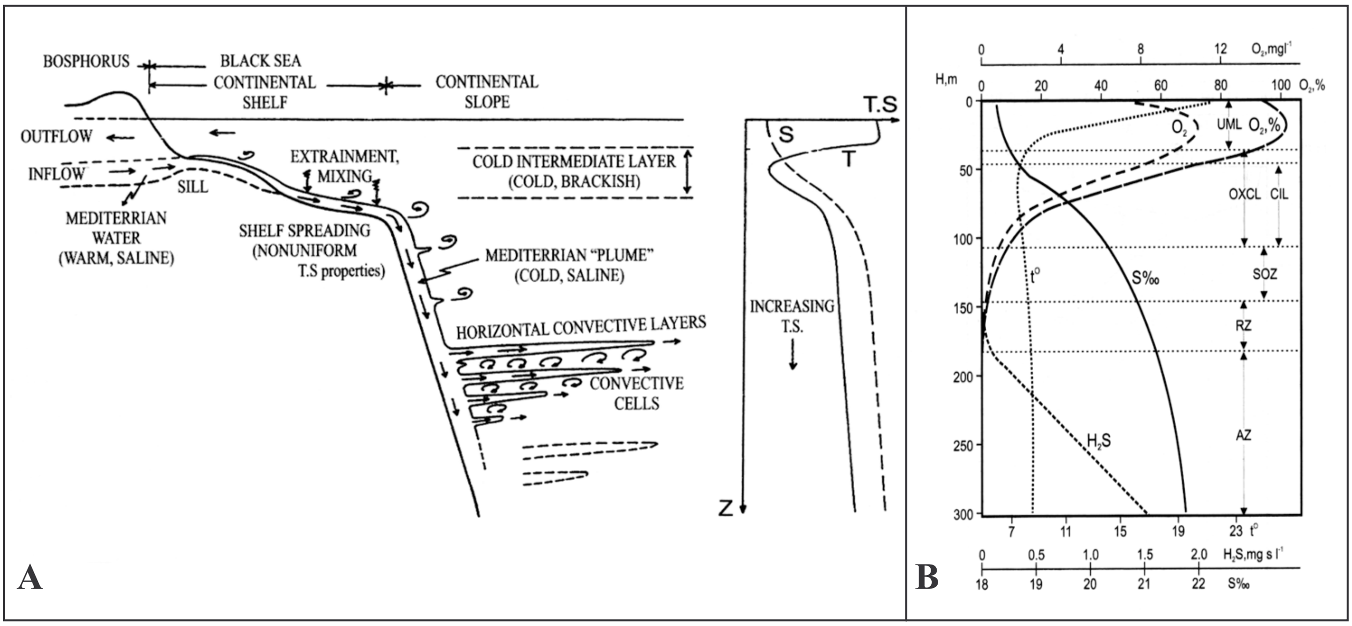

Fig. 8. Mechanisms and hydrochemistry of the Black Sea water column. *A.* Temperature profile in the SW Black Sea, which demonstrates intrusions of transformed warm Mediterranean waters below the main pycnocline. *B.* Profiles of dissolved oxygen, temperature, salinity, H_2S and boundaries of main layers reflecting thermohaline and redox stratification of Black Sea in summer; *designations of layers:* UML-upper mixed layer, CIL-cold intermediate layer, OXCL-oxycline layer, SOZ-suboxic zone, RZ-redox transition zone (chemocline), AZ-anoxic (H_2S) zone. (Sorokin 2002)

Despite the unlikeliness of bacterial photosynthesis in the Black Sea chemocline, at light intensities far lower than in any other known photosynthetic bacterial community, a green sulfur bacterium was successfully enriched from the Black Sea chemocline in 1989 (Overmann et al. 1992). The 2 µm long, rod-shaped, brown-colored bacterium was enriched from a depth of ~80 m and grew only under strongly reducing conditions (400 µM $Na_2S_2O_4$, E'=-560 mV) in the absence of oxygen (Overmann et al. 1992). On the basis of cell morphology and pigment composition, the brown-colored bacterium was assigned to the *Chlorobiaceae* and was named *Chlorobium phaeobacteroides* MN-1. Even though the bacterium could be highly enriched, the culture could not be purified from a colorless bacterium, which was isolated and identified as a sulfate-reducing strain (Overmann et al. 1992). The strain was shown to be extremely well adapted to very low light intensities, since photosynthetic activity was shown at light intensities as low as 0.25 µmol Quanta m^{-2} s^{-1} (Overmann et al. 1992). At light intensities below 1 µmol Quanta m^{-2} s^{-1} its growth rate exceeded those of the morphologically similar green sulfur bacterium *Chlorobium phaeobacteroides* 2430, DSM 266^T. Above 200 µEinstein m^{-2} s^{-1} growth of *Chlorobium phaeobacteroides* MN-1 was light inhibited (Overmann et al. 1992).

THE BLACK SEA AS A MODEL FOR PAST SULFIDIC OCEANS

The Black Sea may represent the largest and closest contemporary analogue to past sulfidic oceans and therefore constitutes a useful model system for the study of the biogeochemical cycles and the (micro) organisms that are associated with these environments.

The Proterozoic ocean ($2.3 \cdot 10^9 - 5.42 \cdot 10^8$ years b.p.), after oxygenation of the atmosphere, may have consisted of sulfidic deep water covered by a potentially 100 m-thick oxygenated surface layer, in contrast to the present ocean. This has been inferred from the fraction of reactive iron and the degree of pyritization, the S isotopic composition of sulfides in the sediments, the evidence for decreased organic carbon burial, and a limited abundance and diversity of protists (Anbar and Knoll 2002). The sulfidic pelagial of the Proterozoic ocean may have persisted over 1000 million years. Extended water column anoxia may have occurred as well during the Phanerozoic (from $4.88 \cdot 10^8$ years b.p.). Derivatives of carotenoids and bacteriochlorophylls of the obligately anoxygenic phototrophic green sulfur bacteria have been detected in sediments deposited during several eras in the Phanerozoic (Koopmans et al. 1996, Hartgers et al. 1994, Grice et al. 1996, Kohnen et al. 1992).

From the presence and the distinct carbon isotopic composition of these bacterial biomarkers it has been deduced that the anoxic layers in paleoceans frequently reached the photic zone. These findings have caused broad interest in the structure and function of such large oceanic oxic/anoxic environments.

PHYLOGENY OF GREEN SULFUR BACTERIA

Chlorobiaceae comprise a distinct phylum in the 16S rRNA phylogenetic tree (Fig. 4), which shares a common root with the *Bacteroidetes* (Cytophaga-Flavobacteria-Bacteroides) group (Garrity and Holt 2001). The group of green sulfur bacteria consists of very closely related members (Imhoff 2003). Initially, the taxonomy of green sulfur bacteria was based on phenotypic attributes, such as color, shape and unusual morphotypes (Schlegel 1992). Latest genetic analyses, however, revealed the need for revision of the systematics of green sulfur bacteria (Overmann and Tuschak 1997, Imhoff 2003).

The 16S rRNA gene is the most frequently used gene for the analysis of phylogenetic relationships between bacterial species (Pace et al. 1986, Woese 1987). But there is also the possibility of lateral gene transfer between species, and intraorganismal heterogeneity due to multiple copies of 16S rRNA gene sequences in a single organism (Rothschild et al. 1986). Overmann und Tuschak (1997) found similarity values of > 90.1% and K_{nuc} values of < 0.11 within the group of green sulfur bacteria indicating a close phylogenetic relatedness among species. Denaturing gradient gel electrophoresis did not result in a differentiation of 16S rDNA fragments of different species. But species could be distinguished when using dispersed repetitive DNA sequences as targets in PCR (Overmann and Tuschak 1997).

A green sulfur bacterial phylogeny based on 16S rRNA gene sequences was first published by Figueras et al. (1997) and included 18 species, at that time all known species of green sulfur bacteria. They found that *Chlorobiaceae* can be subdivided in brown and green colored species and that there is a separate cluster for saline strains (Figueras et al. 1997).

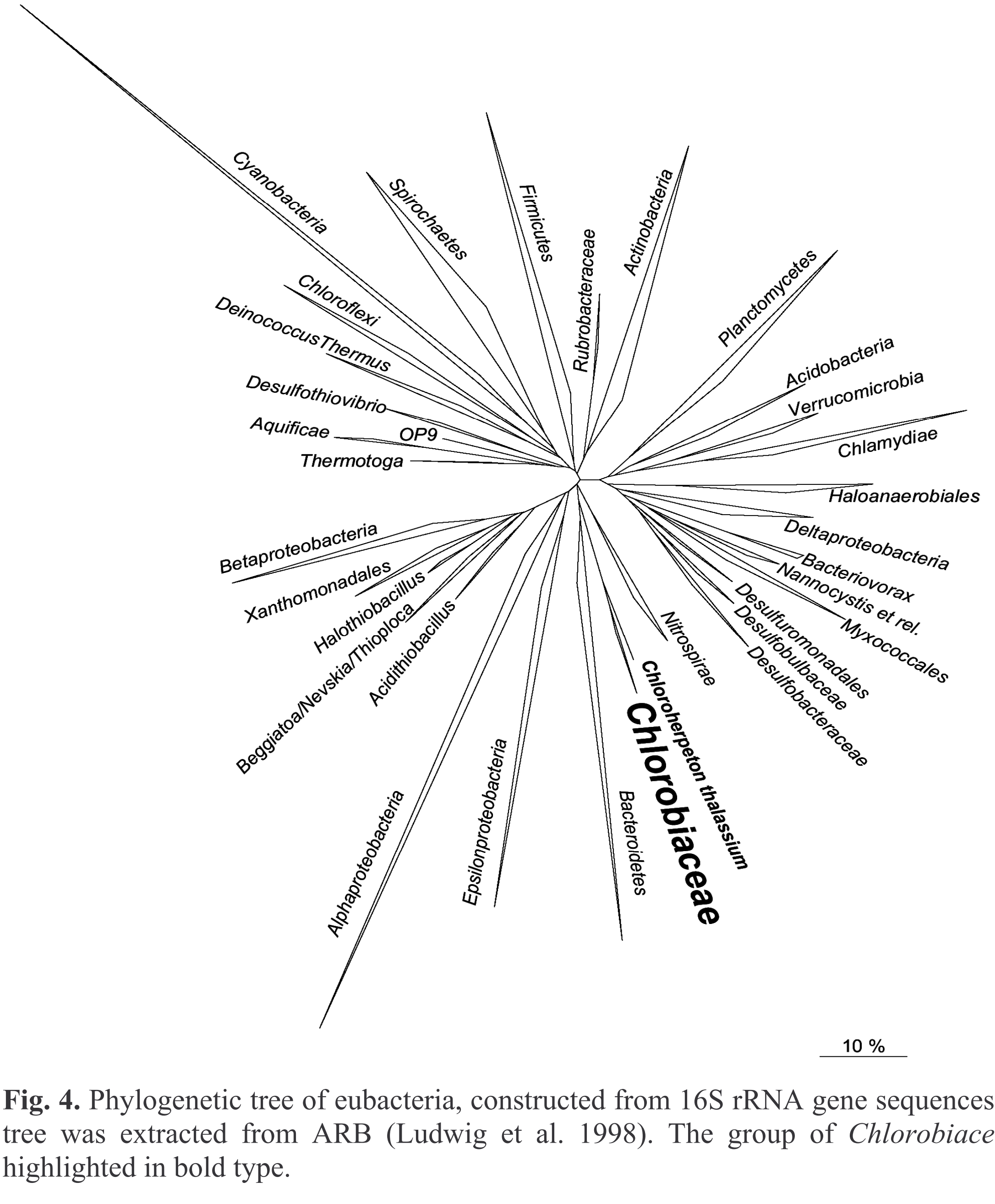

Fig. 4. Phylogenetic tree of eubacteria, constructed from 16S rRNA gene sequences. The tree was extracted from ARB (Ludwig et al. 1998). The group of *Chlorobiaceae* is highlighted in bold type.

Another study focused on the phylogenetical evaluation of BchG amino acid sequences of 14 strains of GSB (Garcia-Gil et al. 2003). Garcia-Gil et al. (2003) found that the phylogenetic relationships of green sulfur bacteria are congruent with taxonomic features, including cell shape, NaCl requirement and the presence of gas vacuoles (Overmann 2001). Evaluating the *bchG* gene sequences, they observed only very short phylogenetic distances within the GSB group, but several distinct clusters. These clusters contained either brown colored species, gas-vacuolated species, prosthecated species, or members of the genus *Chlorobium*. Like in the 16S rRNA tree (Figueras et al. 1997) a subdivision of saltwater strains was found (Garcia-Gil et al. 2003).

A phylogenetic study on green sulfur bacteria published in 2002 was based on 16S rRNA gene sequences and Fenna-Matthews-Olson protein (FMO) amino acid sequences (Fig. 1) (Alexander et al. 2002). Alexander et al. (2002) found a congruence of the phylogenetic trees built from 31 16S rRNA gene sequences and FMO amino acid sequences. They found several distinct clusters, e.g. a separate cluster of marine strains, and at the same time showed the incongruence of the phylogenetic trees with the current taxonomic classification. Thus, a new taxonomy of the *Chlorobiaceae* based on 16S rRNA gene and FMO amino acid sequences was proposed introducing new genus and species names (Imhoff 2003).

The 16S-23S ribosomal RNA intergenic spacer region (ITS) was shown to be a useful marker for the rapid identification of bacteria by Jensen et al. (1993) due to the presence of highly conserved regions as well as of highly variable regions. The ITS region is located between the 16S rRNA gene and the 23S rRNA gene on the bacterial chromosome and contains the information for the tRNA_{Ile} and the tRNA_{Ala} in green sulfur bacteria (Fig. 5). In *Chlorobaculum tepidum*, two alleles were found, one having an insert of 59 bp compared with the other allel. The ITS region was used as a phylogenetic tool by Aakra et al., who used it for studying the ecological diversity of ammonia-oxidizing bacteria *in situ* (Aakra et al. 1999).

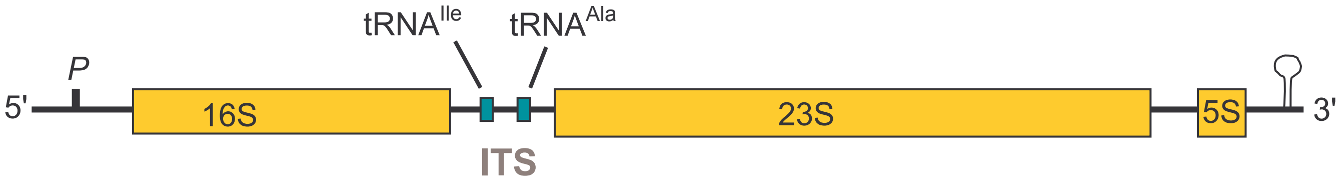

Fig. 5. The 16S-23S ribosomal RNA intergenic spacer region (ITS) of green sulfur bacteria.

Gruber and Bryant (1997) used group 1 sigma factors of the σ^{70}-type as a phylogenetic tool for the investigation of evolutionary relationships among eubacteria (Fig. 6), but only a single sequence of green sulfur bacteria was included. Group 1 sigma factors are the primary sigma factors in all known eubacteria and are elementary for cell viability (Lonetto et al. 1992). The σ^{70}-type sigma factor is part of the eubacterial RNA polymerase holoenzyme (Burgess et al. 1969) and assigns the promoter specificity for transcription initiation onto the core enzyme.

Primary sigma factors are useful for phylogenetic comparisons because these sigma factors possess highly conserved amino acid sequences due to functional constraints (Helmann and Chamberlin 1988) as well as regions that are variable even among close relatives. Gruber and Bryant (1997) analyzed the phylogenetic affiliation of protein sequences of group 1 σ^{70}-type sigma factors from a variety of eubacterial groups including one sequence of green sulfur bacteria (*Chlorobaculum tepidum* TLS, previously known as *Chlorobium tepidum*). Their analysis showed a congruence of the phylogenetic clustering of sequences with the clusters found in 16S rRNA trees and a remote position of the green sulfur bacterial sequence (Fig. 6; Gruber and Bryant 1997).

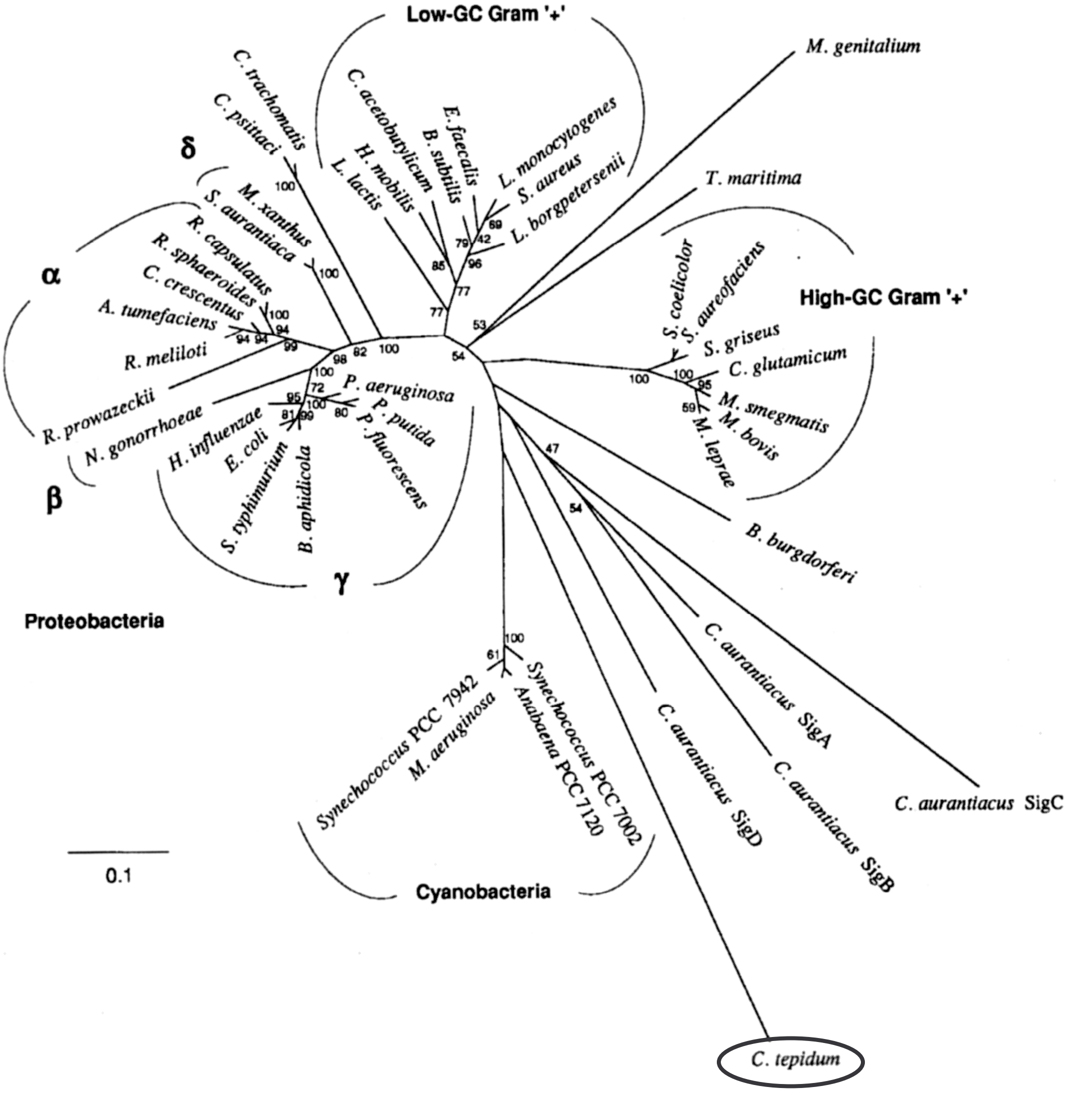

Fig. 6. Fitch-Margoliash tree for group 1 sigma factors of different bacterial groups (Gruber & Bryant 1997). The sequence of the group 1 sigma factor (*SigA*) of *Chlorobaculum tepidum* (*C. tepidum*) differs strongly from other group 1 sigma factors (Gruber and Bryant 1997).

SCOPE OF THE PRESENT WORK

The main organism for this study was *Chlorobium* sp. BS-1 from the Black Sea chemocline.

At the beginning of this thesis, water samples as well as sediment samples from the Black Sea chemocline and sediments were available, obtained during the cruise of the R/V Meteor in December 2001. Trials for enrichment cultures had been set up by Jens Glaeser and Martina Sterz in advance and huge amounts of Black Sea water had been filtered onto polycarbonate filters (0.1 µm pore size).

First aim of the study was the enrichment of the green sulfur bacterium from the Black Sea chemocline and the isolation of the strain, since the strain MN-1 from 1989 was lost previously. For the enrichment, several different media had been prepared known from experience with the strain MN-1, enriched in 1989 (Overmann et al. 1992).

Since the Black Sea strain was shown to be extremely well adapted to its low-light environment (Overmann et al. 1992) we wanted to gain further insight into the mechanisms of adaptation. It was shown that the Black Sea strain differs in pigment content and maintenance energy requirement μ_e from the morphologically similar green sulfur bacterium *Chlorobium phaeobacteroides* 2430, DSM 266^T (Overmann et al. 1992). Furthermore, unusual bacteriochlorophyll homologs, the geranyl esters of bacteriochlorophyll *e*, had been detected in the chemocline before but not assigned to any organism (Repeta and Simpson 1991). Since the Black Sea strain is thought to represent the only green sulfur bacterium in the Black Sea, the geranyl esters of bacteriochlorophyll *e* are likely to be produced by that bacterium, even though they have not been detected in other brown-colored green sulfur bacteria (Glaeser et al. 2002). Therefore, a correlation of the presence of atypical bacteriochlorophyll homologs and a low-light adapted species seemed obvious. Pigment analyses of the Black Sea strain by HPLC and carbon assimilation experiments at different light intensities were expected to give more insight into the physiological adaptations of that strain.

The phylogenetical affiliation of the Black Sea strain had not been determined before. For that purpose, chemocline samples for the extraction of DNA had been prepared, as well as filter samples for the quantification of the Black Sea strain in the chemocline by fluorescence-*in situ*-hybridization (FISH). With the information of the *in situ* cell density of the Black Sea strain and the quantification of pigment content in the chemocline, the specific pigment content in the cell was calculated.

Sediment cores of up to 7 m length had been taken at three different stations by Jens Glaeser and Martina Sterz. Two of the cores at ~2000 m water depth from the western basin, one of them at 468 m depth at the northwestern shelf edge. The cores had been sampled onboard the vessel and the samples were stored at –20°C. By determining the 16S rRNA gene sequence of the Black Sea strain, the specific genetic fingerprints were detected in the sediment samples by a PCR-DGGE (denaturing gradient gelelectrophoresis) approach. With this approach the biodiversity of green sulfur bacteria in the Black Sea over prolonged periods of time can be investigated as well as the presence of the chemocline strain. The phylogenetical diversity was assessed by grouping the sequences obtained with this approach in a phylogenetic tree based on present sequences of green sulfur bacteria. Pigment analyses showed the presence of green sulfur bacteria in the Black Sea in the past (Sinninghe Damsté et al. 1993), but they could not be assigned to any green sulfur bacterial species. With the information of fingerprints of the different populations of green sulfur bacteria the history of the Black Sea basin can possibly be reconstructed in much more detail.

From the isolated Black Sea strain, genomic DNA was extracted for whole genome sequencing. Since only one whole genome in the group of *Chlorobiaceae*, the genome of *Chlorobaculum tepidum*, has been sequenced so far, only limited genetic information is available on the group of green sulfur bacteria. To date the whole genomes of six species are in the process of sequencing and the results will give more insights into the phylogenetic affiliation of green sulfur bacteria.

Since the phylogenetic system of green sulfur bacteria was not up to date at the beginning of this work, I needed a proper phylogenetic analysis of all environmental and cultured species of green sulfur bacteria to embed my data within. In the last years numerous environmental sequences of green sulfur bacteria had been published (www.ncbi.nlm.nih.gov), but not analyzed with respect to their phylogenetic position. Most of the available green sulfur bacterial sequences were 16S rRNA gene sequences. Phylogenetic trees employing 16S rRNA gene sequences, however, did not show a high resolution.

Since the green sulfur bacteria are all very closely related (Garcia-Gil et al. 2003), different genetic markers should give more resolution to the phylogenetical system. The green sulfur bacterial phylogeny had been revised by Imhoff (2003) based on 16S rRNA gene sequences and *fmoA* gene sequences from only a limited number of cultured species. The systematics based on the 16S rRNA gene sequences of published species and cultures present in our laboratory were refined by determining the sequences of the ITS region, the *sigA* gene, and the *bchG* gene sequences of as many cultured species as possible. Combining these data with released data withdrawn from the EMBL database (www.ncbi.nlm.nih.gov) a comprehensive phylogeny of green sulfur bacteria was constructed. The genus names proposed by Imhoff et al. (2003) can either be supported or rejected by the use of our analyses, and help resolve the confusion about green sulfur bacterial names. Also, a basis for a new discussion about bacterial phylogeny is created.

The enrichment and characterization of a green sulfur bacterium, *Chlorobium bathyomarinum* (GSB1), which was isolated from the Ty vent, a deep-sea hydrothermal vent in the Pacific Ocean helped me to get further insight into the oxygen tolerance of green sulfur bacteria. The search for photosynthetic bacteria round the vent was preceded by the finding that light was emitted from that vent, which could possibly support photosynthesis (van Dover et al. 1989, Pelli and Chamberlain 1989). The finding of green sulfur bacteria in oxygenated water evolved the question if a green sulfur bacterium can resist high amounts of oxygen in their habitat. This project was done in cooperation with Tom Beatty, who sent us the the first enrichment culture of GSB1.

LITERATURE

(1) **Aakra, A., Utaker, J.B., and I.F. Nes.** 1999. RFLP of rRNA genes and sequencing of the 16S-23S rDNA intergenic spacer region of ammonia-oxidizing bacteria: a phylogenetic approach. Int J Syst Bacteriol. **49** Pt 1:123-30.

(2) **Addlesee, H.A., Fiedor, L., and C.N. Hunter.** 2000. Physical mapping of *bchG*, orf427, and orf177 in the photosynthesis gene cluster of *Rhodobacter sphaeroides:* functional assignment of the bacteriochlorophyll synthetase gene. J. Bacteriol. **182:**3175-82.

(3) **Anbar, A.D. and A.H. Knoll.** 2002. Proterozoic ocean chemistry and evolution: a bioinorganic bridge? Science 297: 1137-1142.

(4) **Alexander, B., J. H. Andersen, R. P. Cox, and J. F. Imhoff.** 2002. Phylogeny of green sulfur bacteria on the basis of gene sequences of 16S rRNA and of the Fenna-Matthews-Olson protein. Arch. Microbiol. 178:131-140.

(5) **Blankenship, R. E., J. M. Olson, and M. Miller.** 1995. Antenna complexes from green photosynthetic bacteria. In: Blankenship R.E., Madigan M.T., Bauer C.E. (eds) Anoxygenic photosynthetic bacteria. Kluwer, Dordrecht, pp. 399-435.

(6) **Bird, D. F. and Karl, D. M.** 1991. Microbial biomass and population diversity in the upper water column of the Black Sea. Deep-Sea Res. 38 (Suppl 2): S1069-S1082

(7) **Borrego, C. M. and L. J. Garcia-Gil.** 1995. Rearrangement of light harvesting bacteriochlorophyll homologs as a response of green sulfur bacteria to low light intensities. Photosynth. Res. **41:**53-65.

(8) **Borrego, C. M., J. B. Arellano, C. A. Abella, T. Gillbro, and L. J. Garcia-Gil.** 1999. The molar extinction coefficient of bacteriochlorophyll *e* and the pigment stoichiometry in *Chlorobium phaeobacteroides.* Photosynth. Res. **60:**257-264.

(9) **Burgess, R.R., Travers, A.A., Dunn, J.J. und E.K.F. Bautz.** 1969. Factor stimulating transcription by RNA polymerase. Nature **221**: 43-46.

(10) **Canfield, D.E., Lyons, T.W., Ralswell, R.** 1996. A model for iron deposition to euxinic Black Sea sediments. Am. J. Sci. 296: 818-834

(11) **Codispoti, L. A., G. E. Friederich, J. W. Murray, and C. M. Sakamoto.** 1991. Chemical variability in the Black Sea: implications of continuous vertical profiles that penetrated the oxic/anoxic interface. Deep Sea Res. 38 (suppl.2): S691-S710

(12) **Fenna, R. E., B. W. Matthews, J. M. Olson, and E. K. Shaw.** 1974. Structure of a bacteriochlorophyll-protein from the green photosynthetic bacterium *Chlorobium limicola*: Crystallographic evidence for a trimer. J. Mol. Biol. 84:231-240.

(13) **Francke, C. and J. Amesz.** 1997. Isolation and pigment composition of the antenna system of four species of green sulfur bacteria. Photosynth. Res. 52:137-146.

(14) **Frigaard, N.-U.** 1997. Light-harvesting structures in green sulfur bacteria. PhD thesis. Odense University, Odense, Denmark.

(15) **Frigaard, N.-U., Voigt, G. D. and D. A. Bryant.** 2002. *Chlorobium tepidum* mutant lacking bacteriochlorophyll *c* made by inactivation of the *bch*K gene, encoding bacteriochlorophyll *c* synthase. J. Bacteriol. 184:3368-76.

(16) **Frigaard, N.-U. and D. A. Bryant.** 2004. Seeing green bacteria in a new light: genomics-enabled studies of the photosynthetic apparatus in green sulfur bacteria and filamentous anoxygenic phototrophic bacteria. Arch. Microbiol. **182**:265-276.

(17) **Garcia-Gil, L.J., Gich, F.B., and X. Fuentes-Garcia.** 2003. A comparative study of *bchG* from green photosynthetic bacteria.Arch Microbiol. **179:**108-15.

(18) **Garrity, G. M. and J. G. Holt.** 2001. Phylum BXI. Chlorobi phy. nov. In: Boone D. R., Castenholz, R. W. (eds). Bergey's manual of systematic bacteriology, vol 1, 2nd edn. Springer, New York, Berlin, Heidelberg, pp. 601-623.

(19) **Glaeser, J., L. Bañeras, H. Rütters, and J. Overmann.** 2002. Novel bacteriochlorophyll *e* structures and species-specific variability of pigment composition in green sulfur bacteria. Arch. Microbiol. **177:**476-485.

(20) **Grice, K., R. Gibbison, J. E. Atkinson, L. Schwark, C. B. Eckhardt, and J. R. Maxwell.** 1996. Maleimides (1*H*-pyrrole-2,5-diones) as molecular indicators of anoxygenic photosynthesis in ancient water columns. Geochim. Cosmochim. Acta 60: 3913-3924

(21) **Hartgers, W. A., J. S. Sinninghe Damsté, A. G. Requejo, J. Allan, J. M. Hayes, and J.W. de Leeuw.** 1994. Evidence for only minor contributions from bacteria to sedimentary organic carbon. Nature 369: 224-227

(22) Helmann, J.D. and M.J. Chamberlin. 1988. Structure and function of bacterial sigma factors. Annu Rev Biochem.**57:**839-72.

(23) **Imhoff, J. F.** 1995. Taxonomy and physiology of phototrophic purple bacteria and green sulfur bacteria, p. 1-15. In R. E. Blankenship, M. T. Madigan, and C. E. Bauer (ed.), Anoxygenic photosynthetic bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.

(24) **Imhoff, J. F.** 2003. Phylogenetic taxonomy of the family *Chlorobiaceae* on the basis of 16S rRNA and fmo (Fenna-Matthews-Olson protein) gene sequences. Int. J. Syst. Evol. Microbiol. **53:**941-951.

(25) **Kohnen, M.E.L., Schouten, S., Sinninghe Damsté, J.S., DeLeeuw, J.W., Merritt, D.A., and J.M. Hayes.** 1992. Recognition of paleochemicals by a combined molecular sulfur and isotope geochemical approach. Science 256: 358-362

(26) **Konovalov, S.K., L.I. Ivanov, and A.S. Samodurov.** 2001. Fluxes and budgets of sulphide and ammonia in the Black Sea anoxic layer. J. Mar. Syst. 31: 203-216

(27) **Koopmans, M.P., Köster, J., van Kaam-Peters, H.M.E., Kenig, F., Schouten, S., Hartgers, W.A., de Leeuw, J.W., and J.S. Sinninghe Damsté.** 1996. Diagenetic and catagenic products of isorenieratene: Molecular indicators for photic zone anoxia. Geochim. Cosmochim. Acta 60: 4467-4496

(28) **Lonetto, M., Gribskov, M., and C.A. Gross.** 1992. The sigma 70 family: sequence conservation and evolutionary relationships. J Bacteriol.**174**:3843-9.

(29) **Ludwig, W., O. Strunk, S. Klugbauer, N. Klugbauer, M. Weizenegger, J. Neumann, M., Bachleitner and K. H. Schleifer.** 1998. Bacterial phylogeny based on comparative sequence analysis. Electrophoresis **19**:554-568.

(30) **Madigan, M. T.** 1988. Microbiology, physiology, and ecology of phototrophic bacteria, p 39-111. In A. J. B. Zehner (ed.), Biology of anaerobic microorganisms. John Wiley & Sons, New York, NY.

(31) **Montesinos, E., R. Guerrero, C. Abella, and I. Esteve.** 1983. Ecology and physiology of the competition for light between *Chlorobium limicola and Chlorobium phaeobacteroides* in natural habitats. Appl. Environ. Microbiol. 46: 1007-1016

(32) **Murray, J. W., H. W. Jannasch, S. Honjo, R. F. Anderson, W. S. Reeburgh, Z. Top, G. E. Friederich, L. A. Codispoti, and E. Izdar.** 1989. Unexpected changes in the oxic/anoxic interface in the Black Sea. Nature **338:**411-413.

(33) **Otte S.C.M, van de Meent, E.J., van Veelen, P.A., Pundsnes, A.S. and J. Amesz.** 1993. Identification of the major chlorosomal bacteriochlorophylls of the green sulfur bacteria *Chlorobium vibrioforme* and *Chlorobium phaeovibrioides*; their function in lateral energy transfer. Photosynth. Res. **35**:159-169

(34) **Overmann, J., H. Cypionka, and N. Pfennig.** 1992. An extremely low-light-adapted green sulfur bacterium from the Black Sea. Limnol. Oceanogr. **37**:150-155.

(35) **Overmann, J.** 1997. Mahoney Lake: a case study of the ecological significance of phototrophic sulfur bacteria. Adv. Microbial. Ecol. **15:** 251-288

(36) **Overmann, J. and C. Tuschak.** 1997. Phylogeny and molecular fingerprinting of green sulfur bacteria. Arch Microbiol. **167**:302-9.

(37) **Overmann, J., and F. Garcia-Pichel.** 2000. The Phototrophic Way of Life. In: Dworkin, M. et al. (eds.) The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community, 3rd edition, Springer, New York, 2000 (http://ep.springer-ny.com:6336/contents/)

(38) **Overmann, J., C. Tuschak, J. M. Fröstl, and H. Sass**. 1998. The ecological niche of the consortium *"Pelochromatium roseum"*. Arch. Microbiol. 169:120-128.

(39) **Parkin, T.B. and Brock, T.D.** 1980. Photosynthetic bacterial production in lakes: the effects of light intensity. Limnol. Oceanogr. 25: 711-718

(40) **Pelli, D. G., and S. C. Chamberlain.** 1989. The visibility of 350°C black-body radiation by the shrimp *Rimicaris exoculata* and man. Nature, **337:**460-461.

(41) **Pfennig, N.** 1989. Ecology of phototrophic purple and green sulfur bacteria, p. 81-96. In H. G. Schlegel and B. Bowien (ed.), Autotrophic bacteria. Springer-Verlag, New York, NY.

(42) **Pierson, B. K., and J. M. Olson.** 1987. Photosynthetic bacteria, p. 21-42. In J. Amesz (ed.), Photosynthesis. Elsevier Science Publishers, B. V. Amsterdam, The Netherlands.

(43) **Pierson, B. K. and R. W. Castenholz**. 1995. Taxonomy and physiology of filamentous anoxygenic phototrophs. In: Anoxygenic Photosynthetic Bacteria, pp. 31±47. Edited by R. E. Blankenship, M. T. Madigan & C. E. Bauer. Dordrecht: Kluwer.

(44) **Psencik, J., Arellano, J. B., Ikonen, T. P., Borrego, C. M., Laurinmaki, P. A., Butcher, S. J., Serimaa, R. E. and R. Tuma.** 2006. Internal structure of chlorosomes from brown-colored *Chlorobium* species and the role of carotenoids in their assembly. Biophys J. **91**:1433-40. Epub 2006 May 26

(45) **Repeta, D.J., Simpson, D.J., Jørgensen, B.B., Jannasch, H.W.** 1989. Evidence for anoxygenic photosynthesis from the distribution of bacteriochlorophylls in the Black Sea. Nature. 342:69-72

(46) **Repeta, D.J. and Simpson, D.J.** 1991. The distribution and recycling of chlorophyll, bacteriochlorophyll and carotenoids in the Black Sea. Deep-Sea Res. 38 (Suppl. 2) S969-S984

(47) Rothschild, L.J., Ragan, M.A., Coleman, A.W., Heywood, P. and S.A. Gerbi. 1986. Are rRNA sequence comparisons the Rosetta stone of phylogenetics? Cell. **47**:640.

(48) **Schlegel, H. G.** 1992. Allgemeine Mikrobiologie. Thieme, Stuttgart, New York.

(49) **Sinninghe Damsté, J. S., Wakeham, S. G., M. E. L. Kohnen, Hayes, J. M., and J. W. de Leeuw.** 1993. A 6,000-year sedimentary molecular record of chemocline excursions in the Black Sea. Nature. **362**:827-829.

(50) **Sorokin, Yu. I.** 2002. The Black Sea. Ecology and oceanography. Backhuys Publishers, Leiden, The Netherlands.

(51) **Steensgaard, D.B., H. Wackerbarth, P. Hildebrandt, and A.R. Holzwarth** (2000) Diastereoselective control of bacteriochlorophyll *e* aggregation. 3^1-S-BChl *e* is essential for the formation of chlorosome-like aggregates. J. Phys. Chem. B **104:** 10379–10386

(52) **Van Dover, C.L., Szuts, E.Z., Chamberlain, S.C., and J. R. Cann.** 1989. A novel eye in 'eyeless' shrimp from hydrothermal vents of the Mid-Atlantic Ridge. Nature. **337**:458-60.

(53) **van Gemerden, H., and H. H. Beeftink.** 1983. Ecology of phototrophic bacteria, p. 146-185. In J. G. Ormerod (ed.), The phototrophic bacteria. University of California Press, Berkeley.

(54) **van Gemerden, H., and J. Mas.** 1995. Ecology of phototrophic sulfur bacteria, pp. 49-85. In: Blankenship R.E., Madigan M.T., and C.E. Bauer (eds), Anoxygenic photosynthetic bacteria, Advances in Photosynthesis, Vol. II. Kluwer Academic Publishers, Dordrecht, The Netherlands.

(55) **van Rossum B-J., D. B. Steensgard, F. M. Mulder, G. J. Boender, K. Schaffner, A.R. Holzwarth, and H. J. M. de Groot.** 2001. A refined model of the chlorosomal antennae of the green sulfur bacterium *Chlorobium tepidum* from proton chemical shift constraints obtained with high-field 2-D and 3-D MAS NMR dipolar correlation spectroscopy. Biochemistry **40:**1587-1595.

CHAPTER 2

DISCUSSION

1. The green sulfur bacterium from the Black Sea (cf. Chapter 4)

It was shown using isolation techniques as well as PCR and FISH hybridization that the Black Sea strain MN-1, which was enriched in 1988 from the Black Sea chemocline (Overmann et al. 1992), still persisted in the chemocline in 2001 (*Chlorobium* sp. BS-1, this study). It was the only phylotype of green sulfur bacteria in the investigated water samples.

16S rRNA gene and ITS sequence comparison showed that the strain MN-1, enriched in 1988 and *Chlorobium* sp. BS-1, enriched in 2001, are identical. No physiological differences were found either. The same strain was found in May 2004 (R/V Marion Dufresne; A. K. Manske, unpublished) indicating the persistence of that population for at least 16 years.

The used cultivation techniques were successful for enriching *Chl.* sp. BS-1 from the water column. In contrast, no 16S rRNA genes of green sulfur bacteria were detected in clone libraries established after PCR amplification with eubacterial primers (Vetriani et al. 2003).

In this study it was shown that a maximal pigment content in cells of *Chl.* sp. BS-1 is reached at ~220 µgBChl*e*·(mg protein)$^{-1}$. Hence, from pigment analyses of bacteriochlorophyll *e* (≤ 0.8 mg BChl *e* m^{-2}) and FISH hybridization of the sampled water column a biomass of *Chl.* sp. BS-1 was calculated which is orders of magnitude lower than in any other green sulfur bacterial habitat so far (25 – 2000 mg BChl *e* m^{-2}) (van Gemerden and Mas, 1995). The resulting low fraction of BS-1 therefore may have prevented its detection in clone libraries constructed with eubacterial primers (Vetriani et al. 2003). The group-specific PCR protocol used in the present study permits the amplification of 16S rRNA genes from less than 200 bacterial cells (Glaeser and Overmann, 2004) and therefore provides a much higher sensitivity for the detection of green sulfur bacteria.

Phylogenetic studies showed that the green sulfur bacterium from the Black Sea chemocline is a marine bacterium, which has to the publication date, not been detected in any other habitat. Recently, a phylotype showing 99% sequence similarity (16S rRNA) was found in environmental clones from the Sippewissett saltmarsh in the U.S. (clone K-207, Alexander and Imhoff 2006) and was shown to group within the phylogenetic cluster of marine green sulfur bacteria. The relationship of that environmental clone to BS-1, however, will need to be verified in the future by physiological and molecular biological methods.

The persistence of that strain in the chemocline as well as the monospecific assemblage are indicators for a special adaptation of the strain BS-1 to the extreme low light conditions prevailing in a depth of 100 m.

2. Biogeochemical significance of anoxygenic photosynthesis in the Black Sea (cf. Chapter 4)

To evaluate the biogeochemical significance of anoxygenic photosynthesis by green sulfur bacteria in the Black Sea, two parameters were calculated from the obtained data. The first parameter is the *in situ* light intensity in the Black Sea water column, the second one is the CO_2-fixation rate *in situ*. *In situ* light intensities were calculated by using light transmission data in the water column and global irradiance and average day length in the Black Sea region at summer and winter solstice, taken from the Solar Irradiance Data Utility (http://sunbird.jrc.it/pvgis/apps/radday.php). CO_2-fixation rates were then interpolated using the experimental data measured at light intensites of ≥ 0.015 µmol Quanta m^{-2} s^{-1} considering the biomass of green sulfur bacteria present at each depth of the water column. The calculated integrated anoxygenic photosynthetic rate was 211 ngC m^{-2} d^{-1} for a clear December day (corresponding to a doubling time t_d of 26 years), and 1760 ngC m^{-2} d^{-1} during summer solstice (t_d=3.1 years). Compared to other anoxygenic phototrophic bacteria in stratified environments the calculated values are at least thousandfold lower (Chapter 3, Fig. 2). The overall phytoplankton primary production in the central Western basin was calculated to equal 575 mgC m^{-2} d^{-1} (Karl and Knauer, 1991). Hence, green sulfur bacteria in the Black Sea chemocline likely contribute far less than 1% to total photosynthesis in the Black Sea

The data gained during this study can also be used to infer the significance of anoxygenic photosynthesis for sulfide turnover in the Black Sea. A sulfide oxidation rate of 8.8 nmol H_2S m^{-2} d^{-1} (sulfate) to 293.3 nmol H_2S m^{-2} d^{-1} (elemental sulfur) was deduced from the integrated anoxygenic photosynthetic rate. Based on the vertical concentration profiles and the coefficient for turbulent diffusivity, the sulfide flux from below the chemocline into the green sulfur bacterial layer is much higher (370 – 530 µmol sulfide m^{-2} d^{-1}) (Konovalov et al. 2001). Hence, green sulfur bacteria account for less than 0.1% of total sulfide oxidation in the Black Sea chemocline. They are therefore not responsible for the existence of the suboxic zone, as postulated by Jørgensen et al. (1991).

The models of the upward fluxes of sulfide in the Black Sea suggest that sulfide is actually consumed within the anoxic zone between the chemocline and 150 m depth, but not within the chemocline (Konovalov et al. 2001). Massive lateral injections of oxygen-enriched Mediterranean waters through the Bosporus plume lead to the formation and sedimentation of particulate MnO_2 in the chemocline, which in turn serves as the oxidant of ~70% of the sulfide diffusing upwards (Konovalov et al. 2003). Together with the molecular diffusion of O_2 from upper water layers (accounting for ~10% of the sulfide oxidation) (Konovalov et al. 2001) these two factors would be sufficient for oxidizing most of the sulfide in the Black Sea anoxic zone.

3. Low-light adaptation of BS-1 (cf. Chapter 4)

The habitat of *Chlorobium* sp. BS-1 provides a light intensity of a little candle at 50 m distance in otherwise total darkness. Compared to other environments (van Gemerden and Mas, 1995), *Chl.* sp BS-1 experiences by far the most severe light limitation ever recorded for any phototrophic organism. *Chlorobium bathyomarinum* GSB1 another green sulfur bacterium, isolated from a deep sea hydrothermal vent (Chapter 5), is even suspected to survive with geothermal light only. This theory has not been verified yet, though. Further experiments will give more insight into the physiology of *Chl. bathyomarinum* to verify this exciting theory.

The obligately photolithoautotrophic green sulfur bacteria typically fix CO_2 via the reverse citric acid cycle (Sirevåg 1995). This results in a biomass depleted by 2.5-12 ‰ relative to CO_2 (van der Meer 1998) and membrane lipids and photosynthetic pigments which are enriched in ^{13}C by 1-15 ‰ relative to the total biomass (Glaeser and Overmann 2003, van der Meer 1998). Our data show that farnesol is the dominant lipid biomarker of the green sulfur bacteria in the Black Sea chemocline. Since the $\delta^{13}C$ values of farnesol and dissolved CO_2 measured in the present study are nearly identical, *Chl.* sp. BS-1 most likely fixes CO_2 via the reverse citric acid cycle, like most of the green sulfur bacteria. Hence, a photolithotrophic growth *in situ* is most certain. Also, growth in the dark was never observed in cultures of *Chl.* sp BS-1 (A. Manske, unpublished data) but it was shown by ^{13}C-NMR that *Chl.* sp BS-1 did not acquire an alternative, chemoorganoheterotrophic mode of growth.

Farnesol is not only typical for green sulfur bacteria but can, in small traces, also be found in *Archaea* (Tornabene et al. 1979). Archaeal 16S rRNA gene sequences have been found in the Black Sea chemocline (Vetriani et al. 2003), but no information on the abundance of archaeal cells in the chemocline is currently available. Since farnesol occurs in only few archaeal species and only in species which are only distantly related to those found in the Black Sea chemocline (Vetriani et al. 2003), it seems unlikely that farnesol deriving from archaeal cells contributed to the isotopic signal.

Low-light adaptation of *Chl.* sp BS-1 was shown to coincide with an increased specific BChl *e* content before (Fuhrmann et al. 1993, Overmann et al. 1992). But only cells incubated at light intensities above 0.25 µmol Quanta m^{-2} s^{-1} had been studied so far, which reached a specific pigment content of ~220 µgBChl*e*·(mg protein)$^{-1}$ (Overmann et al. 1992). Similar values for cells grown at even lower light intensities, below 1 µmol Quanta m^{-2} s^{-1}, were obtained by this study. A cellular pigment content of $3.1 \cdot 10^{-5}$ ngBChl*e* cell^{-1} in cells grown at 0.1 µmol Quanta m^{-2} s^{-1} was determined. This is comparable to the *in situ* values of $1.7 - 3.5 \cdot 10^{-5}$ ng cell^{-1}. Hence, the specific cellular pigment of strain BS-1, grown at very low light intensities is doubled as compared to the brown colored reference strain *Chl. phaeobacteroides* DSM 266 (Fuhrmann et al. 1993, Overmann et al. 1992). The *in situ* values were derived from the pigment concentrations in the Black Sea chemocline and the numbers of green sulfur bacteria determined by quantitative PCR (E. Marschall, personal communication). The cellular pigment content in the cells was not increased any further under *in situ* light limiting conditions, as compared to laboratory conditions. Hence, the pigment content in the cells has reached its maximum value at 0.1 µmol Quanta m^{-2} s^{-1}.

Surprisingly, the maximum concentrations of BChl *e* in the chemocline determined in this study (2001) were decreased by a factor of 14 as compared to the value detected in 1988 (68 ng·l^{-1} versus 940 ng·l^{-1}; Bird and Karl 1991, Repeta et al. 1989, Overmann et al 1992). These values correlate with the numbers of cells detected. In 1988, the green sulfur bacteria represented 10% of the total cell numbers (Bird and Karl 1991), in 2001 only 0.5 to 1 % (E. Marschall, personal communication). Thus, the green sulfur bacterial population shows a significant decrease over the last decade. This is in accordance with the vertical displacement of their biomass maximum, from 74 m depth in 1988 to 100 m in 2001. This is supported by investigations of a location in the Black Sea basin at the northwestern slope where the chemocline is located at a depth of 140 m. Only traces of BChl e were detected at that depth. This implies that *Chl.* sp BS-1 reaches its lower limits for phototrophic growth between 100 and 140 m in the Black Sea chemocline.

Due to its capability to survive under severe light limiting conditions *Chl.* sp BS-1 is a valuable model for studying the molecular basis of low light adaptation. In this study it was shown that changes in pigment composition can be observed when *Chl.* sp BS-1 is shifted from a light intensity of 3 µmol Quanta m^{-2} s^{-1} to 0.1 µmol Quanta m^{-2} s^{-1}. These changes involve a loss of the

BChl *e* homolog [E,M]-Bchle_F accompanied by an increase in [I,E]-Bchle_F. A comparable mechanism had been observed before in cells of *Chlorobium phaeobacteroides* strain Dagow III (Glaeser et al. 2002) and may represent an adaptative trait to increase the photosynthetic efficiency. Since the alkyl side chain of porphyrin ring III is directly involved in the aggregation of BChl molecules (van Rossum et al. 2001), a high degree of alkylation leads to a red shift of the Qy absorption maximum by 7-11 nm (Borrego and Garcia-Gil 1995). This may facilitate the channeling of excitation energy towards the reaction center, resulting in an increased energy transfer efficiency of the chlorosomes (Borrego and Garcia-Gil 1995). Recently, differences in chlorosome formation in green colored green sulfur bacteria as compared to brown colored species were observed, involving carotenoids and secondary homologs of bacteriochlorophylls (Psencik et al. 2006). Psencik et al. found that brown-colored species produce rough, multi-domain lamellar chlorosomes, which may enhance their light harvesting capability. Between the lamellae, carotenoids and possibly quinones are located. It was also shown that hydrophobic interactions between carotenoids and esterifying alcohols of secondary homologs are the major driving force in aggregate assembly (Klinger et al. 2004). A low carotenoid content and a shift in bacteriochlorophyll secondary homologs thus are stereochemically essential for *Chl.* sp BS-1 at very low light conditions.

The enhanced light harvesting features of *Chl.* sp BS-1 could be helpful in the development of biomimetic solar cells in the future. First steps in this direction have already been made (Marek and Balaban 2006). Therefore, the population of *Chl.* sp. BS-1 in the Black Sea is not only the most extremely low-light-adapted population of phototrophic organisms documented to date but causes also industrial interest.

4. Subfossil 16S rRNA fingerprints in the Black Sea sediments (cf. Chapter 6)

4.1. BS-1 and other green sulfur bacteria

The Black Sea sediments were supposed to support fermentative growth of phototrophic bacteria by supplying high amounts of carbon (Hashwa and Trüper 1978). However, the high hydrostatic pressure would allow only for low turnover rates (Jannasch et al 1971, 1976).

Live cells of green sulfur bacteria could be detected in Black Sea surface sediments from 2210 m water depth during this study. This confirms earlier results of Hashwa and Trüper (1978), who obtained enrichment cultures of *Chlorobium phaeobacteroides* from sediments at 660 and 2240 m water depth. Besides the live cells, genetic fingerprints and other fossil biomarkers of green sulfur bacteria at different sediment depths should portray the history of the overlying water column. Fossil biomarkers specific for green sulfur bacteria are diaromatic carotenoids and their sulfur-linked derivatives. They have been used as indicator for the presence of green sulfur bacteria in past depositional environments, which are now located on land like the Messinian Vena del Gesso basin (Northern Appenines, Italy) (Kohnen et al. 1991).

In the present study we were able to amplify the subfossil 16S rRNA gene sequences of nine species of green sulfur bacteria from sediments of Units I, II, and III. The species could be assigned to saltwater as well as to freshwater groups of green sulfur bacteria (Chapter 7, Imhoff 2003, Alexander and Imhoff 2006). The highest diversity of phylotypes was found in sediments of Unit IIa (Chapter 7). Only three of the detected green sulfur bacterial 16S rRNA gene sequences clustered with the true marine species of green sulfur bacteria (group 1, Imhoff 2003). Two of these species fingerprints could only be found in Unit IIa, the sediment layer formed during a period of high input of terrigenous material and lower productivity (Hay 1988, Hay et al. 1991). The third green sulfur bacterial sequence was assigned to *Chl.* sp BS-1, the strain currently found as sole phylotype in the Black Sea chemocline (Chapter 4). It was found in sediments of Units I, II, and III. These results suggest a photic zone anoxia during all deposition stages. This is in contrast to earlier data, based on the typical green sulfur bacterial carotenoids isorenieratene (Repeta 1993) and sulfurized isorenieratane (Sinninghe Damsté et al. 1993, Wakeham et al. 1995), that proposed photic zone anoxia only during deposition of Units I and IIb. Our results are surprising since Unit III was deposited during a freshwater period of the Black Sea water column (Degens and Ross 1972). On the other hand, it is as well surprising to find green sulfur bacterial sequences which do not cluster with the marine species in the Black Sea sediments deposited during saltwater periods. These sequences cluster with epibionts of phototrophic consortia

(Glaeser et al. 2004) and other freshwater or low-salt adapted species (Imhoff 2003). It seems that green sulfur bacteria adapt to their environment, which is reflected in their 16S rRNA gene sequence (Chapter 7, Alexander and Imhoff 2006). Alexander and Imhoff (2006) showed that environmental clones from a saltwater environment constantly cluster with the marine species. Phylogenetic lineages of green sulfur bacteria were also found for other habitats like hot springs (this study). One phylotype found in Units II and III of the Black Sea sediments represents a ubiqitous phylotype, i.e. a phylotype found in several habitats and sediments worldwide (Chapter 7). It was also found in Mediterranean sapropel layers (Coolen and Overmann 2007). It is not surprising, though, that we found sequences of green sulfur bacteria that are alrady isolated and described, since the majority of green sulfur bacterial sequences is known today (see below). This was shown by a comprehensive phylogenetic analysis of 16S rRNA gene sequences as well as of *sigA* gene sequences, *bchG* gene sequences and ITS sequences (see below).

These findings lead to the conclusion that most of the green sulfur bacterial sequences did not derive from the Black Sea water column but are of allochthonous origin. *Chl.* sp BS-1, clustering with the saltwater species, is the only green sulfur bacterial species which is shown to be present in the chemocline as as well as in Black Sea sediments and Eastern Mediterranean sediments (E. Marschall, personal communication). The population found in the chemocline today may have their origin in the Mediterranean, and could have entered the Black Sea basin during the post-glacial sea-level rise leading to an inundation of the Black Sea basin with saltwater from the Mediterranean (Repeta 1993, Wakeham et al. 1995, Canfield et al. 1996). In the Black Sea water column, only a very small fraction of the green sulfur bacteria from the chemocline reaches the deep-sea sediments, as sediment traps (Repeta and Simpson 1991) and the vertical concentration profiles of bacterial pigments (Repeta et al. 1989, Repeta and Simpson 1991) showed. The analysis of fossil 16S rRNA gene sequences of green sulfur bacteria in sediments showed that biofossils have to be interpreted carefully. The sedimentary record does not necessarily reflect the situation in the overlying water column.

An allochthonous origin of sequences to the sediments is plausible, even a transport through oxygenated water. Gorlenko et al. (2005) enriched green colored *Chlorobiaceae* from sediments at a shallow water depth (64 m), and another green sulfur bacterium (*Chlorobium bathyomarinum*) was isolated from oxygenated water near a hydrothermal vent in the Pacific, in a water depth of ~2000 m (see below). Oxygen resistance over prolonged periods of time was shown for this strain (see below) as well as for other green sulfur bacteria (Henßge 2006, K. Zikeli, personal communication).

Nevertheless, the 16S rRNA gene sequence of the extremely low-light adapted strain *Chl.* sp BS-1 is suited as specific biomarker for photic zone anoxia, also in other parts of the world. The recently found sequence of an environmental clone showing 99% sequence similarity to *Chl.* sp BS-1 in the Sippewissett saltmarsh (Alexander and Imhoff 2006) could give more information on this topic, provided that physiological and phylogenetical investigations show that this strain is identical to *Chl.* sp BS-1. Also, other biomarker molecules such as the ITS sequence and the *sigA* gene sequence of *Chl.* sp BS-1 should be considered.

4.2. Close relatives of green sulfur bacteria

The phylogenetic analyses of 16S rRNA gene sequences derived from Black Sea sediments with primers specific for green sulfur bacteria revealed the presence of closely related sequences which do not cluster with the green sulfur bacteria but at the root of the green sulfur bacterial phylogenetic tree. The new cluster shows a high diversity with up to 33 % sequence variability. Sequence comparisons with the data base showed that these sequences were found all over the world in environmental clones from diverse habitats, but not a single strain has been isolated to date carrying that 16S rRNA fingerprint.

The enrichment culture of *Chl.* sp BS-1 obtained during this study, derived from sediment top layers at a water depth of 2210 m, did not contain “deep-branching” green sulfur bacteria but bacteria of the *Cytophaga* group and the *Fusobacteriaceae*.

Hence, growth conditions must differ from that of green sulfur bacteria, or the sequences originated from dead cells or debris. The ecological role or physiology of these bacteria therefore remains unclear. Further investigations will have to show if these bacteria are photosynthetic or have something else in common with green sulfur bacteria. Furthermore, it cannot be concluded that the Black Sea sediments represents a habitat for live “deep-branching green sulfur bacteria”.

Based on this study, first attempts were made to develop a nucleotide probe for the group of “deep-branching green sulfur bacteria“, but the high diversity within the group made it impossible to develop a probe which would detect all of the members of the group (Henßge, 2006). With the proposed oligonucleotides, bacteria of the phyla *Bacteroidetes*, *Firmicutes* und *Proteobacteria* would have also been detected. The cluster of “deep-branching green sulfur bacteria” is possibly not monophyletic but must be subdivided and positioned at the bases of different phylogenetic branches.

5. Phylogeny of green sulfur bacteria (cf. Chapter 7)

So far, the phylogenetic system of green sulfur bacteria was based on only few 16S rRNA gene sequences of green sulfur bacteria (Imhoff 2003), even though a lot more sequences were available from the data bases. The first indication of a separate cluster for marine strains came from Figueras et al. (1997). Other markers used for phylogenetic classification of green sulfur bacteria are *bchG* gene sequence analyses (Garcia-Gil et al. 2003) and FMO gene sequences (Imhoff 2003). The investigation of the Black Sea sediments, though, showed the need for the establishment of a much more comprehensive phylogenetic system of green sulfur bacteria, not only to understand the origin of the sequences found in the Black Sea sediments.

The 246 green sulfur bacterial 16S rRNA gene sequences investigated were used to create a phylogenetic tree. Rarefaction analysis showed that our investigation covers almost the whole spectrum of 16S rRNA gene sequences of green sulfur bacteria which can be found in the sampled habitats and that the cultured species represent not even half of the biodiversity of the green sulfur bacteria. Hence, newly found environmental sequences will most probably be identical to sequences already investigated. The 16S rRNA gene phylogenetic tree showed the presence of several clusters which, in most cases, match the seven clusters as described previously by Imhoff (1, 2a/b, 3a/b, 4a/b; Imhoff 2003). It supports four of the basic clusters, corresponding to the main groups 1, 2a, 2b, and 3b, as proposed by Imhoff (2003), and additional species and environmental sequences with uncertain positions. In this analysis groups 3a, 4a and 4b are clusters with only low bootstrap values. Some of their included species were positioned at a different position in the tree, depending on the algorithm used (DNAML, DNAPARS, NJ), therefore these species cannot clearly be assigned to either of the groups. This is also supported by analysis of the ITS, *sigA* and *bchG* gene sequences.

The four trees contructed from the ITS sequences, the 16S rRNA, *sigA*, and *bchG* gene sequences, were analyzed by a matrix comparison (Mantel test). Additionally, *fmoA* gene sequences withdrawn from the database where included. In the Mantel test the correlation of the 16S rRNA gene sequence with the other marker genes was analyzed by comparing the distance matrices. A high correlation was found for *bchG* and *fmoA*, a smaller, but significant correlation for the ITS region, and no significant correlation for *sigA*. *SigA* therefore represents the marker gene with the highest potential for receiving more information on phylogenetic relationships of green sulfur bacterial species. It also seems to be different from other bacterial sigma factors, which is a hint for a functional change (Gruber and Bryant, 1997). The other marker genes will not give any more information, as other studies have already shown (Garcia-Gil et al. 2003, Imhoff 2003, Alexander and Imhoff 2006). The ITS region, however, might be useful in the construction of probes for environmental samples since it is highly specific for green sulfur bacteria. They might be even more specific than 16S rRNA gene sequences, since with the primer pairs specific for the 16S RNA gene sequence close relatives of green sulfur bacteria had also been detected in Black Sea sediments (see above). Since these markers, when used separately, did not give any further information on phylogenetic distances between species, and whole genome sequences are still very rare, a concatenated tree was constructed. For that tree, sequence information of three markers (ITS, *sigA*, *bchG*) were taken together, to imitate part of the whole genome. Comparisons with the 16S rRNA tree showed that five clusters are visible, but only three of the clusters are stable, as shown by high bootstrap values.

In the group of green sulfur bacteria, phylogeny seems to be correlated to physiological adaptations. The cluster “group 1” is the most stable and distant cluster amongst the other green sulfur bacterial clusters. A close phylogenetic relationship of species in that cluster was shown before (Figueras et al. 1997) and a special physiological adaptation to saltwater is very likely (Alexander et al. 2006). Species in the cluster “group 1” are all dependent on salt in the medium (Garcia-Gil et al. 2003, Imhoff 2003), and, the other way round, species from saltwater habitats do most likely cluster within “group 1” (Alexander and Imhoff 2006). A highly coherent branching pattern of 16S rRNA tree and concatenated tree support the thesis of a common ancestor.

The cultured species in group 2a and 2b require >1% salt (Alexander et al. 2002). In this cluster, also some sequences of Mariager Fjord, a stratified marine environment (Teske et al. 1996) are placed, reflecting their low-salt adaptation. Interestingly, sequences of the Mariager Fjord are also placed within group 4a, next to species requiring low salt conditions (Alexander et al. 2002) as well as within group 1a, where species are placed which need 2-5% salt. Surprisingly, sequences from Mariager Fjord are also found in group 4b, which is occupied by cultured species without salt requirement. These sequences might be introduced allochthonously to the Fjord. An allochthonous introduction was also suspected for freshwater sequences that were found in marine sediments of the Black Sea (Chapter 6). However, sequences from Mariager Fjord are not found within group 3. Isolates that cluster within groups 3a and 3b live in rather moderate environments like the chemocline of freshwater lakes worldwide. Group 3b seems to comprise an ubiquitous phylotype, which is reflected by a high number of isolates. Species of that group also might be easy to enrich and grown in laboratory conditions. All but one of the sequences from hot springs can be found in group 4b, suggesting that species in this cluster have special physiological adaptations.

Interestingly, two species did not have the same positions in the 16S rRNA gene phylogenetic tree and the concatenated tree. Different positions of *Chl. phaeobacteroides* UdG 6047 in the concatenated and the 16S rRNA tree show a possible origin within the group of moderate freshwater species (group 3b) and a subsequent specialization like species in group 2b (>1 % salt requirement). *Chl. limicola* C1/C2 also has different positions in the concatenated tree and in the 16S rRNA tree. It could represent a primordial species, representing the root of group 4, or a more specialized species.

Some species did not cluster within the main clusters but had uncertain positions in the 16S rRNA gene phylogenetic tree. *Chl. phaeobacteroides* DSM 266^{T} and *Chl. chlorochromatii* CaD, an epibiont of phototrophic consortia (Glaeser et al. 2004; Vogl et al. 2005), are two of them. They seem to be more distant to other green sulfur bacteria, most probably due to special adaptations. Like *Chl. chlorochromatii* CaD, the 16 S rRNA gene sequences of most epibionts did not cluster with any of the distinct clusters, but had an unstable position between group 1 and the other groups. The presence of some sequences of epibionts in group 3a, could be an artifact or could be ascribed to an uncertain basis of the group.

Our analysis showed that 16S rRNA gene sequences are well suited to classify green sulfur bacteria. Nevertheless, members of the group have adapted to very special habitats or physiological needs. To account for these adaptive traits, protein coding regions proved to be useful, especially the *sigA* gene, providing additional information on phylogenetic distances. Lateral gene exchange of protein coding regions rather than 16S rRNA changes might play a major role during the process of adaption to a given habitat. The special adaptations are therefore a major driving force for the biodiversity of green sulfur bacteria. However, a correlation of the BChl *e* expression or the presence of gas vacuoles and the phylogenetic relation of species was not found, which is in contrast to an investigation by Garcia-Gil et al. (2003), which was based on only a small number of isolates. In this study, a huge set of 16S rRNA gene sequences of cultured species and environmental sequences were investigated, providing a broad basis for the discussion of green sulfur bacterial phylogeny.

On the basis of this analysis the phylogenetic system could be revised. A phylogenetic revision, based on 16S rRNA gene sequences and *fmoA* gene sequences was done recently by Imhoff (2003). It seems to be very difficult, though, to account for the phylogeny as well as for the physiological aspects of green sulfur bacteria. A system depending only on 16S rRNA gene sequences does most likely not reflect the phylogenetic history which may rest upon also on lateral gene exchange as well as on special adaptations in protein coding regions. The shortly expected whole genome sequences of several green sulfur bacteria and cultivation setups will give more insight into this genetically very shallow, but physiologically very diverse group.

6. An oxygen-tolerant green sulfur bacterium from a deep-sea hydrothermal vent (cf. Chapter 5)

In a collaborative project, a green sulfur bacterium was enriched from water samples of a location near a deep sea hydrothermal vent in the Pacific ocean. The East Pacific Rise is an area of high volcanic activity with vents that support charcteristic ecosystems (van Dover 2000, Haymon et al. 1991). The nonmotile green colored green sulfur bacterium was named *Chlorobium bathyomarinum* GSB1. Phylogenetic analyses of the FMO protein sequence and 16S rRNA gene sequence shows that this bacterium represents a previously unknown marine species (Chapter 7) and is most closely related to *Chlorobium* and *Prosthecochloris* marine species. The sample had not been taken from a site typical for green sulfur bacteria, but was taken directly from the effluent plume within 50 cm above the orifice of the TY black smoker in 2,391 m in depth. The surrounding water is oxygenated and lacks a source of reduced S and light. *Chl. bathyomarinum* GSB1, like all green sulfur bacteria, requires anaerobiosis, light, H_2S or elemental S, and CO_2 for growth. Growth experiments also showed that of 104 substances tested only acetate, propionate, peptone, and elemental S enhanced growth in minimal medium SL10. It is surprising to find green sulfur bacteria in oxygenated environments, like the vicinity of the effluent plume. Under experimental conditions the presence of light and H_2S reduced viability of cultures of *Chl. bathyomarinum* GSB1 exposed to air, but it was shown that GSB1 is resistant to exposure to air in the absence of light and H_2S. No significant loss in viability was observed even after 2 weeks, as also found with an anoxic control. The resistance of GSB1 to the toxic effects of O_2 in air is consistent with survival in the fluctuating environment of deep-sea hydrothermal vents (Fornari et al. 1998). *Chl. bathyomarinum* GSB1 may depend on the ability to survive translocation from decaying to nascent vents in the dark, oxygenated ocean depths. Green sulfur bacteria possess mechanisms that protect them from being oxidized (Frigaard and Bryant 2004, Blankenship et al. 1995), which makes the transport of live green sulfur bacteria even over long distances possible.

The strain may have traveled a long way from the coast of Costa Rica (~2,250 km), the nearest locale that could provide solar light and H_2S, or its origin may be a microbial mat (Overmann 2004, Overmann and Garcia-Pichel 2004) or similar microenvironments within centimeters of the

TY vent orifice. Microbial respiration or spontaneous chemical reactions between oxygen and reduced substances present in the vent effluent could provide anaerobiosis. Other samples, taken from different and more distant locations of the vent did not yield growth of green sulfur bacteria, supporting the origin of that strain at a vent microenvironment. The vent effluent or SO_4-reducing bacteria may supply reduced S for growth. The detection of a green-colored green sulfur bacterium at a deep-sea hydrothermal vent, but not in surrounding waters suggests that growth of green sulfur bacteria might be even supported by geothermal light. Geothermal light has been reported to support growth in previous studies (White et al. 2002). The light intensity detected was greatest at wavelengths in excess of 700 nm (i.e., thermal or blackbody radiation) (White et al. 2002). The photon flux at 750 ± 50 nm is $\approx 10^8$ photons cm^{-2} s^{-1} sr^{-1} at the orifice of a 370 °C black smoker was (White et al. 2000). This wavelength corresponds to the long wavelength absorption peak of light-harvesting BChl *c* in GSB1. The flux in the 400- to 500-nm range (short wavelength BChl and chlorobactene absorption peaks) was $\approx 10^4$ photons cm^{-2} s^{-1} sr^{-1} (White et al. 2002, White et al. 2002b) (6×10^{13} photons cm^{-2} s^{-1} = 1 µmole photons cm^{-2} s^{-1}; the term *sr* refers to a solid angle measured in steradians). Within 1–2 cm of 332 °C flange pools on black smoker chimneys, the total photon flux ($\approx 10^{11}$ photons cm^{-2} s^{-1} sr^{-1}) over the 600- to 1,000-nm range was estimated to be of the same order of magnitude as the solar photon availability for *Chl.* sp BS-1 at 80 m depth in the Black Sea (White et al. 2000, Overmann et al. 1992, Chapter 4). One major difference of these bacteria is the presence of BChl *e* and isorenieratene in the brown-colored *Chl.* sp BS-1 from the Black Sea, and the presence of BChl *c* and the absence of isorenieratene in the green-colored *Chl. bathyomarinum* GSB-1 from the TY vent. This is in accordance with the geothermal light wavelengths at the vent emitting realtively little light in the green (550 nm) region of the visible spectra. However, these extremely low light intensities would support only low rates of photosynthesis and cell divison times comparable to that of *Chl.* sp BS-1 from the Black Sea (2.8 years; Overmann et al. 1992).

The present study shows that green sulfur bacteria represent a highly diverse group of bacteria, inhabiting a multitude of habitats. Representatives have adapted to saltwater, very low light intensities and possibly also developed the ability of photosynthesis using geothermal light. Therefore this group of bacteria should be in the focus of research in future studies on photosynthesis and adaptation of species to diverse habitats.

LITERATURE

(1) **Alexander, B. and Imhoff, J. F.** 2006. Communities of green sulfur bacteria in marine and saline habitats ana lyzed by gene sequences of 16S rRNA and Fenna-Matthews-Olson protein. Int. Microbiol. 9:259-266

(2) **Anbar, A.D. and A.H. Knoll.** 2002. Proterozoic ocean chemistry and evolution: a bioinorganic bridge? Science 297: 1137-1142

(3) **Blankenship, R. E., J. M. Olson, and M. Miller.** 1995. Antenna complexes from green photosynthetic bacteria. In: Blankenship R.E., Madigan M.T., Bauer C.E. (eds) Anoxygenic photosynthetic bacteria. Kluwer, Dordrecht, pp. 399-435.

(4) **Biebl, H. and N. Pfennig.** 1978. Growth yields of green sulfur bacteria in mixed cultures with sulfur and sulfate reducing bacteria. Arch. Microbiol. 117:9-16

(5) **Bird, D.F. and Karl, D.M.** 1991. Microbial biomass and population diversity in the upper water column of the Black Sea. Deep-Sea Res. 38 (Suppl 2): S1069-S1082

(6) **Borrego, C. M. and L. J. Garcia-Gil.** 1995. Rearrangement of light harvesting bacteriochlorophyll homologs as a response of green sulfur bacteria to low light intensities. Photosynth. Res. **41:**53-65.

(7) **Canfield, D.E., Lyons, T.W., Ralswell, R.** 1996. A model for iron deposition to euxinic Black Sea sediments. Am. J. Sci. 296: 818-834

(8) **Codispoti, L. A., G. E. Friederich, J. W. Murray, and C. M. Sakamoto.** 1991. Chemical variability in the Black Sea: implications of continuous vertical profiles that penetrated the oxic/anoxic interface. Deep Sea Res. 38 (suppl.2): S691-S710.

(9) **Coolen, M. J. L. and J. Overmann.** 2007. 217 000-year-old DNA sequences of green sulfur bacteria in Mediterranean sapropels and their implications for the reconstruction of the paleoenvironment. Environ. Microbiol. 9:238-49.

(10) **Degens, E.T. and P. Stoffers.** 1976. Stratified waters as a key to the past. Nature. 263:22-27.

(11) **Fenna, R. E., B. W. Matthews, J. M. Olson, and E. K. Shaw.** 1974. Structure of a bacteriochlorophyll-protein from the green photosynthetic bacterium *Chlorobium limicola*: Crystallographic evidence for a trimer. J. Mol. Biol. 84:231-240.

(12) **Francke, C. and J. Amesz.** 1997. Isolation and pigment composition of the antenna system of four species of green sulfur bacteria. Photosynth. Res. 52:137-146.

(13) **Frigaard, N.-U.** 1997. Light-harvesting structures in green sulfur bacteria. PhD thesis. Odense University, Odense, Denmark.

(14) **Frigaard, N.-U. and D. A. Bryant.** 2004. Seeing green bacteria in a new light: genomics-enabled studies of the photosynthetic apparatus in green sulfur bacteria and filamentous anoxygenic phototrophic bacteria. Arch. Microbiol. **182**:265-276.

(15) **Fuhrmann S., J. Overmann, N. Pfennig, and U. Fischer.** 1993. Influence of vitamin B_{12} and light on the formation of chlorosomes in green- and brown-colored *Chlorobium* species. Arch. Microbiol. **160**: 193-198

(16) **Fornari, D. J., Shank, T., Damm, K. L. V., Gregg, T. K. P., Lilley, M., Levai, G., Bray, A., Haymon, R. M., Perfit, M. R. & Lutz, R.** (1998) Earth Planet Sci. Lett. **160**, 419–431.

(17) **Garrity, G. M. and J. G. Holt.** 2001. Phylum BXI. Chlorobi phy. nov. In: Boone D. R., Castenholz, R. W. (eds). Bergey's manual of systematic bacteriology, vol 1, 2nd edn. Springer, New York, Berlin, Heidelberg, pp. 601-623.

(18) **Glaeser, J., L. Bañeras, H. Rütters, and J. Overmann.** 2002. Novel bacteriochlorophyll *e* structures and species-specific variability of pigment composition in green sulfur bacteria. Arch. Microbiol. **177:**476-485.

(19) **Glaeser, J., and J. Overmann.** 2003. Characterization and *in situ* carbon metabolism of phototrophic consortia. Appl. Environ. Microbiol. **69:**3739-3750.

(20) **Glaeser, J., and J. Overmann.** 2004. Biogeography, evolution and diversity of the epibionts in phototrophic consortia. Appl. Environ. Microbiol. **70**: 4821-4830

(21) **Grice, K., R. Gibbison, J. E. Atkinson, L. Schwark, C. B. Eckhardt, and J. R. Maxwell.** 1996. Maleimides (1*H*-pyrrole-2,5-diones) as molecular indicators of anoxygenic photosynthesis in ancient water columns. Geochim. Cosmochim. Acta 60: 3913-3924

(22) **Gruber, T. M. and D. A. Bryant.** 1997. Molecular systematic studies of eubacteria, using σ^{70}-type sigma factors of group 1 and group 2. J. Bac. 179:1734-1747.

(23) **Hartgers, W. A., J. S. Sinninghe Damsté, A. G. Requejo, J. Allan, J. M. Hayes, and J.W. de Leeuw.** 1994. Evidence for only minor contributions from bacteria to sedimentary organic carbon. Nature 369: 224-227

(24) **Hashwa, F.A. and Trüper, H.G.** 1978. Viable phototrophic sulfur bacteria from the Black-Sea bottom. Helgoländer wiss. Meeresunters. 31:249-253

(25) **Hay, B.I.** 1988. Sediment accumulation in the central western Black Sea over the past 5100 years. Paleoceanogr. 3: 491-508

(26) **Hay, B.I. and Arthur, M.A.** 1991. Sediment deposition in the Holocene Black Sea. Deep-Sea Res. 38 (suppl 2): S1211-S1235

(27) **Haymon, R. M., Fornari, D., Edwards, M., Carbotte, S., Wright, D. & Macdonald, K. C.** (1991) Earth Planet Sci. Lett. **104**, 513–534.

(28) **Henßge, U.** 2006. Physiologie Grüner Schwefelbakterien und Paläomikrobiologie im Schwarzen Meer. Diplomarbeit. Department Biologie I, Bereich Mikrobiologie, LMU München

(29) **Imhoff, J. F.** 2003. Phylogenetic taxonomy of the family *Chlorobiaceae* on the basis of 16S rRNA and fmo (Fenna-Matthews-Olson protein) gene sequences. Int. J. Syst. Evol. Microbiol. **53:**941-951.

(30) **Imhoff, J. F.** 1995. Taxonomy and physiology of phototrophic purple bacteria and green sulfur bacteria, p. 1-15. In R. E. Blankenship, M. T. Madigan, and C. E. Bauer (ed.), Anoxygenic photosynthetic bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.

(31) **Jannasch, H.W., Eimhjellen, K., Wirsen, C.O., Farmanfamaian, A.** 1971. Microbial degradation of organic matter in the deep sea. Science. 171:672-675.

(32) **Jannasch, H.W., Wirsen, C.O., Taylor, C.D.** 1976. Undecompressed microbial populations from the deep sea. Appl. environ. Microbiol. 32:360-367

(33) **Jørgensen, B. B., H. Fossing, C. O. Wirsen and H. W. Jannasch.** 1991. Sulfide oxidation in the anoxic Black Sea chemocline. Deep-Sea Res. **38:**S1083-S1103.

(34) **Karl, D.M. and G.A. Knauer.** 1991. Microbial production and particle flux in the upper 350 m of the Black Sea. Deep-Sea Res. 38 (Suppl): S921-S942

(35) **Klinger, P., J. B. Arellano, F. E. Vacha, J. Hala, and J. Psencik**. 2004. Effect of carotenoids and monogalactosyl diglyceride on bacteriochlorophyll c aggregates in aqueous buffer: implications for the self-assembly of chlorosomes. Photochem. Photobiol. 80:572–578.

(36) **Kohnen, M.E.L., Schouten, S., Sinninghe Damsté, J.S., DeLeeuw, J.W., Merritt, D.A., and J.M. Hayes.** 1992. Recognition of paleochemicals by a combined molecular sulfur and isotope geochemical approach. Science 256: 358-362

(37) **Konovalov, S.K., L.I. Ivanov, and A.S. Samodurov.** 2001. Fluxes and budgets of sulphide and ammonia in the Black Sea anoxic layer. J. Mar. Syst. 31: 203-216

(38) **Konovalov, S. K., G. W. Luther, G. E. Friederich, D. B. Nuzzio, B. M. Tebo, J. W. Murray, T. Oguz, B. Glazer, R. E. Trouwborst, B. Clement, K. J. Murray, and A. S. Romanov.** 2003. Lateral injection of oxygen with the Bosporus plume – fingers of oxidizing potential in the Black Sea. Limnol. Oceanogr. **48:**2369-2376.

(39) **Koopmans, M.P., Köster, J., van Kaam-Peters, H.M.E., Kenig, F., Schouten, S., Hartgers, W.A., de Leeuw, J.W., and J.S. Sinninghe Damsté.** 1996. Diagenetic and catagenic products of isorenieratene: Molecular indicators for photic zone anoxia. Geochim. Cosmochim. Acta 60: 4467-4496

(40) **Ludwig, W., O. Strunk, S. Klugbauer, N. Klugbauer, M. Weizenegger, J. Neumann, M., Bachleitner and K. H. Schleifer.** 1998. Bacterial phylogeny based on comparative sequence analysis. Electrophoresis **19**:554-568.

(41) **Madigan, M. T.** 1988. Microbiology, physiology, and ecology of phototrophic bacteria, p 39-111. In A. J. B. Zehner (ed.), Biology of anaerobic microorganisms. John Wiley & Sons, New York, NY.

(42) **Madigan, M. T**. 1995. Microbiology of nitrogen fixation in photosynthetic bacteria, p. 915-928. In R. E. Blankenship, M. T. Madigan, and C. E. Bauer (ed.), Anoxygenic photosynthetic bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.

(43) **Marek, P. and T.S. Balaban.** 2006. Biomimetische Solarzellen. Nachrichten aus der Chemie. **54:**1072-77

(44) **Montesinos, E., R. Guerrero, C. Abella, and I. Esteve.** 1983. Ecology and physiology of the competition for light between *Chlorobium limicola and Chlorobium phaeobacteroides* in natural habitats. Appl. Environ. Microbiol. 46: 1007-1016

(45) **Murray, J. W., H. W. Jannasch, S. Honjo, R. F. Anderson, W. S. Reeburgh, Z. Top, G. E. Friederich, L. A. Codispoti, and E. Izdar.** 1989. Unexpected changes in the oxic/anoxic interface in the Black Sea. Nature **338:**411-413.

(46) **Overmann, J., H. Cypionka, and N. Pfennig.** 1992. An extremely low-light-adapted green sulfur bacterium from the Black Sea. Limnol. Oceanogr. **37**:150-155.

(47) **Overmann, J.** 1997. Mahoney Lake: a case study of the ecological significance of phototrophic sulfur bacteria. Adv. Microbial. Ecol. **15:** 251-288

(48) **Overmann, J.** 2004. in The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community (Springer, New York), http://141.150.157.117:8080/prokPUB/chaprender/jsp/showchap.jsp?chapnum=323.

(49) **Overmann, J., and F. Garcia-Pichel.** 2000. The Phototrophic Way of Life. In: Dworkin, M. et al. (eds.) The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community, 3rd edition, Springer, New York, 2000 (http://ep.springer-ny.com:6336/contents/)

(50) **Overmann, J., C. Tuschak, J. M. Fröstl, and H. Sass**. 1998. The ecological niche of the consortium *"Pelochromatium roseum"*. Arch. Microbiol. 169:120-128.

(51) **Parkin, T.B. and Brock, T.D.** 1980. Photosynthetic bacterial production in lakes: the effects of light intensity. Limnol. Oceanogr. 25: 711-718

(52) **Pfennig, N.** 1989. Ecology of phototrophic purple and green sulfur bacteria, p. 81-96. In H. G. Schlegel and B. Bowien (ed.), Autotrophic bacteria. Springer-Verlag, New York, NY.

(53) **Pierson, B. K., and J. M. Olson.** 1987. Photosynthetic bacteria, p. 21-42. In J. Amesz (ed.), Photosynthesis. Elsevier Science Publishers, B. V. Amsterdam, The Netherlands.

(54) **Psencik, J., Arellano, J.B., Ikonen, T.P., Borrego, C.M., Laurinmaki, P.A., Butcher, S.J., Serimaa, R.E., and R. Tuma.** 2006. Internal structure of chlorosomes from brown-colored chlorobium species and the role of carotenoids in their assembly. Biophys J. **91**:1433-40. Epub 2006 May 26.

(55) **Repeta, D.J., Simpson, D.J., Jørgensen, B.B., Jannasch, H.W.** 1989. Evidence for anoxygenic photosynthesis from the distribution of bacteriochlorophylls in the Black Sea. Nature. 342:69-72

(56) **Repeta, D.J. and Simpson, D.J.** 1991. The distribution and recycling of chlorophyll, bacteriochlorophyll and carotenoids in the Black Sea. Deep-Sea Res. 38 (Suppl. 2) S969-S984

(57) **Repeta, D.J.** 1993. A high resolution historical record of Holocene anoxygenic primary production in the Black Sea. Geochim. Cosmochim. Acta. 57:4337-4342

(58) **Ross D.A. and E.T. Degens.** 1970. Black Sea: Recent sedimentary history. Science **170:**163-165

(59) **Schlegel, H.** G. 1992. Allgemeine Mikrobiologie. Thieme, Stuttgart, New York.

(60) **Sinnighe Damsté, J. S., Wakeham, S. G., M. E. L. Kohnen, Hayes, J. M., and J. W. de Leeuw.** 1993. A 6,000-year sedimentary molecular record of chemocline excursions in the Black Sea. Nature. **362**:827-829.

(61) **Sirevåg, R.** 1995. Carbon metabolism in green bacteria. p.871-883. *In* R. E. Blankenship, M. T. Madigan, and C. E. Bauer (eds.), Anoxygenic photosynthetic bacteria. Advances in photosynthesis, vol. II. Kluwer Academic Publishers, Dordrecht, The Netherlands.

(62) **Sorokin, IuI.** 2002. The Black Sea. Ecology and oceanography. Backhuys Publishers, Leiden, The Netherlands.

(63) **Steensgaard, D.B., H. Wackerbarth, P. Hildebrandt, and A.R. Holzwarth** (2000) Diastereoselective control of bacteriochlorophyll *e* aggregation. 3^1-S-BChl *e* is essential for the formation of chlorosome-like aggregates. J. Phys. Chem. B **104:** 10379–10386

(64) **Tornabene, T.G., T.A. Langworthy, G. Holzer, and J. Oró.** 1979. Squalenes, phytanes and other isoprenoids as major neutral lipids of methanogenic and thermoacidophilic "Archaebacteria". J. Mol. Evol. **13:** 73-83

(65) **van der Meer, M. T. J., S. Schouten and J. S. Sinninghe Damsté.** 1998. The effect on the reversed tricarboxylic acid cycle on the ^{13}C contents of bacterial lipids. Org. Geochem. 28: 527-533

(66) **Van Dover, C. L.** (2000) The Ecology of Deep-Sea Hydrothermal Vents (Princeton Univ. Press, Princeton).

(67) **van Gemerden, H., and J. Mas.** 1995. Ecology of phototrophic sulfur bacteria, pp. 49-85. In: Blankenship R.E., Madigan M.T., and C.E. Bauer (eds), Anoxygenic photosynthetic bacteria, Advances in Photosynthesis, Vol. II. Kluwer Academic Publishers, Dordrecht, The Netherlands.

(68) **van Rossum B-J., D. B. Steensgard, F. M. Mulder, G. J. Boender, K. Schaffner, A.R. Holzwarth, and H. J. M. de Groot.** 2001. A refined model of the chlorosomal antennae of the green sulfur bacterium *Chlorobium tepidum* from proton chemical shift constraints obtained with high-field 2-D and 3-D MAS NMR dipolar correlation spectroscopy. Biochemistry **40:**1587-1595.

(69) **Vetriani, C., H.V. Tran, and L.J. Kerkhof.** 2003. Fingerprinting microbial assemblages from the oxic/anoxic chemocline of the Black Sea. Appl. Environ. Microbiol. **69:** 6481-6488.

(70) **White, S. N., Chave, A. D., Reynolds, G. T., Gaidos, E. J., Tyson, J. A. & VanDover, C. L.** (2000) Geophys. Res. Lett. **29**, 1151–1154.

(71) **White, S. N., Chave, A. D., Reynolds, G. T. & VanDover, C. L.** (2002) Geophys. Res. Lett. **29**, 10/1029/2002GL014977.

(72) **White, S. N., Chave, A. D. and G. T. Reynolds.** 2002b. J. Geophys. Res. **107**, doi:10.1029/2000JB000015.

CHAPTER 3

ANOXYGENIC PHOTOTROPHIC BACTERIA IN THE BLACK SEA CHEMOCLINE

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Currently, the Black Sea is the largest anoxic water body on the planet and represents the closest contemporary analogue to past sulfidic oceans. For the understanding of such oxic/anoxic environments, green sulfur bacteria are significant, since they (1) can substantially alter the carbon and sulfur cycles and (2) may be used as indicator organisms for the reconstruction of past photic zone anoxia. In the chemocline of the Black Sea, brown-colored green sulfur bacteria form an extremely dilute, but detectable population. Based on analysis of their 16S rRNA gene sequences, these bacteria represent a single and novel phylotype. Measurements of light intensities in the chemocline and experiments with laboratory cultures revealed that the strain from the Black Sea is extraordinarily low-light adapted and grows with doubling times of several years. Fossil 16S rRNA gene sequences of the chemocline green sulfur bacterium have been detected in different sediment layers, indicating past photic zone anoxia during which the chemocline must have been positioned at depth of up to 150 m. Although not significant in the carbon and sulfur cycles of the Black Sea, the particular type of green sulfur bacterium present in the chemocline thus represents a valuable indicator organism for the reconstruction of the paleoenvironmental conditions of the Black Sea.

Keywords: Chemocline | green sulfur bacteria | 16S rRNA | low-light adaptation | primary productivity | photic zone anoxic | paleomicrobiology

1. INTRODUCTION

In the present day ocean, anoxic sediments cover ~ 0.3% of the seafloor (44), corresponding to ~0.22% of the surface of the earth. An even smaller area of the planet is covered by sulfidic water bodies like the Black Sea (0.083%), the Cariaco Basin as the second largest basin (~0.0022%) (62), the Gotland Basin of the Baltic Sea, some smaller anoxic fjords, and numerous coastal lagoons (Chapters Ia.1, 5, 7; 8). Since 87-92% of the water body of the Black Sea is permanently anoxic (11, 38, 69), the latter currently contains the largest volume of anoxic water on the planet.

In contrast to the present situation, the entire Proterozoic ocean may have consisted of sulfidic deep water covered by a possibly 100 m-thick oxygenated surface layer. This has been inferred from the S isotopic composition of sedimentary sulfides, the fraction of reactive iron and the degree of pyritization, the evidence for decreased organic carbon burial, and a limited abundance and diversity of protists (1). The sulfidic pelagial of the Proterozoic ocean may have persisted over 1000 million years. Extended water column anoxia may have occurred also during the Phanerozoic. Derivatives of bacteriochlorophylls and carotenoids of the obligately anoxygenic phototrophic green sulfur bacteria have been detected in sediments deposited throughout this period (40), starting with late Ordovician marine black shales, and including Upper Devonian (24), Permian and Mid-Triassic shales (21) and Messinian Marl (37) sediments. From the presence and the distinct carbon isotopic composition of these bacterial biomarkers, it has been deduced that the anoxic layers in paleoceans frequently extended into the photic zone. These findings have sparked a considerable interest in the structure and function of such large oceanic oxic/anoxic environments. The Black Sea may represent the largest and closest contemporary analogue to past sulfidic oceans (15), and hence constitutes a valuable model system for the study of the biogeochemical cycles and the (micro)organisms which become relevant in these environments.

Anoxygenic phototrophic bacteria typically occur where light reaches sulfidic water layers. Among the anoxygenic phototrophic bacteria, green sulfur bacteria are especially well adapted to low-light habitats due to their large photosynthetic antennae, their lower maintenance energy requirements and higher sulfide tolerance (56). Green sulfur bacteria are significant for the understanding of oxic/anoxic oceans in two respects. Firstly, like other phototrophic sulfur bacteria, they may substantially alter the carbon and sulfur cycles by reoxidizing a major portion of the biogenic sulfide, thereby efficiently recycling electrons and feeding additional organic carbon into the pelagic carbon cycle (51). In order to be able to assess the role of green sulfur bacteria in anoxic oceans, however, the regulation of photosynthetic activity by environmental factors like light, sulfide and temperature first needs to be quantified for those types which are typical for euxinic water bodies. Secondly, green sulfur bacteria have been used as indicator organisms for past photic zone anoxia when reconstructing oceanic paleoenvironments (40) (compare chapters III.2 by Sinninghe Damsté and III.4 by Coolen). Green sulfur bacteria are especially well suited for this purpose since their photosynthetic pigments are well preserved in anoxic sediments and since the origin even of pigment derivatives can be verified by their distinct carbon isotopic signatures (37). Still, a correct interpretation of fossil green sulfur bacterial biomarkers and a detailed reconstruction of environmental conditions in ancient oceans require a better knowledge of the physiology of green sulfur bacteria typical for the marine oxic/anoxic pelagial. From a more general perspective, green sulfur bacteria represent excellent model systems for the study of low-light adaptation and of mechanisms of adaptation towards extreme energy-limited environments in general. As outlined below, the green sulfur bacteria recovered from the Black Sea chemocline represent a particularly well suited model system for the investigation of these basic scientific questions.

2. ANOXYGENIC PHOTOTROPHIC BACTERIA IN THE BLACK SEA

The first report on the occurrence of phototrophic sulfur bacteria in the Black Sea dates back to 1953, when Kriss and Rukina (41) described the enrichment of purple sulfur bacteria from the dark water layers between 500 and 2000 m depths. These findings were corroborated by microscopic observations of cells resembling *Thiocapsa roseopersicina* which occurred in water samples from 160 and 200 m depth (16). Similar reports were made by others (79), and it was suggested that these bacteria may be major primary producers in the Black Sea (66).

However, the presence of anoxygenic phototrophic bacteria in the water column could not be confirmed in two parallel studies in which pure cultures of *Chromatium warmingii* and *Thiocapsa roseopersicina* and enrichment cultures of *Chlorobium phaeovibrioides* were obtained exclusively from 660 m and 2240 m-deep sediment layers, but not from 129 different pelagic water samples (25, 30). Because of the low salt requirements for growth of these strains, and because of the absence of light and the presence of high amounts of organic carbon in the deep sediments it was proposed that the cells of phototrophic sulfur bacteria in sediments originate in coastal lagoons of the Black Sea and survive by means of a fermentative metabolism (25). Active proliferation still seems unlikely due to extremely low turnover rates at deep sea hydrostatic pressures (29, 31). The presence of large spherical cells below the chemocline observed by Dickman and Artuz (16) was confirmed more recently (4, 69). These cells constituted 5-10% of the total living biomass, but resemble *Achromatium* and thus most likely are not phototrophic.

More specific evidence for the presence of phototrophic sulfur bacteria became available more than a decade ago. Bacterial cells exhibiting autofluorescence were observed at the oxic/anoxic interface (5). Since cells of green sulfur bacteria become red fluorescent upon treatment with fixative due to the formation of free bacteriopheophytins (73), the bacterial cells accumulated in the chemocline most likely contained bacteriochlorophylls and consequently were identified as green sulfur bacteria. These cells were reported to account for 10% of the total bacterial cell number (5). Traces of different bacteriochlorophyll *e* homologues were directly detected by the U.S.-Turkish expedition on the RV Knorr in May 1988 (60). These pigments are characteristic for the obligate anaerobic photolithotrophic green sulfur bacteria *Chlorobium phaeobacteroides* and *Chl. phaeovibrioides*. Pigment concentrations reached 940 ng BChl*e* l^{-1} at a depth of 74 m in the central western basin (60).

From water samples obtained during the same U.S.-Turkish expedition on the RV Knorr, the first successful enrichment of green sulfur bacteria could be established in 1989 (strain MN1; 54). The inoculum was retrieved in the central western basin from a water depth of 80 m where oxygen and hydrogen sulfide concentrations were below detection limit. A very similar green sulfur bacterial strain (BS-1) was isolated again from chemocline water samples recovered in 2001 from a depth of 95 m in the central western basin (45).

In a recent survey of 16S rRNA genes of chemocline bacteria, no green sulfur bacteria-like sequences could be detected (75) despite the presence of the specific pigment biomarkers. This discrepancy may be attributed to the clonal bias in the *E. coli* library used for 16S rRNA gene analyses and, together with the very low concentrations of green sulfur bacterial pigments, indicate that specific detection methods need to be employed for a molecular identification of the green sulfur bacteria present in the Black Sea. Primers specific for 16S rRNA gene sequences of green sulfur bacteria have become available (55) and permit a highly selective PCR amplification of 550 bp-gene fragments from as little as 100 cells (19). Employing these specific primers and separation of the PCR products by denaturing gradient gel electrophoresis yielded a single and novel type of 16S rRNA gene sequence from the 2001 chemocline water samples, phylogenetically clustering with the other marine strains of green sulfur bacteria (Fig. 1). This sequence was identical to that from strains MN1 and BS-1, indicating that the *in situ* population exclusively consists of this one type of green sulfur bacterium which persisted at least over a time period of 13 years. The successful isolation of the green sulfur bacterium permits detailed laboratory investigations of the physiological adaptations to the specific environmental conditions in the Black Sea chemocline.

FIG. 1. Phylogenetic position of the strain BS-1, isolated from the Black Sea chemocline, within other brown colored green sulfur bacteria. The partial sequence obtained from the sediment at 109 cm below sea floor (*Env. sequence A*) is identical to that from the chemocline. *Bar* represents 0.1 fixed nucleotide exchanges.

3. ENVIRONMENTAL CONDITIONS IN THE BLACK SEA CHEMOCLINE

Within the chemocline of the Black Sea, salinity values of 20 - 21 ‰ and temperatures of 8 - 9 °C have been measured. Chemical analysis of samples frrom 70 to 100 m showed suboxic concentrations of molecular oxygen (0 - 3.8 µM), concentrations of nitrate of up to 8 µM, of nitrite < 0.3 µM, of ammonia between 0.2 and 8.8 µM, and of phosphate between 1 and 7 µM. Sulfide concentrations reach 15 µM and elemental sulfur 0.2 µM in the chemocline (12, 32, 33, 49, 60).

Whereas the above environmental factors correspond to those in other oxic/anoxic habitats, the Black Sea chemocline is characterized by a very extreme light climate. The chemocline is positioned much deeper in the water column than in other environments of phototrophic sulfur bacteria described so far. Typically, accumulations of phototrophic sulfur bacteria have been observed between 2 and 20 m, rarely up to 30 m depth (74). In such environments, values for the light transmission to populations of phototrophic sulfur bacteria range from 0.015 to 10% (57, 74). Due to the lower position of the chemocline, the *in situ* light intensities in the Black Sea were expected to be significantly lower and consequently represent the limiting factor for the growth of the green sulfur bacteria (60, 61).

A first estimate for the light transmission to the top of the population of green sulfur bacteria at 78 m depth was based on Secchi disk measurements and arrived at a value of 0.2% of surface light intensity (5). Another estimate, similarly derived from Secchi depth readings amounted to 0.0006% of total incident solar radiation, corresponding to 0.012 µmol Quanta m^{-2} s^{-1} (12). Based on a more detailed calculation which took into consideration the chlorophyll-specific attenuation coefficient (52), the light transmission reported by Repeta et al (60) and data from irradiance measurements at Odessa (54), the underwater irradiance available for photosynthesis in the Black Sea chemocline was estimated to be 0.003 µmol Quanta m^{-2} s^{-1} (max. in June 0.01 µmol Quanta m^{-2} s^{-1}). This corresponds to 0.0005 % (0.002 %) of the surface irradiance (600 µmol Quanta m^{-2} s^{-1} in winter) (54).

However, the actual values of the light intensities available to the green sulfur bacteria present in the Black Sea chemocline remained obscure until 2001, when the first direct measurement of light intensities in the Black Sea chemocline, then positioned between 90 and 110 m depth, was made with an integrating quantum meter (45). According to these recent measurements, *In situ* light intensities during winter reach 0.001 µmol Quanta m^{-2} s^{-1} in the chemocline, corresponding to 0.0007% of surface light intensity. These measurements hence confirm the earlier estimates.

4. SPECIFIC LOW-LIGHT ADAPTATION OF GREEN SULFUR BACTERIA

Previous to the discovery of green sulfur bacteria in the Black Sea chemocline, the deepest populations of green sulfur bacteria were detected at depths of 25 - 30 m in some freshwater lakes (20, 23, 48, 74). So far, all attempts to discover photosynthetic activity in natural samples from the Black Sea chemocline have failed (5, 33, 68) with only one possible exception where light-dependent $^{14}CO_2$-incorporation was observed once and at intensities above *in situ* values (12). It has therefore been questioned whether the green sulfur bacteria in this environment are at all photosynthetically active (69). As an independent line of evidence, modeling of sulfide fluxes revealed that direct and indirect oxidation by molecular oxygen accounts for most, if not all of the sulfide removal in and beneath the Black Sea chemocline (38, 39). In accordance with these results, a stimulation of sulfide oxidation by light could not be detected in natural water samples (60). As a consequence, anoxygenic photosynthesis most likely does not represent a biogeochemically important process in this system (see below). Furthermore, a large portion of microorganisms present in water layers below 50 m depth may be moribund or dead based on their low specific ATP content (5).

However, oxygenic coralline red algae have been observed at a depth of 268 m in the Caribbean where *in situ* light intensities reach only as little as 0.007-0.025 µmol Quanta m^{-2} s^{-1} (0.0005% of the surface light intensity) (42). These values are comparable to those determined for the Black Sea chemocline (see above). Since they strictly depend on sulfide-containing environments and are obligately phototrophic, at least some green sulfur bacterial lineages would be expected to have experienced a high selection pressure towards low-light adaptation. The fact that fluorescence measurements of pigment extracts demonstrated the presence of intact bacteriochlorophyll *e* pigments, together with the very low abundance of bacteriopheophytins (12, 60) contradicts the assumption that green sulfur bacterial cells in the Black Sea chemocline are in a moribund state. Further indirect evidence for a light dependence of anoxygenic photosynthetic bacteria comes from the variation of their population densities with depth of the chemocline. Whereas pigment concentrations of 940 ng BChl*e* l^{-1} were determined at 74 m at central station in the western basin, much lower concentrations of bacteriochlorophyll *e* (214 ng l^{-1}) were found closer to the Turkish coast, where the chemocline was located at a greater depth of 100 m depth (60). This pronounced decrease in the chemocline bacteriochlorophyll *e* concentrations towards the coastal sampling stations was fully confirmed by the recent comparison of the population densities of green sulfur bacteria at 100 m and 150 m depths (45).

Direct evidence for a specific adaptation of green sulfur bacteria dwelling in the chemocline of the Black Sea comes from the study of five strains of brown-colored green sulfur bacteria which were isolated from chemocline water sampled during the U.S.-Turkish expedition with the RV Knorr in May 1988 (54). All strains contained bacteriochlorophyll *e* as the main photosynthetic pigment and revealed an extreme low-light adaptation compared to 12 other green and purple sulfur bacterial strains. One isolate was chosen for a detailed study of this low-light adaptation. Under severely limiting light intensities ≤ 1 µmol Quanta m^{-2} s^{-1}, the Black Sea isolates grew significantly faster than related green sulfur bacteria. In contrast, growth rates at light saturation were lower than in other phototrophic sulfur bacteria.

Most notably, the Black Sea bacterium is capable of growing at 0.25 µmol Quanta m^{-2} s^{-1} of daylight fluorescent tubes (0.04 % of surface irradiance), which is too low to support growth of most other anoxygenic phototrophic bacteria. Only two strains of the brown-colored *Chlorobium phaeovibrioides* could also grow at this low quantum flux, albeit at much slower rate than the Black Sea strain. Since not only the growth rate, but also the sulfide oxidation rates of whole cells were significantly higher under light limitation (54), the photosynthetic reaction itself must be more efficient in the Black Sea strain, which has been attributed to an increase in light-harvesting pigments by a factor of ~2 as compared to other green sulfur bacteria. This might also be an explanation for the high concentrations of bacteriochlorophyll compared to rather low amounts of biomass detected in this water depth (5, 60). Green sulfur bacteria employ specialized intracellular structures, so-called chlorosomes, for photosynthetic light-harvesting (56). As demonstrated by ultrathin sectioning, the Black Sea strain MN1 produced two-fold larger chlorosomes than another *Chlorobium phaeobacteroides* strain (DSMZ 266^T) (18). The number of chlorosomes per cell was found to be constant and independent of light intensity. Changes in chlorosome volume, hence cellular pigment content, are due to changes in chlorosome length, but not width (18). A second unusual feature of the Black Sea green sulfur bacterium is its extraordinarily low maintenance energy requirement which is commensurate with the extremely low doubling time of 2.8 years calculated for green sulfur bacterial cells under *in situ* conditions (54). Recent assimilation experiments with ^{14}C-labeled bicarbonate revealed that the green sulfur bacteria from the Black Sea are capable of anoxygenic photosynthesis even at light intensities as low as 0.015 - 0.08 µmol Quanta m^{-2} s^{-1} depending on the light adaptation state of the culture (45).

However, growth of the green sulfur bacteria under the extremely low light intensities *in situ* may also be supported by the assimilation of organic carbon compounds. Under suboptimal light conditions, organic carbon substrates strongly influence the phototrophic growth of different green sulfur bacteria (2, 3). Green sulfur bacteria are capable of incorporating acetate into cellular components in the CO_2-dependent reaction of pyruvate synthase (64). This reaction requires reduced ferredoxin and hence depends on light for the regeneration of reducing power. While the photosynthetic rate is decreased in the presence of organic substrates, the lowest light intensity supporting growth of a brown-colored *Chlorobium phaeobacteroides* isolated from Lake Kinneret is decreased from 1.0 µmol Quanta m^{-2} s^{-1} in the absence to 0.3 µmol Quanta m^{-2} s^{-1} in the presence of acetate (2). The hypolimnion of Lake Kinneret contains acetate at concentrations of 3.3 µM (2). At present, the concentrations and types of low molecular weight organic compounds in the chemocline of the Black Sea have not been investigated.

From the *in situ* data mentioned in the preceding section, it can be calculated that each bacteriochlorophyll *e* molecule present in the chemocline layer absorbs one photon every 8 hours. The ecological situation of phototrophic organisms in the Black Sea chemocline is comparable to that of growing plants at a distance of 50 m from a little candle in an otherwise pitch black glass house. The evidence accumulated to date indicates that the particular strain of green sulfur bacteria dwelling in the Black Sea chemocline is indeed capable of exploiting this minute light intensity. The green sulfur bacterium dwelling in the Black Sea chemocline so far represents the most extreme case of low-light adaptation. It therefore represents a valuable model system for the study of the molecular basis of low light adaptation. Comparison of its 16S rRNA gene sequence with all known sequences in the databases revealed that the Black Sea bacterium so far is unique and has not been discovered in any other system (Fig. 1).

5. BIOGEOCHEMICAL SIGNIFICANCE OF ANOXYGENIC PHOTOSYNTHESIS

Karl and Knauer (34) determined a total rate of oxic primary productivity in the photic zone of 575 mg C m^{-2} day^{-1}. Because the total amount of bacteriochlorophyll *e* determined in the chemocline of the Black Sea surpasses the amount of chlorophyll *a* in the overlying oxygenated water layers, it has been argued that anoxygenic photosynthesis has become a significant process in the Black Sea carbon cycle (60, 61). In many oxic/anoxic aquatic ecosystems, anoxygenic phototrophic bacteria reoxidize a major fraction of the sulfide produced in the chemocline or in deeper water layers (74). Since the electrons from sulfide oxidation are almost completely transferred onto CO_2, anoxygenic photosynthesis couples the carbon and sulfur cycles much more efficiently than chemolithotrophic sulfide oxidation. Phototrophic sulfur bacteria hence may substantially alter the pelagic carbon and sulfur cycles (51), for example via the accumulation of a high microbial biomass which may enter the aerobic pelagic food web.

One attempt to determine the ecological significance of green sulfur bacteria in the Black Sea was made by Repeta et al. (60). They tried to translate the measured bacteriochlorophyll values into biomass with POC data (particulate organic matter) and estimated a total photosynthetic bacterial biomass of 0.5 g m^{-2} in the chemocline compared to a total phytoplankton biomass in the upper water layers of 0.9 g m^{-2}. They also detected the highest rates of H_2S oxidation *in situ* at the base of the bacteriochlorophyll maximum which shows a correlation to anoxygenic photosynthetic bacteria. However, a significant light induced increase in bacterial photosynthesis rates *in situ* could not be shown.

The light intensity determined *in situ* was 0.001 µmol Quanta m^{-2} s^{-1} in winter (Section 3). Based on the very slow growth, the lowest light intensity which could be employed in growth experiments with laboratory cultures was 0.25 µmol Quanta m^{-2} s^{-1} (54) and hence far higher than the *in situ* light intensity values. Therefore, $^{14}CO_2$ assimilation rates were recently assessed at much decreased intensities of as low as 0.006 µmol Quanta m^{-2} s^{-1}, and then used to calculate photosynthesis rates and sulfide oxidation rates (see below) in the chemocline of the Black Sea (45). These calculations yielded a rate of anaerobic primary production of 211 ngC m^{-2} day^{-1} under *in situ* conditions. Considering the integrated biomass of green sulfur bacteria in the chemocline of 798 µg BChl*e* m^{-2} (Fig. 3), equal to 2.89 mgC m^{-2}, this primary production corresponds to an average growth rate of $7.31 \cdot 10^{-5}$ d^{-1} and a doubling time of 26 years.

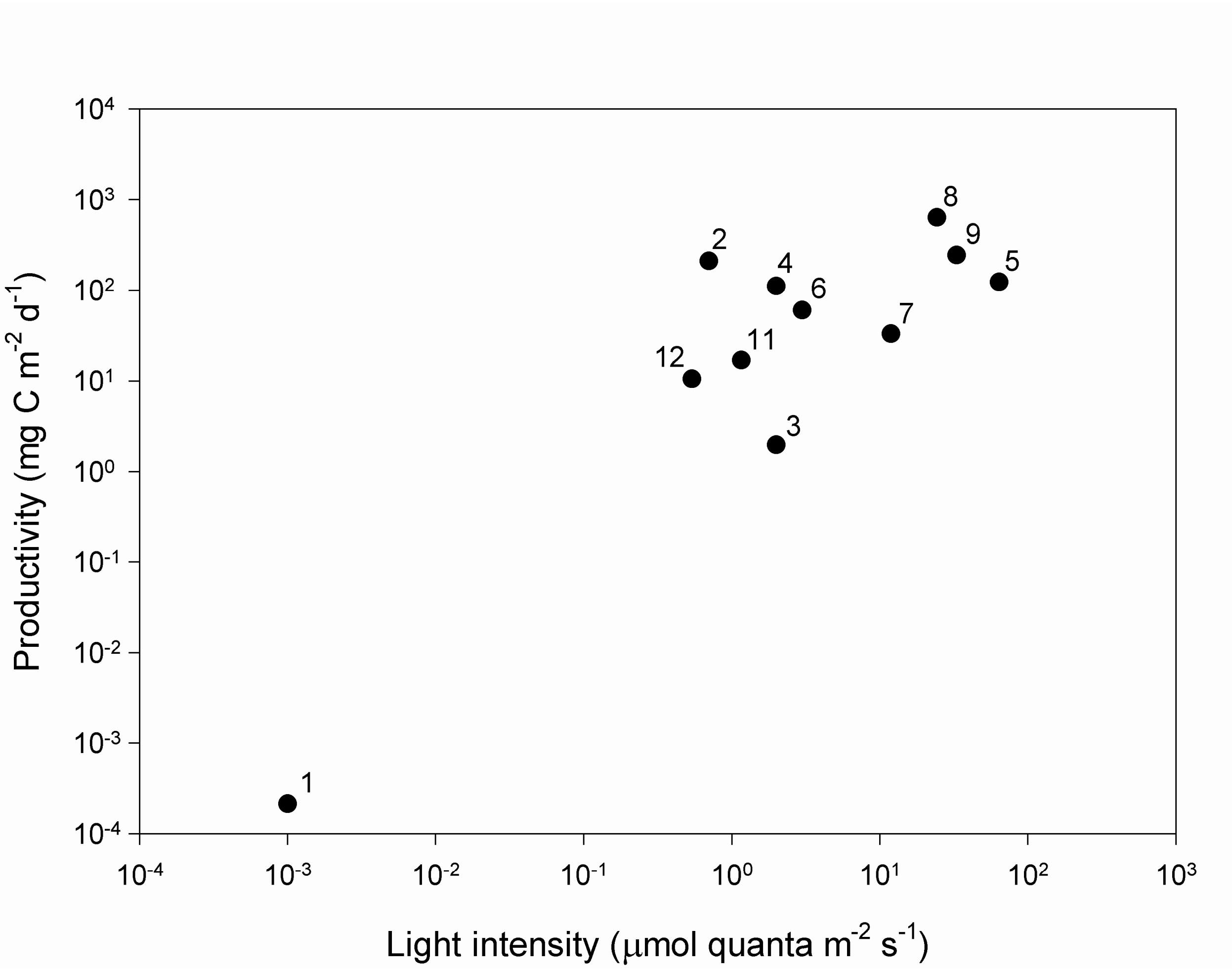

FIG. 2. Relation between productivity and available light intensities (determined directly above the bacterial layer) in different anoxygenic photosynthetic communities. 1 Black Sea (45); 2 Big Soda Lake (10); 3 Mary (6, 57); 4 Knaack Lake (58); 5 Mahoney Lake (50); 6 Mirror Lake (6, 57); 7 Rose Lake (6, 57), 8 Lake Cisó (22); 9 Lake Vilar (22); 11 Paul (6, 57); 12 Peter (6, 57).

Although experimental conditions in the laboratory cultures may be suboptimal, and anoxygenic photosynthesis will be higher during summertime, green sulfur bacteria in the chemocline of the Black Sea clearly form the least dense and slowest growing population known to date as revealed by a cross-system comparison of stratified aquatic ecosystems (Figs. 2, 3). Specific adaptations, like the assimilation of organic carbon substrates generated by accompanying bacteria could theoretically result in a higher growth rate as calculated from CO_2-incorporation alone. Auxiliary metabolic reactions and the role of interactions with accompanying bacteria should therefore be the focus of future investigations of bacterial physiology in the Black Sea chemocline.

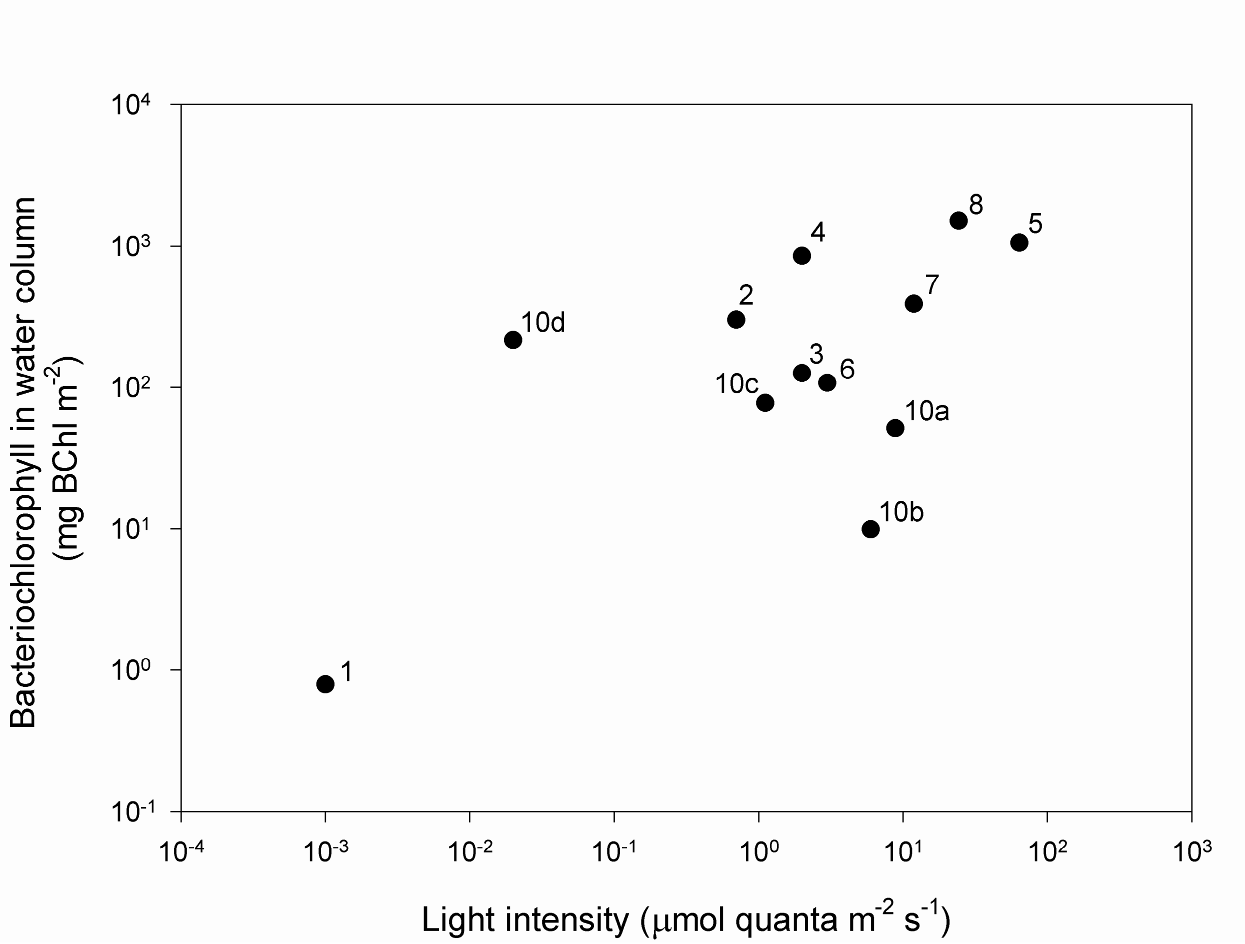

FIG. 3. Relation between bacteriochlorophyll amount in the water column and light intensity reaching the bacterial plate for different anoxygenic phototrophic communities. *1* Black Sea (45); *2* Big Soda Lake (10); *3* Mary Lake (57); *4* Knaack Lake (58); *5* Mahoney Lake (50); *6* Mirror Lake (57); *7* Rose Lake (57); *8* Lake Cisó (22, 47); *10a* Banyoles, BChl *a* plate (August); *10b* Banyoles, BChl *a* plate (May); *10c* Banyoles, BChl *e* plate (August); *10d* Banyoles, BChl *e* plate (May) (7).

From the very low growth rates calculated for green sulfur bacteria in the chemocline, it has also to be concluded that losses of these bacteria must be extraordinarily small in order to explain the persistence of these bacteria even in winter. Indeed, ciliates of the order Scuticociliatida and isokont flagellates are confined to the upper layers of the H_2S-containing zone (80). Since their population densities are rather low (maximum of 2 cells per ml), it appears unlikely that they cause significant losses of the bacterial population by grazing. Similarly, losses by sedimentation appear to be very low, since no biomarkers of green sulfur bacteria could be detected in sediment traps incubated below the chemocline of the Black Sea (see Section 6 below).

Besides CO_2-fixation, green sulfur bacteria could theoretically be significant for sulfide oxidation in the Black Sea chemocline. Jørgensen et al. (33) observed a peak maximum of H_2S oxidation in a depth of 85 m at the top of the sulfide zone. It was accompanied by a similarly sharp maximum of dark CO_2 fixation and of total bacterial numbers. Sulfide oxidation experiments with $H_2{}^{35}S$ with samples from that depth showed maximum rates of up to 1.4 mmol m^{-2} h^{-1} (33) which confirmed previous data (67). Chemical oxidation of sulfide by oxidized metals such as iron and manganese could only account for <0.1% of the measured H_2S oxidation rates as they calculated from the iron fluxes into the chemocline.

Field and laboratory data can be used to infer the significance of anoxygenic photosynthesis for sulfide turnover in the Black Sea chemocline. Based on the vertical profiles of sulfide concentrations determined at two stations in the western basin of the Black Sea (45), and employing a coefficient for turbulent diffusivity of $1.0 \cdot 10^{-5}$ m^2 s^{-1} (at 100 m depth) (38), the sulfide flux into the layer of green sulfur bacteria amounts to 0.37 – 0.53 mmol m^{-2} d^{-1}. This value is very similar to that estimated earlier for a depth of 150 m (0.421 mmol m^{-2} d^{-1}) (38). The results of modeling the upward fluxes of sulfide suggest that sulfide is actually consumed within the anoxic zone between the chemocline and 150 m depth, but not within the chemocline (38). Most likely molecular oxygen enters the anoxic zone by massive lateral injections of oxygen-enriched Mediterranean waters through the Bosporus plume, and leads to the formation and sedimentation of particulate MnO_2 in the chemocline which in turn serves as the oxidant of ~70% of the sulfide diffusing upwards (39). Together with molecular diffusion of O_2 from upper water layers (accounting for ~10% of the sulfide oxidation) (38), the fluxes of molecular oxygen thus are sufficient to explain most of the sulfide oxidation in the Black Sea. This indirect evidence suggests that anoxygenic photosynthesis is of little importance for the sulfur cycle in the Black Sea. Presently it is unknown, whether the intrusion of oxygen has direct effects on the physiological activity of green sulfur bacteria in the chemocline of the Black Sea.

Support for this conclusion comes from specific sulfide oxidation rates which were determined in laboratory cultures of the green sulfur bacterium from the chemocline of the Black Sea (54). An extrapolation from data obtained at higher light intensities to *in situ* light levels yielded sulfide oxidation rates of anoxygenic photosynthesis which could account for not more than 4 to 13% of total sulfide oxidation in the chemocline during summer (54). Using the most recent data for the rate of anoxygenic photosynthesis (211 ngC m^{-2} day^{-1}, or 17.6 nmol m^{-2} day^{-1}, see above) a sulfide oxidation rate of 8.8 – 35.2 nmolH_2S m^{-2} day^{-1} can be inferred depending on the oxidation product formed (sulfate or elemental sulfur, respectively). This range of predicted oxidation rates accounts for 0.002 - 0.01% of total sulfide oxidation in the Black Sea chemocline during winter.

Nevertheless, the significance of anoxygenic photosynthesis in the carbon and sulfur cycles may actually be higher due to a vertical displacement of the chemocline caused by internal waves (35). Such short-term exposure of green sulfur bacteria to higher quantum fluxes can significantly enhance their contribution to photosynthetic carbon assimilation (2).

6. MOLECULAR FOSSILS OF GREEN SULFUR BACTERIA AND THEIR IMPLICATIONS FOR THE RECONSTRUCTION OF THE PALEOENVIRONMENT

Caused by the postglacial sea-level rise, the Black Sea was first inundated with Mediterranean water about 9000 yr BP, leading to the deposition of a sequence of sediment horizons (9), which indicates a conversion of the freshwater lake to an anoxic marine basin (65). Most of the paleoceanographic data obtained to date cover the last 7000 – 8000 years (radiocarbon dating) of Black Sea history (69). Only few excursions yielded sediment cores long enough to document earlier periods. Two of them were the Glomar challenger deep sea drilling operation (63) and the recent excursion of the french research vessel Marion Dufresne in May 2004.

In the Black Sea, oxic freshwater conditions started 22,000 years BP and lasted for a period of over 13,000 years (14). After deposition of this sediment Unit III, a microlaminated sapropel layer formed at about 8200 yr BP (59) (or 7500, or 5000 yr BP depending on the dating method, see 77), probably indicating the onset of euxinic conditions in the center of the basin. Unit II is an organic rich (12 – 15% organic matter, 77) sapropel and was deposited during an early stage as the Black Sea and Mediterranean Sea were reconnected. In sediments on the slope, deposition of this sapropel started significantly later. The rise of the O_2-H_2S-interface from the bottom at 2200 m depth to a water depth of 500 m has been calculated from the rate of sedimentation of organic matter and on the chemical characteristics of contemporaneous sediment layers at the two depths, and probably lasted between 2300 to 3000 years (15). The bottom part of Unit II (Unit IIb) has a higher organic carbon content and was deposited during a period of higher productivity, whereas the layers above (Unit IIa) contain a higher fraction of terrigenous material deposited under a period of lower primary productivity and stronger terrestrial inputs due to an intensified erosion of the Eurasian continent (26, 27). During the deposition of Unit IIa, however, the water column was probably largely oxic (65).

Increasing salinity allowed the final invasion of the marine coccolithophorid Emiliania huxleyi between 3500 and 1600 yr BP (depending on the dating method). This event initiated the deposition of finely laminated coccolith ooze, defined as Unit I. These microlamina are thought to represent varves (63) of annual events like spring blooms (28). The sediments contain 3-5% organic carbon, with the white laminae consisting mainly of coccoliths (over 90 weight %) deposited during spring and fall blooms. The intervening dark laminae are enriched in terrigenous material. These laminated layers are separated from an earlier invasion (at the very beginning of Unit I; 65) by a several cm-thick transition sapropel which consists largely of terrigenous material and contains 9% TOC. The transition sapropel was deposited over a time interval of 400-1000 years.

Independent evidence for the vertical extent of the anoxic zone may come from fossil biomarkers of green sulfur bacteria. Diaromatic carotenoids and their sulfur-linked derivatives have been used as indicators of the presence of green sulfur bacteria in various past depositional environments, even those which are now located on land like the Messinian Vena del Gesso basin (Northern Apennines, Italy) (36). Similarly, isorenieratene (59) and sulfurized isorenieratane (65, 77) have been detected in the analyses of organic sulfur fractions from Black Sea sediments. In Unit IIb, free isorenieratene was found (17, 65, 72) as well as farnesane with $\delta^{13}C$ values typical for green sulfur bacteria which use the reverse TCA cycle for CO_2 fixation. Isorenieratene reaches ranges between 2-72 µg/gdw in Unit I and up to 1.4 µg/gdw in Unit IIb (59). Compared to free isorenieratene, sulfurized isorenieratane reached lower concentrations in Unit I (8.3 – 13.0 $\mu g \cdot (g \text{ dry weight})^{-1}$) but higher concentrations in Unit IIb (50.0 $\mu g \cdot (g \text{ dry weight})^{-1}$) (65, 77). Taken together, these data indicate that photic zone anoxia, hence a large anoxic water body, developed during the deposition of Unit IIb and Unit I.

Recently, the 16S rRNA gene sequences of *Chlorobium* sp. BS-1 have been detected in sediment layers of the Black Sea by PCR employing specific primers (46). The occurrence of fossil 16S rRNA gene sequences of the extremely low-light adapted green sulfur bacterium may now be used as a more specific biomarker to infer photic zone anoxia and hence the vertical extent of the oxic zone more specifically.

Currently, the vertical position of the anoxic interface in the Black Sea is positioned between ~60 and 200 m depending on sampling location, and also varies over time (9). The chemocline is bent towards the shelf edges similar to the situation during the deposition of Unit I and II (70, 71). If the maximum penetration of light in the marine environment is considered (42) green sulfur bacteria could theoretically colonize water layers down to 250 m depth. Since the vertical attenuation is usually higher in oxic/anoxic environments, populations of green sulfur bacteria would be expected to occur in shallower depths. In the Black Sea, a depth of 121 m has been considered the maximum depth for photoautotrophic growth (59). The compensation depth for the extremely low-light adapted green sulfur bacteria in the Black Sea may actually be even lower, since bacteriochlorophyll *e* has recently been detected even at a sampling location where the chemocline was positioned at 150 m (45). In the Cariaco Basin, where lipids and pigments of anoxygenic phototrophic bacteria are entirely absent, the oxic/anoxic interface is positioned at 275-300 m depth (43, 76). In conclusion, the presence of specific 16S rRNA gene sequence biomarkers of the low-light adapted green sulfur bacterium in a potentially oxic/anoxic marine environment most likely indicates photic zone anoxia down to a maximum of 150 m depth.

However, a more detailed analysis of the fossil DNA sequences of green sulfur bacteria present in the Black sea sediments suggests that molecular markers have to be interpreted with caution.

Firstly, in contrast to other oxic/anoxic environments with accumulations of phototrophic sulfur bacteria (53), the vertical concentration profile of bacterial pigments showed an unusually sharp drop below the chemocline, where BChl *e* concentrations declined below the detection limit at a depth of 120 m (60, 61). Secondly, chlorophyll *a*, but no pigments of green sulfur bacteria were detected in the sedimenting matter (at 87.5 hours deployment of sediment traps) (61) although the total amount of bacteriochlorophyll *e* determined in the chemocline of the Black Sea surpasses the total chlorophyll *a* in the overlying oxygenated water layers (60). Both observations indicate that only a very small fraction of the green sulfur bacteria present in the Black Sea chemocline actually reach the deep-sea sediments. Similarly, biomarkers of archaea involved in anaerobic methane oxidation could not be detected in sinking particulate matter and even not in underlying sediments (78), indicating that important bacterial or archaeal biomarkers may be entirely absent or underrepresented in Black Sea sediments. On the opposite, 16S rRNA gene sequences have been detected in sediments from the Eastern Mediterranean as old as 240,000 years (13). All the Mediterranean sequences, however, show identity or strong similarity to sequences from freshwater strains and therefore may not represent indigenous low-light indicators for photic zone anoxia. In comparison to aromatic carotenoids, fossil DNA sequences with their significantly larger information content thus permit a more differentiated view of past environmental conditions. It is also in this respect that the Black Sea serves as the most important contemporary model system for photic zone anoxia in the past.

7. REFERENCES

(1) **Anbar, A.D. and Knoll, A.H.** 2002. Proterozoic ocean chemistry and evolution: a bioinorganic bridge? Science 297: 1137-1142

(2) **Bergstein, T., Henis, Y., Cavari, B.Z.** 1979. Investigations on the photosynthetic sulfur bacterium *Chlorobium phaeobacteroides* causing seasonal blooms in Lake Kinneret. Can. J. Microbiol. 25: 999-1007

(3) **Bergstein, T., Henis, Y., Cavari, B.Z.** 1981. Uptake and metabolism of organic compounds by *Chlorobium phaeobacteroides* isolated from Lake Kinneret. Can. J. Microbiol. 27: 1087-1091

(4) **Bird, D.F. and Karl, D.M**. 1988. Microbial stratification within oxygenated, suboxic and sulfidic zones of the Black Sea. EOS 69: 1241

(5) **Bird, D.F. and Karl, D.M.** 1991. Microbial biomass and population diversity in the upper water column of the Black Sea. Deep-Sea Res. 38 (Suppl 2): S1069-S1082

(6) **Borrego, C.M., Garcia-Gil, J., Cristina, X.P., Vila, X., Abella, C.A.** 1998. Occurrence of new bacteriochlorophyll *d* forms in natural populations of green photosynthetic sulfur bacteria. FEMS Microbiol. Ecol. 26:257-267

(7) **Borrego, C.M., Bañeras, L., Garcia-Gil, J.** 1999. Temporal variability of *Chlorobium phaeobacteroides* antenna pigments in a meromictic karstic lake. Aquat. Microb. Ecol. 17:121-129

(8) **Burnett, W.C., Landing, W.M., Lyons, W.B., Orem, W.** 1989. Jellyfish Lake, Palau: A model anoxic environment for geochemical studies. EOS 70: 777-779

(9) **Canfield, D.E., Lyons, T.W., Ralswell, R.** 1996. A model for iron deposition to euxinic Black Sea sediments. Am. J. Sci. 296: 818-834

(10) **Cloern J.E., Cole B.E. and R.S. Oremland.** 1983. Autotrophic processes in meromictic Big Soda Lake, Nevada. Limnol. Oceanogr. 28:1049-1061

(11) **Codispoti, L.A., Friederich, G.E., Murray, J.W., Sakamoto, C.M.** 1991. Chemical variability in the Black Sea: implications of continuous vertical profiles that penetrated the oxic/anoxic interface. Deep Sea Res. 38 (suppl.2): S691-S710

(12) **Coble, P.A., Gagosian, R.B., Codispoti, L.A., Friedrich, G.E., Christensen, J.P.** 1991. Vertical distribution of dissolved and particulate fluorescence in the Black Sea. Deep-Sea Res. 38 (Suppl. 2): S985-S1001

(13) **Coolen M.J.L., Overmann J.** 2004. 240'000 year-old DNA sequences indicate different origins of green sulfur bacteria in mediterranean sapropels.(submitted)

(14) **Degens, E.T. and Ross, D.A.** 1972. Chronology of the Black Sea over the last 25,000 years. Chem. Geol. 10:1-16

(15) **Degens, E.T. and Stoffers, P.** 1976. Stratified waters as a key to the past. Nature. 263:22-27.

(16) **Dickman, M. and Artuz, I.** 1978. Mass mortality of photosynthetic bacteria as a mechanism for dark lamina formation in sediments of the Black Sea. Nature. 275:191-195.

(17) **Freeman, K.H., Wakeham, S.G., Hayes, J.M.** 1994. Predictive isotopic biogeochemistry: hydrocarbons from anoxic marine basins. Org. Geochem. 21:629-644.

(18) **Fuhrmann, S., Overmann, J., Pfennig, N., Fischer, U.** 1993. Influence of vitamin B_{12} and light on the formation of chlorosomes in green- and brown-colored *Chlorobium* species. Arch. Microbiol. 16: 193-198

(19) **Glaeser, J. and Overmann, J.** 2004. Biogeography, evolution and diversity of the epibionts in phototrophic consortia. Appl. Environ. Microbiol. 70: 4821-4830

(20) **Gorlenko, V.M.** 1988. Ecological niches of green sulfur and gliding bacteria. In: Olson, J.M., Ormerod, J.G., Amesz, J., Stackebrandt, E., Trüper, H.G. (eds) Green photosynthetic bacteria. Plenum Press, New York, London. P. 257-267

(21) **Grice, K., Gibbison, R., Atkinson, J.E., Schwark, L., Eckhardt, C.B., Maxwell, J.R.** 1996. Maleimides (1H-pyrrole-2,5-diones) as molecular indicators of anoxygenic photosynthesis in ancient water columns. Geochim. Cosmochim. Acta 60: 3913-3924

(22) **Guerrero, R., Montesinos, E., Pedrós-Alió, C., Esteve, I., Mas, J., Van Gemerden, H., Hofman, P.A.G., Bakker, J.F.** 1985. Phototrophic sulfur bacteria in two Spanish lakes: Vertical distribution and limiting factors. Limnol. Oceanogr. 30:919-931

(23) **Guerrero, R.., Pedrós-Alió, C., Esteve, I., Mas, J.** 1987. Communities of phototrophic bacteria in lakes of the Spanish Mediterranean region. Acta Acad. Aboensis 47: 125-151

(24) **Hartgers, W.A., Sinninghe Damsté, J.S., Requejo, A.G., Allan, J., Hayes, J.M., de Leeuw, J.W.** 1994. Evidence for only minor contributions from bacteria to sedimentary organic carbon. Nature 369: 224-227

(25) **Hashwa, F.A. and Trüper, H.G.** 1978. Viable phototrophic sulfur bacteria from the Black-Sea bottom. Helgoländer wiss. Meeresunters. 31:249-253

(26) **Hay, B.I.** 1988. Sediment accumulation in the central western Black Sea over the past 5100 years. Paleoceanogr. 3: 491-508

(27) **Hay, B.I. and Arthur, M.A.** 1991. Sediment deposition in the Holocene Black Sea. Deep-Sea Res. 38 (suppl 2): S1211-S1235

(28) **Hay, B.I., Honjo, S., Kempe, S.** 1990. Interannual variability in the particle flux in SW Black Sea. Deep-Sea Res. 37 (6A):911-928

(29) **Jannasch, H.W., Eimhjellen, K., Wirsen, C.O., Farmanfamaian, A.** 1971. Microbial degradation of organic matter in the deep sea. Science. 171:672-675.

(30) **Jannasch, H.W, Trüper, H.G., Tuttle, J.H.** 1974. Microbial sulfur cycle in Black Sea. In: Degens, E.T., Ross, D.A. (eds) The Black Sea – Geology, Chemistry, and Biology. Am. Ass. Petroleum Geologists. Tulsa, Oklahoma. 419-425 (Memoir. 20)

(31) **Jannasch, H.W., Wirsen, C.O., Taylor, C.D.** 1976. Undecompressed microbial populations from the deep sea. Appl. environ. Microbiol. 32:360-367

(32) **Jørgensen, B.B. and Fossing, H.** 1990. Black Sea Cruise, Knorr-88-2. Preliminary data report.

(33) **Jørgensen, B.B., Fossing, H., Wirsen, C.O., Jannasch, H.W.** 1991. Sulfide oxidation in the Black Sea chemocline. Deep-Sea Res. 38 (Suppl):S1083-S1103

(34) **Karl, D.M. and Knauer, G.A.** 1991. Microbial production and particle flux in the upper 350 m of the Black Sea. Deep-Sea Res. 38 (Suppl): S921-S942.

(35) **Kempe, S., Liebezeit, G., Dierks, A.-R., Asper, V.** 1990. Water balance in the Black Sea. Nature 346: 419

(36) **Kohnen, M.E.L., Sinninghe Damsté, J.S., DeLeeuw, J.W.** 1991. Biases from natural sulphurization in palaeoenvironmental reconstruction based on hydrocarbon biomarker distribution. Nature 349: 775 - 778

(37) **Kohnen, M.E.L., Schouten, S., Sinninghe Damsté, J.S., DeLeeuw, J.W., Merritt, D.A., Hayes, J.M.** 1992. Recognition of paleochemicals by a combined molecular sulfur and isotope geochemical approach. Science 256: 358-362

(38) **Konovalov, S.K., Ivanov, L.I., Samodurov, A.S.** 2001. Fluxes and budgets of sulphide and ammonia in the Black Sea anoxic layer. J. Mar. Syst. 31: 203-216

(39) **Konovalov, S.K., Luther, G.W., Friederich, G.E., Nuzzio, D.B., Tebo, B.M., Murray, J.W., Oguz, T., Glazer, B., Trouwborst, R.E., Clement, B., Murray, K.J., Romanov, A.S.** 2003. Lateral injection of oxygen with the Bosporus plume – fingers of oxidizing potential in the Black Sea. Limnol. Oceanogr. 48: 2369-2376

(40) **Koopmans, M.P., Köster, J., van Kaam-Peters, H.M.E., Kenig, F., Schouten, S., Hartgers, W.A., de Leeuw, J.W., Sinninghe Damsté, J.S.** 1996. Diagenetic and catagenic products of isorenieratene: Molecular indicators for photic zone anoxia. Geochim. Cosmochim. Acta 60: 4467-4496

(41) **Kriss, A.E. and Rukina, E.A.** 1953. Purple sulfur bacteria in deep sulfureous water of the Black Sea. Dokl. Akad. Nauk. SSSR 93: 1107-1110

(42) **Littler, M.M., Littler, D.S., Blair, S.M., Norris, J.N.** 1985. Deepest known plant life discovered on an uncharted seamount. Science 227: 57-59

(43) **Lyons, T.W., Werne, J.P., Hollander, D.J., Murray, R.W.** 2003. Contrasting sulfur geochemistry and Fe/Al and Mo/Al ratios across the last oxic-to-anoxic transition in the Cariaco Basin, Venezuela. Chem. Geol. 195: 131-157

(44) **Manheim, F.T.** 1974. Molybdenum. In: Handbook of Geochemistry (ed. K.H. Wedepohl), Springer-Verlag

(45) **Manske, A.K., Glaeser, J., Overmann, J.** 2005a. Phylogenetic identification and low light limits of green sulfur bacteria from the Black Sea chemocline (submitted).

(46) **Manske, A.K. and Overmann, J.** 2005b. Reconstruction of paleoceanography of the Black Sea based on subfossil DNA of green sulfur bacteria (submitted)

(47) **Mas, J., Pedrós-Alió, C., Guerrero, R.** 1990. *In situ* specific loss and growth rates of purple sulfur bacteria in Lake Cisó. FEMS Microbiol. Ecol. 73:271-281

(48) **Montesinos, E., Guerrero, R., Abella, C., Esteve, I.** 1983. Ecology and physiology of the competition for light between *Chlorobium limicola* and *Chlorobium phaeobacteroides* in natural habitats. Appl. Environ. Microbiol. 46: 1007-1016

(49) **Murray, J.W., Jannasch, H.W., Honjo, S., Anderson, R.F., Reeburgh, W.S., Top, Z., Friederich, G.E., Codispoti, L.A., Izdar, E.** 1989. Unexpected changes in the oxic/anoxic interface in the Black Sea. Nature 338: 411-413

(50) **Overmann, J.** 1991. Standortspezifische Anpassung bei phototrophen Schwefelbakterien. Dissertation der Universität Konstanz. ISBN 3-89191-494-6

(51) **Overmann, J.** 1997. Mahoney Lake: a case study of the ecological significance of phototrophic sulfur bacteria. Adv. Microbial. Ecol. 15: 251-288

(52) **Overmann, J. and Tilzer, M.M.** 1989. Control of primary productivity and the significance of photosynthetic bacteria in a meromictic kettle lake (Mittlerer Buchensee, West Germany). Aquat. Scie. 51:261-278

(53) **Overmann, J., Beatty, J.T., Hall, K.J., Pfennig, N., Northcote, T.G.** 1991. Characterization of a dense, purple sulfur bacterial layer in a meromictic salt lake. Limnol. Oceanogr. 36: 846-859

(54) **Overmann, J., Cypionka, H., Pfennig, N.** 1992. An extremely low-light-adapted phototrophic sulfur bacterium from the Black Sea. Limnol. Oceanogr 37:150-155

(55) **Overmann, J., Coolen, M.J.L., Tuschak, C.** 1999. Specific detection of different phylogenetic groups of chemocline bacteria based on PCR and denaturing gradient gel electrophoresis of 16S rRNA gene fragments. Arch. Microbiol. 172: 83-94

(56) **Overmann, J., Garcia-Pichel, F.** 2000. The Phototrophic Way of Life. In: Dworkin, M. et al. (eds.) The Prokaryotes: An Evolving Electronic Resource for the Microbiological

Community, 3rd edition, Springer, New York, 2000 (http://ep.springer-ny.com:6336/contents/)

(57) **Parkin, T.B. and Brock, T.D.** 1980. Photosynthetic bacterial production in lakes: the effects of light intensity. Limnol. Oceanogr. 25: 711-718

(58) **Parkin, T.B. and Brock, T.D.** 1981. Photosynthetic bacterial production and carbon mineralization in a meromictic lake. Arch. Hydrobiol. 91:366-382

(59) **Repeta, D.J.** 1993. A high resolution historical record of Holocene anoxygenic primary production in the Black Sea. Geochim. Cosmochim. Acta. 57:4337-4342

(60) **Repeta, D.J., Simpson, D.J., Jørgensen, B.B., Jannasch, H.W.** 1989. Evidence for anoxygenic photosynthesis from the distribution of bacteriochlorophylls in the Black Sea. Nature. 342:69-72

(61) **Repeta, D.J. and Simpson, D.J.** 1991. The distribution and recycling of chlorophyll, bacteriochlorophyll and carotenoids in the Black Sea. Deep-Sea Res. 38 (Suppl. 2) S969-S984

(62) **Richards, F.A.** 1975. The Cariaco Basin (Trench). Oceanogr. Mar. Biol. Ann.Rev. 13: 11-67

(63) **Ross, D.A. and Degens, E.T.** 1974. The Black Sea – Geology, Chemistry and Biology (eds Degens E. and Ross D.) 183-199. (Memoir No. 20. Am Ass. Petrol. Geol., Tulsa, Oklahoma).

(64) **Sadler, W.R. and Stanier, R.Y.** 1960. The function of acetate in photosynthesis by green bacteria. Proc. Natl. Acad. Sci. USA 46: 1328-1334

(65) **Sinninghe Damsté, J.S., Wakeham, S.G., Kohnen, M.E.L., Hayes, J.M., de Leeuw, J.W.** 1993. A 6000-year sedimentary record of chemocline excursions in the Black Sea. Nature 362: 827-829

(66) **Sorokin, Yu.I.** 1964. On primary production and microbial activity in the Black Sea. Journ. du Cons. Expl. Mer 29: 41-54

(67) **Sorokin Yu. I.** 1972. The bacterial population and the processes of hydrogen sulphide oxidation in the Black Sea. J. du Conseil Int. pour l'exploitation de la Mer. 34:423-454.

(68) **Sorokin, Yu. I.** 1982. The Black Sea: nature and resources. Nauka. Moscow. 216 pp.

(69) **Sorokin, Yu. I.** 2002. The Black Sea. Ecology and oceanography. Backhuys Publishers, Leiden, The Netherlands, 875 pp.

(70) **Spencer, D. W. and Brewer P. G.** 1971. Redox potential as control on the vertical distribution of Mn in waters of the Black Sea. J. geophys. Res. 76:5877-5892.

(71) **Spencer, D. W., Brewer, P. G., Sachs, P. L.** 1972. Aspects of the distribution and trace element composition of suspended matter in the Black Sea. Geochim. cosmochim. Acta. 36:71-86.

(72) **Summons R.E. and Powell T.G.** 1987. Identification of aryl isoprenoids in source rocks and crude oils: biological markers for the green sulfur bacteria. Geochim. Cosmochim. Acta 59:521-533

(73) **Tuschak, C., Glaeser, J., Overmann, J.** 1999. Specific detection of green sulfur bacteria by *in situ*-hybridization with a fluorescently labeled oligonucleotide probe. Arch. Microbiol. 171: 265-272

(74) **van Gemerden, H., Mas. J.** 1995. Ecology of purple sulfur bacteria. In Blankenship RE, Madigan MT, Bauer CE (Hrsg.) Anoxygenic Photosynthetic Bacteria. Kluwer Academic Publishers, S. 49-85

(75) **Vetriani, C., Tran, H.V., Kerkhof, L.J.** 2003. Fingerprinting microbial assemblages from the oxic/anoxic chemocline of the Black Sea. Appl. Environ. Microbiol. 69: 6481-6488

(76) **Wakeham, S.G.** 1995. Lipid biomarkers for heterotrophic alteration of suspended particulate organic matter in oxygenated and anoxic water columns of the ocean. Deep-Sea Res. 42: 1749-1771

(77) **Wakeham, S.G., Sinnighe Damsté, J.S., Kohnen, M.E.L., DeLeeuw, J.W.** 1995. Organic sulfur compounds formed during early diagenesis in Black Sea sediments. Geochim. Cosmochim. Acta 59: 521-533

(78) **Wakeham, S.G., Lewis, C.M., Hopmans, E.C., Schouten, S., Sinninghe Damste, J.S.** 2003. *Archaea* mediate anaerobic oxidation of methane in deep euxinic waters of the Black Sea. Geochim. Cosmochim. Acta 67: 1359 - 1374

(79) **Zenkewitch, L.** 1963. Biology of the Seas of the U.S.S.R. Allen and Unwin, London, pp.

(80) **Zubkov, M.V., Sazhin, A.F., Flint, M.V.** 1992. The microplankton organisms at the oxic-anoxic interface in the pelagial of the Black Sea. FEMS Microbiol. Ecol. 101: 245-250

CHAPTER 4

PHYSIOLOGY AND PHYLOGENY OF GREEN SULFUR BACTERIA FORMING A MONOSPECIFIC PHOTOTROPHIC ASSEMBLAGE AT 100 M DEPTH IN THE BLACK SEA

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The biomass, phylogenetic composition and photoautotrophic metabolism of green sulfur bacteria in the Black Sea was assessed *in situ* and in laboratory enrichments. In the center of the western basin, bacteriochlorophyll *e* (BChl *e*) was detected between 90 and 120 m depth and reached maxima of 54 and 68 ng l^{-1}. HPLC analysis revealed a dominance of farnesyl esters and the presence of four unusual geranyl ester homologs of BChl *e*. Only traces of BChl *e* (8 ng l^{-1}) were found at the northwestern slope of the Black Sea basin, where the chemocline was positioned at a significantly greater depth of 140 m. Stable carbon isotope fractionation values of farnesol indicated an autotrophic growth mode of the green sulfur bacteria. For the first time, light intensities in the Black Sea chemocline were determined employing an integrating quantum meter, which yielded maximum values between 0.0022 to 0.00075 µmol Quanta m^{-2} s^{-1} at the top of the green sulfur bacterial layer around solar noon in December. These values represent by far the lowest values reported for any habitat of photosynthetic organisms. Only one single 16S rRNA gene sequence type was detected in the chemocline using PCR primers specific for green sulfur bacteria. This previously unknown phylotype groups with the marine cluster of the Chlorobiaceae and was successfully enriched in a mineral medium containing sulfide, dithionite and freshly prepared yeast extract. Under precisely controlled laboratory conditions, the enriched green sulfur bacterium proved to be capable of exploiting light intensities as low as 0.015 µmol Quanta m^{-2} s^{-1} for photosynthetic $^{14}CO_2$ fixation. Calculated *in situ* doubling times of the green sulfur bacterium range between 3.1 to 26 years depending on the season, and anoxygenic photosynthesis contributes only 0.002 - 0.01 % to total sulfide oxidation in the chemocline. The stable population of green sulfur bacteria in the Black Sea chemocline thus represents the most extremely low-light-adapted and slowest growing type of phototroph known to date.

Keywords: Black Sea chemocline | green sulfur bacteria | anoxygenic photosynthesis |
low light adaptation | bacteriochlorophyll *e* | carbon cycle | sulfur cycle

INTRODUCTION

The Black Sea represents the largest extant anoxic water body worldwide. Its stratified water column comprises a ~60 m thick oxic top layer, a ~40 m thick suboxic intermediate zone devoid of sulfide and oxygen, and a ~2000 m deep sulfidic bottom zone (32). Bottom water anoxia was initiated 7000 – 8000 years ago by the intrusion of saltwater from the Mediterranean via the Bosporus strait (44). Within the subsequent 3000 years, the O_2-H_2S-interface rose from the bottom at 2200 m depth toward the surface (13). The presence of green sulfur bacterial photosynthetic pigments and their degradation products in subfossil sediments suggests that photic zone anoxia occurred already more than 6000 years ago (41, 47).

Different bacteriochlorophyll *e* (Bchl *e*) homologs, as well as the carotenoids isorenieratene and β-isorenieratene were detected in chemocline samples (43). These photosynthetic pigments are specific for brown-colored species of the green sulfur bacteria. Red autofluorescent cells became visible after fixation of chemocline samples (4). Since this autofluorescence is due to the formation of free bacteriopheophytins from bacteriochlorophylls (52), it was used to quantify green sulfur bacterial cell numbers, yielding a fraction of 10% of the total bacterial cell numbers (corresponding to $\leq 8\cdot104$ cells$\cdot$ml^{-1}; 4). Theoretically, the green sulfur bacteria could be metabolically active in the chemocline and thus be relevant for the biogeochemical cycles in the Black Sea (43).

A brown-colored green sulfur bacterium, *Chlorobium phaeobacteroides* MN1, was previously enriched from chemocline water samples (34). First growth experiments indicated that this bacterium is adapted to low-light conditions and could divide at a light intensity of 0.25 µmol Quanta m^{-2} s^{-1}, but was inhibited at ≥ 200 µmol Quanta m^{-2} s^{-1}. No data are available on the physiology of this bacterium at lower light intensities. To date, the *in situ* light conditions in the Black Sea chemocline have not been determined. It is also unknown, whether the enriched bacterium is representative for the natural assemblage of green sulfur bacteria present in the chemocline.

In order to quantify anoxygenic photosynthesis in the chemocline and to elucidate its role in the carbon and sulfur cycles of the Black Sea, the *in situ* light intensities and the photosynthetic metabolism of the dominant phototrophic organisms need to be elucidated under natural conditions. The Black Sea also serves as a model system for past oceanic anoxic events and green sulfur bacterial pigments have been used as indicators for photic zone anoxia (2, 28). A better understanding of the ecophysiology of the low-light-adapted green sulfur bacteria in the Black Sea therefore would also contribute to a more precise reconstruction of the biogeochemical processes in past sulfidic oceans.

MATERIALS AND METHODS

Study sites and sampling procedures. Water samples were obtained during cruise No. 51, Leg 51-4, of the R/V *Meteor* between December 12 and December 28, 2001. Two of the sampling sites were located in the center of the western basin. Station GeoB No. 7605 was positioned at a water depth of 2162 m (42°30.7'N, 30°14.7'E) and station GeoB No. 7620 (42°56.2'N, 30°01.9'E) at a depth of 2006 m. A third station (Station GeoB No. 7617; 43°38.9'N, 30°04.1'E) was located at the Bulgarian shelf edge at a water depth of 468 m.

Water samples were obtained by means of an integrated pumpcast-system (Seabird Electronics, Bellevue, WA) mounted on a 12-bottle Hydrobios rosette water sampler (29), allowing to sample the water column at 3 m intervals. Water samples for pigment and DNA analyses were collected in 20 L polyethylene containers, those for physiological experiments in sterile 1 L glass bottles. Bacterials cells for the extraction of genomic DNA were collected from 120 L of water by a Pellicon 2 tangential flow device (Millipore, Bedford, MA) equipped with a VVPP-C-filter (0.1 µm pore size, Millipore), and further concentrated by subsequent filtration onto polycarbonate filters (diameter 47 mm, pore size 0.1 µm, Millipore) in a sterilized filtration unit (Sartorius).

Physicochemical parameters and total cell numbers. Conductivity, temperature and molecular oxygen concentration were determined with sensors mounted to the Hydrobios water sampler. Underwater light intensities were measured with a LI-190SZ quantum sensor and a LI-200SZ pyranometer sensor, both connected to a LI-1400 data logger (LiCor Biosciences GmbH, Bad Homburg, Germany) and encased in a custom-built stainless steel pressure-proof chamber closed with a perspex lid. Light meters were calibrated at the sea surface. The equipment was then lowered through the water column down to a depth of 130 m and the integrated light intensity was recorded in vertical intervals of 10 m above the chemocline, and in intervals of 3 m within the chemocline. At each depth, integration lasted for one minute. This method increased the sensitivity of the light measurements by three orders of magnitude, resulting in a detection limit of $(3.9 \pm 2.3) \cdot 10^{-4}$ µmol Quanta m^{-2} s^{-1}.

Sulfide concentrations were determined in water samples of 20 to 50 ml. Samples were directly injected via syringe needles from the pumpcast-system into 100 ml serum bottles preloaded with 0.7 ml Zn-acetate (20%, w/v) and 0.2 ml sodium hydroxide (4 %, w/v), kept under a nitrogen atmosphere and sealed with 1 cm thick butyl rubber stoppers to avoid oxidation. Precipitated sulfide was quantified with the methylene blue method (10).

For the determination of total bacterial cell numbers, samples were fixed in 2 % glutaraldehyde and stored in autoclaved 22 ml screw cap glass tubes at 4°C. Total bacterial cell numbers were determined by epifluorescence microscopy on polycarbonate filters (Millipore) after staining with 4',6-diamidino-2-phenylindol (DAPI) (22).

Analysis of photosynthetic pigments. Seven liters from each sampling depth were filtered onto GF/F filters (Whatman) and the filters stored in the dark at -20°C. Although GF/F filters have a nominal pore size of 0.7 µm and theoretically may not retain all green sulfur bacterial cells (length, 1.8 ± 0.5 µm; width 0.65 ± 0.1 µm; see Results section), losses were found to be insignificant due to rapid clogging of the filters by manganese oxides and colloidal organic matter present in the chemocline water. Filters were lyophilized for 6 h and subsequently extracted overnight with 4 ml of a methanol:acetone mixture (2:7, v/v) (18). The solvent was evaporated by a nitrogen flow. Total BChl *e* was quantified photometrically at a wavelength of 650 nm (Lambda 25 spectrophotometer, Perkin Elmer), after redissolving the pigments in acetone, employing the molar extinction coefficient of 48.9 mM^{-1} cm^{-1} (58.6 l g^{-1} cm^{-1}; 6). For separation of different homologs, pigments were redissolved in 500 µl of acetone:methanol (1:5, v/v; for HPLC analysis). 150 µl of the sample were mixed with 15 µl of a 1 M ammonium acetate solution as ion pairing agent and injected into a Dionex HPLC system equipped with a P580 pump, an STH585 column oven, a PDA-100 photo diode array detector and an RF2000 online fluorescence detector (Dionex Softron). For separation, a Spherisorb ODS2 column (3 µm, 250 mm x 4.6 mm) in-line with a pre-column packed with the same material (CS Chromatographie Service, Langerwehe, Germany) was employed. The mobile phase consisted of a linear gradient of two different mixtures of acetonitrile, methanol, 0.01 M ammonium acetate and ethyl acetate at a flow rate of 0.7 ml min^{-1} (Method B; 1). Homologs were identified based on their retention time (18, 42). Fluorometric detection of pigments was carried out at an excitation wavelength of 476 nm and an emission at 676 nm. For standardization, extracts from *Chl. phaeovibrioides* DSMZ260^{T} and *Chl. phaeobacteroides* Dagow III (18) were used.

Lipid analysis. Particulate organic matter for lipid analyses was collected by *in situ* filtration of large volumes (~1000 L) of water through 292 mm diameter, precombusted (at 450ºC) GF/F filters using *in situ* pumps. The wet GF/F filters were extracted for 24 h with dichloromethane:methanol (7.5:1, v/v) in a Soxhlet extractor to obtain the total lipid extracts. Aliquots of the total extracts were saponified after addition of an internal standard (containing 1-nonadecanol, nonadecanoic acid, 5α-cholestane and hexatriacontane) with aqueous 0.5 N KOH in methanol (3h at 80 ºC). Nonsaponifiable (neutral lipids) and acid fractions were sequentially extracted with hexane at pH = 14 and pH = 2, respectively.

The neutral fractions were silylated with N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) in pyridine and analyzed by gas chromatography-mass spectrometry (GC-MS) for identification and quantification of farnesol. Repeated concentration measurements were within ±10%. GC-isotope ratio monitoring MS (irmMS) was performed as described previously (46).

Isotope analysis of DIC. Water samples for dissolved inorganic carbon (DIC) analysis were treated with 100 µM $HgCl_2$ to inhibit biological activity. $\delta^{13}C$-analysis of the total DIC in the Black Sea water column was performed by analysis of the headspace after the addition of 100 µl H_3PO_4 and an equilibration period of 2 hours at 40°C. The headspace was analyzed six times using a Multiflow system connected to an Isoprime irmMS system (GV instruments, Manchester, UK), with typical standard deviations of 0.1 to 0.2‰. Stable carbon isotope ratios were determined relative to lab standards calibrated on NBS-19 carbonate and CO-8 (IAEA) and reported in the Vienna Pee Dee Belemnite (VPDB) notation.

Enrichment of green sulfur bacteria. Artificial seawater medium (12) was adjusted to the ionic strength of the Black Sea chemocline. The medium contained (per liter) 14.1 g NaCl, 2.9 g $MgCl_2{\cdot}6\ H_2O$, 0.6 g $CaCl_2{\cdot}2\ H_2O$, 0.4 g KCl, 2.3 g Na_2SO_4, 2.4 g HEPES, 0.2 ml selenite-tungstate solution (58), 100 mg KBr, 24.7 mg H_3BO_3, 23.8 mg $SrCl_2$, 21.4 mg NH_4Cl, 5.4 mg KH_2PO_4, 2.9 mg NaF. After autoclaving, 1.4 mM $NaHCO_3$, 1 mM $Na_2S{\cdot}9\ H_2O$, 1 ml of trace element solution SL 10 (58), 0.1 ml of 10 vitamins solution (3) and 200 µM dithionite were added. The pH was adjusted to 7.2 and the medium distributed into 50 ml screw cap bottles. Different enrichment conditions were established by combining a variety of electron donors and carbon sources. As sulfur sources sulfide (final concentration, 1 or 2.5 mM), polysulfides (1 mM $S_{3.25}{}^{2-}$) (39) or thiosulfate (1 mM) were added. Acetate, pyruvate, glutamate, 2-oxoglutarate (each 1 mM), propionate, glycerol, malate (each 0.5 mM) served as carbon sources. Yeast extract was freshly prepared according to (30) and employed at a dilution of 1% (v/v). The substrate combinations were also tested in standard saline SL10 medium (58) and in sterile filtered chemocline water.

For inoculation, 200 ml of freshly sampled chemocline water from 95 m depth at station 7620 was filtered through nitrocellulose filters (25 mm, pore size 0.2 µm, Sartorius) and one filter each was added to the different media. All sample manipulations were done in an anaerobic chamber (AtmosBag, Sigma-Aldrich) onboard the research vessel in order to maintain anoxic conditions throughout. Enrichments were incubated at 5 µmol Quanta m^{-2} s^{-1} and at 15°C. Successful enrichments were used to inoculate deep agar dilution series (40) and liquid dilution series for purification of the cultures.

Absorption spectra of whole cells were recorded after resuspending cell pellets in 50 µl of medium which was then mixed with 500 µl of saturated sucrose solution. Absorption spectra were recorded between 400 and 1100 nm (Lambda 25, Perkin Elmer).

DNA extraction and PCR. For amplification of 16S rRNA genes from laboratory cultures, cell pellets were resuspended in 10 mM Tris·HCl (pH 8.5) and 0.5 µl of the suspension were added to each PCR reaction. Bacterial cells on filters were extracted according to Fuhrman et al. (14) and the extracts concentrated with Centricon YM-50 centrifugal filter devices (Millipore). DNA concentrations were determined using Pico Green (Molecular Probes).

The 16S rRNA gene fragments and 16S – 23 S ribosomal RNA intergenic transcribed spacer regions (ITS) of green sulfur bacteria were amplified using primer combinations specific for green sulfur bacteria (8f/GSB822r, GC341f/GSB822r, GSB822f/L1R) (36; Supplemental Tables 1 and 2). For the analysis of 16S rRNA gene sequences of chemocline bacteria, the primer combination GC341f/907r was employed (33). Standard conditions for PCR comprised 20 ng DNA or 0.5 µl of a cell suspension, 10 µM of each primer, 5 µl of GeneAMP 10x PCR buffer, 0.2 mM of each dNTP, 3.5 mM $MgCl_2$ and 2.5 U AmpliTaq Gold polymerase (Applied Biosystems) in a total volume of 50 µl. All reactions were run in a GeneAMP 9600 thermocycler PCR system (Applied Biosystems). Amplification products were analyzed by standard agarose gel electrophoresis.

Denaturing gradient gel electrophoresis (DGGE). PCR products were loaded onto 6 % (w/v) polyacrylamide gels in 1x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 7.4) containing a linear gradient of formamide and urea as denaturant (33). Gels were stained with SybrGold (Molecular Probes) for 45 min, the gel images were captured with a digital camera (Spot RT color, Diagnostic Instruments Inc., MI) and processed with the Spot RT software (version 3.1). Individual DNA bands were excised from the gel and eluted in 40 µl of 10 mM Tris·HCl (pH 8.5) at 65°C for 45 min. For subsequent sequencing 1 µl of the eluate was reamplified. The reamplification products were purified with the QiaQuick PCR purification kit (Qiagen).

Supplemental Table 1. Primers used in the present study. Numbering of the primers refers to the accordant position in the *E. coli* 16S rRNA gene sequence. L1r binds to the 5´end of the 23S rRNA gene.

Primer name	Sequence	Reference
8f	5'-AGA GTT TGA TCC TGG CTC AG-3'	Lane 1991
GC341f	5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG-3'	Muyzer et al. 1993
Uni341f	5'-CC TAC GGG AGG CAG CAG-3'	Muyzer et al. 1993
Uni515f	5'-GTG CCA GCA GCC GCG G-3'	Lane 1991, modified
517r	5'-ATT ACC GCG GCT GCT GGC-3'	Lane 1991
GSB532f	5'-GAT ACA GGG GTG GCA-3'	Overmann et al. 1999
GSB532r	5'-TGC CAC CCC TGT ATC-3'	Overmann et al. 1999
GSB822f	5'-AAT ACT AGA TGT TGG TCA T-3'	Overmann et al. 1999
GSB822r	5'-ATG ACC AAC ATC TAG TAT T-3'	Overmann et al. 1999
Uni907r	5'-CCG TCA ATT CCT TTG AGT TT-3'	Lane 1991
926f	5'-AAA CTC AAA GGA ATT GAC GG-3'	Lane 1991
1055r	5'-AGC TGA CGA CAG CCA T-3'	Amann et al. 1995
Uni1492r	5'-GGT TAC CTT GTT ACG ACT T-3'	Weisburg et al. 1991
1525f	5'-GG(CT) TGG A(GC)C ACC TCC TT-3'	Lane 1991
L1r	5'-CAA GGC ATC CAC CGT-3'	Aakra et al. 1999

Supplemental Table 2. PCR conditions and step down PCR[a]

Primer combination	Annealing (Step down PCR)		Extension
Specific for green sulfur bacteria			
8f – GSB822r, GC341f – GSB822r, Uni341f – GSB822r	55°C, 45 sec (10x)	50°C, 45 sec (30x)	72°C, 30 sec
GSB532f – GSB822r	55°C, 45 sec (10x)	50°C, 45 sec (25x)	72°C, 30 sec
Uni515f – GSB822r	60°C, 45 sec (10x)	55°C, 45 sec (30x)	72°C, 30 sec
Uni341f – GSB532r	57°C, 45 sec (10x)	52°C, 45 sec (25x)	72°C, 30 sec
GSB822f –L1r	55°C, 60 sec (10x)	50°C, 60 sec (25x)	72°C, 60 sec
Universal			
GC341f – Uni907r	61°C, 45 sec (10x)	56°C, 45 sec (20x)	72°C, 60 sec
Uni515f – 1055r	63°C, 60 sec (10x)	57°C, 60 sec (25x)	72°C, 60 sec

[a] Each PCR started with a hot start (95°C, 5 min) and ended with a 4°C hold. Each cycle was preceded by a melting step (94°C, 30 sec).

Sequencing and phylogenetic analyses. For sequencing of 16S rRNA genes, primers 8f, 517r, 341f, 515f, GSB532f, GSB822r, 907r, 926f, 1055r, 1492r were used (Supplemental Table 1). The ITS region was sequenced with the primers 1525f and L1r (Supplemental Table 1). Cycle sequencing was performed with the AmpliTaq FS BigDyeTerminator cycle sequencing kit (Applied Biosystems) following the protocol supplied by the manufacturer. Samples were analyzed on a capillary sequencer (ABI Prism377 DNA sequencer, Applied Biosystems).

The 16S rRNA gene sequences were analyzed using the ARB phylogeny software package (31). The Fast Aligner V1.03 tool was used for automatic alignment. The resulting alignments were then corrected based on the 16S rRNA secondary structure information for *Chlorobium vibrioforme* DSMZ 260^T, as available through The Comparative RNA Web (CRW) Site (www.rna.icmb.utexas.edu; 9). Phylogenetic trees were constructed including 16S rRNA gene sequences of available strains and environmental sequences. First, sequences longer than 1100 bp were used for the calculation, employing the MAXIMUM LIKELIHOOD algorithm (Fast DNA_ML). The shorter environmental sequences were inserted afterwards without changing overall tree topology employing the PARSIMONY INTERACTIVE tool implemented in the ARB software package.

Light dependence of photosynthesis. Green sulfur bacterial cells were grown to a titer of 10^8 – 10^9 cells ml^{-1} in artificial seawater medium supplemented with 200 µM dithionite and 1 mM malate at 0.1 or 3 µmol Quanta m^{-2} s^{-1}. The protein content of the cultures was determined (21) and the fraction of green sulfur bacteria was determined by fluorescence *in situ* hybridization, using the green sulfur bacteria-specific Cy3-labeled probe GSB-532 (MWG, Ebersberg, Germany) (52).

Photosynthetic rates were determined in a light cabinet consisting of seven compartments with light intensities adjusted between 0.006 and 11.3 µmol Quanta m^{-2} s^{-1}. The compartments were illuminated from below by two daylight fluorescent tubes (Osram daylight 5000 de luxe, 18 W). Aliquots of the enrichment culture were distributed into 22 ml glass scintillation vials and sealed with screw caps and teflon-coated butyl rubber septa. Four parallels were incubated at each light intensity. Dark controls consisted of vials coated with two layers of aluminum foil. The average light intensity I_a in the culture vials was calculated from the light intensity at the bottom of the vial (I_1) and at its top (I_2, measured in a vial without screw cap), according to

$$I_a = (I_1 - I_2) / \ln (I_1/I_2) \qquad (1)$$

(55). Each vial was supplemented with 370 kBq of [^{14}C]-sodium bicarbonate (Hartmann Analytics, Braunschweig, Germany) and incubated for 6.5 hours at 15°C. After incubation, 2 to 4 ml aliquots were filtered onto nitrocellulose filters (pore size 0.22 µm, diameter 23 mm; Sartorius), the filters were transferred to scintillation tubes and inorganic carbonates were removed by acidification with hydrochloric acid. After evaporation of the acid, the filters were air dried before adding 10 ml of scintillation solution (Ultima Gold F; Packard). For the determination of total radioactivity, 500 µl aliquots from each vial were mixed with 10 ml scintillation liquid (Ultima Gold; Packard). Radioactivity was determined in a liquid scintillation counter (Packard Tri-Carb, GMI, Albertville, MN).

As a reference strain, the brown-colored *Chlorobium phaeovibrioides* DSMZ 269^T was used since this strain is the most low-light-adapted strain amongst all cultured green sulfur bacterial species (34). Cultures were grown at 3 µmol Quanta m^{-2} s^{-1} in standard saline SL 10 medium (34) supplemented with 1 mM acetate.

Nucleotide accession numbers. 16S rRNA gene and ITS sequences obtained in this study are available at the EMBL under accession numbers AJ972456 and AM039431, respectively.

RESULTS

Environmental conditions and total bacterial cell counts in the Black Sea water column. At all three stations, the typical stratification of the Black Sea water column was observed (Fig. 1). According to conductivity and temperature profiles the thermocline and halocline were positioned between 50 and 60 m depth at stations 7605 and 7620 (Fig. 1A and 1D). Anoxic conditions prevailed below 80 m depth and hydrogen sulfide was detected at 100 m and 120 m at stations 7605 and 7620, respectively (Fig. 1C and 1F). The oxic and sulfidic zones were thus separated by a suboxic zone of 20 – 30 m thickness. Light intensities decreased exponentially at both stations (Fig. 1C and 1F). The lowest light intensities which were detectable with the integrating quantum meter amounted to $5.7 \cdot 10^{-4}$ µmol Quanta m^{-2} s^{-1} (station 7605) and $3.9 \cdot 10^{-4}$ (station 7620) at a depth of 108 and 105 m, respectively.

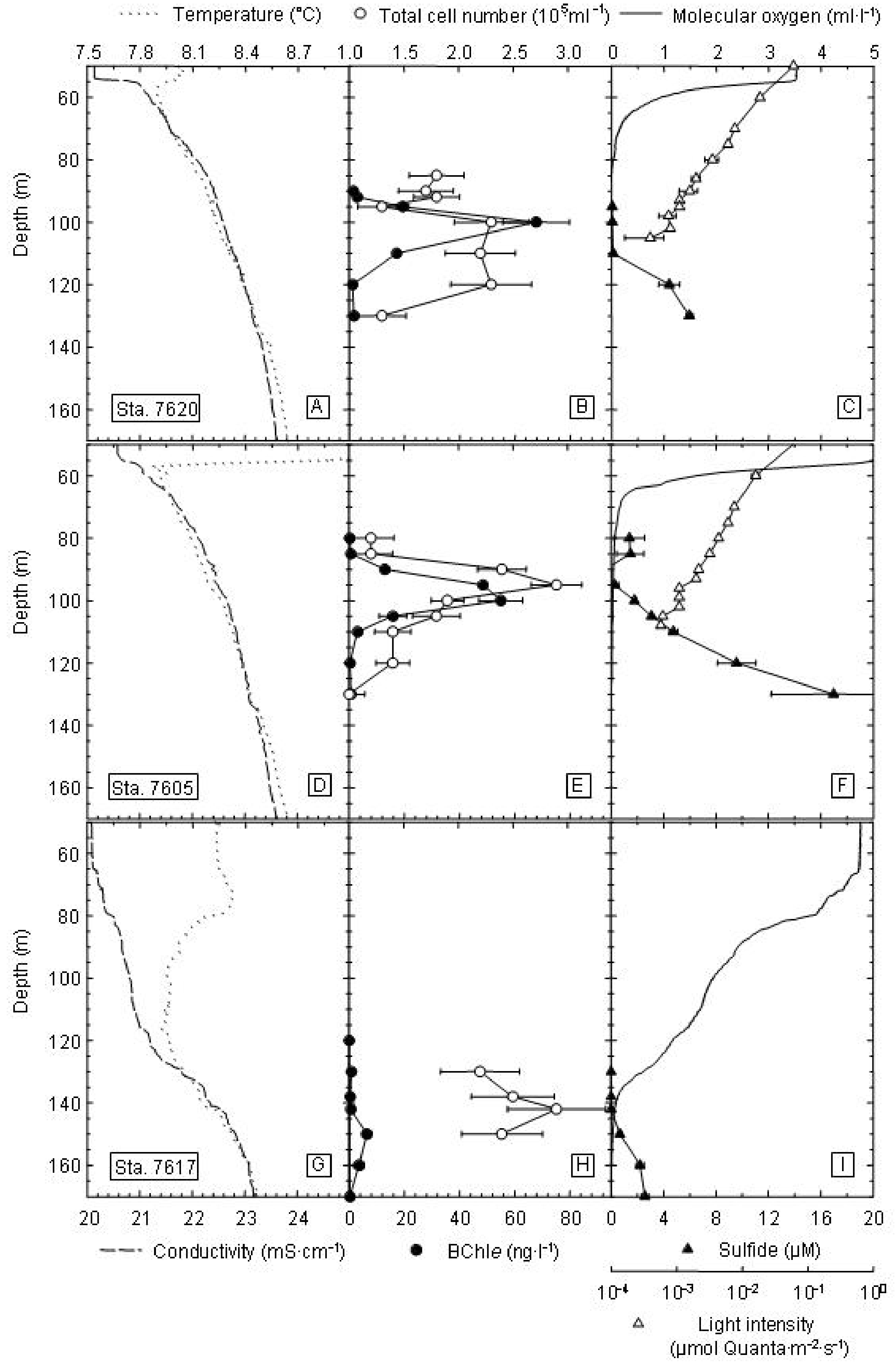

Figure 1. Depth profiles of physicochemical parameters and bacterial biomass in the chemocline of the Black Sea at two stations in the western anoxic basin of the Black Sea (Sta. 7620 and Sta. 7605) and one station at the edge of the northwestern shelf (Sta. 7617). Horizontal bars denote standard deviation.

The chemocline in the Black Sea is bowed, reaching the shallowest depths in the gyre centers. Towards the shelf areas, the discharge of the Danube, Dnjepr and Dnjestr rivers lead to an accumulation of freshwater, causing a deeper position of the chemocline and enhanced vertical mixing (49). Accordingly, the chemocline was located at significantly greater depth at the edge of the northwestern shelf (station 7617) than at the deep-water sites. Temperature and conductivity gradients were less pronounced, and the steepest increase in conductivity was observed at 130 m (Fig. 1G). The oxic and the sulfidic zones overlapped slightly at 140 m depth (Fig. 1I). In contrast to the other two stations, underwater light intensity in the chemocline was below the detection limit.

Maximum bacterial cell numbers were comparable between the three stations, ranging from $2.3 \cdot 10^5$ to $3 \cdot 10^5$ cells ml^{-1}. At stations 7620 and 7605, maximum cell counts were detected at the top of the sulfidic zone at 100 - 120 m depth and at 95 m depth (Fig. 1B, E). At station 7617, comparable cell numbers were determined at the oxic/anoxic interface in 140 m depth (Fig. 1H).

Green sulfur bacteria in the chemocline. In the center of the western basin, green sulfur bacteria reached their biomass maximum at 100 m depth, where maximum concentrations of BChl *e* were between 54 and 68 ng l^{-1} (Fig. 1B and 1E). The integrated BChl *e* content of the Black Sea water column was 701 µg BChl *e* m^{-2} (Sta. 7605) and 798 µg BChl *e* m^{-2} (Sta. 7620). In pronounced contrast, a value of only 8 ngBChl*e* l^{-1} and an integrated amount of BChl *e* of 113 µg BChl *e* m^{-2} were measured at 140 m depth at station 7617 (Fig. 1H).

A series of BChl *e* homologs were detected by HPLC analysis of particulates from the chemocline (Fig. 2C). Dominant homologs were the ethyl/ethyl, propyl/ethyl and isobutyl/ethyl homologs of BChl*e* farnesyl esters ([E,E]-BChl e_F, [P,E]-BChl e_F, [I,E]-BChl e_F), which together constituted 82.7 % of the total BChl*e*. In addition, considerable amounts of the geranyl ester isobutyl/ethyl [I,E]-BChl e_G with its typical very short retention time (42) were detected (Fig. 2C). Only traces of secondary BChl*e* homologs (mostly ethyl/ethyl-BChl e_{Hen} and isobutyl/ethyl-BChl e_{Hen}, esterified with hexadecenol) were present.

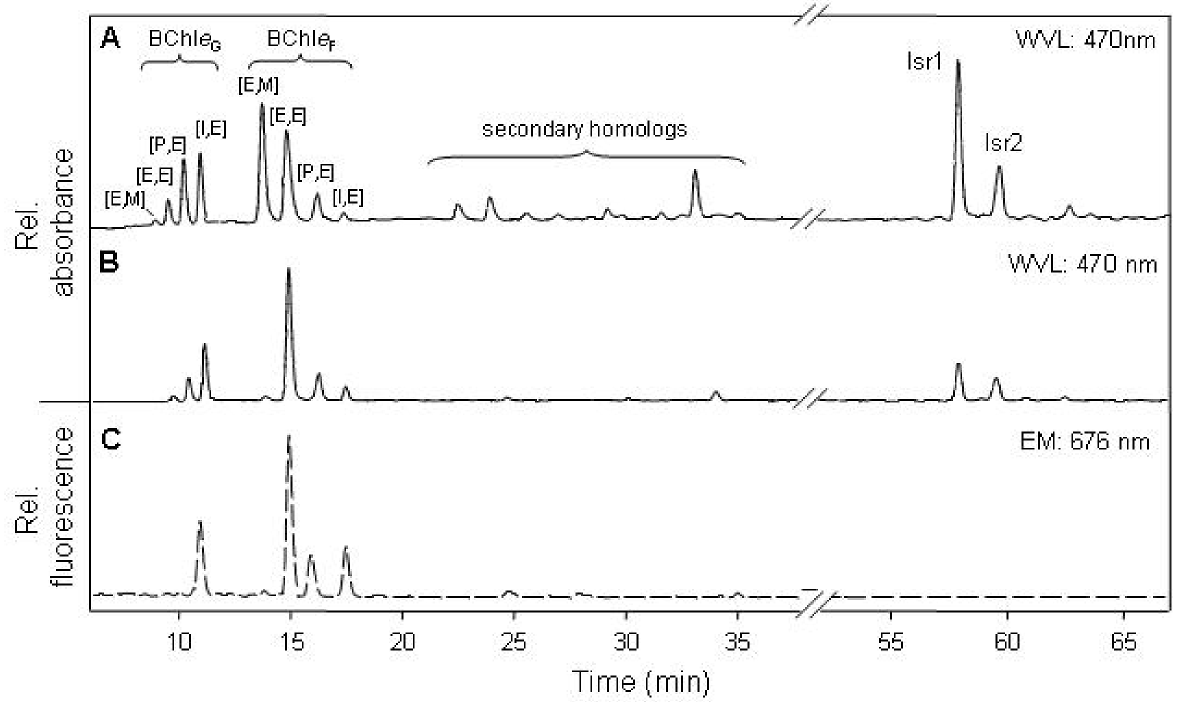

Figure 2. Comparison of HPLC traces of photosynthetic pigments extracted from BS-1 enrichment cultures and from a chemocline water sample. **A**. Pigment extract from a BS-1 culture grown at 3 µmol Quanta m^{-2} s^{-1}. Absorption detected at 470 nm. **B.** Pigment extract from a BS-1 culture grown at 0.1 µmol Quanta m^{-2} s^{-1}. Absorption detected at 470 nm. **C.** Pigment extract from a chemocline water sample from 100 m depth (Station 7620). Due to the low concentrations in these samples, tetrapyrrol pigments could only be detected by fluorescence. Excitation wavelength was set to 476 nm, emission was recorded at 676 nm. The tentatively identified (see text) geraniol homologs ethyl/methyl [E,M]-BChl e_G, ethyl/ethyl [E,E]-BChl e_G, propyl/ethyl [P,E]-BChl e_G and isobutyl/ethyl [I,E]-BChl e_G eluted first. Homologs esterified with farnesol were ethyl/methyl [E,M]-BChl e_F, ethyl/ethyl [E,E]-BChl e_F, propyl/ethyl [P,E]-BChl e_F, isobutyl/ethyl [I,E]-BChl e_F. Trace amounts of the secondary homologs (mostly ethyl/ethyl-BChle_{HEN} and isobutyl / ethyl -BChle_{HEN} esterified with hexadecenal) were also detected. Isr1, isorenieratene, Isr2, β-isorenieratene.

Farnesol concentrations in the chemocline reached maxima between 0.90 and 5.89 ng l^{-1} in the center of the western basin. $\delta^{13}C$ values for farnesol determined at the depth of the maximum farnesol concentrations were -12.1‰ and -11.4‰ (vs. VPDB) at stations 7605 and 7620, respectively. The $\delta^{13}C$ values of dissolved inorganic carbon (DIC) were -1.3 to -1.4‰ in the chemocline, corresponding to $\delta^{13}C$ values of dissolved CO_2 of -12.3 to -12.4‰ (45).

16S rRNA gene fingerprinting of green sulfur bacteria revealed the presence of only one single phylotype (melting type 5, Fig. 3A). The nucleotide sequence of this fragment grouped with the marine cluster of the *Chlorobiaceae* and was most closely related to environmental clones recovered from various marine habitats (Fig. 3B). Most notably, it was identical to the sequences of the phylotype BS-1 enriched in the present study (see below), and of *Chlorobium phaeobacteroides* MN1, which had been enriched 13 years earlier from the Black Sea chemocline (34) (Fig. 3B).

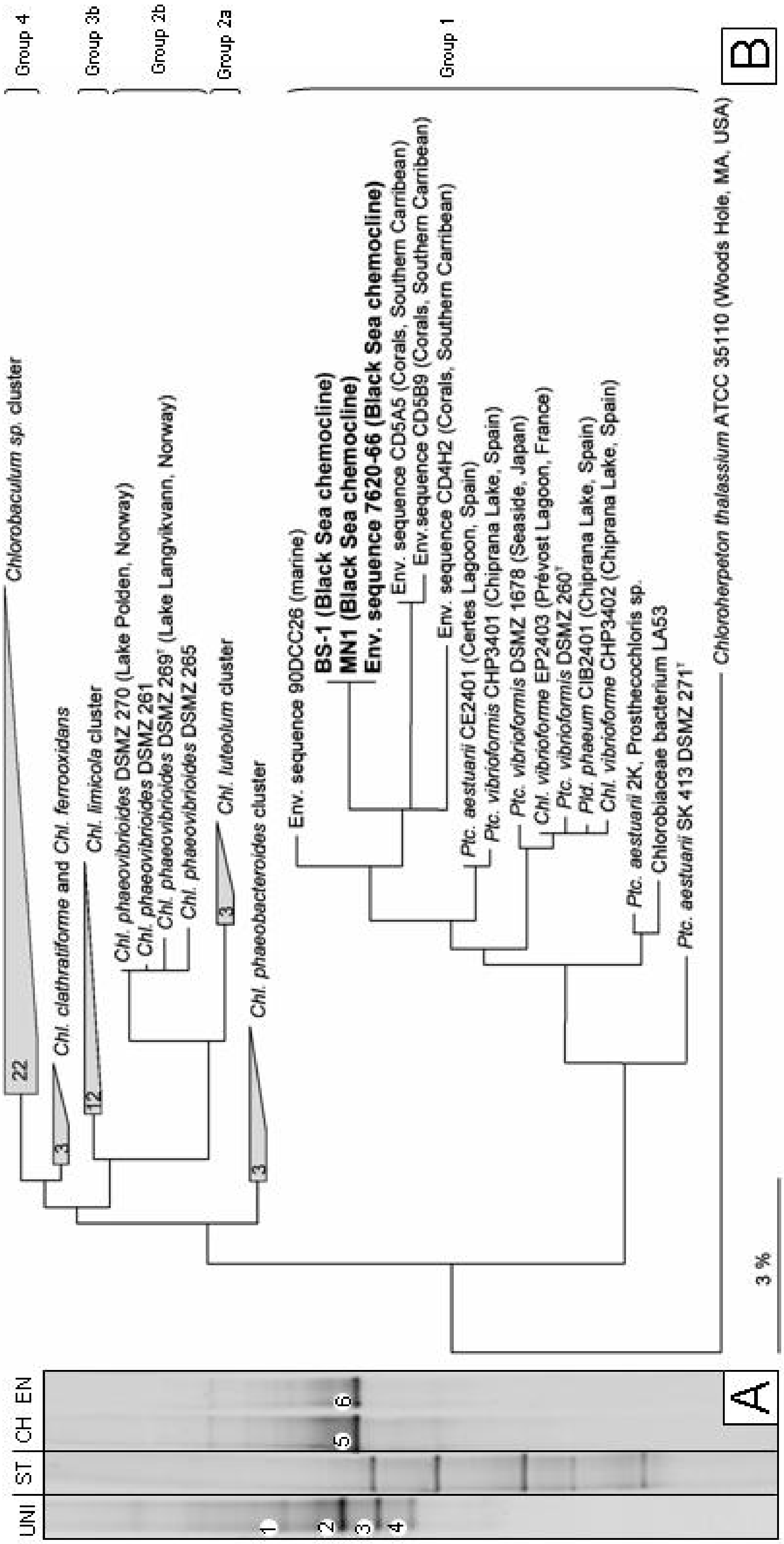

Figure 3. A. DGGE fingerprinting of the 16S rRNA gene fragment amplified with green sulfur bacterial primers from the chemocline (band 5, lane CH), as compared to the fingerprint from BS-1 in the green sulfur bacterial enrichment culture (band 6, lane EN). Bacterial fingerprints amplified with primer pair GC341f/907r are depicted in lane UNI. Melting type 2 corresponded to the chemocline green sulfur bacterium. In addition, 16S rRNA gene sequences of two *Cytophaga* (bands no. 1 and 3, 97% similarity to Genbank Accession No. AJ011658, and 96% similarity to AJ296576, respectively) and a relative of *Ilyobacter polytrophus* DSM 2926^T (94% similarity, band 4) were identified. *ST* DGGE-Standard. **B.** Phylogenetic position of the green sulfur bacteria BS-1 and MN1 enriched from the chemocline of the Black Sea and of the sequence obtained directly from the chemocline (7620-66, from band 5). Sequences retrieved in the present study given in bold face. For comparison, phylogenetic subgroups as proposed by Imhoff (23) are indicated. Bar denotes fixed substitutions per nucleotide.

Selective enrichment of green sulfur bacteria. Using chemocline water samples collected at a depth of 100 m at station 7620, the green sulfur bacterium could be selectively enriched. A total of 29 combinations of electron donors and carbon substrates were tested in two different media and in sterile filtered chemocline water. Of all the combinations, only one single enrichment culture was successful, indicating a very low culturability of the cells in natural samples. This particular cultivation medium contained artificial seawater medium supplemented with 2.5 mM sulfide and 1% (v/v) of freshly prepared yeast extract solution. In contrast, no enrichment was observed in media containing 1 mM sulfide, 1 mM polysulfides or 1 mM thiosulfate, or a combination of 1 mM thiosulfate plus 1 mM acetate, 1 mM polysulfides plus 1 mM acetate, or in media containing 1 mM acetate, 0.5 mM propionate, 0.5 mM glycerol, 0.5 mM malate or a carbon source mixture (0.5 mM pyruvate, 0.5 mM glutamate, 0.5 mM 2-oxoglutarate). Similarly, fermented rumen extract (1% , v/v, of the extract) yielded no enrichment.

Unlike other green sulfur bacteria, the bacterium from the chemocline did never grow in deep agar dilution series. However, the enrichment culture could be partially purified in liquid dilution series. A total of 68 different carbon substrates were tested for stimulation of growth of the green sulfur bacterium. In contrast to the primary enrichment experiments, growth in culture could be stimulated by addition of either malate, acetate, L-aspartate or L-glutamate in the presence of sulfide as electron-donating substrate. Therefore, sulfide was employed as electron donor and 1 mM malate was routinely added as a carbon source for subsequent cultivation and physiological characterization.

DGGE analyses of the enrichment confirmed that the green sulfur bacterium present in the chemocline of the Black Sea had been selectively enriched. When the 16S rRNA gene fragment of the green sulfur bacterium was specifically amplified with primer pair GC341f/ GSB822, its melting behavior was identical to that of the phylotype in the chemocline (Fig. 3A, compare bands 5 and 6). Green sulfur bacteria constituted ≥ 80% of the cells in the culture as determined by fluorescent *in situ* hybridization (FISH) using the specific probe GSB-532. In order to identify the accompanying bacteria in the enrichment culture (compare Fig. 4B, arrows), a PCR/DGGE analysis with the eubacterial primer pair GC341f/907r was conducted (Fig. 3A, lane UNI). In addition to the fingerprint of the green sulfur bacterium (band 2) this analysis revealed the presence of three additional phylotypes. One phylotype (band 1) exhibited 97% sequence similarity to an unidentified *Cytophaga* sp. The second phylotype (band 3) showed 96% similarity to an environmental clone of the *Cytophagales*. The third additional phylotype (band 4) showed 94% similarity to *Ilyobacter polytrophus* DSM 2926^{T} (*Fusobacteriaceae*), a fermentative bacterium specialized in the degradation of hydroaromatic compounds.

Employing group specific PCR primers, the almost complete 16S rRNA gene (1388 bp) and the ITS region (468 bp length) of the green sulfur bacterium BS-1 were selectively amplified and sequenced. The 16S rRNA gene and ITS sequences were both identical to the sequences determined in parallel for a conserved sample of *Chl. phaeobacteroides* MN1, which had been enriched from the Black Sea chemocline 13 years earlier (Fig. 3B) (34).

Characterization of the green sulfur bacterium BS-1. Cells of the enriched green sulfur bacterium were non-motile short rods, 2.8 ± 1.2 µm long and 0.65 ± 0.1 µm wide when grown at 3 µmol Quanta m^{-2} s^{-1} (Fig. 4A). After growth at 0.1 µmol Quanta m^{-2} s^{-1}, cells were significantly (p<0.001) shorter (1.8 ± 0.5 µm).

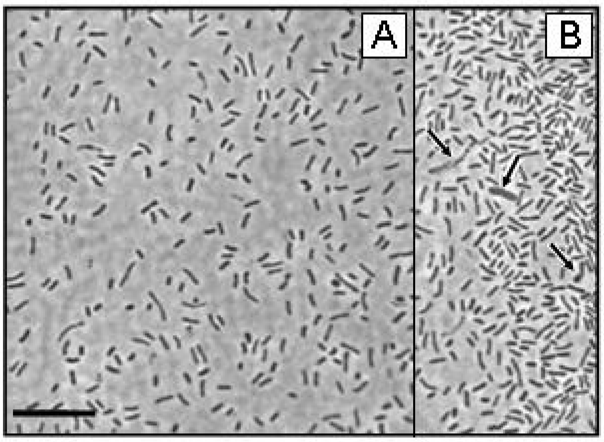

Figure 4. A. Phase contrast photomicrograph of the green sulfur bacterium BS-1 in the enrichment culture from the Black Sea chemocline. **B.** Microscopic field showing three cells of chemotrophic contaminants (arrows).

Absorption spectra of whole cells and crude pigment extracts (Fig. 5) were similar to those of other brown-colored green sulfur bacteria (6) and identical to the spectra reported for *Chl. phaeobacteroides* MN1 (34). The pigment composition of the cells changed with incubation light intensity (Fig. 2A, B), whereby the BChl *e* homolog composition of cells grown at 0.1 µmol Quanta m^{-2} s^{-1} approached that of samples from the chemocline (Fig. 2C). The BChl *e* farnesyl esters [E,E]-, [P,E]-, and [I,E]-BChl e_F amounted to 52 – 64% of the total BChl *e* and thus represented the dominant homologs. A fourth farnesyl homolog, [E,M]-BChl e_F could be detected in cells grown at 3 µmol Quanta m^{-2} s^{-1} where it constituted 44% of all farnesyl homologs (Fig. 2A). In contrast, the relative amount of [E,M]-BChl e_F was significantly decreased in cells grown at 0.1 µmol Quanta m^{-2} s^{-1} (2.3% of all farnesyl homologs; Fig. 2B), as well as in the chemocline (1.1% of all farnesyl homologs; Fig. 2C).

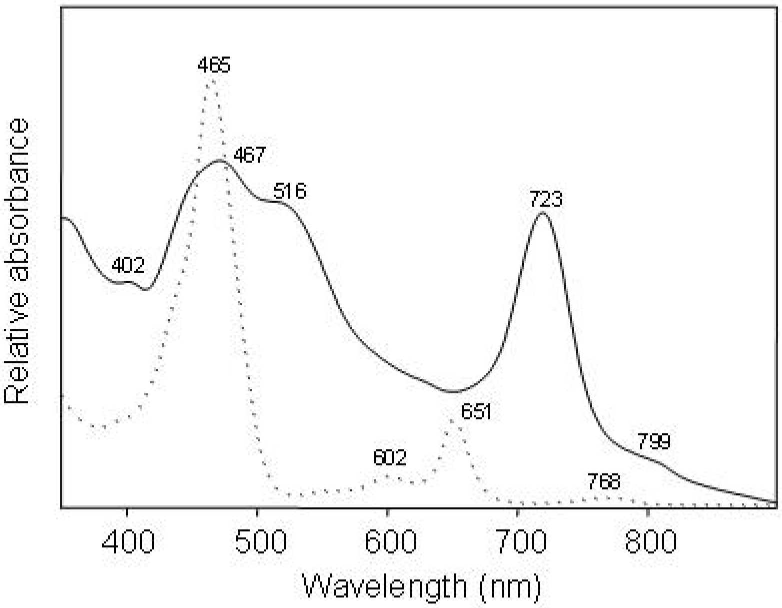

Figure 5. Absorption spectra of whole cells (—) and acetone pigment extracts (·····) of the enrichment culture of phylotype BS-1, grown at 0.1 µmol Quanta m^{-2} s^{-1}.

Similar to the chemocline population, the geranyl ester isobutyl/ethyl [I,E]-BChl e_G occurred also in the enrichment cultures. However, two other geranyl homologs were also detected and tentatively identified as ethyl/methyl-, ethyl/ethyl- and propyl/ethyl-BChl e_G based on their retention times. Their relative amount was strongly influenced by the incubation light intensity. Cells grown at light saturation (i.e. 3 µmol Quanta m^{-2} s^{-1}) contained [E,E]-BChl e_G , [P,E]-BChl e_G and [I,E]-BChl e_G at a ratio of 17:40:43 (Fig. 2A). This ratio changed towards a strong dominance of [I,E]-BChl e_G (6:28:66) in low-light adapted cells (Fig. 2B).

In order to determine the specific pigment content of the green sulfur bacterial cells, their cellular protein was estimated from the total protein content of the enrichment, and from the fraction of green sulfur bacterial among the cells as determined by FISH. This yielded a specific bacteriochlorophyll content in the enrichment culture of (97.4 ± 36) µg (mg protein)$^{-1}$ for cells grown at 3 µmol Quanta m^{-2} s^{-1} and increased values of (224 ± 95) µg (mg protein)$^{-1}$ for cultures grown at 0.1 µmol Quanta m^{-2} s^{-1}.

Low light threshold of anoxygenic photosynthesis. Due to the extremely low biomass density of green sulfur bacteria in the chemocline, photosynthetic CO_2-fixation could not be determined directly in natural samples (data not shown), which is inconsistent with the results of another recent investigation (19). Our culture contained only one single 16S rRNA gene sequence type of green sulfur bacteria and no other phototrophs. Furthermore, photosynthetic pigments other than those of green sulfur bacteria were never detected by HPLC. Therefore the enrichment was used to assess the light dependence of anoxygenic photosynthesis of the green sulfur bacteria by $^{14}CO_2$ fixation.

The light intensities that were measured at the top of the green sulfur bacterial layer around solar noon were 0.0022 to 0.00075 µmol Quanta m^{-2} s^{-1} at stations 7605 and 7620, respectively (Fig. 1C and 1F). These values correspond to 0.0009 - 0.0003 % of the surface light intensities (236-239 µmol Quanta m^{-2} s^{-1} on the sampling date). It was therefore mandatory to use a light cabinet which permitted the precise control of very low light intensities during the measurement of photosynthetic rates.

Cells precultured at 3 µmol Quanta m^{-2} s^{-1} exhibited significant photosynthetic activity at light intensities as low as 0.022 µmol Quanta·m^{-2}·s^{-1} (Fig. 6). Cultures grown at 0.1 µmol Quanta ·m^{-2}·s^{-1} exhibited a different light dependence of photosynthetic CO_2-fixation and showed detectable photosynthetic activity at 0.015 µmol Quanta·m^{-2}·s^{-1} (7.5% of the maximum rate) (Fig. 6B). However, the radiocarbon method was not suited to determine even lower rates of CO_2-incorporation.

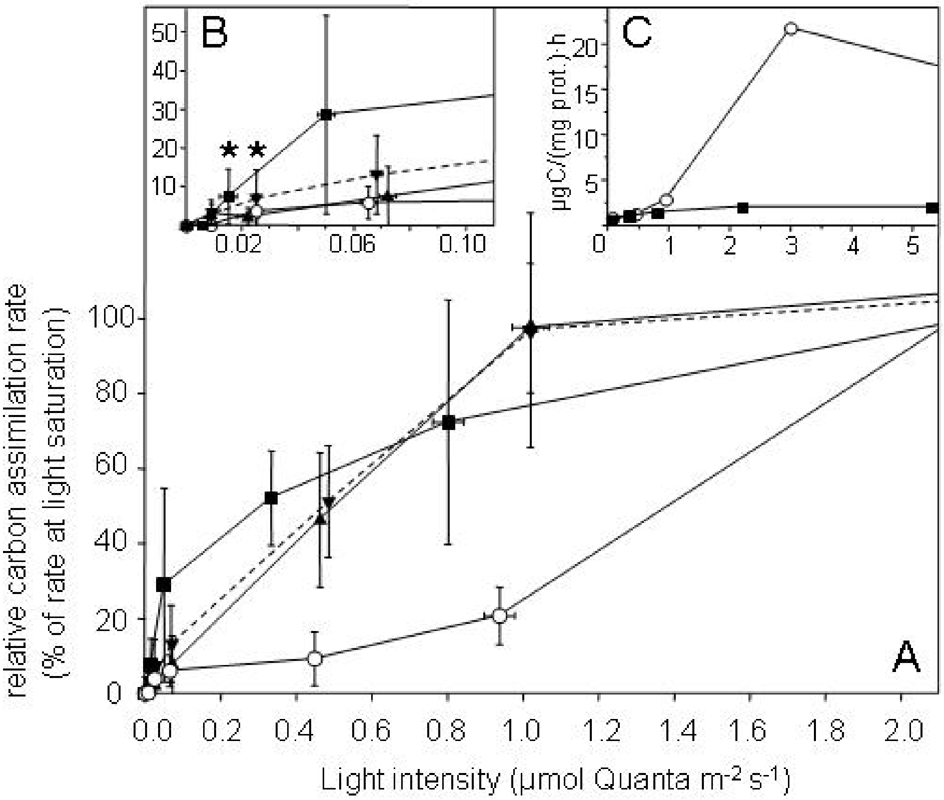

Figure 6. Light dependence of [14C]-bicarbonate assimilation in the culture of phylotype BS-1 and Chlorobium phaeovibrioides DSMZ 269T. A. Relative assimilation rates at light intensities between 0 and 2 µmol Quanta m-2 s-1 given as percentages of the maximum photosynthetic rate at light saturation. Bars represent one standard deviation. B. Expanded view of curves measured below 0.1 µmol Quanta m-2 s-1. Asterisks denote values which were significantly higher than dark controls (p = 0.1). C. Comparison of absolute values of biomass-specific photosynthetic rates. ○ Chl. phaeovibrioides DSMZ 269T, cells grown at 3 µmol Quanta m-2 s-1, ■ BS-1, grown at 0.1 µmol Quanta m-2 s-1, ▼ BS-1, grown at 3 µmol Quanta m-2 s-1 (experiment 1), ▲ BS-1, grown at 3 µmol Quanta m-2 s-1 (experiment 2).

The green sulfur bacterium from the Black Sea chemocline reached light saturation of photosynthesis at 1 µmol Quanta m^{-2} s^{-1}. In contrast, *Chl. phaeovibrioides* DSMZ 269^T showed only marginal (4% of the maximum photosynthetic rate) photosynthetic CO_2-incorporation at the low light intensities tested in the present study and reached only 20.7% of the maximum photosynthetic rate at 1 µmol Quanta·m^{-2}·s^{-1}. Photosynthesis of this latter strain was light saturated at 3 µmol Quanta m^{-2} s^{-1} (Fig. 6C). The light-saturated specific photosynthetic rates of the Black Sea bacterium were markedly lower than those of the reference strain DSMZ 269^T (Fig. 6C). At the same time, the fraction of growing cells in cultures of the Black Sea bacterium was unusually low (most probable numbers ~ 0.1% of total cell counts; U. Henßge, J. Overmann unpublished) compared to cultures of other green sulfur bacteria. Therefore, the low values for light-saturated photosynthetic rates most likely are due to a large number of photosynthetically inactive cells in the culture.

DISCUSSION

A single phylotype of green sulfur bacteria persists in the Black Sea chemocline. The biomass of phototrophic sulfur bacteria in the Black Sea ($\leq$ 0.8 mg BChl *e* m^{-2}) is orders of magnitude lower than in any other environment studied so far (25 – 2000 mg BChl *e* m^{-2}; 54). In a previous study, 16S rRNA genes of green sulfur bacteria could not be detected in clone libraries established after PCR amplification with eubacterial primers (57). Obviously, the limited number of clones tested and the low frequency of green sulfur bacteria prevented their detection by non-specific molecular methods. The group-specific PCR protocol employed in the present work permits the highly specific amplification of 16S rRNA genes from $\leq$ 200 target cells (17) thereby providing the required sensitivity. Only a single green sulfur bacterial phylotype was detected. Comparison of the 16S rRNA gene sequence with all known sequences in the databases revealed that the Black Sea bacterium so far is unique and has not been discovered in any other system.

Based on 16S rRNA gene and ITS sequence comparison, the green sulfur bacterium BS-1 is identical to a green sulfur bacterium enriched 13 years earlier from chemocline samples of the Black Sea (34). The same bacterium was detected again in chemocline samples obtained in May 2004 (R/V*Marion Dufresne*; A. K. Manske, unpublished). Also, no physiological differences were detected between strains MN1 and BS-1. These data indicate that a single phylotype of green sulfur bacteria continuously persisted in the chemocline of the Black Sea over a time period of at least 16 years. Both, the monospecific assemblage and persistence indicate a particular adaptation of BS-1 to the extreme low-light conditions.

Low-light adaptation of BS-1. The light intensity in the Black Sea chemocline corresponds to that available at a distance of 50 m from a little candle in otherwise total darkness. As compared to other environments (54), phylotype BS-1 thus experiences the by far most severe light limitation ever recorded for any phototrophic organism.

Theoretically, phylotype BS-1 could have acquired an alternative, chemoorganoheterotrophic mode of growth. However, growth of phylotype BS-1 was never observed in cultures incubated in the dark. All known green sulfur bacteria are obligate photolithoautotrophs which use the reverse citric acid cycle for CO_2 fixation (48) resulting in green sulfur bacterial biomass which is depleted by 2.5 – 12‰ in ^{13}C relative to CO_2 (53). Balancing this depletion, membrane lipids and photosynthetic pigments are enriched in ^{13}C by 1 – 15‰ relative to green sulfur bacterial biomass (16, 53). Based on our HPLC analyses, farnesol represents the dominant lipid biomarker of green sulfur bacteria in the Black Sea chemocline. The $\delta^{13}C$ values of farnesol and dissolved CO_2 measured in the present study are nearly identical, suggesting that green sulfur bacteria in the Black Sea chemocline indeed fix CO_2 via the reverse citric acid cycle. In conclusion, our data suggest that phylotype BS-1 grows photolithoautrophically *in situ*. Theoretically, farnesol could also originate from *Archaea* which have been detected based on their 16S rRNA gene sequences in the chemocline (57). No information on the abundance of archaeal cells in the chemocline is presently available. However, only few archaea and only species which are phylogenetically distant to those occurring in the chemocline of the Black Sea (57) contain very small traces of farnesol (51). It is therefore unlikely that archaeal farnesol contributed to the isotopic signal.

Previous studies indicated that low-light adaptation of the green sulfur bacterium from the Black Sea chemocline includes an increase in the specific BChl *e* content (15, 34). So far, only cells incubated at ≥ 0.25 µmol Quanta m^{-2} s^{-1} had been studied and reached a specific pigment content of ~220 µgBChl*e*·(mg protein)$^{-1}$. In the present study, similar values were determined for cells grown at lower light intensities. This specific pigment content of strain BS-1 surpasses that of other green sulfur bacteria (34). The cellular pigment content in our cultures grown at 0.1 µmol Quanta m^{-2} s^{-1} was $3.1\cdot10^{-5}$ ngBChl*e* cell^{-1}. This value is comparable to *in situ* values of 1.7 - $3.5\cdot10^{-5}$ ng cell^{-1} as derived from the pigment concentrations and the numbers of green sulfur bacteria determined by quantitative PCR in the Black Sea chemocline. Thus, the cellular pigment content is not increased further under the severe light limitation in the chemocline, and hence represents the maximum pigment content the cells can attain.

Compared to the results reported for the year 1988 (4,43), the maximum concentrations of Bchl *e* determined by us 13 years later were decreased by a factor of 14 (68 ng·l^{-1} versus 940 ng·l^{-1}). In 1988, green sulfur bacteria amounted to 10% of the total bacterial cell numbers (4), whereas a quantification of the cell number of phylotype BS-1 by real-time PCR indicated that this bacterium constituted only 0.5 to 1% of the total cell number in the chemocline in 2001 (E. Marschall and J. Overmann, unpublished). Both lines of evidence indicate a significant decrease in the population density of the green sulfur bacteria over the last decade, concomitant to the vertical displacement of their biomass maximum from 74 to 100 m depth. At the northwestern continental slope, the chemocline was located at a depth of 140 m and only traces of BChl *e* were detected. This pronounced dependence of green sulfur bacterial biomass on the vertical position of the chemocline implies that phylotype BS-1 reaches its lower limits for phototrophic growth between 100 and 140 m in the Black Sea.

Its capability to exploit minute light intensities renders phylotype BS-1 a valuable model system for the study of the molecular basis of low-light adaptation. Based on our data, this adaptation involves changes in pigment composition. Green sulfur bacteria use chlorosomes as light-harvesting structures. The latter contain rod-shaped aggregates of antenna bacteriochlorophylls. Similar to phylotype BS-1, a loss of [E,M]-Bchle_F with a concomitant increase in [I,E]-Bchle_F upon transfer to light limiting conditions has previously been documented for *Chlorobium phaeobacteroides* strain Dagow III (18) and may represent an adaptative trait to increase the photosynthetic efficiency. The alkyl side chain of porphyrin ring III is directly involved in the aggregation of BChl molecules (56). A high degree of alkylation therefore leads to a red shift of the Q_Y absorption maximum by 7-11 nm (5), which has been hypothesized to facilitate the channeling of excitation energy towards the reaction center, thereby causing an increase in energy transfer efficiency of the chlorosomes (5). A conspicuous feature of the low-light adaptation of phylotype BS-1 is the presence of geranyl homologs of BChl *e* which have never been described for any isolate of the green sulfur bacteria (5,8,18). The cellular concentrations of the geranyl ester homologs ([E,E]-BChl e_G and [P,E]-BChl e_G) were decreased in cells grown at 0.1 µmol Quanta m^{-2} s^{-1} and absent in the severely light-limited chemocline population. Within the aggregates, bacteriochlorophylls seem to be arranged in a bilayer with the alcohol tails of bacteriochlorophylls in the inner layer oriented towards the central cavity of the rod (50). Because of spatial constraints, the structure of esterifying alcohols may therefore influence the stability of the rod structure and the function of the chlorosome as a whole (18).

Biogeochemical significance of anoxygenic photosynthesis in the Black Sea. Based on our data, CO_2-fixation rates of green sulfur bacteria in the chemocline can be estimated. For the calculation of the daily rate of anoxygenic photosynthesis, global irradiance and average day length in the Black Sea region at summer and winter solstice were taken from the Solar

Irradiance Data Utility (http://sunbird.jrc.it/pvgis/sunraddayframe.php). *In situ* light intensities were then calculated using our light transmission data for the Black Sea water column. Experimentally, photosynthetic CO_2 fixation rates could only be determined at ≥ 0.015 µmol Quanta m^{-2} s^{-1}. Therefore, rates of anoxygenic photosynthesis were calculated for each depth by linear interpolation of laboratory data to *in situ* light intensities, and considering the biomass of green sulfur bacteria present at each depth. This yielded an integrated anoxygenic photosynthetic rate of 211 ng C m^{-2} d^{-1} (corresponding doubling time t_d=26 years) for a clear December day, and a maximum value of 1760 ng C m^{-2} d^{-1} (t_d=3.1 years) during summer solstice. These values are at least thousandfold lower than the productivity of anoxygenic phototrophic bacteria in any other stratified environment investigated to date (Fig. 7). By comparison, the phytoplankton primary production in the center of the Western basin amounts to 575 mg m^{-2} d^{-1} (25). Similar to laboratory enrichments, the cultivation success for green sulfur bacterial cells from natural samples was very low, indicating a low fraction of photosynthetically active cells in the chemocline. Even if this fraction was significantly higher in the chemocline than in the cultures used for the determination of photosynthetic activity, integrated anoxygenic photosynthesis would still contribute far less than 1% of total photosynthesis in the Black Sea.

It has been discussed previously (24) that the suboxic zone may be the result of sulfide oxidation by anoxygenic photosynthetic bacteria. Our data can be used to infer the significance of anoxygenic photosynthesis for sulfide turnover in the Black Sea. From the integrated anoxygenic photosynthetic rate, a sulfide oxidation rate of 8.8 – 293.3 nmol H_2S m^{-2} d^{-1} can be deduced, depending on the oxidation product formed (sulfate or elemental sulfur). By comparison, the sulfide flux from below into the green sulfur bacterial layer is 370 – 530 µmol sulfide m^{-2} d^{-1} based on the vertical concentration profiles and the coefficient for turbulent diffusivity (27).

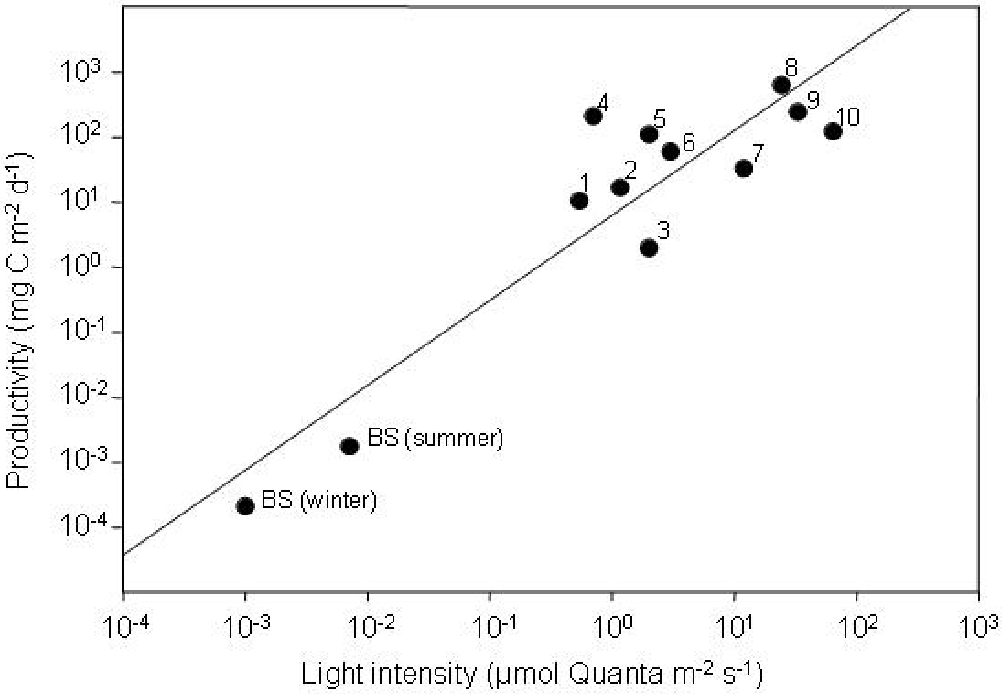

Figure 7. Cross-system comparison of the integrated productivity of anoxygenic phototrophs in different chemocline environments. BS data for Black Sea chemocline (this study) estimated for light intensities available in summer and winter; 1 Peter Lake (7,37); 2 Paul Lake (7, 37); 3 Mary Lake (7, 37); 4 Big Soda Lake (11); 5 Knaack Lake (38); 6 Mirror Lake (7, 37); 7 Rose Lake (7, 37); 8 Lake Cisó (20); 9 Lake Vilar (20); 10 Mahoney Lake (35). Line depicts a linear regression of all data.

Hence, green sulfur bacteria account for ≤ 0.1% of total sulfide oxidation in the Black Sea chemocline. The results of modeling the upward fluxes of sulfide suggest that sulfide is actually consumed within the anoxic zone between the chemocline and 150 m depth, but not within the chemocline (27). Most likely molecular oxygen enters the anoxic zone by massive lateral injections of oxygen-enriched Mediterranean waters through the Bosporus plume, and leads to the formation and sedimentation of particulate MnO_2 in the chemocline, which in turn serves as the oxidant of ~70% of the sulfide diffusing upwards (26). Together with molecular diffusion of O_2 from upper water layers (accounting for ~10% of the sulfide oxidation) (27), the fluxes of molecular oxygen thus are sufficient to explain most of the sulfide oxidation in the Black Sea.

In conclusion, the monospecific assemblage of green sulfur bacteria in the Black Sea represents the most extremely low-light-adapted population of phototrophic organisms documented to date. Although not of significance for the carbon and sulfur cycles in the Black Sea, its specific adaptation renders phylotype BS-1 an indicator of marine low light habitats. Based on our results, the ITS sequence of BS-1 is the most promising biomarker which can be used to detect extreme light limitation during photic zone anoxia.

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REFERENCES

(1) **Airs, R. L., J. E. Atkinson, and B. J. Keely.** 2001. Development of a high resolution liquid chromatographic method for the analysis of complex pigment distributions. J. Chromatogr. A **917:**167-177.

(2) **Anbar, A.D. and A.H. Knoll.** 2002. Proterozoic ocean chemistry and evolution: a bioinorganic bridge? Science **297:** 1137-1142

(3) **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43:**260-296.

(4) **Bird, D. F. and D. M. Karl.** 1991. Microbial biomass and population diversity in the upper water column of the Black Sea. Deep-Sea. Res. **38:**1069-1082.

(5) **Borrego, C. M. and L. J. Garcia-Gil.** 1995. Rearrangement of light harvesting bacteriochlorophyll homologs as a response of green sulfur bacteria to low light intensities. Photosynth. Res. **41:**53-65.

(6) **Borrego, C. M., J. B. Arellano, C. A. Abella, T. Gillbro, and L. J. Garcia-Gil.** 1999. The molar extinction coefficient of bacteriochlorophyll *e* and the pigment stoichiometry in *Chlorobium phaeobacteroides*. Photosynth. Res. **60:**257-264.

(7) **Borrego, C. M., J. Garcia-Gil, X. P. Cristina, X. Vila, and C. A. Abella.** 1998. Occurrence of new bacteriochlorophyll d forms in natural populations of green photosynthetic sulfur bacteria. FEMS Microbiol. Ecol. **26:**257-267.

(8) **Borrego, C. M., L. J. Garcia-Gil, X. Vila, X. P. Cristina, J. B. Figueras, and C. A. Abella.** 1997. Distribution of bacteriochlorophyll homologs in natural populations of brown-colored phototrophic sulfur bacteria. FEMS Microbiol. Ecol. 24:301-309.

(9) **Cannone, J. J., S. Subramanian, M. N. Schnare, J. R. Collett, L. M. D'Souza, Y. Du, B. Feng, N. Lin, L. V. Madabusi, K. M. Muller, N. Pande, Z. Shang, N. Yu, and R. R. Gutell** 2002. The Comparative RNA Web (CRW) Site: An Online Database of Comparative Sequence and Structure Information for Ribosomal, Intron, and other RNAs. BioMed Central Bioinformatics, 3:2. [Correction: BioMed Central Bioinformatics. 3:15.]

(10) **Cline, J. D.** 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. **14:**454-458.

(11) **Cloern J. E., B. E. Cole, and R.S. Oremland.** 1983. Autotrophic processes in meromictic Big Soda Lake, Nevada. Limnol. Oceanogr. **28:**1049-1061.

(12) **Coolen, M. J. L. and J. Overmann.** 2000. Functional exoenzymes as indicators of metabolically active bacteria in 124,000-year-old sapropel layers of the eastern Mediterranean Sea. Appl. Environ. Microbiol. **66:**2589-2598.

(13) **Degens, E.T. and Stoffers, P. 1976.** Stratified waters as a key to the past. Nature **263**:22-27

(14) **Fuhrman, J. A., D. E. Comeau, Å. Hagström, and A. M. Chan.** 1988. Extraction from natural planktonic microorganisms of DNA suitable for molecular biological studies. Appl. Environ. Microbiol. **54:**1426-1429.

(15) **Fuhrmann S., J. Overmann, N. Pfennig, and U. Fischer.** 1993. Influence of vitamin B_{12} and light on the formation of chlorosomes in green- and brown-colored *Chlorobium* species. Arch. Microbiol. **160**: 193-198

(16) **Glaeser, J., and J. Overmann.** 2003. Characterization and *in situ* carbon metabolism of phototrophic consortia. Appl. Environ. Microbiol. **69:**3739-3750.

(17) **Glaeser, J., and J. Overmann.** 2004. Biogeography, evolution and diversity of the epibionts in phototrophic consortia. Appl. Environ. Microbiol. **70**: 4821-4830

(18) **Glaeser, J., L. Bañeras, H. Rütters, and J. Overmann.** 2002. Novel bacteriochlorophyll *e* structures and species-specific variability of pigment composition in green sulfur bacteria. Arch. Microbiol. **177:**475-485.

(19) **Gorlenko, V.M., P.V. Mikheev, I.I. Rusanov, N.V. Pimenov, and M.V. Ivanov.** 2005. Ecophysiological properties of photosynthetic bacteria from the Black Sea chemocline zone. Microbiology **74:** 201-209

(20) **Guerrero, R., E. Montesinos, C. Pedrós-Alió, I. Esteve, J. Mas, H. Van Gemerden, P. A. G. Hofman, and J. F. Bakker.** 1985. Phototrophic sulfur bacteria in two Spanish lakes: Vertical distribution and limiting factors. Limnol. Oceanogr. **30:**919-931

(21) **Hartree, E. F.** 1972. Determination of protein: a modification of the LOWRY-method that gives a linear photometric response. Anal. Biochem. **48:**422-427.

(22) **Hobbie, J. E., R. J. Daley, and S. Jaspers.** 1977. Use of nucleopore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33:**1225-1228.

(23) **Imhoff, J.F.** 2003. Phylogenetic taxonomy of the family *Chlorobiaceae* on the basis of 16S rRNA and fmo (Fenna-Matthews-Olson protein) gene sequences. Int. J. Syst. Evol. Microbiol. **53:** 941-951

(24) **Jørgensen, B. B., H. Fossing, C. O. Wirsen and H. W. Jannasch.** 1991. Sulfide oxidation in the anoxic Black Sea chemocline. Deep-Sea Res. **38:**S1083-S1103.

(25) **Karl, D.M. and G.A. Knauer.** 1991. Microbial production and particle flux in the upper 350 m of the Black Sea. Deep-Sea Res. **38** (Suppl): S921-S942

(26) **Konovalov, S. K., G. W. Luther, G. E. Friederich, D. B. Nuzzio, B. M. Tebo, J. W. Murray, T. Oguz, B. Glazer, R. E. Trouwborst, B. Clement, K. J. Murray, and A. S. Romanov.** 2003. Lateral injection of oxygen with the Bosporus plume – fingers of oxidizing potential in the Black Sea. Limnol. Oceanogr. **48:**2369-2376.

(27) **Konovalov, S. K., L. I. Ivanov, and A. S. Samodurov.** 2001. Fluxes and budgets of sulphide and ammonia in the Black Sea anoxic layer. J. Mar. Syst. **31:**203-216.

(28) **Koopmans, M.P., J. Köster, H.M.E. van Kaam-Peters, F. Kenig, S. Schouten, W.A. Hartgers, J.W. de Leeuw, and J.S. Sinninghe Damsté.** 1996. Diagenetic and catagenic products of isorenieratene: Molecular indicators for photic zone anoxia. Geochim. Cosmochim. Acta **60:** 4467-4496

(29) **Krüger, S., T. Leipe, F. Pollehne, and P. Wlost.** 2001. First development of an Integrated Pumpcast-CTD System. In: C. Hemleben, K. Hoernle, B. B. Jørgensen and W. Roether (eds), Ostatlantik-Mittelmeer-Schwarzes Meer 2001, Meteor-Berichte 03-1, Leitstelle Meteor, Universität Hamburg 4-31 - 4-33.

(30) **Leadbetter, J. R., T. M. Schmidt, J. R. Graber, and J. A. Breznak.** 1999. Acetogenesis from H_2 plus CO_2 by spirochetes from termite guts. Science 283:686-689.

(31) **Ludwig, W., O. Strunk, R. Westram , L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. Konig, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, K.H. Schleifer.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32**:1363-71.

(32) **Murray, J. W., H. W. Jannasch, S. Honjo, R. F. Anderson, W. S. Reeburgh, Z. Top, G. E. Friederich, L. A. Codispoti, and E. Izdar.** 1989. Unexpected changes in the oxic/anoxic interface in the Black Sea. Nature **338:**411-413.

(33) **Muyzer, G., S. Hottenträger, A. Teske, and C. Waver.** 1995. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA – a new molecular approach to analyse the genetic diversity of mixed microbial communities. In: Akkermans A. D. L, Van Elsas J. D. , De Bruijn F. J. (eds) Molecular microbial ecology manual, 2nd edn. Kluwer, Dordrecht, pp 3.4.4.1-3.4.4.22.

(34) **Overmann, J., H. Cypionka, and N. Pfennig.** 1992. An extremely low-light-adapted green sulfur bacterium from the Black Sea. Limnol. Oceanogr. **37:**150-155.

(35) **Overmann, J., J.T. Beatty, K.J. Hall, N. Pfennig and T.G. Northcote.** 1991. Characterization of a dense, purple sulfur bacterial layer in a meromictic salt lake. Limnol. Oceanogr. **36:** 846-859

(36) **Overmann, J., M. J. L. Coolen, and C. Tuschak.** 1999. Specific detection of different phylogenetic groups of chemocline bacteria based on PCR and denaturing gradient gel electrophoresis of 16S rRNA gene fragments. Arch. Microbiol. **172:**83-94.

(37) **Parkin, T.B. and Brock, T.D.** 1980. Photosynthetic bacterial production in lakes: the effects of light intensity. Limnol. Oceanogr. **25:**711-718.

(38) **Parkin, T.B. and Brock, T.D.** 1981. Photosynthetic bacterial production and carbon mineralization in a meromictic lake. Arch. Hydrobiol. **91:**366-382.

(39) **Pfennig, N. and H. Biebl.** 1976. *Desulfomonas acetoxidans* gen. nov. and sp. nov., a new anaerobic, sulfur-reducing, acetate-oxidizing bacterium. Arch. Microbiol. **110:**2-12.

(40) **Pfennig.** 1978. *Rhodocyclus purpureus* gen. nov. and spec. nov, a ring-shaped, vitamin B12-requiring member of the family Rhodospirillaceae. Int. J. Syst. Bact. **28:**283-288.

(41) **Repeta, D. J.** 1993. A high resolution historical record of Holocene anoxygenic primary production in the Black Sea. Geochim. Cosmochim. Acta **57:**4337-4342.

(42) **Repeta, D. J. and D. J. Simpson.** 1991. The distribution and recycling of chlorophyll, bacteriochlorophyll and carotenoids in the Black Sea. Deep-Sea Res. **38:**969-984.

(43) **Repeta, D. J., D. J. Simpson, B. B. Jørgensen, and H. W. Jannasch.** 1989. Evidence for anoxygenic photosynthesis from the distribution of bacteriochlorophylls in the Black Sea. Nature **342:**69-72.

(44) **Ross D.A. and E.T. Degens.** 1970. Black Sea: Recent sedimentary history. Science **170:**163-165.

(45) **Schouten, S., M. Strous, M. M. M. Kuypers, W. I. C. Rijpstra, M. Baas, C. J. Schubert, M. S. M. Jetten and J. S. Sinninghe Damsté.** 2004. Stable carbon isotopic fractionations associated with inorganic carbon fixation by anaerobic ammonium-oxidizing bacteria. Appl. Env. Microbiol. **70:**3785-3788.

(46) **Schouten, S., W. C. M. Klein-Breteler, P. Blokker, N. Schogt, K. Grice, W. I. C. Rijpstra, M. Baas, and J. S. Sinninghe Damsté.** 1998. Biosynthetic effects on the stable isotopic compositions of algal lipids: implications for deciphering the carbon isotopic biomarker record. Geochim. Cosmochim. Acta **62:**1397-1406.

(47) **Sinninghe Damsté, J. S., Wakeham, S. G., M. E. L. Kohnen, Hayes, J. M., and J. W. de Leeuw.** 1993. A 6,000-year sedimentary molecular record of chemocline excursions in the Black Sea. Nature **362**:827-829.

(48) **Sirevåg, R.** 1995. Carbon metabolism in green bacteria. p.871-883. *In* R. E. Blankenship, M. T. Madigan, and C. E. Bauer (eds.), Anoxygenic photosynthetic bacteria. Advances in photosynthesis, vol. II. Kluwer Academic Publishers, Dordrecht, The Netherlands.

(49) **Spencer, D. W., P. G. Brewer and P. L. Sachs.** 1972. Aspects of the distribution and trace element composition of suspended matter in the Black Sea. Geochim. Cosmochim. Acta. **36:**71-86.

(50) **Steensgaard, D.B., H. Wackerbarth, P. Hildebrandt, and A.R. Holzwarth** (2000) Diastereoselective control of bacteriochlorophyll *e* aggregation. 3^1-S-BChl *e* is essential for the formation of chlorosome-like aggregates. J. Phys. Chem. B **104:** 10379–10386

(51) **Tornabene, T.G., T.A. Langworthy, G. Holzer, and J. Oró.** 1979. Squalenes, phytanes and other isoprenoids as major neutral lipids of methanogenic and thermoacidophilic "Archaebacteria". J. Mol. Evol. **13:** 73-83

(52) **Tuschak, C., J. Glaeser, and J. Overmann.** 1999. Specific detection of green sulfur bacteria by *in situ* hybridization with a fluorescently labeled oligonucleotide probe. Arch. Microbiol. **171:**265-272.

(53) **van der Meer, M. T. J., S. Schouten and J. S. Sinninghe Damsté.** 1998. The effect on the reversed tricarboxylic acid cycle on the ^{13}C contents of bacterial lipids. Org. Geochem. 28: 527-533

(54) **van Gemerden, H., and J. Mas.** 1995. Ecology of phototrophic sulfur bacteria. In: Madigan MT, Bauer CE (Eds.): Anoxygenic Photosynthetic Bacteria: pp 49-85, Kluwer Academic Publishers.

(55) **van Liere, E. and A. E. Walsby.** 1982. Interactions of cyanobacteria with light. In: Carr, N. G., Whitton, B. A. (eds). The biology of cyanobacteria. Blackwell Scientific Publications, Oxford. p. 9-45.

(56) **van Rossum B-J., D. B. Steensgard, F. M. Mulder, G. J. Boender, K. Schaffner, A.R. Holzwarth, and H. J. M. de Groot.** 2001. A refined model of the chlorosomal antennae of the green sulfur bacterium *Chlorobium tepidum* from proton chemical shift constraints obtained with high-field 2-D and 3-D MAS NMR dipolar correlation spectroscopy. Biochemistry **40:**1587-1595.

(57) **Vetriani, C., H.V. Tran, and L.J. Kerkhof.** 2003. Fingerprinting microbial assemblages from the oxic/anoxic chemocline of the Black Sea. Appl. Environ. Microbiol. **69:** 6481-6488.

(58) **Widdel, F., G. W. Kohring, and F. Mayer.** 1983. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. III. Characterization of the filamentous gliding *Desulfonema limicola* gen. nov., sp. nov., and *Desulfonema magnum* sp. nov. Arch. Microbiol. **134:**286-293.

CHAPTER 5

AN OBLIGATELY PHOTOSYNTHETIC BACTERIAL ANAEROBE FROM A DEEP-SEA HYDROTHERMAL VENT

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Abbreviation: BChl, bacteriochlorophyll.

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The abundance of life on Earth is almost entirely due to biological photosynthesis, which depends on light energy. The source of light in natural habitats has heretofore been thought to be the sun, thus restricting photosynthesis to solar photic environments on the surface of the Earth. If photosynthesis could take place in geothermally illuminated environments, it would increase the diversity of photosynthetic habitats both on Earth and on other worlds that have been proposed to possibly harbor life. Green sulfur bacteria are anaerobes that require light for growth by the oxidation of sulfur compounds to reduce CO_2 to organic carbon, and are capable of photosynthetic growth at extremely low light intensities. We describe the isolation and cultivation of a previously unknown green sulfur bacterial species from a deep-sea hydrothermal vent, where the only source of light is geothermal radiation that includes wavelengths absorbed by photosynthetic pigments of this organism.

Keywords: photosynthesis | anoxygenic | green sulfur bacterium | evolution | habitat

INTRODUCTION

Light energy from the sun drives photosynthesis to provide the primary source of nearly all of the organic carbon that supports life on Earth (1). An exception to this are deep-sea hydrothermal vents, such as black smokers located far below the photic zone in the oceans, where unusual microbial and invertebrate populations exist on organic material from CO_2 reduction by chemotrophic bacteria that oxidize inorganic compounds (2). Hydrothermal vents may resemble the environment in which life evolved (3), and the discovery of geothermal light at otherwise dark deep-sea vents led to the suggestion that such light may have provided a selective advantage for the evolution of photosynthesis from a chemotrophic microbial ancestor that used light-sensing molecules for phototaxis toward nutrients associated with geothermal light (4, 5). However, it was not clear whether the photon flux emanating from hydrothermal vents could support the existence of an obligately photosynthetic organism.

A bacterium that appears to use light as an auxiliary source of energy to supplement an otherwise chemotrophic metabolism was isolated from the general vicinity of a deep-sea hydrothermal vent (6, 7), but we wished to determine whether an obligately photosynthetic microbe might exist in close proximity to a vent orifice. The discovery of such an organism in this environment would indicate that volcanic or geothermal light is harvested to drive photosynthetic reactions in the absence of light from the sun. The possibility of geothermal light-driven photosynthesis on Earth relates to speculations about the existence of extraterrestrial life on planets and moons far from the sun in the solar system (8) and, conceivably, in other galaxies. Bulk DNA may be isolated from natural environments, and gene sequences may be used to infer the presence of microbial species and potential metabolic activities (9). Because these methods do not necessarily reveal the existence of viable cells or genuine physiological properties of an organism, we instead used a cultivation approach to search for living cells of obligately photosynthetic microbes at a deep-sea hydrothermal vent. Here, we report the capture and initial description of an anaerobic green sulfur phototrophic bacterium from a deep-sea black smoker.

MATERIALS AND METHODS

Enrichment, Isolation, and Cultivation. Water samples (1 ml) were added to completely filled 16.5-ml screw-cap (anaerobic) tubes containing ‘‘Medium 1 for Cultivation of Green and Purple Sulfur Bacteria’’ as described for cultures from marine habitats (10), supplemented with 0.02% yeast extract and 0.1% thiosulfate. Tubes were incubated at ≈25°C on a bench top, and continuously illuminated with weak fluorescent light (from ceiling fixtures) supplemented with a 60-W incandescent lamp to yield a combined intensity of ≈10 μmole photons$\cdot$m$^{-2}\cdot$s^{-1} (measured with a Li-Cor quantum sensor equipped with LI-190SB probe). In subsequent cultivations, GSB1 was grown anaerobically in saline SL10 medium, a minimal medium containing H_2S as the sole electron donor and CO_2 as the sole source of carbon (11), at 21 °C and illuminated with 100 μmol photons$\cdot$m$^{-2}\cdot$s^{-1} provided by incandescent lamps. Saline SL10 medium was solidified with agar (1.5%) to obtain colonies in tubes for purification of GSB1. A positive effect on growth was observed when the saline SL10 medium was supplemented with 5 mM acetate, 5 mM propionate, 0.05% peptone, or elemental sulfur (S^0; a few milligrams per 10 ml). No growth stimulation was observed with any of 100 other substances added to the saline SL10 medium (see Supporting Text, which is published as supporting information on the PNAS web site).

PCR Amplification and Sequence Analyses. DNA was obtained from pure cultures of GSB1 using standard methods, and used in PCR amplification with degenerate oligonucleotide primers FMO1fd (5'-WCWAAHGACRYNACVACCGC-3') and FMO4rd (5'-CGCTCCAGCGRT-AYTCYTCRAGG- 3') for the FMO gene. The PCR conditions were 30 cycles of 98 °C for 1 min, 52°C for 1 min, and 72 °C for 2 min. The 16S rRNA gene segment was amplified by using primers 27f (5'-GAGTTTGATCCTGGCTCAG- 3') and 1525r (5'-AGAAAGGAGGTGA-TCCAGCC-3') (12). The PCR conditions were 10 cycles of 94 °C for 30 s, 59 °C for 45 s, and 72 °C for 60 s, followed by 20 cycles of 94 °C for 30 s, 54 °C for 45 s, and 72 °C for 60 s. The PCR products were sequenced, the sequences were analyzed by BLAST of the NCBI databases, and alignments were created by using CLUSTALW in BIOEDIT for FMO or the online RDP-II alignment tool for 16S rRNA (13–15). MEGA2 was used to construct neighbor-joining trees based on the Jukes and Cantor model (16). The GenBank accession numbers of FMO/16S rRNA sequences used as representatives are as follows: *Prosthecochloris aestuarii* 2K, AJ290823/AJ290835; *P. aestuarii* DSM 271, AJ391151/Y07837; *Chlorobium phaeovibriodes* DSM 1678, AJ391163/AJ290833; *Chlorobium vibrioforme* DSM 260^T , AJ391145/M62791; *Chlorobium limicola f. thios.* DSM 249, X83529/Y08102; *Chlorobium phaeobacteroides* 1549, AJ306184/AJ299413; *Chlorobium tepidum* ATCC 49652, L13700/M58468; *Chlorobium vibrioforme f. thios.* NCIB 8346, AJ391161/AJ290830; *Chlorobium limicola* UdG 6044, AJ306190/Y10645;

Chlorobium limicola DSM 246, AJ391142/AJ290824; *Chlorobium phaeobacteroides* DSM 266, AJ391148/Y08104*; Pelodictyon luteolum* DSM 273, AJ391152/Y08107; *Chlorobium phaeovibrioides* DSM 269, AJ391150/Y08105; and *Pelodictyon phaeoclathratiforme* DSM 5477, AJ290822/Y08108. Additional accession numbers of 16S rRNA sequences are as follows: *Chlorobium vibrioforme* DSM 262, Y08103; *Chlorobium limicola. f. thios.* 9330, AJ290827; *Chlorobium ferrooxidans* DSM 13031, Y18253; *Clathrochloris sulfurica*, X53184; and *Chloroherpeton thalassium*, AF170103.

Pigment Analyses. Absorption and fluorescence emission (excitation at 460 nm) spectra of intact cells were obtained at room temperature with a Shimadzu UV-2501PC spectrophotometer and Photon Technology International fluorometer, respectively. Pigments were extracted from cells in acetone/methanol (7:2) overnight at -20°C and injected onto a 4.6 x 150 mm Waters Symmetry C8 (3.5 μm) column attached to an Agilent Technologies Model 1100 HPLC equipped with diode array absorption and scanning fluorometer detectors. Pigment spectra of individual HPLC peaks were taken from the data stored by the detector by using software provided by Agilent Technologies. Carotenoids were collected from HPLC peaks, dried under N_2, and analyzed by MALDI-TOF mass spectrometry using terthiophene as the matrix (17).

Electron Microscopy. Negatively stained (2% aqueous uranyl acetate) cells were examined in a Hitachi H-7600 TEM. For thin sections, cells were fixed in 2.5% glutaraldehyde buffered with 0.1 M cacodylate buffer, pH 7.4, postfixed in cacodylate buffered 1% osmium tetroxide, dehydrated through an ethanol series, then infiltrated in a Spurr-Epon resin mix and polymerized at 60 °C overnight. The processing was performed by using a Pelco Laboratory Microwave as described (www.emlab.ubc.ca/protocol.htm). Thin sections were cut with a diamond knife on a Leica Ultracut T microtome and put onto 300-mesh uncoated copper grids, stained in 2% uranyl acetate for 12 min and lead citrate for 6 min, and examined by using a Hitachi H-7600 TEM. For scanning electron microscopy, cells were fixed as above and collected on a 0.4- μm nucleopore filter. The cells were then postfixed in cacodylate buffered 1% osmium tetroxide, dehydrated through an ethanol series, critical point-dried by using liquid carbon dioxide, and examined in a Hitachi S-4700 scanning electron microscope.

Survival of GSB1 During Exposure to Air. Before exposure to air, all manipulations were conducted under an atmosphere of 95% N_2/5% H2. Cells were harvested by centrifugation and resuspended in an anoxic, H2S-free, artificial seawater medium (18) buffered with 10 mM Hepes, pH 6.7, to $\approx 10^8$ cells per ml. For air exposure times of up to 24 h, 80 ml of cell suspension was transferred to 125-ml glass serum bottles that were sealed with butyl rubber stoppers in an anaerobic chamber. The serum bottles were incubated without agitation at room temperature in the dark, and the experiment was started by continuously sparging the suspension with filter-sterilized synthetic air (Messer-Griessheim, A bis Zet, Nu¨rnberg, Germany). Aliquots of 200 µl were taken aseptically with a syringe at time intervals and immediately transferred to saline SL10 medium supplemented with 1 mM sodium acetate. These samples were serially diluted 1:10 in screw-cap tubes containing the same medium, for enumeration by the most probable number (MPN) method (19, 20). After dilution, the inoculated MPN tubes were kept in the dark at 15 °C overnight to allow for complete reduction of O_2 carried over during inoculation, and then incubated at 21 °C with illumination as described above. For long-term (up to 2 weeks) exposure to air, 80-ml cell suspensions in the artificial seawater medium were incubated in 250-ml baffled Erlenmeyer flasks closed with cotton plugs. After withdrawing the initial sample, the flasks were incubated at 250 rpm on a rotary shaker in darkness at 15 °C under ambient air. Samples were taken and serially diluted for MPN enumeration as described above. Tubes were incubated for 1 month, the number of positive tubes for each dilution was scored, and MPN values were calculated by using the tables of de Man (19, 20).

RESULTS AND DISCUSSION

On this cruise, we visited the East Pacific Rise, which is an area of high volcanic activity with a variety of vents that support characteristic ecosystems (2, 21). A water sample was obtained directly from the effluent plume within 50 cm above the orifice of the TY black smoker (2,391 m in depth; 9° 49.63' N, 104° 17.37' W), using a 1-liter capacity Niskin sampler on the ALVIN submersible. After return to the ship, 1-ml portions of the sample were used to inoculate culture media designed to enrich for different types of bacteria. A phototrophic sulfur bacterial enrichment medium incubated anaerobically with illumination gave rise to green-pigmented turbidity, which appeared to be due to a small, nonmotile bacterium that is called GSB1. Twenty-two other samples collected on this cruise, at various depths ranging from the surface to the ocean floor, failed to yield microbial growth in this enrichment medium, although growth of other organisms was obtained in other media.

After isolation of GSB1 in pure culture, the in vivo absorption spectrum (Fig. 1) was found to be similar to that of green sulfur bacteria such as those in the genera *Chlorobium* and *Prosthecochloris* (22), with a major peak at ≈750 nm indicating the presence of light-harvesting bacteriochlorophyll (BChl) *c*, and absorption in the 450 nm region due to a BChl Soret band and light-harvesting carotenoid pigments (23). The in vivo fluorescence emission spectrum (Fig. 1) contained a major peak at ≈775 nm, indicative of BChl *c* (23). The GSB1 pigments were extracted into an organic solvent, resolved in HPLC, and determined to be very similar to the pigments of a *Chlorobium tepidum* control (24). Thus, the quantitatively major chlorophylls of GSB1 are BChls *c* on the basis of absorption/fluorescence spectra and HPLC elution times, and mass spectrometry indicated that the major carotenoid is chlorobactene (data not shown).

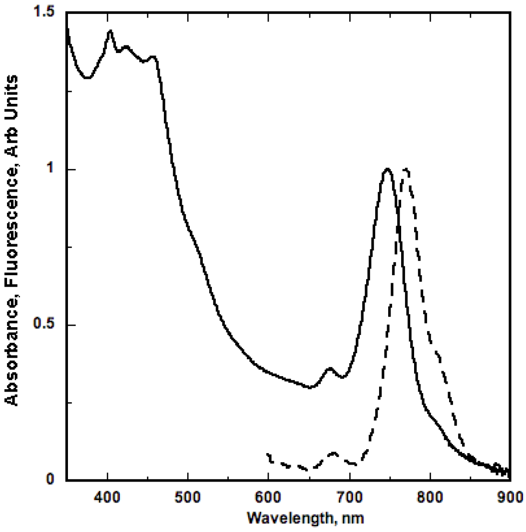

Fig. 1. Absorption (solid line) and fluorescence emission (broken line) spectra of GSB1 intact cells. Vertical axis gives absorbance/fluorescence (arbitrary units) and horizontal axis gives wavelengths in nanometers.

Electron microscopy (Fig. 2) showed that GSB1 is rod-shaped (≈0.3 x 1 µm), and revealed the presence of chlorosomes (light-harvesting structures found in green sulfur bacteria) (22) appressed against the inner membrane of these Gram-negative cells. The constrictions of some cells indicate that cell division occurs by binary transverse fission, and the absence of flagella is consistent with the lack of motility in liquid media.

Green sulfur bacteria uniquely contain a light-harvesting protein called the Fenna–Matthews–Olson (FMO) protein (23). Oligonucleotide primers were designed by using conserved sequences of FMO genes, and GSB1 DNA was used to PCR-amplify a 970-bp DNA segment. The DNA sequence of this PCR product encoded a 323-aa sequence that was from 71% to 91% identical in alignments with FMO sequences from 14 species of green sulfur bacteria. A tree of FMO sequences (Fig. 3a) indicates that the GSB1 FMO protein is most closely related to the FMO proteins of Chlorobium and Prosthecochloris marine species. PCR was also used to amplify a ≈1.5-kb segment of the GSB1 16S rRNA gene, and a tree of 19 bacterial 16S rDNA sequences

(Fig. 3b) again places GSB1 in a cluster that includes *Chlorobium* and *Prosthecochloris* marine species. We conclude that the GSB1 isolate is a previously unknown marine species of the green sulfur bacteria that is related to organisms classified as in the *Chlorobium* and *Prosthecochloris* genera (22). The capture of GSB1 from a deep-sea sample is unexpected, because viable green sulfur bacteria were thought to be found only in environments where light from the sun is available (22, 25).

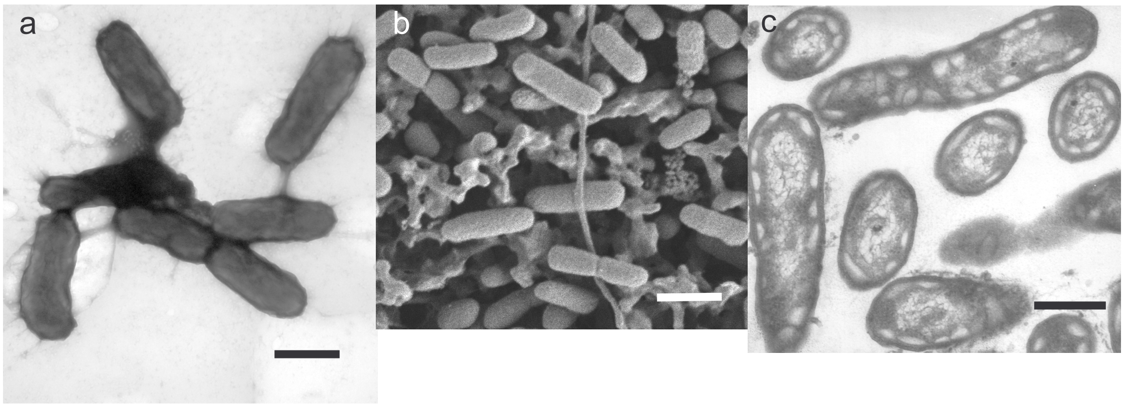

Fig. 2. Morphology and ultrastructure of GSB1 cells. (a) Negatively stained cells viewed by transmission electron microscopy. (Bar, 500 nm.) (b) Cells deposited on a filter and viewed by scanning electron microscopy. (Bar, 800 nm.) (c) Thin section through cells viewed by transmission electron microscopy with electron-transparent structures characteristic of chlorosomes. (Bar, 300 nm.)

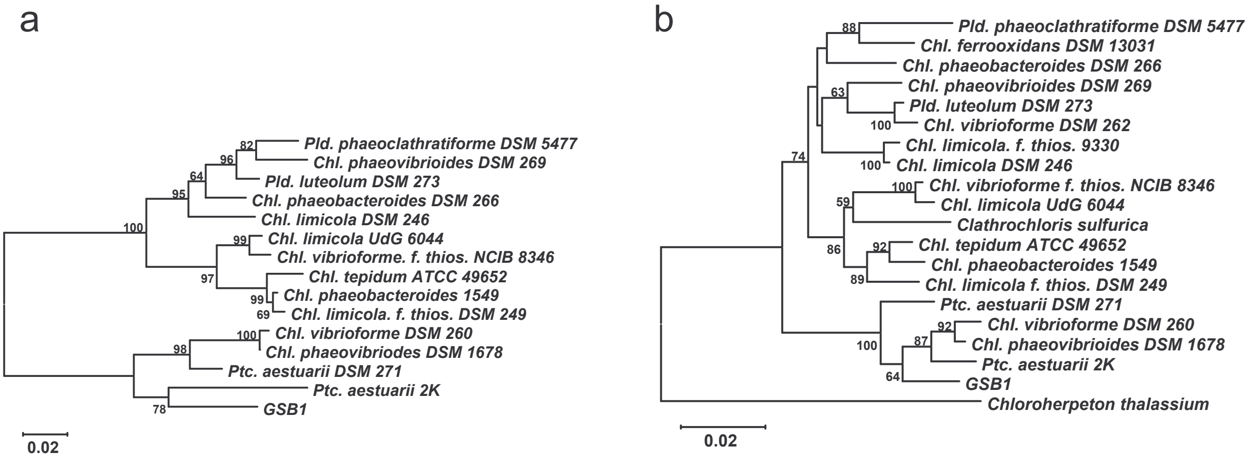

Fig. 3. Phylogenetic analyses of GSB1. (a) Tree of FMO protein amino acid sequences. (b) Tree of 16S rDNA sequences. Support values at nodes are given as percentages, and scale bars represent the expected number of changes per residue position.

The growth of GSB1 requires anaerobiosis, light, H_2S or elemental S, and CO_2. Of 104 substances tested, only acetate, propionate, peptone, and elemental S stimulated photosynthetic growth in the minimal medium SL10 (see Materials and Methods and Supporting Text). Exposure of cultures to air in the presence of light and H_2S reduced viability, but we found that GSB1 is resistant to exposure to air in the absence of light and H_2S (Fig. 4). There was no significant loss in GSB1 viability after 2 weeks, as also found with an anoxic control. The resistance of GSB1 to the toxic effects of O_2 in air is consistent with survival in the fluctuating environment of deep-sea hydrothermal vents (26), which could depend on the ability to survive translocation from decaying to nascent vents in the dark, oxygenated ocean depths (see below).

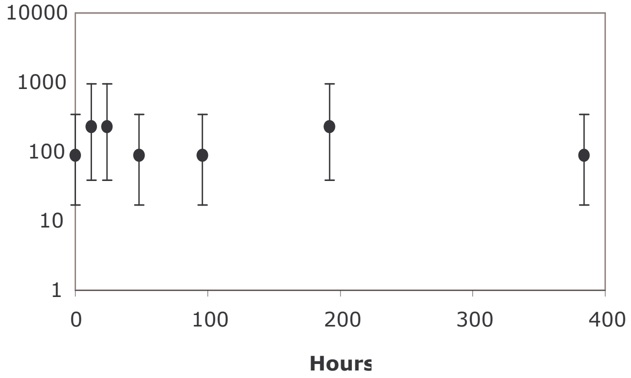

Fig. 4. Survival of GSB1 during exposure to air in darkness and the absence of H_2S. The vertical axis ($\log_{10}$ scale) gives percentages of viable cells based on most probable number (MPN) enumerations relative to microscopic counts (19, 20), and the horizontal axis gives the time of incubation. Points give average values, and vertical bars indicate 95% confidence limits.

Although the sample that contained GSB1 was obtained directly from a black smoker plume, it could be argued that this bacterium grows in the surrounding bulk water. We view this possibility as unlikely because the surrounding water is oxygenated and lacks a source of reduced S and light. The nearest locale (on the coast of Costa Rica) that could provide solar light and H_2S to support the anaerobic photosynthetic growth of green sulfur bacteria is ≈2,250 km distant from the TY black smoker, and the chance that GSB1 was directly transported by currents over that distance to be captured in a vent plume sample at >2 km depth appears to be vanishingly small. Instead, we suggest that the nonmotile GSB1 was shed from a microbial mat (22, 25) or similar microenvironment within centimeters of the TY vent orifice, and was swept into the turbulent plume. In such a microenvironment, microbial respiration or spontaneous chemical reactions between reduced substances present in the vent effluent and O_2 in the surrounding seawater could provide anaerobiosis, and a steep temperature gradient from the ≈2 °C surrounding water and the ≈300 °C interior of a vent could allow for survival close enough to geothermal light by harvesting of photons for photosynthesis. The source of reduced S could be either SO_4-reducing bacteria in a mat, or the vent effluent. Multiple samples obtained at several locations and depths, and a second 1-ml portion of the 1-liter sample that contained GSB1 did not yield growth under the same enrichment conditions. Thus, GSB1-like bacteria were not found at distances from a vent, although it appears that GSB1 was a minor component of the microbial community at the TY black smoker at the time of this cruise.

The properties of GSB1 are consistent with its deep-sea survival being enhanced by geothermal light that has been reported at several hydrothermal vents (27). Although there are vent-to-vent differences, in general the light intensity detected was greatest at wavelengths in excess of 700 nm (i.e., thermal or blackbody radiation) (27). The photon flux at 750 ± 50 nm (corresponding to the long wavelength absorption peak of light-harvesting BChl c in GSB1) at the orifice of a 370 °C black smoker was $\approx 10^8$ photons$\cdot$cm$^{-2}\cdot$s$^{-1}\cdot$sr^{-1} (28); the flux in the 400- to 500-nm range (short wavelength BChl and chlorobactene absorption peaks) was $\approx 10^4$ photons$\cdot$cm$^{-2}\cdot$s$^{-1}\cdot$sr^{-1} (27, 29) (6 x 10^{13} photons$\cdot$cm$^{-2}\cdot$s^{-1} = 1 μmole photons$\cdot$cm$^{-2}\cdot$s^{-1}; the term sr refers to a solid angle measured in steradians). Within 1–2 cm of 332 °C flange pools on black smoker chimneys, the total photon flux ($\approx 10^{11}$ photons$\cdot$cm$^{-2}\cdot$s$^{-1}\cdot$sr^{-1}) over the 600- to 1,000-nm range was estimated to be of the same order of magnitude as the solar photon availability for a green sulfur bacterium living at 80 m depth in the Black Sea (28, 30). The Black Sea bacterium is a brown-colored strain of the green sulfur bacteria, and contains BChl *e* and isorenieratene carotenoids that are thought to improve the harvesting of solar green light that penetrates to such depths (31, 32). There is relatively little light in the green (≈550 nm) region of spectra measured at deep-sea hydrothermal vents, and so the absence of isorenieratene carotenoids and the presence of BChl *c* in GSB1 are in accordance with the geothermal light wavelengths that have been measured at vents. The in situ cell division time of the Black Sea bacterium was calculated to be 2.8 years (30). Because of similarly low light intensities at deep-sea vents, GSB1 may be thought of as eking out an existence by infrequent harvesting of rare geothermal photons; this is in keeping with current ideas about the survival of bacteria in oligotrophic habitats, and in contrast to the relatively vigorous growth obtained under laboratory conditions. Although GSB1 was captured from the TY black smoker effluent, it is unlikely to be the direct descendent of a line of photosynthetic organisms that have continuously occupied this deep-sea hydrothermal vent since the appearance of anoxygenic photosynthesis on Earth >3 x 10^9 years ago (33), before the evolution of oxygenic photosynthesis that led to the accumulation of O_2 in the atmosphere ≈2 x 10^9 years ago (34, 35).

This is because individual hydrothermal vents are ephemeral relative to geological time scales, as observations from repeated visits to black smokers indicated major structural changes over the course of days to decades (26). There is evidence that isorenieratene carotenoid-containing green sulfur bacteria thrived in the North Atlantic Ocean in the Cenomanian/Turonian (C/T) age $\approx 10^8$ years ago during poorly understood ‘‘oceanic anoxic events’’ (36), which may have also occurred in the Pacific Ocean. The oceans have risen and subsided, and land masses have shifted over such time scales, and so the possibility exists of vent-to-shore as well as vent-to-vent exchange of green sulfur bacteria in the past.

Regardless, the capture of GSB1 at a deep-sea hydrothermal vent, but not from surrounding waters, indicates that geothermal light and associated reduced S compounds are sufficient to at least enhance the survival of green sulfur bacteria in the otherwise dark, oxygenated ocean depths. This discovery expands the range of possible environments that could harbor life forms which use light energy to drive endergonic biochemical reactions (1, 25), and frees the thinking of the scientific community from the constraint that any form of life that depends on light energy is necessarily limited to solarly illuminated habitats.

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SUPPORTING INFORMATION

The following supplements to SL10 medium that had no effect on growth: 10 mM acetoin; 5 mM adipate; 5 mM ascorbate; 2 mM benzoate; 2.5 mM butyrate; 2 mM citrate; 0.5 mM caproate; 0.5 and 5 mM caprylate; 0.5 and 5 mM crotonate; 2.5 mM formate; 5 mM fumarate; 5 mM gluconate; 5 mM glyoxylate; 5 mM glycolate; 0.5 and 5 mM heptanoate; 2.5 mM gamma-hydroxybenzoate; 2.5 mM alpha-hydroxybutyrate; 2.5 mM beta-hydroxybutyrate; 2.5 mM gamma-hydroxybutrate; 2.5 mM isobutyrate; 0.5 and 5 mM isovalerate; 5 mM isocitrate; 10 mM lactate; 0.5 and 5 mM levulinate; 5 mM malate; 5 mM maleinate; 5 mM malonate; 0.5 and 5 mM protocatechuate; 0.5 and 5 mM shikimate; 10 mM succinate; 2 mM tartrate; 2 mM trimethoxybenzoate; 0.5 and 5 mM valerate; 5 mM 2-oxoisocaproate; 5 mM 2-oxo-D-gluconate; 5 mM 2-oxoglutarate; 5 mM 2-oxooctanoate; 5 mM 2-oxomalonate; 5 mM 2-oxovalerate; 5 mM oxaloacetate; 2.5 mM pyruvate; 5 mM ethanol; 5 mM butanol; 5 mM 1,2-butanediol; 5 mM 2,3-butanediol; 5 mM ethyleneglycol; 5 mM glycerol; 5 mM methanol; 5 mM propanol; 5 mM 1,2-propanediol; 5 mM L(+) alanine; 5 mM L(+) arginine; 5 mM L(+) asparagine; 5 mM L(+) aspartate; 5 mM L(+) cysteine; 5 mM L(+) glutamate; 5 mM L(+) glutamine; 5 mM L(+) glycine; 5 mM L(+) isoleucine; 5 mM L(+) lysine; 5 mM L(+) methionine; 5 mM L(+) phenylalanine; 5 mM L(+) proline; 5 mM L(+) serine; 5 mM L(+) threonine; 5 mM L(+) tryptophan; 5 mM L(+) tyrosine; 5 mM L-valine; 5 mM ornithine; 5 mM N-acetyl-glucosamine; 5 mM D(+) arabitol; 5 mM L(-) arabitol; 5 mM D(+) arabinose; 5 mM L(-) arabinose; 5 mM D(+) cellobiose; 5 mM D(-) erythrose; 5 mM L-erythrulose; 5 mM fructose; 5 mM D(+) galactose; 5 mM D(+) glucosamine; 5 mM glucose; 5 mM dulcite; 5 mM D(-) lactose; 5 mM beta-D-lactose; 5 mM D(-) lyxose; 5 mM maltose; 5 mM D-mannitol; 5 mM D(+) mannose; 5 mM D(+) melezitose; 5 mM myo-inositol; 5 mM D(+) raffinose; 5 mM ribitol; 5 mM D-sorbitol; 5 mM L-sorbose; 5 mM trehalose; 5 mM xylitol; 5 mM D(+) xylose; 0.05% casamino acids; 0.05% yeast extract; 1 mM thiosulfate.

REFERENCES

(1) **Blankenship, R. E.** (2002) Molecular Mechanisms of Photosynthesis (Blackwell Science, Oxford).

(2) **Van Dover, C. L.** (2000) The Ecology of Deep-Sea Hydrothermal Vents (Princeton Univ. Press, Princeton).

(3) **Martin, W. & Russell, M. J.** (2003) Philos. Trans. R. Soc. London B **358**, 59–85.

(4) **Van Dover, C. L., Reynolds, G. T., Chave, A. D. &Tyson, J. A.** (1996) Geophys. Res. Lett. **23**, 2049–2052.

(5) **Nisbet, E. G., Cann, J. R. & VanDover, C. L.** (1995) Nature (London) **373**, 479–480.

(6) **Yurkov, V. V., Krieger, S., Stackebrandt, E. & Beatty, J. T.** (1999) J. Bacteriol. **181**, 4517–4525.

(7) **Beatty, J. T.** (2002) Photosynth. Res. **73**, 109–114.

(8) **Chyba, C. F. & Hand, K. P.** (2001) Science **292**, 2026–2027.

(9) **Venter, J. C., Remington, K., Heidelberg, J. F., Halpern, A. L., Rusch, D., Eisen, J. A., Wu, D., Paulsen, I., Nelson, K. E., Nelson, W., et al.** (2004) Science **304**, 66–74.

(10) **Pfennig, N. & Trüper, H. G.** (1981) in The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria, eds. Starr, M. P., Stolp, H., Trüper, H. G., Balows, A. & Schlegel, H. G. (Springer, New York), Vol. 1, pp. 279–289.

(11) **Widdel, F., Kohring, G. W. & Mayer, F.** (1983) Arch. Microbiol. **134**, 286–294.

(12) **Rainey, F. A., Ward-Rainey, N., Kroppenstedt, R. M. & Stackebrandt, E.** (1996) Intl. J. Syst. Bact. **46**, 1088–1092.

(13) **Cole, J. R., Chai, B., Marsh, T. L., Farris, R. J., Wang, Q., Kulam, S. A., Chandra, S., McGarrell, D. M., Schmidt, T. M., Garrity, G. M., et al.** (2003) Nucleic Acids Res. **31**, 442–443.

(14) **Hall, T. A.** (1999) Nucleic Acids Symp. Ser. **41**, 95–98.

(15) **Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J.** (1990) J. Mol. Biol. **215**, 403–410.

(16) **Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M.** (2001) Bioinformatics **17**, 1244–1245.

(17) **Brune, D. C.** (1999) Rapid Commun. Mass Spectrom. **13**, 384–389.

(18) **Coolen, M. J. L. & Overmann, J.** (2000) Appl. Environ. Microbiol. **66**, 2589–2598.

(19) **De Man, J. C.** (1977) Eur. J. Appl. Microbiol. **4**, 307–316.

(20) **De Man, J. C.** (1983) Eur. J. Appl. Microbiol. Biotechnol. **17**, 301–305.

(21) **Haymon, R. M., Fornari, D., Edwards, M., Carbotte, S., Wright, D. & Macdonald, K. C.** (1991) Earth Planet Sci. Lett. **104**, 513–534.

(22) **Overmann, J.** (2004) in The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community (Springer, New York), http://141.150.157.117:8080/prokPUB/chaprender/jsp/showchap.jsp?chapnum=323.

(23) **Blankenship, R. E. & Matsuura, K.** (2003) in Light-Harvesting Antennas, eds. Green, B. R. & Parson, W. W. (Kluwer, Dordrecht, The Netherlands), pp. 195–217.

(24) **Frigaard, N.-U. & Bryant, D. A.** (2004) Arch. Microbiol. **182**, 265–276.

(25) **Overmann, J. & Garcia-Pichel, F.** (2004) in The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community (Springer, New York), http://141.150.157.117:8080/prokPUB/chaprender/jsp/showchap.jsp? chapnum=239.

(26) **Fornari, D. J., Shank, T., Damm, K. L. V., Gregg, T. K. P., Lilley, M., Levai, G., Bray, A., Haymon, R. M., Perfit, M. R. & Lutz, R.** (1998) Earth Planet Sci. Lett. **160**, 419–431.

(27) **White, S. N., Chave, A. D., Reynolds, G. T. & VanDover, C. L.** (2002) Geophys. Res. Lett. **29**, 10/1029/2002GL014977.

(28) **White, S. N., Chave, A. D., Reynolds, G. T., Gaidos, E. J., Tyson, J. A. & VanDover, C. L.** (2000) Geophys. Res. Lett. **29**, 1151–1154.

(29) **White, S. N., Chave, A. D. & Reynolds, G. T.** (2002) J. Geophys. Res. **107**, doi:10.1029/2000JB000015.

(30) **Overmann, J., Cypionka, H. & Pfennig, N.** (1992) Limnol. Oceanogr. **370**, 150–155.

(31) **Pfennig, N.** (1967) Annu. Rev. Microbiol. **21**, 285–384.

(32) **Overmann, J., Beatty, J. T., Hall, K. J. & Pfennig, N.** (1991) Limnol. Oceanogr. **36**, 846–859.

(33) **Des Marais, D. J.** (2000) Science **289**, 1703–1705.

(34) **Dismukes, G. C., Klimov, V. V., Baranov, S. V., Kozlov, Y. N., DasGupta, J. & Tyryshkin, A.** (2001) Proc. Natl. Acad. Sci. USA **98**, 2170–2175.

(35) **Kasting, J. F. & Siefert, J. L.** (2002) Science **296**, 1066–1068.

(36) **Sinninghe Damsté, J. S. & Köster, J.** (1998) Earth Planet Sci. Lett. **158**, 165–173.

CHAPTER 6

SUBFOSSIL DNA SEQUENCES OF GREEN SULFUR BACTERIA AS INDICATORS FOR PAST WATER COLUMN ANOXIA IN THE BLACK SEA

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ABSTRACT

Today, only one species of green sulfur bacteria, *Chl.* sp. BS-1, inhabits the Black Sea chemocline. Due to its extreme physiological adaptations exhibiting the lowest cell density and low light adaption recorded in a stable green sulfur bacterial population to date, this strain was suspected to represent a reliable biomarker for photic zone anoxia. In this study, a living population of green sulfur bacteria was enriched from 2000 m deep sediments of the Black Sea. Sediment samples from depths up to 3.5 m below sea floor were shown to contain reamplifiable rRNA of nine phylotypes of green sulfur bacteria, only three of them representing saltwater species. One of the other sequences, which was found in Units IIa, IIb, and III, clusters with a sequence that seems to be ubiquitous in the freshwater habitats of the world and seems to represent the most widespread phylotype amongst the green sulfur bacteria. Another sequence clusters with an environmental sequence from the mediterranean sapropel layers. It was only found in Unit III. The 16S rRNA gene sequence of *Chl.* sp. BS-1 was found in Units I, IIa, IIb and III. The deepest location of that melting type was at station 7620 in a sediment depth of 171 cm, well situated in Unit III. An allochthonous introduction of green sulfur bacterial sequences to the Black Sea sediments seems likely and disproves that green sulfur bacterial pigments in sediments can be used in any case as indicators for photic zone anoxia in the overlying water column. An allochthonous introduction of the strain *Chl.* sp. BS-1 may be the offspring of the population which is now stable in the Black Sea chemocline. Sediments also revealed seven rRNA gene fingerprints of bacteria that are only distantly related to the green sulfur bacteria. They represent a cluster of a not yet characterized closely related bacteria, the so-called "deep-branching green sulfur bacteria". Phylogenetic analyses show a highly diverse group of bacteria, with up to 33 % sequence variability in the 16S rRNA gene. The cluster of "deep-branching green sulfur bacteria" is possibly not monophyletic but must be subdivided and positioned at the bases of different phylogenetic branches. In the enrichment culture from the sediments, however, none of these bacteria were found, indicating a physiology different from that of green sulfur bacteria.

INTRODUCTION

The Black Sea is the largest present day anoxic water body. It is permanently stratified, and 87-92% of its water body is permanently anoxic (Codispoti et al. 1991, Sorokin 2002, Konovalov et al. 2001). Therefore, the Black Sea is regarded as a contemporary analogue to the past sulfidic oceans, and its paleoceanography is well documented in a sequence of sediment horizons (Canfield et al. 1996).

The earliest period of the Black Sea documented by the sedimentary record is a freshwater period that lasted from 22,000 yrs. BP until about 9000 yrs. BP (Degens and Ross 1972). Limnic sediment layers of that period are referred to as Unit III. Unit III is covered by a microlaminated organic-rich sapropel layer (Unit II; Hay et al. 1991a) which was probably formed during a postglacial sea-level rise which occurred between 8200 yr. BP and 5000 yr. BP (depending on the dating method; Repeta 1993, Wakeham et al. 1995, Canfield et al. 1996). The melting of the glaciers lead to a sea level rise in the Mediterranean Sea followed by an inundation of the Black Sea basin by salt water, which turned the Black Sea into an anoxic marine basin (Sinninghe Damsté et al. 1993). Hence, Unit II is an organic rich sapropel layer (12-15% organic matter, Wakeham et al. 1995) which was first deposited in the center of the basin, and which marks the beginning of euxinic conditions in the central basin. Since then a stable chemocline has formed which has constantly risen upwards from 2200 m to 500 m depth over a time period of 2300 – 3000 years (Degens and Stoffers 1976), reaching the photic zone more than 6000 years ago (Repeta 1993, Sinninghe Damsté et al. 1993). Unit II can be further divided into the older Unit IIb with a higher organic carbon content that was deposited during a period of higher productivity, and Unit IIa which was deposited during a period of lower productivity and increased input of terrigenous material (Hay 1988, Hay et al. 1991a). Unit II is covered by a finely laminated coccolith layer (Unit I, Hay et al. 1991a) which indicates the invasion of the marine coccolithophorid *Emiliana huxleyi* accompanying a rise in salinity. Unit I represents the most recent sedimentary deposition in the Black Sea and is covered by a fluffy top layer. Units II and I are separated by a several cm thick transition sapropel, which was deposited over a time interval of 400-1000 years (Hay et al. 1991b).

Extended water column anoxia in the Black Sea basin is supported by bacterial chemofossils detected in diverse sediment units. Investigations in subfossil sediments focused on the presence of green sulfur bacterial photosynthetic pigments and their degradation products (Repeta 1993, Sinninghe Damsté et al. 1993). The group of green sulfur bacteria (*Chlorobiaceae*) is well suited as marker organism for photic zone anoxia since it represents a monophyletic group of very closely related obligately anoxygenic phototrophic bacteria (Imhoff 2003) which inhabit the chemoclines of stratified water bodies worldwide. The green sulfur bacterial carotenoid isorenieratene (Repeta 1993) as well as derivatives like sulfurized isorenieratane (Sinninghe Damsté et al. 1993, Wakeham et al. 1995) are present in Unit IIb and I. Since these pigments occur almost exclusively in obligately anaerobic phototrophic green sulfur bacteria (Sinninghe Damsté et al. 1993, Wakeham et al. 1995) their deposited pigment biomarkers indicate the development of photic zone anoxia and a stable stratification of the water column during deposition of Unit IIb and I. Consequently, photic zone anoxia occurred already more than 6000 years ago (Repeta 1993, Sinninghe Damsté et al. 1993).

Presently, the chemocline of the Black Sea is situated at a depth of ~60 to 200 m (Canfield et al. 1996). In the northwestern basin, at a depth of 80-100 m, a population of green sulfur bacteria was detected, consisting of only a single, novel phylotype (Overmann et al. 1992, Manske et al. 2005). The layer of green sulfur bacteria in the Black Sea chemocline exhibits the lowest cell density and low light adaption recorded in a stable bacterial population to date. Cell densities of $2.3 \cdot 10^5$ to $3 \cdot 10^5$ GSB cells ml^{-1} were counted in the Black Sea chemocline (Manske et al. 2005), depending on only 0.0022 to 0.00075 µmol Quanta m^{-2} s^{-1} at stations 7605 and 7620, respectively (Manske et al. 2005). Today, the Black Sea being stratified, only one phylotype of green sulfur bacteria is detectable in the water column (Manske et al. 2005). A quantification of the cell number of phylotype BS-1 by real-time PCR indicated that this bacterium constituted only 0.5 to 1% of the total cell number in the chemocline in 2001 (E. Marschall et al. in preparation). However, due to the extreme physiological adaptation to its environment, subfossil DNA sequences of this bacterium in the sedimentary record may be used as a more specific biomarker to infer photic zone anoxia and the vertical extent of the oxic zone in more detail.

The presence of green sulfur bacterial DNA in the Black Sea sediments was shown by the isolation of brown colored *Chlorobiaceae* that were not characterized further from the top layers of the sediments (Hashwa and Trüper, 1978). In our study, a viable population of green sulfur bacteria could be enriched from top layers of Black Sea sediment that was shown to be genetically identical to the strain isolated from the chemocline *Chl.* sp. BS-1. This indicates the presence of intact bacterial DNA in the Black Sea sediments.

In the present study, several subfossil 16S rRNA gene sequences of the phylum *Chlorobi* were recovered from deep-sea sediments of Units I, II and III of the sedimentary record. Surprisingly, with the specific primers (Overmann et al. 1999) seven sequences of a not yet characterized group of bacteria were also found in the sediments. These sequences were compared phylogenetically with those of extant GSB and close relatives in the database and revealed the presence of a highly diverse cluster of not yet cultured species, called the "deep-branching green sulfur bacteria". These bacteria represent a distinct phylum in the phylogenetic tree of bacteria which shares a common root with the Bacteroidetes (Garrity and Holt 2001). Primary phylogenetic investigations give a closer look on these bacteria and the basis for further investigations and attempts to enrich first representatives.

MATERIALS AND METHODS

Study sites. Sediment samples were obtained during cruise 51 of the R/V *Meteor* between December 12 and December 28 in 2001. The sampling sites were located on the northwestern continental slope of the Black Sea. GeoB No. 7620 was located at 42°56.2'N, 30°01.9'E (2006 m water depth), GeoB No. 7605 at 42°30.7'N, 30°14.7'E (2162 m water depth). Surface sediment samples up to a depth of 50 cm were obtained with a multicorer. Sediment layers up to a depth of 7 m below seafloor were obtained with a gravity corer. The first core (GeoB No.7620, ~3 m long) was laminated whereas the second (GeoB 7605, ~7 m long) contained numerous turbidites and scattered parts of sapropel. Additional sediment samples for enrichment experiments were retrieved from the top layer (Unit I) of core MD 04-2762 (42°38'89N, 32°45'94E; 2210 m water depth), received during the ASSEMBLAGE cruise in 2004.

Sample preparation. Cores were split longitudinally and subsamples were recovered with sterile plastic syringes or sterile spatula using aseptic techniques after removal of the exposed sections according to Coolen and Overmann (2000). Sediment samples were stored frozen in sterile 50 ml plastic tubes at -20°C until processing.

DNA Extraction. 5 g subsamples from the multicorer and 10 g samples from the gravity cores were used for extraction of genomis DNA. The UltraClean Mega Soil DNA Kit (MO BIO Laboratories, Inc., Solana Beach, CA) was employed following the manufacturer's manual. An additional sonication step was introduced in order to improve bacterial cell lysis and mixing (level 6 for 2 min, on ice; Branson Sonifier-Cell Disruptor B 15, Danbury, CT). For further purification, DNA extracts were passed through Genomic tip-20 columns (Qiagen, Hilden, Germany) after adjusting the ionic strength of the sample with 0.9 M MOPS·KOH (3-N-(morpholino)propanesulfonic acid·KOH), pH 7.0, and 5 M NaCl. Precautions against contamination of the samples with foreign DNA included the use of a laminar flow hood (security level 2), sterilization of all glassware by dry heat (4h, 180°C), and the use of certified pre-sterilized plastic ware. Extraction controls without sediment addition (negative control) showed no DNA contamination.

Genomic DNA from extant planktonic green sulfur bacteria in the Black Sea was obtained by concentrating 120 L of chemocline water by tangential flow (Pellicon 2 tangential flow device; Millipore, Bedford, MA) fitted with a VVPP-C-filter (0.1 µm pore size, Millipore) and subsequent filtration onto polycarbonate filters (diameter 47 mm, pore size 0.1 µm; Millipore) in a sterilized filtration unit (Sartorius). DNA was extracted from the filters using a phenol/chloroform/isoamylalcohol extraction protocol according to Fuhrman et al. (1988).

All DNA extracts were concentrated with Centricon centrifugal filter devices YM-50 (Millipore) according to the manufacturer's instructions and DNA concentrations were determined using PicoGreen (Molecular Probes, Eugene, OR, USA) (Supplementing table 1).

PCR. Standard conditions for PCR were: 20-50 ng of template DNA, 10 µM of each Primer, 5 µl of GeneAMP 10x PCR buffer, 0.2 mM dATP, dCTP, dGTP and dTTP (GeneAMP dNTPs, Applied Biosystems, Weiterstadt, Germany), 3.5 mM $MgCl_2$ and 2.5 U AmpliTaq Gold polymerase (Applied Biosystems) in a total volume of 50 µl. The PCR was preceded by a hot start (5 min at 95°C) and ended with a 4°C hold. All PCRs were run in a thermocycler GeneAMP PCR system 9600 (Applied Biosystems).

For specific amplification of the 16S rRNA gene of green sulfur bacteria in sediment and chemocline samples the universal primer GC341f and the phylum-specific primer GSB822r (Overmann et al. 1999) were used. Cycling parameters were: 95°C for 5 min, followed by 10 cycles of 94°C for 30 sec, 55°C for 45 sec, 72°C for 30 sec, and 30 cycles at 94°C for 30 sec, 50°C for 45 sec, 72°C for 30 sec.

Amplification products were analyzed by standard agarose gel electrophoresis.

Denaturing gradient gel electrophoresis (DGGE). Two to three amplification reactions, depending on the concentration of the products as determined by agarose gel electrophoresis, were combined and concentrated to a volume of 15 µl by vacuum centrifugation. Samples were loaded onto 6 % (w/v) polyacrylamide gels in 1x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 7.4) containing a linear gradient of formamide and urea as denaturants (Muyzer et al. 1995). Gels were stained with SybrGold (Molecular Probes) for 45 min, the gel images were captured with a digital camera (Spot RT color, Diagnostic Instruments Inc., MI) and processed with the Spot RT software (version 3.1). Individual DNA bands were excised from the gel and eluted in 40 µl of 10 mM Tris·HCl (pH 8.5) by incubation at 65°C for 45 min.

Reamplification. Most of the fragments could be reamplified with primers Uni341f/GSB822r (Muyzer et al. 1993/Overmann et al. 1999) using 1 µl of eluted DNA from the DGGE, resulting in fragments of ~500 bp (Table 1). Since not all sequences were retrieved with these primer combinations, the total fragment was reamplified using nested PCR, yielding two fragments with an overlap of ~17 bp. The 3’-end was reamplified with the primer combination Uni341f /GSB

564r (5’-CGC ACC CTT TAC ACC CAG-3’; Marschall et al. in preparation), the 5’-end was reamplified using the primer combinations Uni 515f (5'-GTG CCA GCA GCC GCG G-3', modified after Lane 1991)/GSB822r or GSB532f (Quelle)/GSB822r (Overmann et al. 1999), the second combination being used only in cases of non successful amplification with the first (Table 1). The reamplification products were purified with the QiaQuick PCR purification kit (Qiagen, Hilden, Germany) and eluted in 50 µl 1mM Tris-HCl (pH 8.5).

Table 1. PCR conditions and step down PCR for the reamplification of DGGE bands. Each PCR started with a hot start (95°C, 5 min) and ended with a 4°C hold.

Primer combination	Melting (94°C)	Step down PCR: Annealing (No. of cycles)		Extension
Uni341f/GSB822r	30 s	55°C, 45 s (10)	50°C, 45 s (30)	72°C, 60 s
Uni341f/GSB564r	30 s	66°C, 45 s (10)	64°C, 45 s (25)	72°C, 60 s
GSB532f/GSB822r	30 s	57°C, 45 s (10)	52°C, 45 s (25)	72°C, 60 s
GSB515f/GSB822r	30s	57°C, 45 s (10)	52°C, 45 s (25)	72°C, 60 s

Cloning of reamplification products. In few cases (sequences 7605-32,-33,-39,-40,-44,-84,-91,-96), the analysis of reamplification products on agarose gels showed double bands. To evaluate these sequences, the PCR products were cloned. For the cloning reaction, PCR products were ligated into the vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA) and subsequently cloned into TOP10 chemically competent cells according to the instructions supplied by the manufacturer (Invitrogen). Selective agent on LB agar plates was kanamycin (50 µg/ml). The plasmids were isolated from overnight cultures of the picked clones in liquid LB medium containing kanamycin (50 µg/ml). The isolation and purification of plasmids was done using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Plasmids were checked for sequence differences by restriction analysis using RsaI (restriction site GT/AC; Fermentas, St. Leon-Rot, Germany), according to the protocol provided by the manufacturer. Fragments showing different banding patterns were sequenced.

Sequencing of reamplification products. For sequencing, the same primers were used that yielded a reamplification product (Table 1). Cycle sequencing was performed with the AmpliTaq FS BigDyeTerminator cycle sequencing kit (Applied Biosystems) following the protocol supplied by the manufacturer. In the sequencing reaction 4 µl of reaction mix , 2 µl of sequencing buffer (Applied Biosystems), 0.5 pmol primer, and 1 to 5 µl of purified PCR product were used in a total volume of 20 µl. The following protocol was used for amplification: 10 s at 96°C, 5 s at

50°C and 4 min at 60°C, for primers with melting temperatures higher than 55°C the protocol was 10 s at 96°C, 5 s at 59.5°C and 4 min at 60.5°C. 30 cycles were run, followed by a hold at 4°C. Samples were run on a capillary sequencer (ABI Prism377, Applied Biosystems).

Phylogenetic trees. 16S rRNA gene sequences were phylogenetically analyzed using the ARB phylogeny software package (Ludwig et al. 1998). For the automated alignments the implemented Fast Aligner V1.03 was used. The resulting alignments were corrected according to the 16S rRNA secondary structure information of *Chlorobium vibrioforme* DSMZ 260^T, as available through www.rna.icmb.utexas.edu. Phylogenetic trees were constructed including 16S rRNA gene sequences of available strains and environmental sequences of green sulfur bacteria, as well as closely related sequences of the deep-branching green sulfur bacteria. First, sequences that were longer than 1200 bp were used for calculation employing the *maximum likelihood algorithm* (*Fast DNA_ML*). Shorter sequences were inserted afterwards employing the *parsimony interactive* tool implemented in the ARB software package without changing overall tree topology (Gich et al. 2001). The resulting tree topology was checked for correct distances with a PHYLIP distance matrix using Felsenstein correction. No filter was used, and only the alignment positions were used which were present in all sequences.

Enrichment of green sulfur bacteria from Unit I. Samples for the enrichment of green sulfur bacteria from Black Sea sediments had been taken from Unit I (~15 cm sediment depth) using aseptic techniques as desribed in "sample preparation". The enrichment culture was set up onboard the vessel by inoculating 22.5 ml of sterile medium for the strain BS-1 (Manske et al. 2005) with 0.5 ml of freshly sampled sediments of core MD 04-2762. These sediment slurries were kept at 4°C in the dark until being subjected to liquid dilution series in 22.5 ml gas-tight closed glass tubes with the same medium. The growth medium consisted of artificial seawater (pH 7.2), adjusted to the ionic strength of the Black Sea chemocline, and supplemented with 1% (v/v) fresh yeast extract, 2 mM sodium acetate and 200 µM dithionite (Manske et al. 2005). The series were then incubated at 15°C at ~5 µmol quanta m^{-2} s^{-1} for several weeks. As a control, the same medium without inoculum was incubated at the same conditions. In order to achieve a higher percentage of green sulfur bacteria in the enrichment culture, 2.2 ml of culture was transferred to medium supplemented with 1 mM malate and 200 µM dithionite (Manske et al. 2005), as well as to fresh medium of the same composition, to maintain the original growth conditions.

RESULTS

Bacterial diversity in the Black Sea sediment layers. Genomic DNA was extracted from 21 depths of core 7620 and 25 different depths of core 7605 (Fig. 1 A, B). In addition, samples of the flocculent surface layer (*fluff*) from both deep-water stations were extracted. Amplification with PCR primers specific for green sulfur bacteria and subsequent separation by DGGE yielded 16 different melting types (a – q, Figure 1). Ten of the melting types occurred in both core 7620 and 7605. Not coherent were the melting types 'a','l' and 'n', which occurred only in core 7620 and melting types 'b', 'c' and 'p' which occurred only in core 7605. The two cores therefore showed similar bacterial diversity. 33 bands from core 7620 and 46 bands from core 7605, representative for the different melting types, were reamplified and sequenced (Figure 1, numbered bands).

Green sulfur bacteria. Nine different phylotypes of green sulfur bacteria were found (Environmental sequences A through I; Fig. 2), but six of these phylotypes do not group with the marine species of green sulfur bacteria (Imhoff 2003, Manske et al. in preparation). One sequence, which was detected only in Unit I, clusters with the salt-requiring species *Chlorobium phaeovibrioides* DSM 269^T and *Chlorobium phaeovibrioides* DSM 261 (Env. sequence A; Fig. 2). Another sequence, found in Units IIa, IIb, and III, clusters with a sequence that seems to be ubiquitous in the freshwater habitats of the world (Env. sequence B; Fig. 2). Env. sequence C clusters with an environmental sequence from the Mediterranean sapropel layers (Fig. 2). It was only found in Unit III. Two of the truly green sulfur bacterial sequences cluster with phototrophic consortia (Env. sequences D, E; Fig. 2) and were found in Units I (E only), IIa, IIb, and III (Fig. 4). A novel phylotype was also detected, which has an unstable position in the phylogenetic tree (Env. sequences F; Fig. 2). This phylotype was only found in Unit IIa.

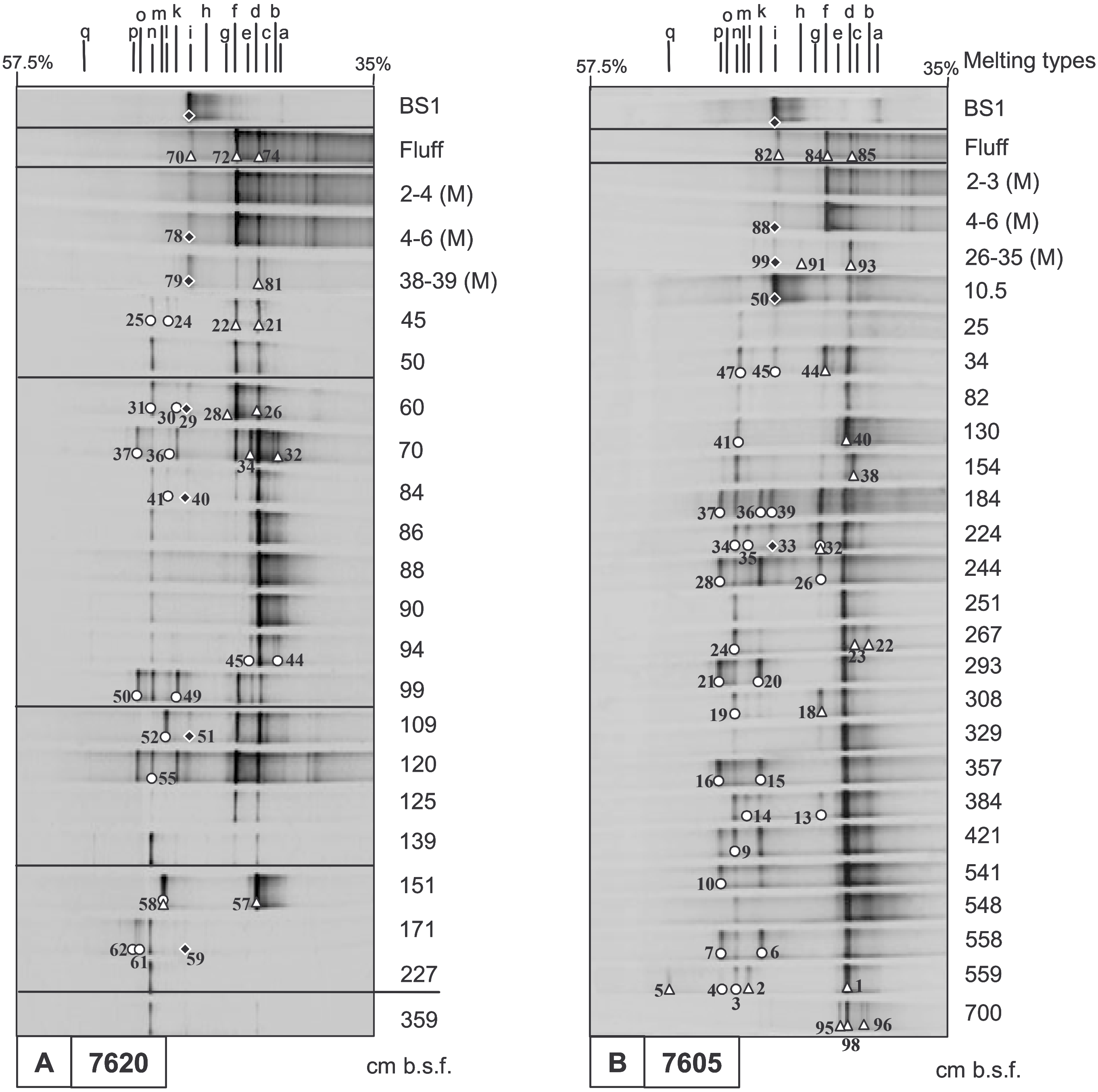

Figure 1. DGGE of 16S rRNA gene sequences in two Black Sea sediment cores. The amplificates were retrieved with primers specific for green sulfur bacteria. *A* – The laminated sediment core GeoB No. 7620, *B* – Sediment core with turbidites GeoB No. 7605, *BS1* – enrichment culture from the chemocline. Numbers to the right indicate sediment depths (cm b.s.f = cm below sea floor), letters on top indicate the different melting types, the percentages (35% - 57.5%) refer to the denaturant concentration in the gel. Symbols refer to the different phylotypes: *circle,* GSB, *black diamond,* BS-1, *triangle,* deep-branching GSB.

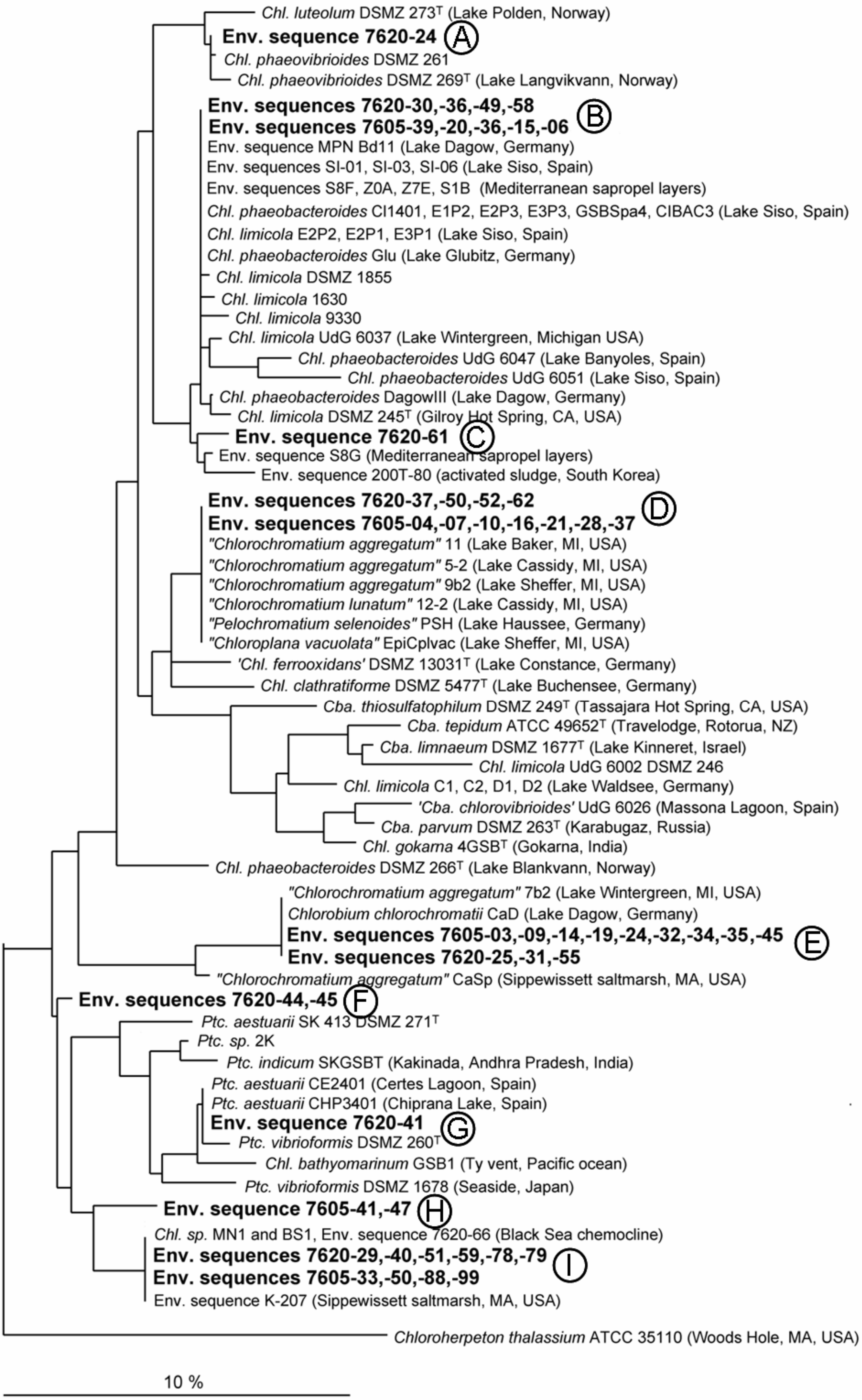

Figure 2. Phylogenetic affiliation of green sulfur bacterial 16S rRNA gene sequences obtained from cores GeoB No. 7620 and 7605. Environmental sequences (phylotypes A-I) obtained during this study are shown in bold type. Numbers refer to the core (7620 or 7605) followed by sequence No. (cf. Fig. 1 A,B).

Real saltwater sequences. Only three of the detected sequences cluster with the real saltwater strains of green sulfur bacteria. The first is identical to *Prosthecochloris aestuarii* CE 2401 (Env. sequence G, Fig. 2) and was recovered from 84 cm depth in Unit IIa (Fig. 1). In the same sediment sample the sequence of the chemocline strain was found. The second one is closely related to *Chl.* sp. BS-1 (Env. sequence H, Fig. 2) and was also isolated from Unit IIa. The third sequence, finally, is the sequence of the chemocline strain BS-1 (Env. sequence I, Fig. 2). It was found in Units I, IIa, IIb and III (Fig. 4)

The chemocline strain in the Black Sea sediments. The melting type corresponding to the green sulfur bacterium in the enrichment culture BS-1 and in the chemocline (melting type 'i') could be detected in 6 depths of core 7620 and in 4 depths of core 7605 (Fig. 1). The deepest location of that melting type was at station 7620 in a sediment depth of 171 cm (Fig. 1), well situated in Unit III. The sequence of the melting type 'i' is in most cases identical to the sequence of the chemocline strain (Env. sequence I) (Figs. 1, 2). In four cases, however, the DNA bands with melting type 'i' contained sequences which group with typical freshwater strains (7605-45, 7605-39; Figs. 1, 2) or with bacteria outside the radiation of the green sulfur bacterial tree (7620-70, 7605-82; Figs. 1, 3). Surprisingly, the DNA sequence of the chemocline strain was not found in the *fluff* layer.

Phylotypes analyzed. In two cases the same DGGE band reveals two different DNA sequences, which were only found by cloning the respective sequence (sequences 7620-58, Fig. 1A and 7605-32, Fig. 1B). Surprisingly, in most cases the phylotypes are found in more than one melting type.

A new group of uncultured bacteria. The 16S rRNA gene sequences detected in the sediments reveal seven DNA fingerprints of bacteria that are only distantly related to the green sulfur bacteria (Environmental sequences K-Q, Fig. 3). These sequences cluster with a group of bacteria that is not yet represented by any cultivated species and which can be positioned at the root of the green sulfur bacterial radiation. Therefore we call them “deep-branching green sulfur bacteria”. Closely related environmental sequences of the “deep-branching green sulfur bacteria” were found worldwide (Fig. 3) but are not characterized further since enrichment cultures are missing. Sequence types L, M, and N (Fig. 3) are very closely related, and probably are an artificial group which represents only one species. These types occur in Units I through III, sequence type N occurs in all analyzed Units, including the Fluff layer. Other sequence types were only found once or twice in the sedimental record (Fig. 4).

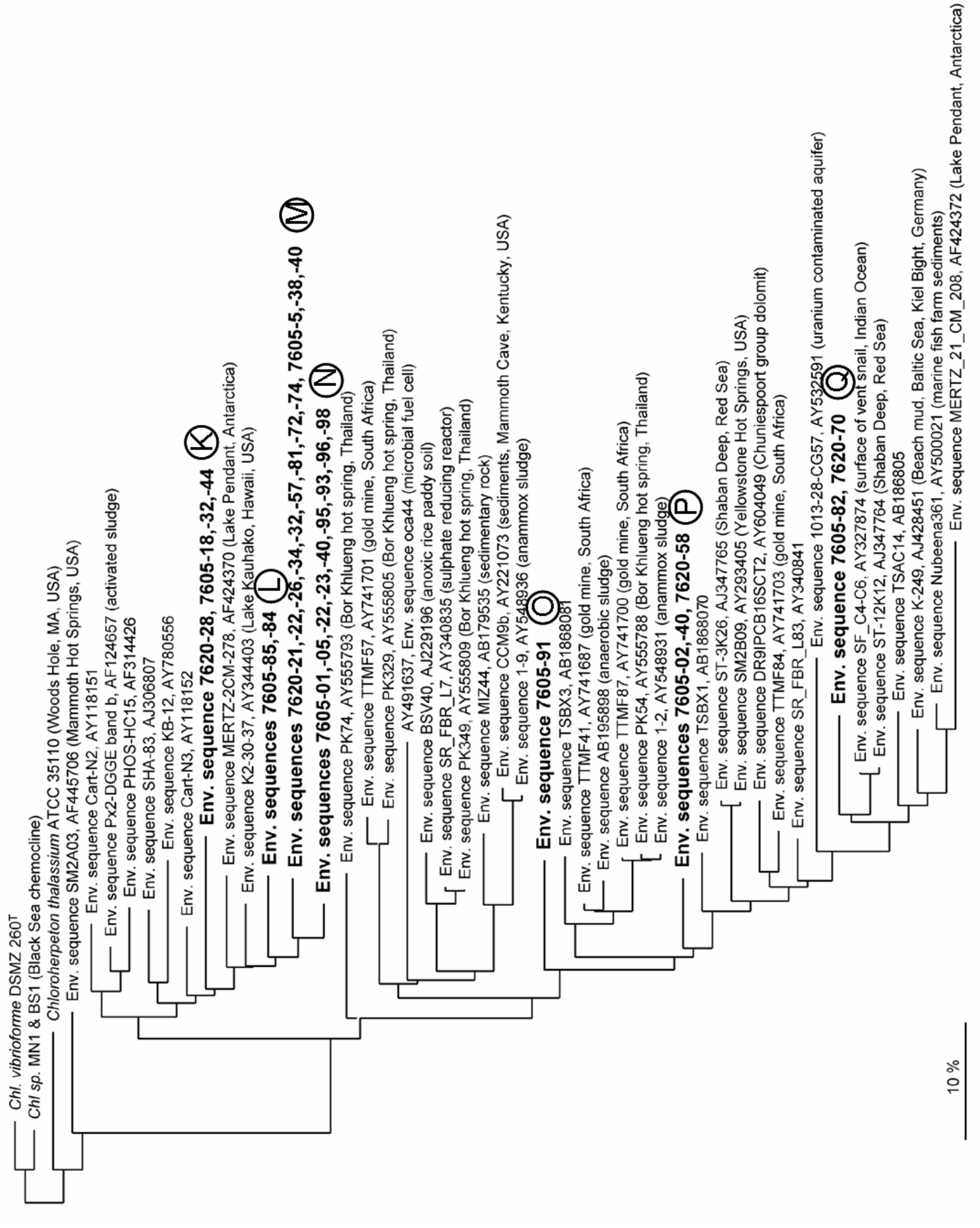

Figure 3. Phylogenetic affiliation of 16S rRNA gene sequences of the so-called "deep-branching green sulfur bacteria" obtained from cores GeoB No. 7620 and 7605. Environmental sequences (phylotypes K-Q) obtained during this study are shown in bold type. Numbers refer to the core (7620 or 7605) followed by sequence No. (cf. Fig. 1 A,B)

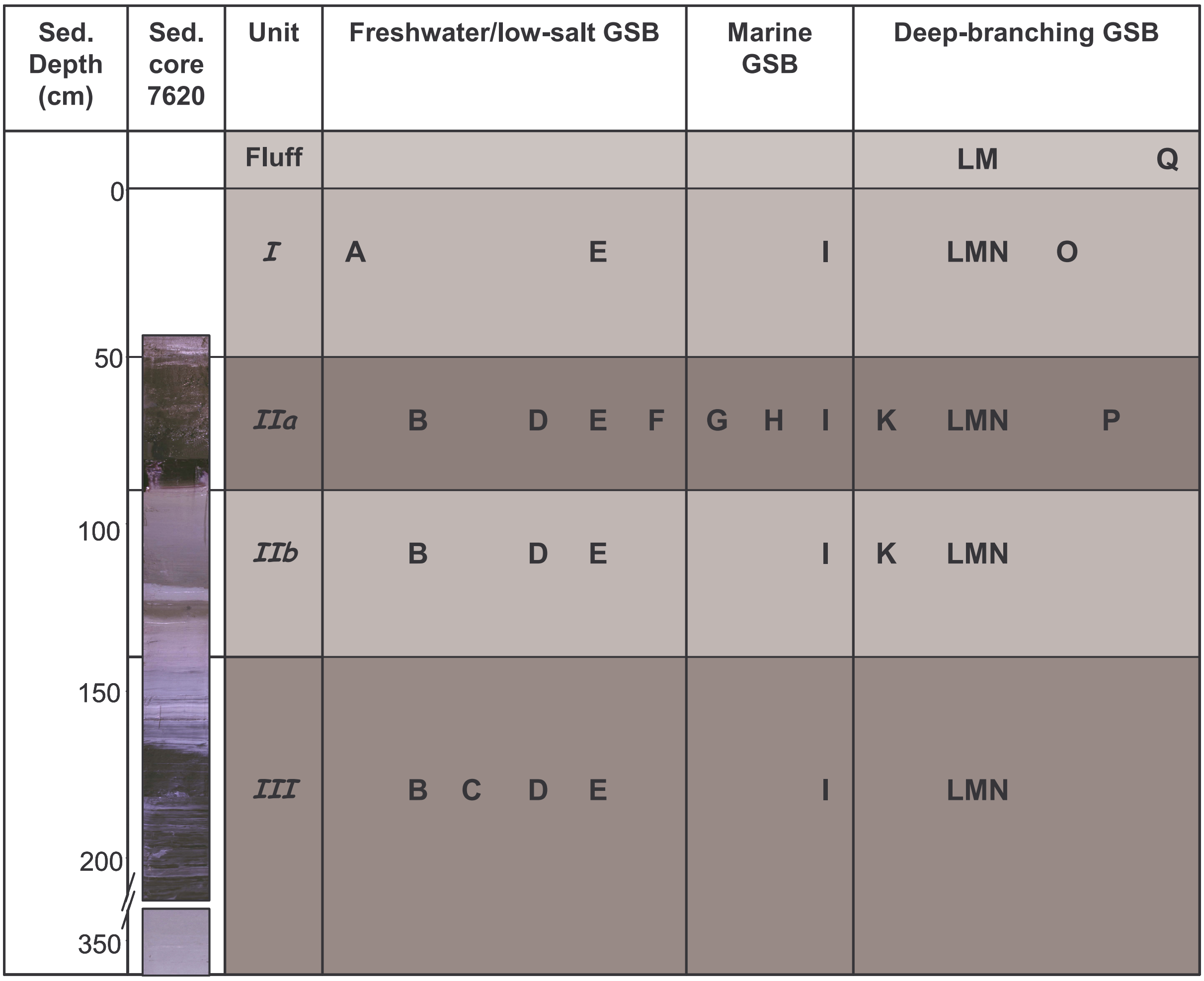

Figure 4. Environmental sequences in the different sediment layers of the Black Sea sediment. Reference is sediment core GeoB No. 7620. The core was retrieved from a water depth of 2006 m and shows the typical lamination (Fluff, Units I-III) of deep Black Sea sediments. Phylotypes derived from phylogenetic analyses (Fig. 2 and 3) of DGGE fingerprints (Fig. 1, stations 7605 and 7620).

A viable population of green sulfur bacteria in the sediments. MPN series set up in 22.5 ml glass tubes with sediment slurries obtained from Unit I of core MD 04-2762 (2210 m water depth) yielded an enrichment culture of brown colored green sulfur bacteria wheras no bacterial growth was observed in the control tube. The enrichment culture was named BS-2. The community was investigated by PCR-DGGE analysis with universal eubacterial primers and a GSB specific primer combination (Fig.5) with subsequent sequence analyses. Four prominent sequences were detected in the enrichment culture BS-2 (melting types 02, 03, 04 and 08, Fig. 5).

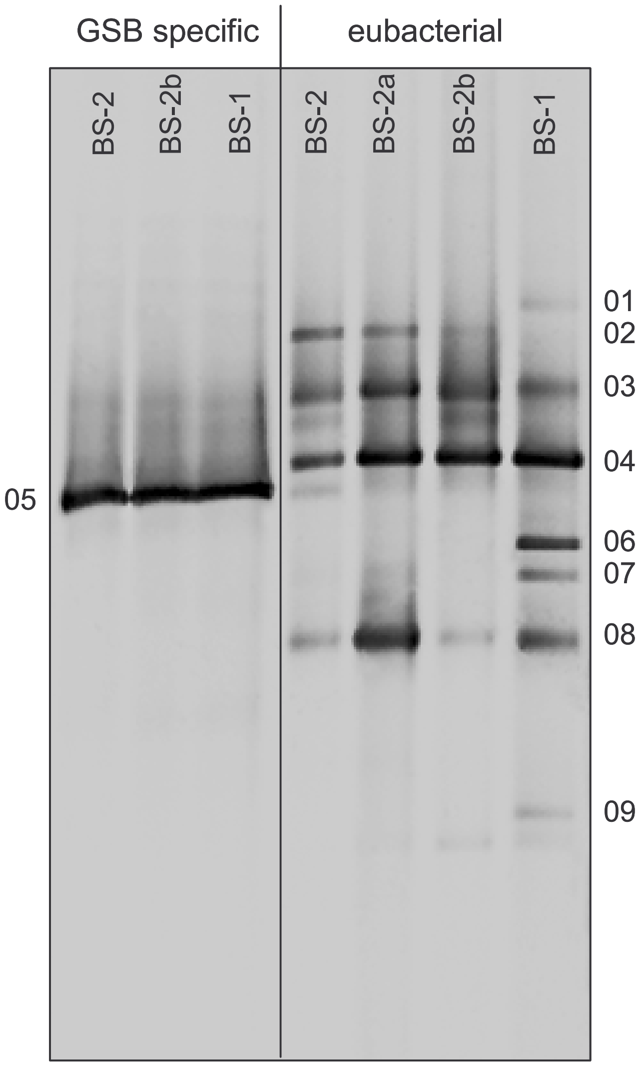

Figure 5. Denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene sequences in enrichment cultures from Black Sea sediments and chemocline. Two sets of primers were used: primers specific for green sulfur bacteria (*GSB specific*) or eubacterial primers (*eubacterial*). BS-1 – enrichment culture from the chemocline, BS-2 – enrichment from the Black Sea sediments, BS-2a – BS-2 culture transferred to medium with acetate and yeast extract, BS-2b – BS-2 culture transferred to medium with malate. Numbers indicate different melting types (see text for details).

The sequence of melting type 04 was 100% identical to the sequence of the Black Sea chemocline strain *Chl.* sp. BS-1, enriched in 2001 from the Black Sea chemocline (Manske et al. 2005) (Table 2). The sequence of *Chl.* sp. BS-1 was the only green sulfur bacterial sequence detected in both sediments and chemocline (melting type 04, table 2).

The sequences accompanying *Chl.* sp. BS-1 were different in the chemocline (melting types 01, 03a, 06, 07 and 09) and in the sediments (02, 03b), except for melting type 08, which was enriched from both sediments and chemocline (Fig. 5). The closest relative to that sequence is *Ilyobacter psychrophilus* FQ50^T (*Fusobacteriaceae*) (Table 2).

The sequence of melting type 03 was different in the enrichment cultures BS-1 (chemocline) and BS-2 (sediment). However, both are closely related to uncultured bacteria, belonging most probably to the CFB group bacteria (Table 2). Melting type 02, which was closely related to the marine *Owenweeksia hongkongensis* (*Bacteroidetes*) (Table 2), was only detected in the sediment, but not in the chemocline enrichment (Fig. 5).

Five sequences (melting types 01, 03a, 06, 07, 09; Fig. 5) were not detected in the sediment enrichment (BS-2), but in the chemocline enrichment (BS-1). Close relatives of these sequences were uncultured bacteria of the *Cytophaga* group, two CFB group bacteria, and an uncultured delta proteobacterium (Table 2). One melting type could not be sequenced (melting type 07).

Table 2. Bacterial sequences in enrichment cultures from the Black Sea chemocline and sediments. The most closely related sequences were withdrawn from the NCBI database, together with information about the isolation source. For melting types cf. Fig. 1.

Melting type	Closest relative	NCBI accession No.	Sequence identity
01	Unidentified bacterium, DGGE amplificate BS6, *Cytophaga* group, (marine sediments with organic carbon input under anaerobic conditions)	AJ011658	98%
02	*Owenweeksia hongkongensis*, *Bacteroidetes*, (marine)	AB125062	91%
03a (BS-1)	Uncultured bacterium, TGGE-band B34 (probably CFB group)	AY328494	93%
03b (BS-2)	Uncultured bacterium, clone Hyd-B2-1, *Cytophagales*, (sediments above gas hydrate (Cascadia Margin, Oregon))	AJ535256	95%
04	***Chl.* sp. BS-1** **(eubacterial primers)**	AJ972456	**100%**
05	***Chl.* sp. BS-1** **(GSB specific primers)**	AJ972456	**100%**
06	Uncultured bacterium GR-WP33-68 (*Cytophaga* group), (uranium mining mill tailing)	AJ296576	96%
07	(not determined)		
08	*Ilyobacter psychrophilus* FQ50^T, *Fusobacteriaceae*, (an anaerobic, psychrophilic strain from Spitsbergen)	AJ877255	99%
09	Uncultured delta proteobacterium clone GoM GC234 630E, (sediments associated with surface-breaching gas hydrate mounds in the Gulf of Mexico)	AY211682	99%

Attempts to further enrich *Chl.* sp. BS-1 from the sediments by inoculating the enrichment to fresh medium were successful. Addition of malate resulted in a decrease of melting types 02 and 08, leading to a higher percentage of the green sulfur bacterium (Fig. 5, lane BS-2b) wheras medium with sodium acetate and yeast extract leaded to an enhanced signal of the melting type 08 (Fig. 5, lane BS-2a). None of the sequences detected in the sediment enrichment, however, represents a member of the deep-branching green sulfur bacteria, despite the presence of their genetic fingerprints in the sediment.

DISCUSSION

Subfossil 16S rRNA fingerprints of green sulfur bacteria in the sediments.

Fossil biomarkers of green sulfur bacteria have been used in the past as evidence for the vertical extent of the anoxic zone. As indicators for the presence of green sulfur bacteria in past depositional environments, which are now located on land like the Messinian Vena del Gesso basin (Northern Apennines, Italy), diaromatic carotenoids and their sulfur-linked derivatives, which are specific for green sulfur bacteria, have been used (Kohnen et al. 1991). In the Black Sea sediments, the carotenoids isorenieratene (Repeta 1993) and sulfurized isorenieratane have been found (Sinninghe Damsté et al. 1993, Wakeham et al. 1995). Free isorenieratene was found in Unit IIb sediments (Freeman et al. 1994, Sinninghe Damsté et al. 1993, Summons and Powell 1987) as well as farnesane with $\delta^{13}C$ values typical for green sulfur bacteria, which use the reverse TCA cycle for CO_2 fixation. Sulfurized isorenieratane reached lower concentrations in Unit I but higher concentrations in Unit IIb (Sinninghe Damsté et al. 1993, Wakeham et al. 1995). Taken together, these data indicate that photic zone anoxia, hence a large anoxic water body, developed during the deposition of Unit IIb and Unit I.

As the presence of pigments specific for green sulfur bacteria proposed, we were able to recover several subfossil 16S rRNA gene sequences of green sulfur bacteria from the deep Black Sea sediments. DGGE analysis showed the presence of a high diversity of genetic fingerprints of green sulfur bacteria from the saltwater and freshwater groups (Imhoff 2003, Manske et al. in preparation) in all sediment layers, showing the highest diversity of phylotypes in Unit IIa. Sequence analyses revealed the fingerprints of nine different species of green sulfur bacteria. Surprisingly, only three of the sequences cluster with the true marine species in the phylogenetic tree of green sulfur bacteria (group 1, Imhoff 2003). One of them is the sequence of the green sulfur bacterium which is found today in the chemocline. The Black Sea strain *Chl.* sp. BS-1 has specific adaptations to its habitat, like very low light adaption and low growth rates (Manske et al. 2005), and is the only known representative of green sulfur bacteria that is proven to be viable in the Black Sea chemocline today (Manske et al. 2005). Its 16S rRNA gene sequence was found in sediments from Unit I, II, and III. The two other marine sequences were found in sediments of Unit IIa, only in a depth of 84 cm. These results support the presence of photic zone anoxia during deposition of Unit I and IIb (Sinninghe Damsté et al. 1993) but also suggest its presence during deposition of Unit IIa and III. Since Unit III was deposited during the freshwater period of the Black Sea (Degens and Ross 1972) these results are surprising and need a more detailed interpretation. One issue is the result that the other green sulfur bacterial 16S rRNA gene sequences that were found cluster with epibionts of consortia (Glaeser et al. 2004) and other freshwater or low-salt-adapted species (Imhoff 2003). One particular sequence from the sediments, clustering with group 3b (Env. sequence B), represents a ubiquitous and the most common phylotype of all species (Manske et al. in preparation) and occurs in both Unit II and Unit III sediments. This sequence was also found in the Mediterranean sapropel layers (Coolen and Overmann 2007). The presence of a specific sequence in the Black Sea sediments as well as in the Mediterranean sediments leads to the conclusion that this sequence derives from the same origin, or that the sequence in the Black Sea derives from the Mediterranean invasion of the Black Sea basin, marked by the formation of Unit II. Another sequence (Env. sequence C) is closely related to the sequence S8G from a deep sapropel layer in the Mediterranean (Coolen and Overmann 2007) and was only found in Unit III. An allochthonous introduction of this sequence to the Black Sea sediments seems obvious.

The detailed analysis of the fossil DNA sequences of green sulfur bacteria present in the Black sea sediments suggests that molecular markers have to be interpreted carefully. One issue is the finding that the DGGE method does not have a high resolution or can detect all possible sequences. In two cases, in the same band there were two different sequences. In contrast, the same sequences can sometimes be found in bands at different detergent concentrations. One possible method to account for that problem is the environmental clone library or the cloning of DGGE band extracts. Future comparisons will show which method is best suited for the sediments of the Black Sea. Another issue is that in the Black Sea, contrary to other oxic/anoxic environments with accumulations of phototrophic sulfur bacteria (Overmann 1991), the vertical concentration profile of bacterial pigments showed a remarkably sharp decrease below the chemocline, where bacteriochlorophyll *e* concentrations drop below the detection limit at a depth of 120 m (Repeta et al. 1989, Repeta and Simpson 1991). Also, chlorophyll *a*, but not bacteriochlorophyll *e* was detected in the sedimenting matter in the Black Sea water column (at 87.5 hours deployment of sediment traps) (Repeta and Simpson 1991) although the total amount of bacteriochlorophyll *e* detected in the chemocline surpasses the total chlorophyll *a* in the upper oxygenated water layers (Repeta et al. 1989). Both findings indicate that only a very small fraction of the green sulfur bacteria existent in the Black Sea chemocline in fact reaches the deep-sea sediments.

Evidence for the horizontal transport of green sulfur bacteria even through oxygenated water bodies, and their survival therein comes from a study by Gorlenko et al. (2005) who enriched green colored *Chlorobiaceae* from sediments at a shallow water depth (64 m) and brown-colored *Chlorobiaceae* from sediments of the central basin. It would be of interest, whether the 16S rRNA gene sequence of the green bacterium from the shelf edge of the Black Sea would cluster with marine or freshwater species, to determine its original habitat. Experiments with green sulfur bacteria show big differences in their oxygen tolerance (Henßge 2006), and there are mechanisms that protect the GSB photosystem from being oxidized (Frigaard and Bryant 2004, Blankenship et al. 1995), which makes the transport of live green sulfur bacteria even over long distances possible. Also, a green-colored green sulfur bacterium (*Chlorobium bathyomarinum*) was found near a hydrothermal vent in the Pacific, in a water depth of ~2000 m, which possibly does not originate from the vent (Beatty et al. 2005). Oxygen resistance over a long period of time was shown for this bacterium (Beatty et al. 2005) and also for other green sulfur bacteria (Uta Henßge, 2006, Kerstin Zikeli, personal communication). Recently, the sequence of *Chl.* sp. BS-1 was found in the Eastern Mediterranean sediments (E. Marschall, personal communication). An allochthonous introduction of green sulfur bacterial sequences to the Black Sea sediments therefore seems more likely and questions that green sulfur bacterial pigments in sediments can be used in any case as indicators for photic zone anoxia in the overlying water column. The 16S rRNA gene sequence of the extremely low-light adapted strain BS-1, however, can be used as a specific biomarker for photic zone anoxia since the Black Sea chemocline is the most extreme habitat for low-light adapted green sulfur bacteria observed so far. An allochthonous introduction of that strain may be the offspring of the population which is now stable in the Black Sea chemocline. The fingerprint of *Chl.* sp. BS-1 could serve as biomarker for photic zone anoxia also in other parts of the world ocean, to reveal photic zone anoxia during deposition of ancient sedimentary records, provided that fingerprints of this strain are found in other parts of the world, like the sequence from the Sippewisset saltmarsh. From our data, we get more insight into the paleoceanographic record of the Black Sea sediments, and we can deduce the history of a green sulfur bacterial population in the Black Sea over a period of several thousand years.

Close relatives of green sulfur bacteria.

Additional to the green sulfur bacterial sequences, 16S rRNA fingerprints of close relatives to green sulfur bacteria were found. These sequences cluster at the root of the green sulfur bacterial tree together with environmental sequences that are not related to any isolated strain. The cluster seems to share a common root with the *Bacteroidetes*. To date, the ecological role and physiology of these bacteria remains unclear, they may be even inactive in the Black Sea. Environmental sequences of this new group of bacteria were found all over the world. Phylogenetic analyses show a highly diverse group of bacteria, with up to 33 % sequence variability in the 16S rRNA gene. All sequences present in the NCBI database are environmental clones from a multitude of diverse habitats. One sequence for instance derives from the surface of a snail, collected from the vicinity of a hot spring in the Indian Ocean (Goffredi et al. 2004). Other sequences derive from microbial consortia that degrade aromatic compounds (von Wintzingerrode et al. 1999, Ulrich et al. 2003) or from the halocline of the Red Sea (Eder et al. 2002). This shows that species within the group are highly diverse also in physiological aspects. A photosynthetic activity, may it be anoxygenic or oxygenic, of some or all of the bacteria remains to be investigated. First attempts were made to develop a nucleotide probe for the group of “deep-branching green sulfur bacteria“, but the high diversity within the group made it impossible to develop a probe which would detect all of the members of the group (Henßge 2006). With the proposed oligonucleotides, bacteria of the phyla *Bacteroidetes*, *Firmicutes* und *Proteobacteria* would have also been detected. The cluster of “deep-branching green sulfur bacteria” is possibly not monophyletic but must be subdivided and positioned at the bases of different phylogenetic branches. To find out more about the habitat and genomes of these bacteria it would be helpful to have probes as a useful tool for a targeted search in environmental samples. This will result in further knowledge about their abundance and physiological properties, and may result in first enrichment cultures and even isolates.

Viable cells of green sulfur bacteria in Black Sea sediments.

It was proposed that cells of phototrophic bacteria survive in the dark sediment layers by means of a fermentative metabolism, since photosynthesis is not possible, and high amounts of carbon would support organotrophic growth (Hashwa and Trüper 1978). The high hydrostatic pressure in the deep sea, however, would lead to only low turnover rates (Jannasch et al 1971, 1976). Indeed, a living population of green sulfur bacteria was enriched from 2000 m deep sediments of the Black Sea, confirming earlier results of enrichment cultures of *Chlorobium phaeobacteroides* from 660 m and 2240 m-deep sediment layers (Hashwa and Trüper 1978). Like in the enrichment culture BS-1 from the chemocline (Manske et al. 2005), the green sulfur bacterium in the enrichment culture from the sediment (BS-2) was accompanied by bacteria of the *Cytophaga* group and the *Fusobacteriaceae*. It remains unclear, whether these bacteria are syntrophic to the green sulfur bacterium or are growing independently. Since “deep-branching” green sulfur bacteria were not enriched together with the chemocline or sediment enrichment culture of *Chl.* sp. BS-1 the growth conditions must differ from that of green sulfur bacteria, or the sequences derive from dead cells or debris. Hence, it cannot be concluded that the Black Sea sediments represents a habitat for growing “deep-branching green sulfur bacteria”. Future investigations with probes used in different habitats will hopefully result in an enrichment culture of these obscure relatives of green sulfur bacteria.

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REFERENCES

(1) **Beatty, J. T., J. Overmann, M. T. Lince, A. K. Manske, A. S. Lang, R. E. Blankenship, C. L. Van Dover, T. A. Martinson, and F. G. Plumley.** 2005. An obligately photosynthetic bacterial anaerobe from a deep-sea hydrothermal vent. PNAS 102:9306-9310

(2) **Blankenship, R. E., J. M. Olson, and M. Miller.** 1995. Antenna complexes from green photosynthetic bacteria. In: Blankenship R.E., Madigan M.T., Bauer C.E. (eds) Anoxygenic photosynthetic bacteria. Kluwer, Dordrecht, pp. 399-435.

(3) **Canfield, D. E., T. W. Lyons, and R. Ralswell.** 1996. A model for iron deposition to euxinic Black Sea sediments. Am. J. Sci. **296:**818-834.

(4) **Codispoti, L. A., G. E. Friederich, J. W. Murray, and C. M. Sakamoto.** 1991. Chemical variability in the Black Sea: implications of continuous vertical profiles that penetrated the oxic/anoxic interface. Deep Sea Res. **38**(suppl.2):S691-S710.

(5) **Coolen, M. J. L. and J. Overmann.** 2000. Functional exoenzymes as indicators of metabolically active bacteria in 124,000-year-old sapropel layers of the eastern Mediterranean Sea. Appl Environ Microbiol **66**:2589-2598.

(6) **Coolen, M. J. L. and J. Overmann.** 2007. 217 000-year-old DNA sequences of green sulfur bacteria in Mediterranean sapropels and their implications for the reconstruction of the paleoenvironment. Environ. Microbiol. 9:238-49.

(7) **Degens, E. T. and D. A. Ross.** 1972. Chronology of the Black Sea over the last 25,000 years. Chem. Geol. **10:**1-16

(8) **Degens, E. T. and P. Stoffers.** 1976. Stratified waters as a key to the past. Nature. **263:**22-27.

(9) **Eder, W., Schmidt, M., Koch, M., Garbe-Schnberg, D., and R. Huber.** 2002. Prokaryotic phylogenetic diversity and corresponding geochemical data of the brine-seawater interface of the Shaban Deep, Red Sea. Environ Microbiol. **4**(11):758-763

(10) **Freeman, K. H., S. G. Wakeham, and J. M. Hayes.** 1994. Predictive isotopic biogeochemistry: hydrocarbons from anoxic marine basins. Org. Geochem. **21:**629-644.

(11) **Frigaard, N.-U. and D. A. Bryant.** 2004. Seeing green bacteria in a new light: genomics-enabled studies of the photosynthetic apparatus in green sulfur bacteria and filamentous anoxygenic phototrophic bacteria. Arch. Microbiol. **182**:265-276.

(12) **Gich, F., J. Garcia-Gil, and J. Overmann.** 2001. Previously unknown and phylogenetically diverse members of the green nonsulfur bacteria are indigenous to freshwater lakes. Arch Microbiol **177**:1-10.

(13) **Glaeser, J. and J. Overmann.** 2004. Biogeography, Evolution, and Diversity of Epibionts in Phototrophic Consortia. Appl. Env. Microbiol. 70:4821-4830.

(14) **Goffredi, S. K., Warén, A., Orphan, V. J., van Dover, C., and R. C. Vrijenhoek.** 2004. Novel Forms of Structural Integration between Microbes and a Hydrothermal Vent Gastropod from the Indian Ocean. Appl Environ Microbiol. 70(5):3082-3290

(15) **Gorlenko, V. M., P. V. Mikheev, I. I. Rusanov, N. V. Pimenov, and M. V. Ivanov.** 2005. Ecophysiological properties of photosynthetic bacteria from the Black Sea chemocline zone. Microbiology 74:201-209. (Translated from Mikrobiologiya 74:239-247, 2005).

(16) **Hashwa, F. A. and H. G. Trüper.** 1978. Viable phototrophic bacteria from the Black-Sea bottom. Helgol. wiss. Meeresunters. **31**:249-253.

(17) **Hay, B. J.** 1988. Sediment accumulation in the central western Black Sea over the past 5100 years. Paleoceanogr. **3:**491-508.

(18) **Hay, B. J. and M. A. Arthur.** 1991a. Sediment deposition in the Holocene Black Sea. Deep-Sea Res. 38 (suppl 2): 1211-1235

(19) **Hay, B. J., M. A. Arthur, W. E. Dean, E. D. Neff, and S. Honjo.** 1991b. Sediment deposition in the Late Holocene abyssal Black Sea with climatic and chronological implications. Deep-Sea Res. 38 (Suppl. 2), S 1211-S 1235.

(20) **Henßge, U.** 2006. Physiologie Grüner Schwefelbakterien und Paläomikrobiologie im Schwarzen Meer. Diplomarbeit. Department Biologie I, Bereich Mikrobiologie, LMU München

(21) **Imhoff, J. F.** 2003. Phylogenetic taxonomy of the family *Chlorobiaceae* on the basis of 16S rRNA and *fmo* (Fenna-Matthews-Olson protein) gene sequences. Int. J. syst. Evol. Microbiol. **53:**941-951.

(22) **Jannasch, H. W., K. Eimhjellen, C. O. Wirsen, and A. Farmanfamaian.**1971. Microbial degradation of organic matter in the deep sea. Science. **171:**672-675.

(23) **Jannasch, H. W., C. O. Wirsen, and C. D. Taylor.** 1976. Undecompressed microbial populations from the deep sea. Appl. environ. Microbiol. **32:**360-367

(24) **Kohnen, M. E. L., J. S. Sinninghe Damsté, and J. W. DeLeeuw.** 1991. Biases from natural sulphurization in palaeoenvironmental reconstruction based on hydrocarbon biomarker distribution. Nature **349:**775 - 778

(25) **Konovalov, S. K., L. I. Ivanov, and A. S. Samodurov.** 2001. Fluxes and budgets of sulphide and ammonia in the Black Sea anoxic layer. J. Mar. Syst. **31:**203-216.

(26) **Lane, D. J.** 1991. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, Chichester, pp 115-175.

(27) **Ludwig, W., O. Strunk, S. Klugbauer, N. Klugbauer, M. Weizenegger, J. Neumann, M., Bachleitner and K. H. Schleifer.** 1998. Bacterial phylogeny based on comparative sequence analysis. Electrophoresis **19**:554-568.

(28) **Manske, A. K., J. Glaeser, M. M. M. Kuypers, and J. Overmann.** 2005. Physiology and phylogeny of green sulfur bacteria forming a monospecific phototrophic assemblage at a depth of 100 meters in the Black Sea. Appl. Environ. Microbiol. 71:8049-8060.

(29) **Manske, A. K., H. Teeling, L. Jesús Garcia-Gil, and J. Overmann1.** In preparation. Biodiversity and phylogeny of the family *Chlorobiaceae* based on comparison of multiple genetic regions.

(30) **Muyzer, G., E. C. De Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59**:695-700.

(31) **Muyzer, G., S. Hottenträger, A. Teske, and C. Waver.** 1995. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA – a new molecular approach to analyse the genetic diversity of mixed microbial communities. In: Akkermans A. D. L, Van Elsas J. D. , De Bruijn F. J. (eds) Molecular microbial ecology manual, 2nd edn. Kluwer, Dordrecht, pp 3.4.4.1-3.4.4.22.

(32) **Overmann, J.** 1991. Standortspezifische Anpassung bei phototrophen Schwefelbakterien. Konstanzer Dissertationen Bd. 321. Hartung-Gorre, Konstanz, Germany.

(33) **Overmann, J.** 2001. Green sulfur bacteria. *In* Boone, D. R., and R. W. Castenholz (eds) Bergey's manual of systematic bacteriology, 2nd edn, vol. 1. Springer, Berlin, Heidelberg, New York, pp.601-623.

(34) **Overmann, J. and N. Pfennig.** 1989. *Pelodictyon phaeoclathratiforme* sp. nov., a new brown-colored member of the *Chlorobiaceae* forming net-like colonies. Arch. Microbiol. **152**: 401-406

(35) **Overmann, J., H. Cypionka, and N. Pfennig.** 1992. An extremely low-light-adapted green sulfur bacterium from the Black Sea. Limnol. Oceanogr. **37**:150-155.

(36) **Overmann, J., M. J. L. Coolen, and C. Tuschak.** 1999. Specific detection of different phylogenetic groups of chemocline bacteria based on PCR and denaturing gradient gel electrophoresis of 16S rRNA gene fragments. Arch Microbiol **172**:83-94.

(37) **Repeta, D. J., D. J. Simpson, B. B. Jørgensen, and H. W. Jannasch.** 1989. Evidence for anoxygenic photosynthesis from the distribution of bacteriochlorophylls in the Black Sea. Nature **342**:69-72.

(38) **Repeta, D. J. and D. J. Simpson.** 1991. The distribution and recycling of chlorophyll, bacteriochlorophyll and carotenoids in the Black Sea.. Deep-Sea Res. **38**: 969-984.

(39) **Repeta, D. J.** 1993. A high resolution historical record of Holocene anoxygenic primary production in the Black Sea. Geochim. Cosmochim. Acta. 57:4337-4342.

(40) **Ross, D. A. and Degens, E. T.** 1974. The Black Sea – Geology, Chemistry and Biology (eds Degens E. and Ross D.) 183-199. (Memoir No. 20. Am Ass. Petrol. Geol., Tulsa, Oklahoma).

(41) **Sinninghe Damsté, J. S., Wakeham, S. G., M. E. L. Kohnen, Hayes, J. M., and J. W. de Leeuw.** 1993. A 6,000-year sedimentary molecular record of chemocline excursions in the Black Sea. Nature. **362**:827-829.

(42) **Sorokin, Yu. I.** 2002. The Black Sea. Ecology and oceanography. Backhuys Publishers, Leiden, The Netherlands, pp. 875.

(43) **Summons, R. E. and T. G. Powell.** 1987. Identification of aryl isoprenoids in source rocks and crude oils: biological markers for the green sulfur bacteria. Geochim. Cosmochim. Acta **59:**521-533

(44) **Ulrich, A. C. and E. A. Edwards**. 2003. Physiological and molecular characterization of anaerobic benzene-degrading mixed cultures. Environ Microbiol. **5**:92-

(45) **von Wintzingerode, F., Selent, B., Hegemann, W., and U. B. Göbel.** 1999. Phylogenetic Analysis of an Anaerobic, Trichlorobenzene-transforming Microbial Consortium. Appl Environ Microbiol. **65**:283-286

(46) **Wakeham, S. G., J. S. Sinninghe Damsté, M. E. L. Kohnen, and J. W. DeLeeuw.** 1995. Organic sulfur compounds formed during early diagenesis in Black Sea sediments. Geochim. Cosmochim. Acta **59:**521-533

SUPPORTING ONLINE MATERIAL

Supplementary table 1. DNA concentrations in sediment extracts

core 7620		**core 7605**	
sediment depth (cm)	DNA concentration in extract (ng/µl)	sediment depth (cm)	DNA concentration in extract (ng/µl)
"Fluff" (M)	1016.2	"Fluff"	970.8
2-4 (M)	1431.6	2-3 (M)	832.2
4-6 (M)	1029.2	4-6 (M)	1070.4
38-39 (M)	328.2	25-35 (M)	667.8
45	68,6	10.5	162.24
50	52.5	25	234.4
60	108,8	34	111.83
70	88.6	62	104.8
84	120.16	130	94.19
86	111.9	154	127.5
88	92.3	184	160.95
90	67,8	224	79.91
94	103.8	244	102.96
99	61.9	251	72.1
109	51.9	267	65.52
120	52.0	293	95.38
125	19.0	308	86.40
139	38,0	329	132.82
151	23.1	357	79.91
171	32.6	384	75.5
227	32,4	421	74.5
359	35,7	542	81.54
		548	84.2
		556	54.81
		559	34.26
		589	12.62
		604	19.87
		700	239.4

(M) sample obtained by multicorer (see materials and methods section)

CHAPTER 7

BIODIVERSITY AND PHYLOGENY OF THE FAMILY CHLOROBIACEAE BASED ON ANALYSES OF DIFFERENT GENOMIC REGIONS

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Running title: Phylogeny and Biodiversity of green sulfur bacteria

KEYWORDS: Green sulfur bacteria, *Chlorobiaceae*, phylogeny, biodiversity, 16S rRNA, 16S-23S rRNA intergenic spacer region, *bchG*, *sigA*, ERIC fingerprinting

Abbreviations: GSB, green sulfur bacteria; *Chl.*, *Chlorobium*; *Cba.*, *Chlorobaculum*; *Ptc.*, *Prosthecochloris*; ERIC, Enterobacterial Repetitive Intergenic Consensus; ITS, 16S-23S rRNA intergenic spacer region

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ABSTRACT

A robust basis for the discussion of the phylogeny of green sulfur bacteria was created by analyzing 246 16S rRNA gene sequences of green sulfur bacteria, including cultured species as well as environmental sequences. By rarefaction analysis it was shown that the presented data represent nearly the whole spectrum of green sulfur bacterial sequences that can be found in the sampled habitats. Sequences of cultured species, however, did not even cover half of the biodiversity. In the 16S rRNA gene tree, different clusters were found, that correlate in most cases with physiological adaptations of the included species. Epibionts of phototrophic consortia are either underrepresented in the analysis, or occupy a very special niche, which is shown by an exclusive position outside the cluster formation. The wide dispersal of green sulfur bacterial species can be seen in one sequence that was found ubiquitously all over the world. In contrast, other species are typical for special habitats like hot springs. To imitate the phylogenetical relationships of whole genome analyses, a concatenated tree was constructed including 33 species and 3 different genetic regions, the *bchG* gene, the *sigA* gene, and the ITS region. Comparison with the 16S rRNA gene tree showed more genetic differences between species, and led to a higher resolution and a more consistent phylogeny. Our data show the close correlation of phylogeny and physiology in green sulfur bacteria. For the phylogeny of *Chlorobiaceae*, physiological gene regions should be analyzed rather than the 16S rRNA gene. With our data, the discussion about new species names for green sulfur bacteria can be restarted, but with more detailed knowledge about phylogenetic relationships of green sulfur bacterial species.

INTRODUCTION

Green sulfur bacteria (*Chlorobiaceae*) typically are immotile obligate photolithoautotrophs which inhabit the chemoclines of stratified water bodies or sediments. All species known use H_2S as electron donor and harbor chlorosomes as specific photosynthetic antenna structures. The chlorosomes contain antenna bacteriochlorophylls surrounded by a galactosyl diglycerid monolayer and are attached to the inner face of the cytoplasmic membrane. Phylogenetically, the green sulfur bacteria form a distinct branch of the Bacteria (Garrity and Holt 2001, Overmann 2000), with non-photosynthetic Bacteroidetes as their closest relatives (Madigan et al. 2000). The Green Sulfur bacterial group comprises very closely related bacteria, the similarity of the 16S rRNA gene of all species except one is >90.1 % ($K_{nuc} < 0.11$) (Overmann, 2000). Only the gliding filamentous marine bacterium *Chloroherpeton thalassium* represents a phylogenetically more deeply branching member of the green sulfur bacteria (85.5 to 87 % similarity) (Overmann 2001).

The latest phylogeny of green sulfur bacteria is based only on genetic information of cultivated species (Imhoff 2003). A green sulfur bacterial phylogeny based on 16S rRNA gene sequences was first published by Figueras et al. (1997) and included 18 species, at that time all known species of green sulfur bacteria. They found that *Chlorobium* species can be subdivided in brown and green colored species and that there is a separate cluster for saline strains (Figueras et al. 1997). But as we know by now, some green sulfur bacterial species have identical 16S rRNA gene sequences but differ in their color (e. g. *Chl. phaeovibrioides* DSM 261 and *Chl. phaeovibrioides* DSM 270) (Imhoff 2003) which must be the result of a difference in the functional genes.

Green sulfur bacteria show typical adaptations to the habitat they live in. Brown colored members of the green sulfur bacteria dominate in extreme low-light environments (Montesinos et al. 1983, Overmann et al. 1992, 1998) and differ in the composition of their bacteriochlorophylls (Glaeser et al. 2002). One example is the extremely low-light adapted strain MN1 that was found in the Black Sea chemocline (Overmann et al. 1992, Manske et al. 2005). That strain is capable of photosynthetic activity at light intensities as low as 0.015 µmol quanta m^{-2} s^{-1} (Manske et al. 2005).

A study which allowed for the importance of functional genes in the phylogeny of green sulfur bacteria focused on the phylogenetical evaluation of *bchG* gene sequences of 14 strains of GSB (Garcia-Gil et al. 2003). The *bchG* gene encodes for bacteriochlorophyll *a* synthase in green sulfur bacteria and belongs to the UbiA enzyme family of polyprenyltransferases. Garcia-Gil et al. (2003) found that the phylogenetic relationships of green sulfur bacteria are congruent with taxonomic features, including cell shape, NaCl requirement and the presence of gas vacuoles (Overmann 2001). Evaluating the *bchG* gene sequences, they observed only very short phylogenetic distances within the GSB group, but several distinct clusters. These clusters contained either brown colored species, gas-vacuolated GSB, prosthecate GSB, or members of the genus *Chlorobium*. Like in the 16S rRNA tree (Figueras et al. 1997) a subdivision of saltwater strains was found (Garcia-Gil et al. 2003).

Group 1 sigma factors of the σ^{70}-type have been used as a phylogenetic tool by Gruber and Bryant (1997) for investigation of evolutionary relationships among eubacteria. Group 1 sigma factors are the primary sigma factors in all known eubacteria and are essential for cell viability (Lonetto et al. 1992). The σ^{70}-type sigma factor is part of the eubacterial RNA polymerase holoenzyme (Burgess et al. 1969) and confers the promoter specificity for transcription initiation onto the core enzyme. They are useful for phylogenetic comparisons because due to structural restrictions these sigma factors possess highly conserved amino acid sequences (Helmann and Chamberlin 1988) as well as regions that are variable even among close relatives. Gruber and Bryant (1997) analyzed the phylogenetic relationship of 45 protein sequences of group 1 σ^{70}-type sigma factors from a variety of eubacterial groups including one sequence of green sulfur bacteria (*Chlorobaculum tepidum*). Their analysis showed a congruence of the phylogenetic clustering of sequences with the clusters found in 16S rRNA trees and an isolated position of the green sulfur bacterial sequence (Gruber and Bryant 1997).

The 16S-23S ribosomal intergenic spacer region (ITS) was shown to be a useful marker for the rapid identification of bacteria by Jensen et al. (1993) due to the presence of highly conserved regions as well as of highly variable regions. The region was used as a phylogenetic tool by Aakra et al. (1999), who used it for studying the ecological diversity of ammonia-oxidizing bacteria *in situ*.

The classical systematics assessed the diversity of *Chlorobiaceae* by using morphological attributes. The resulting system differentiated bacteria forming web-like associations (*Pelodictyon*), species forming star-shaped aggregates (*Prosthecochloris*) and green- or brown colored species (Schlegel 1992, Pfennig 1989, Pfennig and Overmann 2001a,b).

To date, green sulfur bacteria are subdivided into the three genera *Chlorobium*, *Prosthecochloris* and *Chlorobaculum* (Imhoff 2003). Underlying that phylogeny is a study on 16S rRNA gene sequences and Fenna-Matthews-Olson (FMO) protein sequences of cultured GSB species (Alexander et al. 2002). Alexander et al. (2002) included 31 strains of GSB and found a congruence of the phylogenetic trees built from 16S rRNA gene sequences and from FMO protein sequences. The clusters they found showed the incongruence of the phylogenetic trees with the current taxonomic classification of GSB species based on physiological properties, which comprised the six genera *Ancalochloris*, *Chlorobium*, *Chloroherpeton*, *Clathrochloris*, *Pelodictyon*, and *Prosthecochloris* (Overmann 2000). Consequently, a revised taxonomy of the *Chlorobiaceae* based on 16S rRNA gene and FMO protein sequences was proposed introducing new genus and species names (Imhoff 2003). This phylogeny, however, is not yet accepted due to formal reasons.

Only very low genetic information was used for the phylogeny of GSB, since only 16S rRNA gene sequences are available in a reasonable number. Only one whole genome in the group of *Chlorobiaceae*, the genome of *Chlorobium tepidum* TLS, has been sequenced and annotated so far. Therefore, the overall diversity of this phylum is yet unknown due to numerous uncultured species and various habitats of green sulfur bacteria.

For analyzing the overall phylogeny of green sulfur bacteria it is therefore indispensable to use as many genetic information as possible, and to include cultured strains as well as sequences of environmental sequences.

In our phylogenetic study we employed multiple genetic regions as well as 16S rRNA gene sequences, from both cultured strains and environmental sequences. Our study comprises the 16S rRNA gene sequences of 73 green sulfur bacterial species, 40 16S rRNA gene sequences of phototrophic consortia, 133 16S rRNA environmental sequences, 43 gene sequences for the σ^{70}-type sigma factors (*sigA*), 52 sequences for the 16S-23S rRNA intergenic spacer region (ITS) and 37 *bchG* gene sequences.

MATERIALS AND METHODS

Cultures of green sulfur bacteria. Green sulfur bacterial strains were kept in standard saline or freshwater SL10 medium (Widdel et al. 1983). The strain BS-1, which was enriched in 2001, was kept in artificial seawater medium (Coolen and Overmann 2000), adjusted to the ionic strength of the Black Sea chemocline (Manske et al. 2005).

DNA-extraction from cultures and water samples. Genomic DNA of bacterial strains was isolated from exponentially growing cultures by using the DNeasy Tissue Kit (Qiagen, Hilden, Germany) following the instructions supplied by the manufacturer. DNA content was quantified using Pico Green (Molecular Probes, Eugene, OR, USA).

Standard conditions for PCR. Standard conditions for PCR comprised 20 to 50 ng DNA or 0.5 µl of a cell suspension (cell pellet from 2 ml of culture, resuspended in 10 mM Tris·HCl, pH 8.5), 10 µM of each primer, 5 µl of GeneAMP 10x PCR buffer, 0.2 mM of each dNTP (GeneAMP dNTPs, Applied Biosystems, Weiterstadt, Germany), 3.5 mM $MgCl_2$ and 1.25 U AmpliTaq Gold polymerase (Applied Biosystems) in a total volume of 50 µl. Amplifications were performed in a

GeneAMP thermocycler PCR system 2400 or 9700 (Applied Biosystems) (supplemental table 1). Amplification products were analyzed by standard agarose gel electrophoresis.

Amplification of 16S rRNA gene sequences. The primer pair 8f/1492r (supplemental table 2) was used in standard PCR conditions in a step-down PCR to amplify the almost complete 16S rRNA gene sequence (supplemental table 1). For sequencing, the primers 8f, Uni341f, Uni515f, Uni517r, Uni 907r, 926f, 1055r, and 1492r (supplemental table 2) were used.

PCR and cloning of the 16S-23S rRNA intergenic spacer (ITS) region. The ITS region was amplified using standard PCR conditions and the universal ITS primers 1525f and L1r (supplemental tables 1, 2). For mixed cultures, suspected to contain different bacterial species, the primer GSB822f, which is specific for green sulfur bacteria (modified after Overmann et al. 1999), was combined with L1r (supplemental table 2). For sequencing, 1525f and L1r were combined (supplemental table 2).

PCR products were checked for double bands on 1.4 % agarose gels. Strains which yielded more than one operon were selected for cloning of the ITS: *Chl. phaeobacteroides* UdG 6051, *Chl. limicola* DSM 246, *Chl. phaeobacteroides* DSM 266^T, *'Cba. chlorovibrioides'* UdG 6043, *Cba. limnaeum* UdG 6045, *Ptc.* sp. 2K, *Pld. luteolum* E3P2, *Chl. phaeobacteroides* E3P3, *Chl. limicola* DSM 245^T, *Chl. clathratiforme* PG, *Chl. limicola* UdG 6044, *Chl. phaeobacteroides* UdG 6046, *Chl. phaeobacteroides* Glu, *Cba. limnaeum* UdG 6040, *Cba. parvum* DSM 263^T, *Chl. limicola* D1, *Chl. clathratiforme* DSM 5477^T, the Black Sea bacterium BS-1 (Manske et al. 2005) (supplemental table 3).

PCR products were ligated into the vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA) and subsequently cloned into TOP10 chemically competent cells according to the instructions supplied by the manufacturer. Recombinant clones were obtained on LB agar plates supplemented with kanamycin (50 µg/ml), picked and grown in liquid LB medium with kanamycin (50 µg/ml). Plasmids were isolated and purified, randomly using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) or the Quantum Prep Plasmid Miniprep Kit (Bio-Rad, Hercules, CA, USA). The ITS region was sequenced from the extracted plasmid DNA using the primers 1525f and L1r.

Primer design and PCR for group 1 σ^{70}-type sigma factor gene sequences (*sigA*). Sigma factor gene sequences for most of the GSB species were obtained with the primer pairs Sig208f/Sig827r and Sig312f/Sig827r, respectively (supplemental table 2). The PCR reaction (supplemental table 1) comprised 20 – 50 ng DNA, 1 µM of primer (0.5 µM for 312f), 0.2 mM of each dNTP, 5 µl of PCR buffer (10x, incl. 15 mM $MgCl_2$) and 1 U of Taq polymerase (2 U for Sig312f) in a total volume of 50 µl (all chemicals by Perkin Elmer, Foster City, CA, USA).

Since the primer pairs Sig208f/Sig827r or Sig312f/Sig827r did not yield a *sigA* gene sequence in all investigated isolates, another primer pair was designed using an alignment of the available sequences and the *sigA* gene sequences of *Chl. tepidum* TLS and *Chlorobium chlorochromatii* CaD (Vogl et al. 2005). Conserved regions in the alignment were checked for their priming qualities, for self-complementaries, potential hairpin formation, dimer formation and palindromes, using the NetPrimer software (Premier Biosoft International, Palo Alto, CA, USA). Primers were then checked for specificity employing the option of *search for short, nearly exact matches* in the NCBI BLAST database (www.ncbi.nlm.nih.gov/BLAST/). The production of a single amplification product in green sulfur bacteria was demonstrated by PCR for the two primers GSB-SigA-F4 and GSB-SigA-R1 (supplemental table 2). For all subsequently analyzed green sulfur bacteria a pure amplification product was obtained with 50 ng of template DNA in standard PCR conditions (supplemental table 1). The same primer pair was used for the sequencing reaction.

***BchG* gene sequences.** *BchG* gene sequences were amplified using the published primer pair bchG-F/bchG-R (supplemental table 2) with a modified cycling method (supplemental table 1). For sequencing, a shortened reverse primer (BchG-R Seq, supplemental table 2) was used.

Sequencing of PCR products. Cycle sequencing was performed with the AmpliTaq FS BigDyeTerminator cycle sequencing kit (Applied Biosystems, Weiterstadt, Germany) following the protocol supplied by the manufacturer. Samples were analyzed on a capillary sequencer (ABI Prism377 DNA sequencer, Applied Biosystems).

Phylogenetic trees. Additionally to the sequences obtained during this study, a multitude of 16S rRNA gene sequences of green sulfur bacterial strains and environmental sequences was withdrawn from the NCBI database (www.ncbi.nlm.nih.gov) (supplemental table 3). 16S rRNA gene sequences were phylogenetically analyzed using the ARB phylogeny software package (Ludwig et al. 1998). Fast Aligner V1.03 was used for an automatic alignment. The resulting alignments were corrected manually based on 16S rRNA secondary structure information for *Chlorobium vibrioforme* DSM 260^T, available through The Comparative RNA Web (CRW) Site (www.rna.icmb.utexas.edu, Cannone et al. 2002). Nucleotide positions were adjusted in the alignment according to secondary structure constraints. This sequence in its corrected alignment served as a template for the alignment of all other 16S rRNA sequences. Phylogenetic trees were constructed including 16S rRNA gene sequences of all available strains and environmental sequences. First, sequences longer than 1100 bp were used for the calculation, employing the MAXIMUM LIKELIHOOD algorithm (Fast DNA_ML). The shorter environmental sequences were inserted afterwards without changing overall tree topology employing the PARSIMONY INTERACTIVE tool implemented in the ARB software package (Gich et al. 2001). The resulting tree topology was checked for correct distances with a PHYLIP distance matrix using Felsenstein correction. No filter was used, and only the alignment positions were used which were present in all sequences. Using the sequences longer than 1400 bp, bootstrap analyses were performed employing the PHYLIP software package version 3.573c (Felsenstein 1989). 100 bootstraps were calculated using three different algorithms (MAXIMUM LIKELIHOOD (DNAML), MAXIMUM PARSIMONY (DNAPARS) and NEIGHBOR JOINING (NJ, correction: Kimura-2 parameter)) with similar results.

For the ITS sequences, the *bch*G and the *sigA* gene sequences the alignment was performed using ClustalX 1.8 (Thompson et al. 1997), and nucleotide positions were manually corrected using GeneDoc (Nicholas and Nicholas 1997). The trees were calculated using the MAXIMUM LIKELIHOOD method (DNAML) implemented in the PHYLIP software package version 3.573c (Felsenstein 1989).

Concatenated phylogenetic tree. For the concatenated analysis of three different genetic regions (ITS, *sigA*, *bchG*) sequences of overall 33 strains were available, including type strains of all prominent clusters. The sequence lengths of all included organisms in the resulting alignments were customized, and the alignments were combined in one file in the PHYLIP format. Starting from this alignment, a concatenated tree was calculated using the DNAML algorithm implemented in the PHYLIP software package. Bootstrap values (100 bootstraps) were calculated using three different algorithms (DNAML, NJ (correction: Kimura-2 parameter), and DNAPARS), implemented in the PHYLIP software package.

Rarefaction analysis. Coverage of existing sequences was analyzed separately for the isolated strains and all green sulfur bacterial sequences using Software RareFactionAnalysis (HCYP 1999). The collectors curve was calculated using Analytic Rarefaction 1.3 by Steven M. Holland, available at www.uga.edu/~strata/software.

Distance matrix comparisons (Mantel test). For the ITS region, the *bchG* gene, the *sigA* gene and the *fmoA* gene, a matrix comparison analysis was conducted, comparing each of the distance matrices with the 16S rRNA gene distance matrix according to Mantel (1967). For comparison, the distance matrix (symmetric dissimilarity matrix) of each marker was calculated using DNADIST, implemented in PHYLIP. Two matrices were compared and plotted by using MXCOMP, implemented in NTSYSpc 2.02j. In this test, two matrices are multiplied, element by element, and the sum of these products is tested against the expected value of its quantity, the significance of which is evaluated by Monte Carlo techniques (Sokal 1979, Sokal et al. 1986). Since the number of element by element comparisons would be too high, a random sample of permutations (999 permutations) is used. The result is a plot of the product-moment correlation, *r*, which is monotonically related to the Mantel test statistic, *Z* (Smouse et al. 1986), and its permutational distribution. For more than 12 operational taxonomic units (OTU) a correlation greater than 0.5 will be statistically significant at the 1 % level. 100% correlation of permutational distribution would yield an even line with a slope of 1.

Nucleotide accession numbers. DNA sequences obtained in this study are available at the EMBL under accession numbers AM049271-AM049317, AM050117-AM050132, AM050061-AM050096, AM050351-AM050374 (see supplemental table 3).

RESULTS AND DISCUSSION

Rarefaction analysis of the 16S rRNA tree. To calculate how much of the biodiversity of GSB we already covered with our sequence analysis we plotted the "collectors curve", which shows the cumulative No. of phylotypes plotted against the phylotype No. (Fig. 1A). When taking into account all GSB sequences, including the environmental sequences, the curve approaches saturation around a phylotype number of 120 (Fig. 1A). This implies that new environmental sequences will most probably cluster with identical sequences in separate clusters. This is supported by rarefaction analysis which shows a decreasing slope (Fig. 1B).

Our data therefore most probably covers nearly the whole spectrum of sequences of GSB which can be found in the sampled habitats. Hence, it represents a robust basis for a discussion of a new phylogeny of the group. The cultured species, however, represent not even half of the biodiversity of GSB. More cultivation efforts will be necessary to gain a comprehensive picture of the physiological properties and the phylogenetical relationships within the group. Some of the environmental sequences may derive from green sulfur bacteria with new interesting biological properties or special adaptations to their habitat. The phototrophic epibionts of consortia or *Chl. bathyomarinum*, isolated from a deep sea hydrothermal vent, represent only two examples in this respect (Glaeser et al. 2002, Beatty et al. 2005).

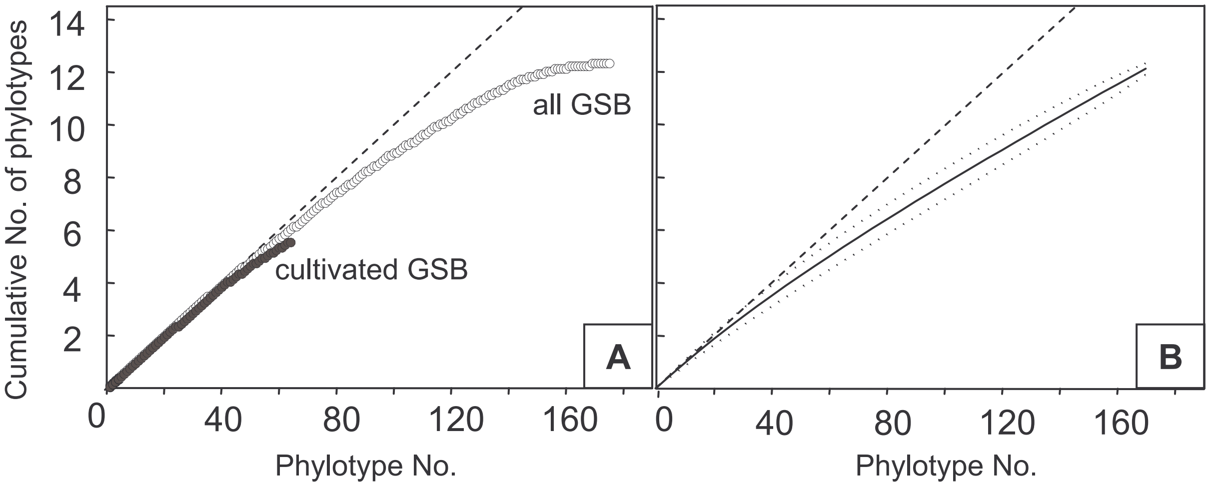

Figure 1. Biodiversity of GSB phylotypes. A. Comparison of the diversity of phylotypes in cultured species (dark cirles) and all available GSB sequences (white circles). The different phylotypes are plotted against the total number of phylotypes. B. Rarefaction of the different phylotypes (solid line). A confidence interval of 95 % is given (dotted lines). For comparison, a dashed line with slope 1 is given in both diagrams.

16S rRNA gene sequence-based phylogeny of GSB. From a total of 246 available green sulfur bacterial 16S rRNA gene sequences a phylogenetic tree was constructed which showed several distinct clusters (Fig. 2, supplemental Fig. 1). The presented 16S rRNA phylogenetic tree comprises five basic clusters, which correspond to the main groups 1, 2a/b, 3a, 3b, and 4a/b, as proposed by Imhoff (2003). In contrast to published data (Imhoff 2003), not all of the included sequences clustered within these groups (Fig. 2). *Chl. phaeobacteroides* DSM 266^T was positioned distant from group 3a. This supports the isolated position and the exclusive species name in Imhoff's publication (Imhoff 2003). Contrarily, in our investigation, the distance from other phylogenetic clusters is supported by seven environmental sequences which form a distinct cluster with the type strain (Fig. 2). Like 29 sequences of phototrophic consortia and two environmental sequences, the '*Chl. phaeobacteroides* DSM 266^T group' cannot be assigned to any of the stable clusters in the 16S rRNA tree. These species therefore have an exceptional position in the phylogenetic tree of GSB.

The species *Chl. limicola* DSM 246 was assigned to group 3b by Imhoff (2003), but this classification was based on low bootstrap values (44-67-33; Imhoff 2003). In our analysis the species *Chl. limicola* DSM 246 is assigned to group 4b, which is supported by high bootstrap values (supplemental Fig. 1). This supports the phylogenetic classification as published by Figueras et al. (1997). Therefore this species should be renamed to *Cba. limnaeum*, to be consistent with the systematics. In conclusion, our analysis supports four of the stable clusters proposed by Imhoff (2003), groups 1, 2a, 2b and 3b

Group 1, denoted by the genus name *Prosthecochloris*, comprises now a whole lot more saltwater sequences (supplemental Fig. 1) and can be subdivided formalistically into six stable sub-clusters (1a-1f). The described species in this cluster require >1% salt for growth (Imhoff 2003) which can therefore be considered the marine cluster (Alexander et al. 2002).

The first cluster is around *Prosthecochloris aestuarii* SK-413, DSM 271^T, which requires 2-5% salt for growth (Imhoff 2003), and contains to date 10 environmental sequences and the type strain (group 1a). The second cluster comprises two *Prosthecochloris* species (*Ptc.* sp. 2K and *Ptc. indicum*) and two environmental sequences (group 1b). Since *Ptc.* sp. 2K is not assigned to any species any more because there is no pure culture available (Imhoff 2003), the species name *Ptc. indicum* 2K would be consistent with the systematics. Group 1c is based on five species and one environmental sequence and comprises the type strain *Ptc. vibrioformis* DSM 260^T as well as the species *Ptc. vibrioformis* DSM 1678 and *Ptc. vibrioformis* CHP3402. The two other species, *Chl. vibrioforme* EP2403 and *Pld. phaeum* CIB2401, have identical 16S rRNA gene sequences and should be renamed to *Ptc. vibrioformis*. The group 1d to date comprises only two species, *Ptc. aestuarii* CE2401 and *Ptc. aestuarii* CHP3401. The fifth cluster comprises a recently published isolate, *Chl. bathyomarinum* GSB1 (Beatty et al. 2005), and two environmental sequences (group 1e). The renaming of *Chl. bathyomarinum* to *Ptc. bathyomarinum* GSB1^T should be considered but postponed, since there is no valid strain description yet. The sixth marine cluster comprises three isolates, *Ptc.* sp. 4Vi, *Ptc.* sp. VK and the green sulfur bacterium from the Black Sea, BS-1, but no valid strain description is available to date (group 1f). Five environmental sequences are also included in this cluster. A new species name will have to be proposed for these isolates in the future.

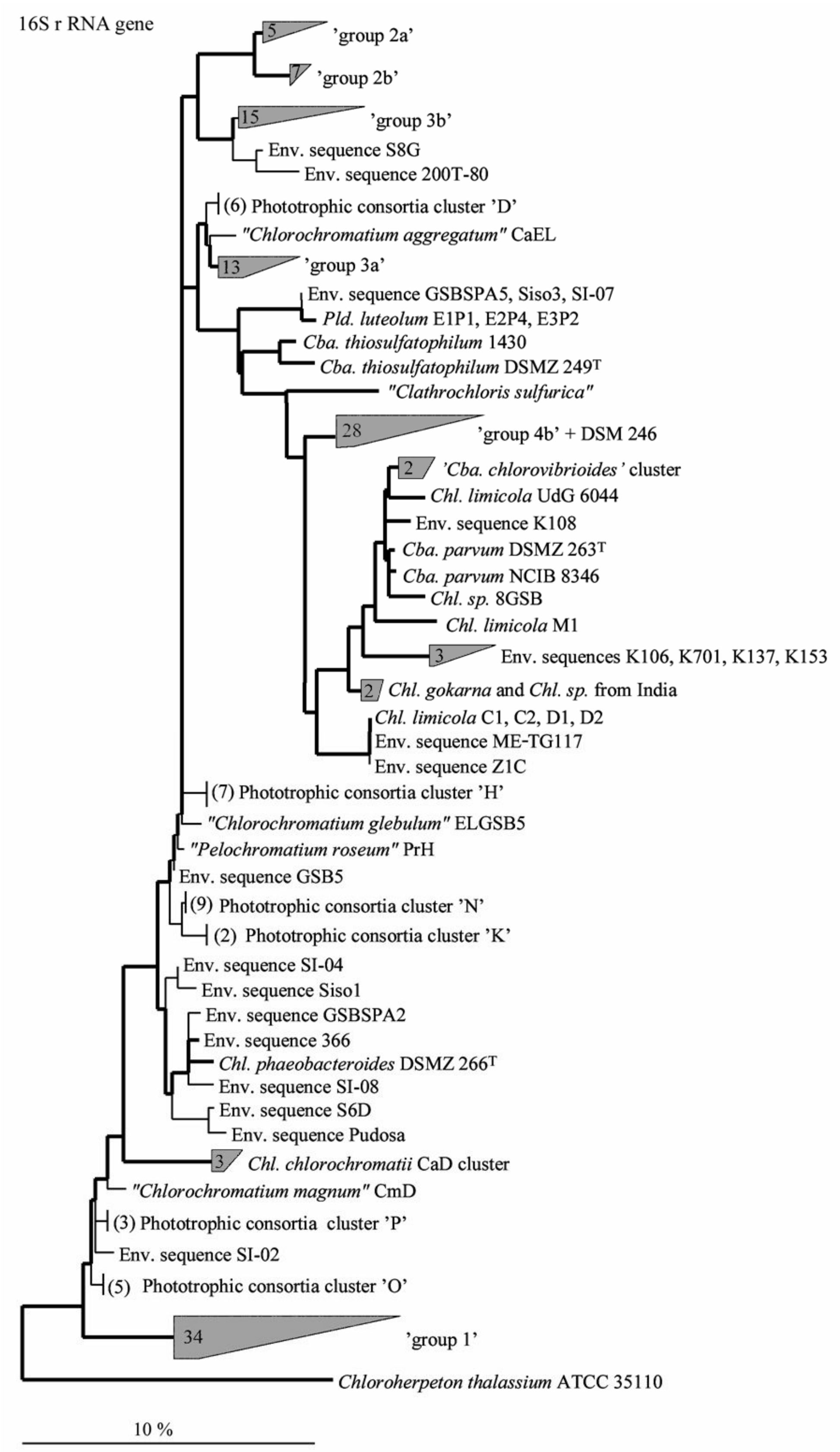

Figure 2. 16S rDNA phylogenetic tree of green sulfur bacteria. The tree was constructed based on sequences longer than 1100 bp in ARB, using the Fast_DNAML algorithm (thick lines). Thin lines indicate branches of environmental sequences, which were inserted with PARSIMONY INTERACTIVE. Highly stable groups (bootstrap values >50%) are collapsed. Naming of the groups refers to Imhoff (2003). For the unfolded tree and bootstrap values see supplemental Figure 1. Bar denotes fixed substitutions per nucleotide.

Group 2 was also extended and the proposed classification into two sub-clusters 2a and 2b (Imhoff, 2003) is supported by our analysis. Group 2a clusters around the type strain *Chl. luteolum* DSM 273^T, and comprises the species *Chl. luteolum* DSM 262 and three environmental species. Group 2b contains the type strain *Chl. phaeovibrioides* DSM 269^T, and the species *Chl. phaeovibrioides* DSM 261, DSM 270 and DSM 265. They are accompanied by three environmental sequences from the Mariager Fjord. Described species in this cluster require >1% salt for growth (Imhoff 2003).

The fourth stable cluster is **group 3b**, which comprises the type strain *Chl. limicola* DSM 245^T. The cultured species in group 3b do not require salt for growth (Imhoff 2003). Other species in this cluster are *Chl. limicola* E2P2, DSM 1855, 1630, 9330, UdG 6037, and *Chl. phaeobacteroides* III, UdG6047, UdG6051, UdG 6046, Glu, Cl1402, and five environmental sequences. *Chl. limicola* DSM 246 is not assigned to group 3b any more (Imhoff 2003) but clusters with group 4b. In group 3b, many identical sequences were found, isolated from all over the world. Indeed, Group 3b seems to comprise a 16S rRNA gene sequence which is found ubiquitously.

Groups 3a, 4a and 4b show only low bootstrap values at their bases. Therefore some of the species were positioned at a different position in the tree, depending on the algorithm used (DNAML, DNAPARS, NJ). These species cannot clearly be assigned to either of the groups. This is supported by analysis of the ITS, *sigA* and *bchG* gene sequences (see below).

Group 3a represents a cluster comprising the type strains *'Chl. ferrooxidans'* DSM 13031^T and *Chl. clathratiforme* DSM 5477^T, which was formerly known as *Pelodictyon phaeoclathratiforme*. This group is supplemented by *Chl. clathratiforme* PG, 11 partial sequences from phototrophic consortia and six environmental sequences. The group comprises both brown and green colored GSB with no salt requirement, species that are characteristically forming three-dimensional nets, or representing epibionts of phototrophic consortia. This group therefore is heterogeneous regarding the morphological properties. *Chl. phaeobacteroides* DSM 266^T did not cluster with group 3a, as published by Imhoff (2003), but has an isolated position in the tree.

Group 4 was divided into subgroups 4a and 4b, but the basis of the subtree 4 cannot be clearly positioned due to low boostrap values (29 with DNAML). Within the group, however, high bootstrap values indicate the internal stability of the group. Group 4b contains the type strains *Cba. thiosulfatophilum* DSM 249^T, *Cba. tepidum* ATCC 49652^T and *Cba. limnaeum* DSM 1677^T. The cultivated species of this cluster do not require any salt for growth. *Cba. thiosulfatophilum* DSM 249^T is positioned at the basis of the subtree, the next relatives are *Pld. luteolum* E1P1, *Cba. thiosulfatophilum* 1430 and *'Clathrochloris sulfurica'*, and one environmental sequence. The closest relatives to *Cba. tepidum* ATCC 49652^T are three environmental sequences from the Mariager Fjord. *Cba. limnaeum* DSM 1677^T clusters with a multitude of strains: *Cba. limnaeum* 1549, *Cba. limnaeum* UdG 6042, *Cba. limnaeum* UdG 6040, *Cba. limnaeum* UdG 6045, *Cba. limnaeum* UdG 6038, *Chl. limicola* UdG 6041 and *Chl. limicola* DSM 246. Interestingly, in our analysis *Chl. limicola* DSM 246 does not cluster with group 3b, as proposed by Imhoff (2003) but with group 4. The cluster surrounding *Cba. limnaeum* DSM 1677^T is the most stable cluster in the subtree 4, which is supported by high bootstrap values. A lot of environmental sequences (16 sequences) are positioned equally distant from *Cba. limnaeum* DSM 1677^T and *Cba. tepidum* ATCC 49652^T. Twelve of these sequences are isolates from a thermal spring in Yellowstone National Park, USA. This is another example for the ability of green sulfur bacteria to adapt to extreme environments and for the awareness that green sulfur bacteria in extreme habitats probably have unusual properties.

The subgroup 4a, around *Cba. parvum* DSM 263^T, arises from subgroup 4b and comprises the species *Cba. parvum* NCIB 8346, *Chl.* sp. 8GSB, *Chl. limicola* M1, *Chl. limicola* C1 (identical to C2, D1, D2), *Chl. limicola* UdG 6044, *'Cba. chlorovibrioides'* UdG 6026, *'Cba. chlorovibrioides'* UdG 6043, as well as two new isolates from India, *Chl. gokarna* 4GSBT and *Chl.* sp. 6GSB. Additionally, the group contains six environmental sequences, from the Sippewisset salt marsh and from Mariager Fjord. This supports the finding that all species in cluster 4a require low salt conditions, as do the cultured species in this cluster.

Matrix comparison (Mantel test). Comparison of the distance matrices of the *bchG* gene with the 16S rRNA gene (Fig. 3A) shows a correlation of 0.698. The correlation of these matrices therefore shows a high value, which can also be derived from the comparably slight scatter of permutational distribution of the genetic distances. Similar values are obtained when comparing the *fmoA* gene distance matrix with the 16S rRNA gene (Fig. 3B). When looking at the comparison of ITS and 16S rRNA gene (Fig. 3C) we see an accumulation of data points around 0 and a very small dispersal around an even line with slope 1. This shows a very small permutational distribution of genetic distances. This can be ascribed to a high number of identical ITS sequences in the phylogenetic investigation, and to only short genetic distances within the distinct phylogenetic clusters (supplemental Fig. 2). However, in most of the GSB, only one ITS allele is found, or two alleles which vary in only few bases. A small correlation value of 0.597 shows a minor correlation of the two matrices. The comparison of *sigA* sequences with 16S rRNA gene sequences shows the smallest correlation value of 0.448, which shows no statistically significant correlation of the two matrices (Fig. 3D). Also the scatter around the even line with slope 1 is greater than in the other correlation plots. Altogether, the matrix comparisons with the

16S rRNA gene show a significant correlation for the genes *bchG* and *fmoA*, a smaller, but still significant correlation for the ITS region, and no significant correlation for *sigA*. The *sigA* gene can therefore be considered as a valuable genetic marker molecule for the classification of GSB that complements the 16S rRNA gene analysis, leading to a higher resolution in the phylogenetic relationships.

The genes *fmoA* and *bchG* are of minor value, since virtually the same evaluation could be done using the 16S rRNA gene only, with a much bigger data base. The ITS region as a complementing phylogeny tool proved not as useful we expected it to be, since the correlation with the 16S rRNA gene analysis is still too high. Still, our analysis shows the usefulness of the published primers pairs for phylogenetic analyses of GSB, as an alternative to 16S rRNA gene sequencing.

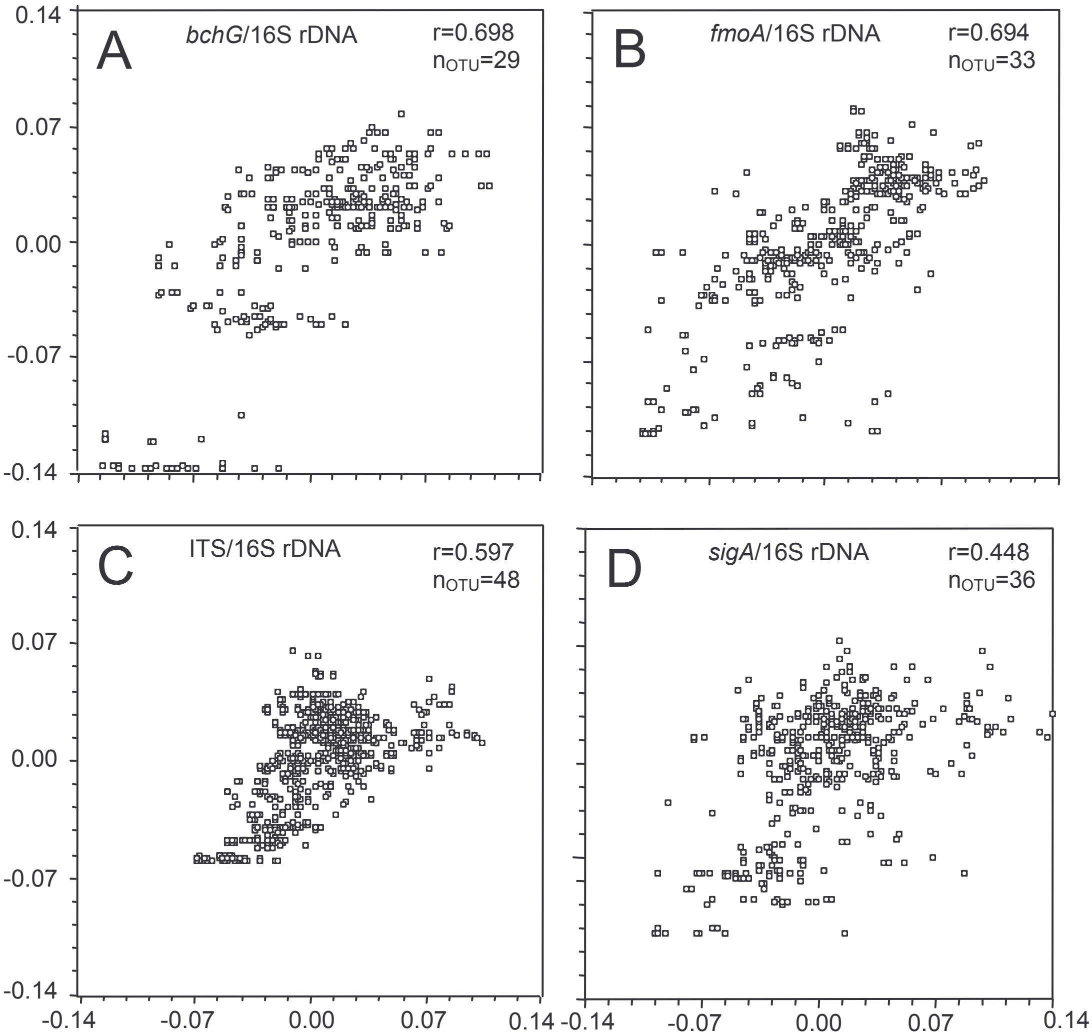

Figure 3. Distance matrix correlation of different genetic markers and the 16S rRNA gene (Mantel test). Distance matrices were calculated in PHYLIP, using DNADIST, of sequences from selected species. A. Comparison of *bchG* genes and 16S rRNA genes. B. Comparison of *fmoA* genes and 16S rRNA genes. C. Comparison of the ITS region and 16S rRNA genes. D. Comparison of *sigA* genes and 16S rRNA genes.
r, product-moment correlation, monotonically related to the Mantel test statistic *Z* (Smouse et al. 1986). Values for $r > 0.5$ show a statistically significant correlation of two matrices. n_{OTU}, number of 'operational taxonomic units' (=sequences). The plots show *r* with its permutational distribution in the distance matrix comparisons.

Comparison of the concatenated tree with the 16S rRNA gene tree. In the concatenated tree three markers (ITS, *sigA*, *bchG*) (supplemental Fig. 2, 3, 4) were combined to reflect the phylogenetic relationships of the whole genomes of the investigated species. 33 species, comprising type strains of all of the prominent clusters, were selected for the analysis (Fig. 2A). From group 3b, multiple species were chosen, accounting for the repeated isolation of species positioned in this cluster.

In the concatenated tree (Fig. 2A) five clusters are visible, but only three of them show high bootstrap values, indicating the internal stability of the cluster. The first cluster is the one that comprises *Chl. luteolum* DSM 273^T and *Chl. phaeovibrioides* DSM 269^T, corresponding to "group 2a" and "group 2b". This group clusters with the second stable group, "group 3b", which comprises 5 identical sequences from *Chl. phaeobacteroides* species (Glu, Cl1401, DagowIII, UdG 6051 and UdG 6046), and *Chl. limicola* E2P2. In contrast, the 16S rRNA gene sequences show a higher diversity in the respective cluster. The functional genes in this group seem to have derived from the same ancestral gene, and give evidence for the close phylogenetic relationship of these strains. At the same time, this cluster comprises a greater number of closely related species than other clusters. This supports the observation that species in this cluster represent a ubiquitous phylotype. *Chl. limicola* DSM 245^T, the type strain for this cluster, has a slightly more distant position, due to differences in the *sigA* and *bchG* gene sequences (supplemental Fig. 3, 4), in contrast to the finding that 16S and ITS are much closer related to the other sequences. This points to a more specialized physiology, which can be explained by its different habitat, which is a hot spring (supplemental table 3, supplemental Fig. 1).

Two species, which build the third stable cluster that is present in all of the trees ("group 3a"), are *Chl. clathratiforme* PG and DSM 5477^T. The position of this cluster, however, varies amongst the trees (Fig. 1, supplemental Fig. 2, 3, 4).

The fourth stable cluster is "group 4", with subgroups 4a and 4b. The two clusters are much more isolated from each other when regarding functional genes and ITS, rather than the 16S rRNA gene. *Chl. limicola* C1/C2 has different positions within groups 4a or 4b, when comparing the concatenated tree and the 16S tree (Fig. 2). But when regarding the ITS region, or *bchG* and *sigA* genes, we see that one of the closest relatives is always *Cba. thiosulfatophilum* DSM 249^T. Only in the 16S rRNA gene tree, the next relative is *Cba. parvum* DSM 263^T. *Chl. limicola* C1/C2 therefore represents either a more specialized species, or, considering its moderate habitat, a more primordial species, representing the root of group 4. The same phenomenon can be observed when regarding *Chl. phaeobacteroides* UdG 6047, which is positioned next to *Chl. phaeovibrioides* DSM 269^T in the concatenated tree, but next to *Chl. phaeobacteroides* UdG 6051 in the 16S rRNA tree (Fig. 2). The 16S rRNA gene analysis indicates an origin of the species within the group of moderate freshwater species (group 3b) followed by a specialization like the species in group 2b which require >1 % salt. *Cba. thiosulfatophilum* DSM 249^T and *Cba. tepidum* ATCC 49652^T are closest relatives in both the 16S and the concatenated tree (Fig. 4A and 4B), which also points to similar physiological adaptations.

The fifth stable cluster is the cluster comprising the real saltwater strains, which once more seems to be the most stable and most distant cluster amongst the GSB. Also in this cluster, the internal grouping differs slightly in the phylogenetic trees (Fig. 4A and 4B, supplemental Fig. 2, 3, 4).

Few species (for example *Chl. phaeobacteroides* DSM 266^T or *Chl. chlorochromatii* CaD, Fig. 4A and 4B) could not be assigned to any of the stable clusters in the 16S rRNA gene tree, and had varying positions in the other calculated trees (supplemental Fig. 2, 3, 4). Long branch lengths in the ITS, *sigA* and *bchG* tree point to an isolated position in the phylogenetic tree of GSB. *Chl. chlorochromatii* CaD is representative for the group of green sulfur bacterial epibionts that are associated in photosynthetic consortia (Glaeser et al. 2004), and the uncertain position in the 16S rRNA tree is symptomatic for most of the 16S rRNA genes of epibionts from phototrophic consortia (supplemental Fig.1). *Chl. phaeobacteroides* DSM 266^T and *Chl. chlorochromatii* CaD may represent species with special adaptations and are expected to possess an unusual physiology, and therefore are exciting study objects (Vogl et al. 2005).

The species with varying positions in the different trees represent examples that 16S rRNA gene analysis or even usage of only one marker gene is not sufficient for the proper classification of the green sulfur bacteria. Another reason for the unstable positioning could be the use of protein coding regions, since these genes can be transferred more easily by lateral gene exchange amongst species, which is directly related to the need for physiological adaptation to a given habitat.

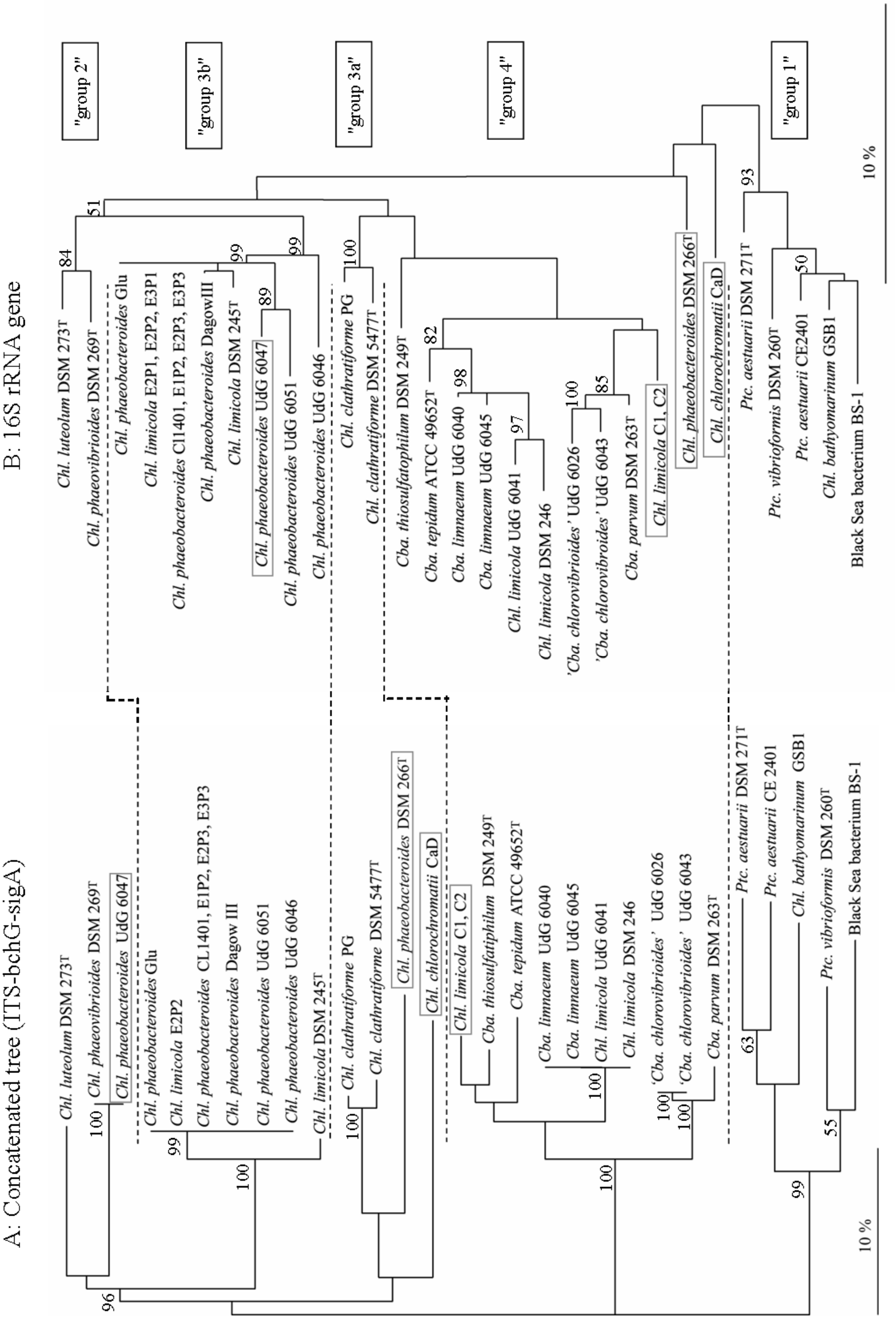

Figure 4. Comparison of a concatenated tree with the 16S rDNA tree. A. Concatenated tree constructed from the ITS region and the *bchG* and *sigA* genes. B. Reduced 16S rDNA tree of GSB, which comprises only the species present in the concatenated tree. Bootstrap values are given for DNAML. Naming of the groups refers to Imhoff (2003). Boxed species are placed in different clusters in the two trees. Bar denotes fixed substitutions per nucleotide.

Biodiversity of GSB. The huge set of 16S rRNA gene sequences investigated gives the chance to discuss coherences of phylogeny and biogeography of GSB. One physiological feature that is reflected in the different clusters in the tree is the salt requirement (Garcia-Gil et al. 2003). The cultured species in group 2a and 2b require >1% salt. This is congruent with the presence of sequences from the Mariager Fjord in this cluster, since this fjord is a stratified marine environment (Teske et al. 1996). Sequences from Mariager Fjord are also positioned in group 4a, next to species requiring low salt conditions, as well as in group 1a, where the closest relative (*Ptc. aestuarii* DSM 271^{T}) requires 2-5 % salt. Surprisingly, sequences from Mariager Fjord are also found in group 4b, which is occupied by cultured species without salt requirement. Equally surprising is the presence of sequences from Mediterranean sapropel layers in group 3b, amongst species without salt requirement. These sequences are most probably introduced allochthonously to the Mediterranean sapropel layers, via lateral transport of freshwater GSB sequences to saltwater habitats. This is supported by investigation of Black Sea sediments, where sequences of freshwater GSB are also present (Manske et al., in preparation). From the Black Sea water column, however, only one strain of GSB was isolated (Overmann et al. 1992, Manske et al. 2005), which is placed well amongst the marine strains (supplemental Fig. 1). Groups 3a and 3b seem to inhabit sequences of strains, which can live in rather moderate environments, like the chemocline of freshwater lakes worldwide. This is reflected by a high number of isolates in group 3b, which seem to represent a ubiquitous phylotype, and an unstable positioning in the phylogenetic tree. In contrast, groups 2a, 2b and 4a occupy a rather isolated position in the phylogenetic tree, and comprise species with requirement for low salt. Interestingly, all but one of the sequences from hot springs can be found in group 4b. Therefore, species in this cluster may have similar physiological adaptations. We also see sequences which do not cluster with other closely related sequences. Two prominent examples are *Chl. phaeobacteroides* DSM 266^{T} and *Chl. chlorochromatii* CaD. For these two species only few closely related sequences were detected worldwide. These sequences are most likely underrepresented in our analyses, and more closely related, interesting species await their isolation.

Specific adaptations are most certainly present in all epibionts of phototrophic consortia (Vogl et al. 2005). Like *Chl. chlorochromatii* CaD, sequences of most epibionts did not cluster with any of the distinct clusters, but had an unstable position between group 1 and the other groups (supplemental Fig. 1). One exception to that rule is the presence of some sequences of epibionts in group 3a, but this could also be an artifact and could be ascribed to an uncertain basis of the group.

A correlation of the BChl *e* expression or the presence of gas vacuoles and the phylogenetic relation of species was not found, in contrast to an investigation by Garcia-Gil et al. (2003), which was based on only a small number of isolates.

Nonetheless, all these findings sustain a close correlation of physiology and phylogeny in the group of GSB, and the advantages of functional genes for classification of bacteria. Sigmafactor A is a promising tool, since it represents the primary sigma factor, and seems to be different from other bacterial sigma factors, which is a hint for a functional change (Gruber and Bryant 1997).

Discussing the new systematics for green sulfur bacteria. Imhoff (2003) introduced genus and species names for the classified bacteria based on two phylogenetic trees, which were calculated using 34 different 16S rRNA gene sequences and 25 *fmoA* gene sequences. In the recent systematics (Imhoff 2003), as a result of the phylogenetic evaluation of 16S rRNA gene and FmoA protein sequences, a lot of descriptive names do not correspond to the physiological or morphological properties of the bacteria any more. With our data, however, we present a more comprehensive basis for the classification of green sulfur bacteria since we included all of the available sequences of isolates as well as environmental sequences. Our study is the most comprehensive study on green sulfur bacterial phylogeny to date. This is supported by a rarefaction analysis of our 16S rRNA data. By using other genetic marker regions we achieved a more reliable basis for the systematic system of the phylogenetically very shallow group of GSB. The usefulness of these markers for phylogenetic analyses was discussed based on Mantel test statistics and a direct comparison of a concatenated analysis of the markers with the 16S rRNA phylogenetic tree. We propose a basis for a revised taxonomy based on 16S rRNA, *fmoA*, *bchG* and *sigA* gene sequences. The systematic system proposed by Imhoff (2003) was premature in the classification of green sulfur bacteria and did not meet all requirements of the naming of bacterial genus and species names. The shortly expected whole genome sequences of several GSB will give more insight into this genetically very shallow, but physiologically very diverse group.

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REFERENCES

1) **Aakra, A., J. B. Utaker, and I. F. Nes.** 1999. RFLP of rRNA genes and sequencing of the 16S-23S rDNA intergenic spacer region of ammonia oxidizing bacteria: a phylogenetic approach. Int J Syst Bacteriol. **49:**123-130.
2) **Alexander, B., J. H. Andersen, R. P. Cox, and J. F. Imhoff.** 2002. Phylogeny of green sulfur bacteria on the basis of gene sequences of 16S rRNA and of the Fenna-Matthews-Olson protein. Arch. Microbiol. 178:131-140.
3) **Amann, R. I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev **59:**143-169.
4) **Beatty J. T., J. Overmann, M. T. Lince, A. K. Manske, A. S. Lang, R. E. Blankenship, C. L. van Dover, T. A. Martinson, and F. G. Plumley.** 2005. An obligately photosynthetic bacterial anaerobe from a deep-sea hydrothermal vent. Proc. Natl. Acad. Sci. USA. 102:9306-9310.
5) **Burgess, R. R., A. A. Travers, J. J. Dunn, and E. K. F. Bautz.** 1969. Factor stimulating transcription by RNA polymerase. Nature (London) 221:43-46.
6) **Cannone, J. J., S. Subramanian, M. N. Schnare, J. R. Collett, L. M. D'Souza, Y. Du, B. Feng, N. Lin, L. V. Madabusi, K. M. Müller, N. Pande, Z. Shang, N. Yu, and R. R. Gutell.** 2002. The comparative RNA web (CRW) site: an online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. BMC Bioinformatics 3(1):2, corrections: 3(1):15, http://www.rna.icmb.utexas.edu.
7) **Coolen, M. J. L. and J. Overmann.** 2000. Functional exoenzymes as indicators of metabolically active bacteria in 124,000-year-old sapropel layers of the eastern Mediterranean Sea. Appl. Environ. Microbiol. 66:2589-2598.
8) **Felsenstein, J.** 1989. PHYLIP - phylogenetic interference package (Version 3.2). Cladistics 5:164-166.
9) **Figueras, J. B., Garcia-Gil, L. J., and C. A. Abella.** 1997. Phylogeny of the genus *Chlorobium* based on 16S rDNA sequence. FEMS Microbiol. Lett. 152:31-36.
10) **Garcia-Gil, L. J., F. B. Gich, and X. Fuentes-Garcia.** 2003. A comparative study of *bchG* from green photosynthetic bacteria. Arch. Microbiol. 179:108-115.
11) **Garrity, G. M., and J. G. Holt.** 2001. Phylum BXI. *Chlorobi* phy. nov., p. 601-623. In D. R. Boone and R. W. Castenholz (ed.), Bergey's manual of systematic bacteriology, 2nd ed., vol. 1. Springer-Verlag, New York, NY.

12) **Glaeser, J. , L. Bañeras, H. Rütters, and J. Overmann.** 2002. Novel bacteriochlorophyll *e* structures and species-specific variability of pigment composition in green sulfur bacteria. Arch. Microbiol. **177:**475-485.

13) **Glaeser, J. and J. Overmann. 2004**. Biogeography, evolution, and diversity of epibionts in phototrophic consortia. Appl Environ Microbiol. **70**:4821-30.

14) **Gruber, T. M. and D. A. Bryant.** 1997. Molecular systematic studies of eubacteria, using σ^{70}-type sigma factors of group 1 and group 2. J. Bac. 179:1734-1747.

15) **Helmann, J. D., and M. J. Chamberlin.** 1988. Structure and function of bacterial sigma factors. Annu. Rev. Biochem. 57:839-872.

16) **Imhoff J. F.** 2003. Phylogenetic taxonomy of the family *Chlorobiaceae* on the basis of 16S rRNA and fmo (Fenna-Matthews-Olson protein) gene sequences. Int. J. Syst. Evol. Microbiol. **53**:941-51.

17) **Jensen, M. A., J. A. Webster, and N. Straus.** 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Appl. Env. Microbiol. 59:945-952.

18) **Lane, D. J.** 1991. 16S/23S rRNA sequencing. In: Stackebrandt E., Goodfellow M. (eds) Nucleic acid techniques in bacterial systematics. Wiley, Chichester, pp. 115-175.

19) **Lonetto, M., M. Gribskov, and C. A. Gross.** 1992. The σ^{70} family: sequence conservation and evolutionary relationships. J. Bacteriol. 174:3843-3849.

20) **Ludwig, W., O. Strunk, S. Klugbauer, N. Klugbauer, M. Weizenegger, J. Neumann, M. Bachleitner, and K. H. Schleifer.** 1998. Bacterial phylogeny based on comparative sequence analysis. Electrophoresis 19:554-568.

21) **Madigan, M. T., J. M. Martinko, and J. Parker.** 2000. Brock – Mikrobiologie. German ed. W. Goebel. Spektrum, Akad. Verlag, Berlin, Germany.

22) **Manske, A. K., J. Glaeser, M. M. M. Kuypers, and J. Overmann.** 2005. Physiology and phylogeny of green sulfur bacteria forming a monospecific assemblage at 100 m depth in the Black Sea. Appl Env Microbiol **71:**8049-8060.

23) **Manske, A. K., Henßge, U., Glaeser, J., and J. Overmann.** Subfossil DNA sequences of green sulfur bacteria as indicators for past water column anoxia in the Black Sea. In preparation.

24) **Mantel, N.** 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. 27:209-220.

25) **Montesinos, E., Guerrero, R., Abella, C. and I. Esteve.** 1983. Ecology and Physiology of the Competition for Light Between *Chlorobium limicola* and *Chlorobium phaeobacteroides* in Natural Habitats. Appl. Environ. Microbiol. **46**:1007-1016.

26) **Muyzer, G., E. C. De Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol **59:**695-700.

27) **Nicholas, K. B. and H. B. Nicholas Jr.** 1997. GeneDoc: a tool for editing and annotating multiple sequence alignments. www.psc.edu/biomed/genedoc. Distributed by the author.

28) **Overmann, J., H. Cypionka, and N. Pfennig.** 1992. An extremely low-light-adapted green sulfur bacterium from the Black Sea. Limnol. Oceanogr. **37:**150-155.

29) **Overmann, J., M. J. L. Coolen, and C. Tuschak.** 1999. Specific detection of different phylogenetic groups of chemocline bacteria based on PCR and denaturing gradient gel electrophoresis of 16S rRNA gene fragments. Arch Microbiol **172:**83-94.

30) **Overmann, J.** 2000. The family *Chlorobiaceae* release 3.1. In M. Dworkin (ed.), The prokaryotes: an evolving electronic resource for the microbiological community, 3rd ed. Springer-Verlag, New York, NY (online). http://141.150.157.117:8080/prokPUB/index.htm

31) **Overmann, J.** 2001. Green sulfur bacteria. *In* Boone, D. R., and R. W. Castenholz (eds) Bergey's manual of systematic bacteriology, 2nd edn, vol. 1. Springer, Berlin, Heidelberg, New York, pp.601-623.

32) **Pfennig, N.** 1989. Green sulfur bacteria. *In* Bergey's Manual of Systematic Bacteriology, vol. 3, pp. 1682-1697. Edited by J. T. Staley, M. P. Bryant, N. Pfennig & J. G. Holt. Baltimore: Williams & Wilkins.

33) **Pfennig, N., and J. Overmann.** 2001a. Genus I. *Chlorobium*. *In* Bergey's Manual of Systematic Bacteriology, 2nd edn, vol. 1, pp. 605-610. Edited by D. R. Boone, R. W. Castenholz & G. M. Garrity. New York: Springer.

34) **Pfennig, N., and J. Overmann.** 2001b. Genus IV. *Pelodictyon*. *In* Bergey's Manual of Systematic Bacteriology, 2nd edn., vol. 1, pp. 614-617. Edited by D. R. Boone, R. W. Castenholz & G. M. Garrity. New York: Springer.

35) **Schlegel, H. G.** 1992. Allgemeine Mikrobiologie. Revised 7th edition. Thieme, Stuttgart, Germany; New York, NY.

36) **Smouse, P. E. J. C. Long, and R. R. Sokal.** 1986. Multiple regression and correlation extensions of the Mantel test of matrix correspondence. Systematic Zool. 35:627-632.

37) **Sokal. R. R.** 1979. Testing statistical significance of geographic variation patterns. Systematic Zool. 28:227-231.

38) **Sokal, R. R., P. E. Smouse, and J. V. Neel.** 1986. The genetic structure of a tribal population, the Yanomama Indians. XV. Patterns inferred by autocorrelation analysis. Genetics 114:259-287.

39) **Teske, A., C. Wawer, G. Muyzer, and N. B. Ramsing.** 1996. Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. Appl. Env. Microbiol. 62(4):1405-1415.

40) **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24:4876-4882.

41) **Vogl, K., J. Glaeser, K. R. Pfannes, and J. Overmann.** 2005. *Chlorobium chlorochromatii* CaD sp. nov., a symbiotic green sulfur bacterium isolated from the phototrophic consortium *"Chlorochromatium aggregatum"*. submitted to Arch. Microbiol.

42) **Widdel, F., G. W. Kohring, and F. Mayer.** 1983. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. III. Characterization of the filamentous gliding *Desulfonema limicola* gen. nov., sp. nov., and *Desulfonema magnum* sp. nov. Arch. Microbiol. 134:286-293.

43) **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. SSU ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173:697-703.

SUPPORTING ONLINE MATERIAL

Supplemental Table 1. PCR conditions and step down PCR. Each PCR started with a hot start (95°C, 5 min) and ended with a 4°C hold.

Target	Primer combination	Melting	Step down PCR: Annealing (No. of cycles)		Extension
16S rRNA gene	Uni8f – 1492r	94°, 30 s	59°C, 45 s (10)	54°C, 45 s (20)	72°C, 60 s
ITS region	GSB822f –L1r, 1525f – L1r	94°, 30 s	55°C, 60 s (10)	50°C, 60 s (25)	72°C, 60 s
Sigma factor A	GSB-SigA-F4 – GSB-SigA-R1[a]	94°, 30 s	61°C, 45 s (10)	56°C, 45 s (20)	72°C, 60 s
	Sig208f – Sig827r[a,b]	94°, 60 s	61°C, 45 s (10)	56°C, 45 s (25)	72°C, 60 s
	Sig312f – Sig827r[a,b]	94°, 45 s	56°C, 45 s (10)	52°C, 45 s (25)	72°C, 60 s
bchG gene	bchG-F – bchG-R	94°, 30 s	57°C, 60 s (10)	50°C, 60 s (20)	72°C, 60 s
ERIC-PCR	ERIC-1R – ERIC-2[b]	95°, 30 s	52°C, 60 s (30)	-	70°C, 8 min

[a] This PCR comprised an additional hold at 72°C (10 min) after the amplification cycles, before cooling to 4°C.

[b] This PCR initiated with a hot start (96°C, 4 min), followed by a hold (80°C, 4 min) where the polymerase was added.

Supplemental Table 2. Primers used in this study

Primer name	**Sequence**	**Reference**
16S rRNA gene[a]		
8f	5'-AGA GTT TGA TCC TGG CTC AG-3'	Lane 1991
Uni341f	5'-CC TAC GGG AGG CAG CAG-3'	Muyzer et al. 1993
Uni515f	5'-GTG CCA GCA GCC GCG G-3'	Lane 1991, modified
517r	5'-ATT ACC GCG GCT GCT GGC-3'	Lane 1991
Uni907r	5'-CCG TCA ATT CCT TTG AGT TT-3'	Lane 1991
926f	5'-AAA CTC AAA GGA ATT GAC GG-3'	Lane 1991
1055r	5'-AGC TGA CGA CAG CCA T-3'	Amann et al. 1995
Uni1492r	5'-GGT TAC CTT GTT ACG ACT T-3'	Weisburg et al. 1991
ITS region[a]		
1525f	5'-GG(CT) TGG A(GC)C ACC TCC TT-3'	Lane 1991
L1r	5'-CAA GGC ATC CAC CGT-3'	Jensen et al. 1993
GSB822f	5'-AAT ACT AGA TGT TGG TCA T-3'	Overmann et al. 1999
Sigma factor A		
Sig208f	5'-AA(CT) CT(GC) CG(CT) (CT)T(GC) GTG GT(CT) TC(GT) GTG GC-3'	Don Bryant, pers. comm.
Sig312f	5'-CAG TA(CT) CAG AA(CT) CAG GG-3'	Don Bryant, pers. comm.
Sig827r	5'-ATC TG(GC) CGG AC(CT) CG(CT) TC(GC) CG(AG) GT(AG) A-3'	Don Bryant, pers. comm.
GSB-SigA-F4	5'-ATT GTG CG(AC) (CT)T(GT) CC-3'	this study
GSB-SigA-R1	5'-AT(AT) GG(CT) ATG GA(CT) AAT CCG CT-3'	this study
***bchG* gene**		
bchG-F	5'-GTC GTG ACT GGG AAA ACA TGA A(AG)C C(AGCT)G T(GCT)A C(AGTC)T GG-3'	Garcia-Gil et al. 2003
bchG-R	5'-ACG GAC TTG AAG TCG TTC ATG (AG)TC ATG A(AT)(AG) CC-3'	Garcia-Gil et al. 2003
BchG-R Seq	5'-ACG GAC TTG AAG TCG TTC ATG-3'	Frederic Gich, pers. comm.

[a] Numbering of the primers refers to the accordant position in the *E. coli* 16S rRNA gene sequence. L1r binds to the end of the ITS region.

Supplemental table 3. References for species used in this study. * sequences obtained in this study.

Species name	**Strain**	**Strain collection no.**	**16S rDNA EMBL Accession no.**	**Length of sequence**	**Sampling site**	16S-23S rRNA ITS	sigA	bchG	fmoA
Cba. limnaeum	UdG6038	-	Y10641	1466	Wintergreen Lake, MI, USA	AM049271*	-	AJ427485	-
Cba. limnaeum	UdG6040	-	Y10642	1465	Puda Spring Mat, Spain	AM049272*	AM050069*	AM050372*	-
Cba. limnaeum	UdG6042	-	Y10644	1467	Lake Vilar, Spain	AM049273*	AM050096*	AM050354*	-
Cba. limnaeum	UdG6045	-	Y10646	1469	Wintergreen Lake, MI, USA	AM049274*	AM050070*	AM050374*	-
Cba. limnaeum	1549	-	AJ299413	1387	-	-	-	-	AJ306184
Cba. limnaeum	-	DSM 1677^{T}	AJ290831	1241	Lake Kinneret, Israel	-	-	-	AJ391149
Cba. parvum	NCIB 8327, UdG6036	DSM 263^{T}	M31769	1504	Karabugaz, Russia	AM049275*, AM049276*	AM050076*	AJ427487	AJ391147
Cba. parvum	NCIB 8346	-	AJ290830	1243	-	-	-	-	AJ391161
Cba. tepidum TLS	-	ATCC 49652^{T}	M58468	1453	Travelodge, Rotorua, NZ	NC_002932 (whole genome)	U67718	AJ427484	L13700
Cba. thiosulfatiphilum	1430	-	AJ290825	1244	-	-	-	-	AJ391144
Cba. thiosulfatiphilum	6230	DSM 249^{T}	AJ391143	1419	Tassajara Hot Spring, CA, USA	AM049277*	AM050073*	AM050370*	AJ391143
"Cba chlorovibrioides"	UdG6026	-	Y10649	1466	Massona Lagoon, Spain	AM049279*	AM050066*	AM050352*	AJ306188/ AJ306182
"Cba. chlorovibrioides"	UdG6043	-	Y10648	1465	Everglades, FL, USA	AM049280*	AM050067*	AM050351*	AJ306193/ AJ306187
"Chl. bathyomarinum"	GSB1	-	AY627756	1379	Ty vent, Pacific Ocean	AM049278*	AM050095*	AM050371*	AY627684
Chl. chlorochromatii	CaD	-	AJ578461	1387	Lake Dagow, Germany	AM049317*	x	Contig1, 2261753-2261967^{a}	Contig1, gene 1127^{a}
Chl. clathratiforme	PG	-	Y08106	1355	Lake Grünenplan, Germany	AM049281*	AM050093*	AM050367*	-
Chl. clathratiforme	BU-1	DSM 5477^{T}	Y08108	1308	Lake Buchensee, Germany	AM049282*	NZ_AAIK01000001	ZP_00589807	AJ290822
Chl. ferrooxidans	KoFox	DSM 13031^{T}	Y18253	1804	Ditch, Konstanz, Germany	AM049283*	-	-	-
Chl. gokarna	4GSB^{T}	-	AJ888464	1287	Gokarna, India	-	-	-	-
Chl. limicola	6330	DSM 245^{T}	Y10113	1359	Gilroy Hot Spring, CA, USA	AM049284*	NZ_AAHJ01000004	ZP_00512032	AJ391153
Chl. limicola	C1	-	AM050125*	1440	Lake Waldsee, Germany	AM049287*	AM050061*	AM050357*	-
Chl. limicola	C2	-	ident. C1	1416	Lake Waldsee, Germany	AM049286*	AM050063*	AM050356*	-
Chl. limicola	D1	-	ident. C1	1406	Lake Waldsee, Germany	AM049289*	AM050064*	-	-
Chl. limicola	D2	-	ident. C1	-	Lake Waldsee, Germany	AM049288*	AM050065*	-	-
Chl. limicola	E2P2	-	AM050126*	1432	Lake Sisó, Spain	AM049290*	AM050082*	AM050359*	-
Chl. limicola	E3P1	-	identical E2P2	-	Lake Sisó, Spain	identical E2P2*	AM050085*	AM050364*	-
Chl. limicola	E2P1	-	identical E2P2	-	Lake Sisó, Spain	identical E2P2*	AM050080*	-	-
Chl. limicola	1630	-	AJ290826	1243	-	-	-	-	AJ391157

Supplemental table 3 (continued).

Species name	**Strain**	**Strain collection no.**	**16S rDNA EMBL Accession no.**	**Length of sequence**	**Sampling site**	16S-23S rRNA ITS	sigA	bchG	fmoA
Chl. limicola	9330	-	AJ290827	1242	-	-	-	-	AJ391158
Chl. limicola	M1	-	AB054671	1465	-	-	-	-	-
Chl. limicola	UdG6002	DSM 246	Y10640	1464	-	AM049285*, AM049291*	AM050072*	AM050355*	AJ391142
Chl. limicola	UdG6037	-	AJ299414	1408	Wintergreen Lake, MI, USA	-	-	-	AJ306186
Chl. limicola	UdG6041	-	Y10643	1468	Lake Coromina, Spain	AM049292*, AM049293*	AM050071*	AM050353*	AJ306183
Chl. limicola	UdG6044	-	Y10645	1465	Round Lake, MI, USA	AM049294*	AM050068*	-	AJ306190
Chl. limicola	-	DSM 1855	AJ290832	1240	-	-	-	-	AJ391162
Chl. luteolum	6132	DSM 262	Y08103	1324	Brackish pond	AM049295*	AM050062*	-	AJ391159
Chl. luteolum	2530	DSM 273^T	Y08107	1403	Lake Polden, Norway	AM049296*	AM050090*	AJ427490	AJ391152
Chl. phaeobacteroides	2430	DSM 266^T	Y08104	1400	Lake Blankvann, Norway	AM049297*	NZ_AAIB0100 0040	ZP_0052777 6	AJ391148
Chl. phaeobacteroides	CL1401	-	AM050127*	1350	Lake Sisó, Spain	AM049298*	AM050077*	AM050362*	-
Chl. phaeobacteroides	E1P2	-	ident. Cl 1401	1438	Lake Sisó, Spain	AM049299*	AM050079*	AM050360*	-
Chl. phaeobacteroides	E2P3	-	ident. Cl 1401	1411	Lake Sisó, Spain	AM049300*	AM050081*	AM050361*	-
Chl. phaeobacteroides	E3P3	-	ident. Cl 1401	1447	Lake Sisó, Spain	AM049301*, AM049302*	AM050084*	AM050363*	-
Chl. phaeobacteroides	Glu	-	AM050128*	1446	Lake Glubitz, Germany	AM049303*	AM050078*	AM050358*	-
Chl. phaeobacteroides	Dagow III	-	AM050129*	1356	Lake Dagow, Germany	AM049304*	AM050086*	AM050365*	-
Chl. phaeobacteroides	UdG6046	-	Y10650	1436	Lake Banyoles, Spain	AM049305*	AM050087*	AM050366*	-
Chl. phaeobacteroides	UdG6047	-	Y10651	1462	Lake Banyoles, Spain	AM049306*	AM050092*	AM050373*	-
Chl. phaeobacteroides	UdG6051	-	Y10652	1461	Lake Sisó, Spain	AM049307*	AM050083*	AM050369*	AJ306191
Chl. phaeovibrioides	-	DSM 270	AJ290834	1240	Lake Polden, Norway	-	-	-	AJ391164
Chl. phaeovibrioides	2631	DSM 269^T	Y08105	1315	Lake Langvikvann, Norway	AM049308*	AM050091*	AJ427489	AJ391150
Chl. phaeovibrioides	2630	DSM 261	AJ290828	1241	-	-	-	-	AJ391146
Chl. phaeovibrioides	-	DSM 265	AJ290829	1241	-	-	-	-	AJ391160
"*Clorobiaceae bacterium*"	BS-1	-	AJ972456	1388	Black Sea (2001)	AM039431*	NZ_AAIC0100 0011	ZP_0053096 8	Contig 2045, gene 1690^a
Chl. sp.	8GSB		AJ888466	1298	salt pan, Gokarna, India	-	-	-	-
Chl. sp.	6GSB		AJ888465	1371	salt pan, Sanikatta, India	-	-	-	-
Chl. vibrioforme	EP2403	-	AM050130*	1399	Prévost Lagoon, France	AM049309*	-	-	-
Chloroherpeton thalassium	-	ATCC 35110	AF170103	1403	Woods Hole, MA, USA	AM049310*	AM050088*	-	-
Pld. luteolum	E1P1	-	AM050131*	1419	Lake Sisó, Spain	AM049311*	AM050074*	-	-
Pld. luteolum	E2P4	-	ident. E1P1	1377	Lake Sisó, Spain	AM049312*	AM050075*	-	-
Pld. luteolum	E3P2	-	ident. E1P1	1446	Lake Sisó, Spain	-	-	-	-
Pld. phaeum	CIB2401	-	AJ291828	1453	Chiprana Lake, Spain	-	-	-	-
Ptc. aestuarii	CE2401	-	AM050132*	1398	Certes lagoon, France	AM049313*	AM050094*	AJ427493	-

Supplemental table 3 (continued).

Species name	**Strain**	**Strain collection no.**	**16S rDNA EMBL Accession no.**	**Length of sequence**	**Sampling site**	16S-23S rRNA ITS	sigA	bchG	fmoA
Ptc. aestuarii	CHP3401	-	AJ291826	1458	Chiprana Lake, Spain	-	-	-	-
Ptc. aestuarii	SK-413	DSM 271^T	Y07837	1345	Lagoon	AM049314*	NZ_AAIJ01000001	ZP_00591434	AJ391151
Ptc. indicum	SKGSB^T		AJ887996	1393	Kakinada, Andhra Pradesh, India	-	-	-	-
Ptc. sp.	VK	-	AJ888467	1373	Visakhapatnam, India	-	-	-	-
Ptc. sp.	2K	-	AJ290835	1240	-	AM049315*	-	AJ427492	AJ306196/ AJ290823
Ptc. sp.	4Vi	-	AJ888468	1328	Visakhapatnam, Rama Krishna Beach, India	-	-	-	-
Ptc. vibrioformis	-	DSM 1678	AJ290833	1242	Seaside, Japan	-	-	-	AJ391163
Ptc. vibrioformis	ATCC 6030	DSM 260^T	M62791	1507	-	AM049316*	AM050089*	AM050368*	AJ391145
Ptc. vibrioformis	CHP3402	-	AJ291827	1464	Chiprana Lake, Spain	-	-	-	AJ306185
"*Clathrochloris sulfurica*"	-	-	X53184	1313	Aldabra Atoll, Seychelles	-	-	-	-
"*Clorobiaceae bacterium*"	LA53	-	AF513460	1460	Hawaii, USA	-	-	-	-

Cba. Chlorobaculum, *Chl. Chlorobium*, *Pld. Pelodictyon*, *Ptc. Prosthecochloris*, n.i. not included, ident. identical, *obtained during this study

Supplemental table 3 (continued).

Name of enrichment culture or env. sequence	Sequence name	Phylotype[b]	EMBL Accession No.	Length of sequence	Sampling site
Phototrophic consortia[a]					
"*Chlorochromatium aggregatum*"	10b-2	N	AJ578420	475	Lake Leach, MI, USA
"*Chlorochromatium aggregatum*"	4b-2	N	AJ578421	470	Round Lake, MI, USA
"*Chlorochromatium aggregatum*"	5-2	A	AJ578407	481	Lake Cassidy, MI, USA
"*Chlorochromatium aggregatum*"	7b-2	M	AJ578419	479	Lake Wintergreen, MI, USA
"*Chlorochromatium aggregatum*"	8a-2	N	AJ578422	482	Lake Wintergreen, MI, USA
"*Chlorochromatium aggregatum*"	9b-2	D	AJ578405	471	Lake Sheffer, MI, USA
"*Chlorochromatium aggregatum*"	CaC	P	AJ578433	487	Lake Coromina, Spain
"*Chlorochromatium aggregatum*"	CaEl	J	AJ272091	448	Lake Echo, WA, USA
"*Chlorochromatium aggregatum*"	CaH	C	AJ578404	480	Lake Haussee, Germany
"*Chlorochromatium aggregatum*"	CaS	P	AJ578432	487	Lake Sisó, Spain
"*Chlorochromatium aggregatum*"	CaSp	L	AJ578418	491	Woods Hole, MA, USA
"*Chlorochromatium glebulum*"	CGElGSB5	F	AJ272092	448	Lake Echo, WA, USA
"*Chlorochromatium lunatum*"	12-2	D	AJ578408	480	Lake Cassidy, MI, USA
"*Chlorochromatium magnum*"	10	H	AJ578412	475	Lake Cassidy, MI, USA
"*Chlorochromatium magnum*"	10a	H	AJ578416	470	Lake Leach, MI, USA
"*Chlorochromatium magnum*"	13-2	H	AJ578414	384	Lake Wintergreen, MI, USA
"*Chlorochromatium magnum*"	14	H	AJ578415	481	Lake Baker, MI, USA
"*Chlorochromatium magnum*"	14-2	H	AJ578413	480	Round Lake, MI, USA
"*Chlorochromatium magnum*"	5-2	D	AJ578401	476	Mud Lake, MI, USA
"*Chlorochromatium magnum*"	9a	H	AJ578411	473	Lake Sheffer, MI, USA
"*Chlorochromatium magnum*"	CmD	E	AJ272094	487	Lake Dagow, Germany
"*Chlorochromatium magnum*"	CmElGSB2	H	AJ272093	448	Lake Echo, WA, USA
"*Chloroplana vacuolata*"	-	D	AJ578405	475	Sheffer Lake, MI, USA
"*Pelochromatium magnum*"	29-2	N	AJ578426	481	Lake Wintergreen, MI, USA
"*Pelochromatium magnum*"	2b	N	AJ578423	470	Lake Cassidy, MI,USA
"*Pelochromatium roseum*"	16a-2	O	AJ578430	467	Lake Cassidy, MI, USA
"*Pelochromatium roseum*"	18a	O	AJ578431	478	Lake Sheffer, MI, USA
"*Pelochromatium roseum*"	18b	N	AJ578427	481	Lake Sheffer, MI, USA
"*Pelochromatium roseum*"	18c-2	B	AJ578402	487	Lake Sheffer, MI, USA
"*Pelochromatium roseum*"	19-2	N	AJ578425	481	Round Lake, MI, USA
"*Pelochromatium roseum*"	1a-2	O	AJ578428	466	Lake Baker, MI, USA
"*Pelochromatium roseum*"	1b-2	N	AJ578424	471	Lake Baker, MI, USA
"*Pelochromatium roseum*"	20	O	AJ578429	473	Lake Leach, MI, USA
"*Pelochromatium roseum*"	25	K	AJ578417	480	Lake Schleinsee, MI, USA
"*Pelochromatium roseum*"	PrDBd7	K	AY247957	486	Lake Dagow, Germany
"*Pelochromatium roseum*"	PrDBd8	N	AY247958	470	Lake Dagow, Germany
"*Pelochromatium roseum*"	PrEl	B	AJ578403	485	Lake Echo, WA, USA

Supplemental table 3 (continued).

Name of enrichment culture or env. sequence	Sequence name	Phylotype[b]	EMBL Accession No.	Length of sequence	Sampling site
"*Pelochromatium roseum*"	PrH	G	AJ578410	488	Lake Haussee, Germany
"*Pelochromatium selenoides*"	PsH	D	AJ578409	488	Lake Haussee, Germany
Environmental clones and DGGE bands					
Env. sequence	1B5		AJ627984	650	Ebro Delta, Spain
	200T-80		AB210277	969	(activated sludge) South Korea
	366		AJ831746	1381	Lake Cadagno, Switzerland
	810		AJ831751	1382	Lake Cadagno, Switzerland
	827		AJ831745	1382	Lake Cadagno, Switzerland
	849		AJ831742	1382	Lake Cadagno, Switzerland
	90dCC26		AF295115	410	(Marine)
	AH24		AJ629863	389	Lake Ahvenlammi, Finland
	AY303358		AY303358	426	Holocene sediments, Vestfold Hills, Ace Lake, Antarctica
	CD4H2		AY038478	611	Corals, Southern Carribean
	CD5A5		AY038425	612	Corals, Southern Carribean
	CD5B9		AY038426	622	Corals, Southern Carribean
	D1-31		AF544880	450	Caribbean coral *Montastrea annularis*
	D1-8		AF544872	450	Caribbean coral *Montastrea annularis*
	EUB42		AY693833	1463	(anaerobic sludge)
	Font Pudosa		AJ809886	473	Font Pudosa, Spain
	Dago99a		AM050117*	477	Lake Dagow, Germany
	GSB5		AJ006182	443	Lake Dagow, Germany
	GSB7		AJ006184	442	Lake Dagow, Germany
	Coromina		AJ809885	473	Lake Coromina, Spain
	gsbspa1		AM050118*	462	Lake Coromina, Spain
	gsbspa11		AM050122*	451	Lake Kirkilai, Lithuania
	gsbspa12		AM050123*	458	Lake Kirkilai, Lithuania
	gsbspa13		AM050124*	436	Lake Kirkilai. Lithuania
	gsbspa2		AM050119*	459	Lake Negre, Spain
	gsbspa3		AM050120*	387	Lake Negre, Spain
	K2-4-2		AY344404	1461	Lake Kauhako, Hawaii, USA
	K-100		AJ428422	1243	Sippewissett saltmarsh, MA, USA
	K-101		AJ428423	1240	Sippewissett saltmarsh, MA, USA
	K-106		AJ428424	1240	Sippewissett saltmarsh, MA, USA
	K-108		AJ428425	1241	Sippewissett saltmarsh, MA, USA
	K-13		AJ428420	1242	Salt lake mud, Isle Mljet, Croatia
	K-137		AJ428426	1240	Sippewissett saltmarsh, MA, USA
	K-153		AJ428427	1240	Sippewissett saltmarsh, MA, USA

Supplemental table 3 (continued).

Name of enrichment culture or env. sequence	Sequence name	Phylotype[b]	EMBL Accession No.	Length of sequence	Sampling site
	K-207		AJ428428	1240	Beach mud, Kiel bight, Baltic Sea, Germany
	K-500		AJ428429	1259	Beach mud, Kiel bight, Baltic Sea, Germany
	K-69		AJ428421	1242	Salt lake mud, Isle Mljet, Croatia
	K-701		AJ428430	1259	Sippewissett saltmarsh, MA, USA
	K-706		AJ428431	1259	Sippewissett saltmarsh, MA, USA
	K-721		AJ428432	1259	Sippewissett saltmarsh, MA, USA
	K-902		AJ428433	1261	Salt lake mud, Death Valley, CA, USA
	K-974		AJ428434	1260	Salt lake mud, Death Valley, CA, USA
	LGP-01-env3		AY394843	497	thermal spring, Yellowstone National Park, USA
	LGP-01-env4		AY394818	504	thermal spring, Yellowstone National Park, USA
	LGP-01-env9		AY394837	495	thermal spring, Yellowstone National Park, USA
	AT-01-env2		AY394830	512	thermal spring, Yellowstone National Park, USA
	BS1-00-36.5-9		AY394810	748	thermal spring, Yellowstone National Park, USA
	BS1-00-env3		AY394805	512	thermal spring, Yellowstone National Park, USA
	BS1-00-env8		AY394804	512	thermal spring, Yellowstone National Park, USA
	BS1-01-env1_2		AY394809	504	thermal spring, Yellowstone National Park, USA
	BS1-01-env10		AY394815	512	thermal spring, Yellowstone National Park, USA
	BS1-01-env2		AY394807	504	thermal spring, Yellowstone National Park, USA
	BS1-01-env4		AY394808	504	thermal spring, Yellowstone National Park, USA
	BS3.2-00-44G_1		AY394814	732	thermal spring, Yellowstone National Park, USA
	MV1-00-44-12_3		AY394833	510	thermal spring, Yellowstone National Park, USA
	MV1-00-44-14_4		AY394832	510	thermal spring, Yellowstone National Park, USA
	MV1-00-44c_1		AY394831	759	thermal spring, Yellowstone National Park, USA
	MV1-00-44e		AY394820	759	thermal spring, Yellowstone National Park, USA
	MV1-00-env2		AY394812	485	thermal spring, Yellowstone National Park, USA
	MV1-00-env9		AY394829	512	thermal spring, Yellowstone National Park, USA
	MV2-00-env1		AY394806	432	thermal spring, Yellowstone National Park, USA
	MV2-00-env2		AY394835	432	thermal spring, Yellowstone National Park, USA
	MV2-00-env3		AY394836	432	thermal spring, Yellowstone National Park, USA
	MV2-00-env4		AY394811	432	thermal spring, Yellowstone National Park, USA
	MV2-00-env5_3		AY394834	400	thermal spring, Yellowstone National Park, USA
	MV2-00-env6		AY394838	403	thermal spring, Yellowstone National Park, USA
	MV2-00-env7		AY394813	401	thermal spring, Yellowstone National Park, USA
	MV4-01-44b_2		AY394824	759	thermal spring, Yellowstone National Park, USA
	MV4-01-44c		AY394816	759	thermal spring, Yellowstone National Park, USA
	MV4-01-44d		AY394828	759	thermal spring, Yellowstone National Park, USA
	MV4-01-44e		AY394817	759	thermal spring, Yellowstone National Park, USA
	MV4-01-env12		AY394839	745	thermal spring, Yellowstone National Park, USA
	MV4-01-env15		AY394827	745	thermal spring, Yellowstone National Park, USA

Supplemental table 3 (continued).

Name of enrichment culture or env. sequence	Sequence name	Phylotype[b]	EMBL Accession No.	Length of sequence	Sampling site
	MV4-01-env17		AY394825	745	thermal spring, Yellowstone National Park, USA
	MV4-01-envf_5		AY394826	753	thermal spring, Yellowstone National Park, USA
	MV4-01-envG		AY394822	753	thermal spring, Yellowstone National Park, USA
	MV4-01-envH		AY394823	753	thermal spring, Yellowstone National Park, USA
	MV4-01-envI		AY394821	753	thermal spring, Yellowstone National Park, USA
	MV4-01-envJ		AY394819	753	thermal spring, Yellowstone National Park, USA
	MV6-02-env2		AY394841	521	thermal spring, Yellowstone National Park, USA
	MV6-02-env4		AY394842	518	thermal spring, Yellowstone National Park, USA
	NG2b-00-env7		AY394840	511	thermal spring, Yellowstone National Park, USA
	ME-JP105		AJ784965	679	Mariager Fjord, Denmark
	ME-JP118		AJ784966	374	Mariager Fjord, Denmark
	ME-JP120		AJ784967	311	Mariager Fjord, Denmark
	ME-JP121		AJ784968	414	Mariager Fjord, Denmark
	ME-JP122		AJ784969	384	Mariager Fjord, Denmark
	ME-JP123		AJ784970	370	Mariager Fjord, Denmark
	ME-JP124		AJ784971	369	Mariager Fjord, Denmark
	ME-TG111		AJ784972	385	Mariager Fjord, Denmark
	ME-TG112		AJ784973	458	Mariager Fjord, Denmark
	ME-TG113		AJ784974	385	Mariager Fjord, Denmark
	ME-TG114		AJ784975	446	Mariager Fjord, Denmark
	ME-TG115		AJ784976	448	Mariager Fjord, Denmark
	ME-TG116		AJ784977	450	Mariager Fjord, Denmark
	ME-TG117		AJ784978	451	Mariager Fjord, Denmark
	ME-TG118		AJ784979	400	Mariager Fjord, Denmark
	ME-TG119		AJ784980	350	Mariager Fjord, Denmark
	MW-JP101		AJ748280	401	Mariager Fjord, Denmark
	MW-JP104		AJ748281	397	Mariager Fjord, Denmark
	MW-JP105		AJ748282	396	Mariager Fjord, Denmark
	MW-JP106		AJ748283	401	Mariager Fjord, Denmark
	MW-JP107		AJ748284	390	Mariager Fjord, Denmark
	MW-JP108		AJ748285	362	Mariager Fjord, Denmark
	MW-JP111		AJ748286	401	Mariager Fjord, Denmark
	MW-JP112		AJ748287	359	Mariager Fjord, Denmark
	MW-JP115		AJ748288	401	Mariager Fjord, Denmark
	MW-JP117		AJ748289	353	Mariager Fjord, Denmark
	MW-TG102		AJ748290	335	Mariager Fjord, Denmark
	MW-TG103		AJ748291	360	Mariager Fjord, Denmark
	MW-TG104		AJ748292	301	Mariager Fjord, Denmark
	MW-TG106		AJ748293	327	Mariager Fjord, Denmark

Supplemental table 3 (continued).

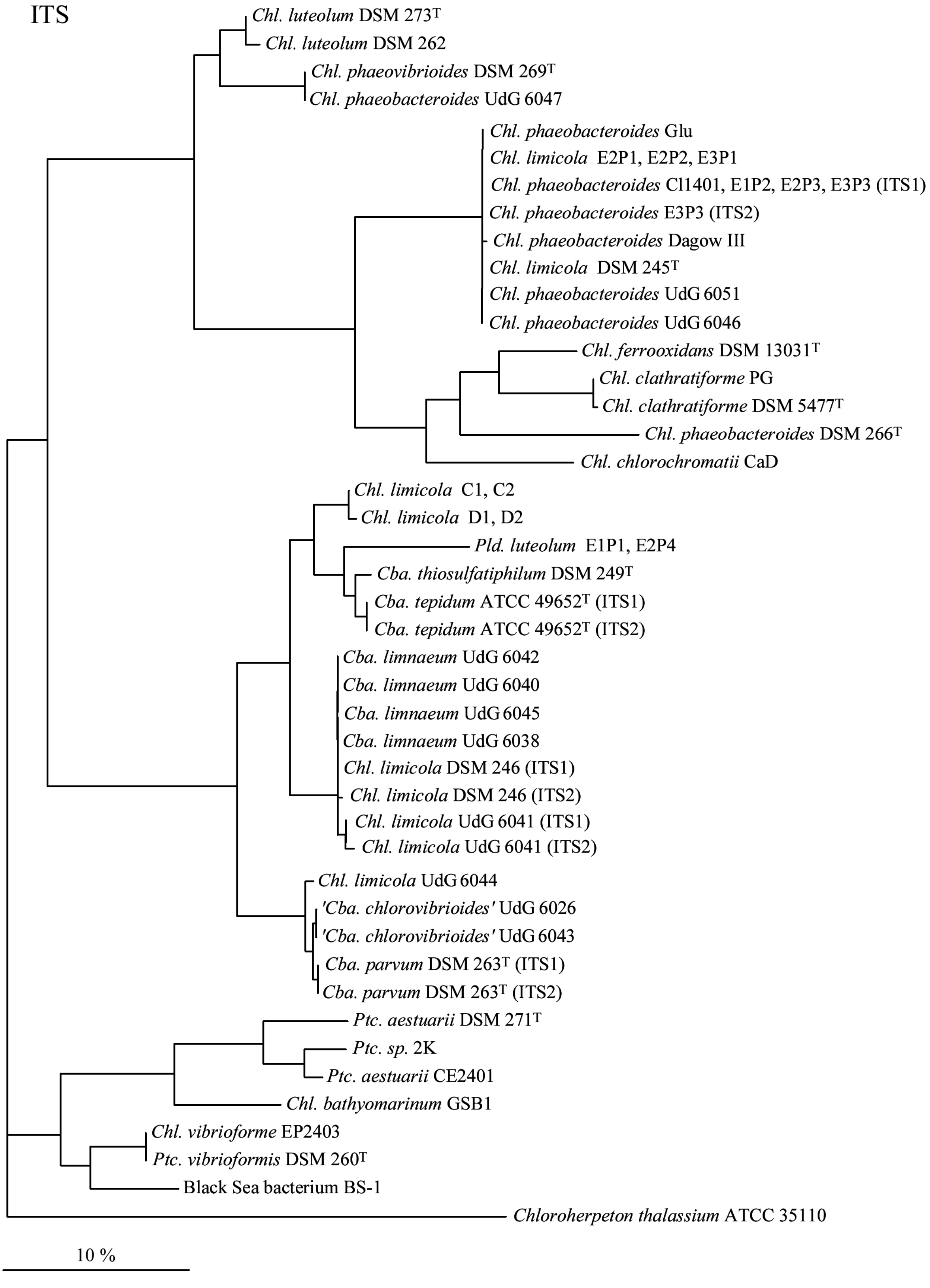
Name of enrichment culture or env. sequence	Sequence name	Phylotype[b]	EMBL Accession No.	Length of sequence	Sampling site
	MW-TG109		AJ748294	362	Mariager Fjord, Denmark
	MW-TG110		AJ748295	362	Mariager Fjord, Denmark
	S6D		AF298534	477	Mediterranean sapropel layers
	S8F		AF298536	477	Mediterranean sapropel layers
	S8G		AF298537	477	Mediterranean sapropel layers
	Z1C		AF298533	477	Mediterranean sapropel layers
	gsbspa5		AM050121*	431	Lake Sisó, Spain
	SI_01		AJ580957	467	Lake Sisó, Spain
	SI_02		AJ580958	465	Lake Sisó, Spain
	SI_03		AJ580959	468	Lake Sisó, Spain
	SI_04		AJ580960	468	Lake Sisó, Spain
	SI_05		AJ580961	461	Lake Sisó, Spain
	SI_06		AJ580962	467	Lake Sisó, Spain
	SI_07		AJ580963	461	Lake Sisó, Spain
	SI_08		AJ580964	466	Lake Sisó, Spain
	Sisó1		AJ809887	473	Lake Sisó, Spain
	Sisó2		AJ809888	476	Lake Sisó, Spain
	Sisó3		AJ809889	473	Lake Sisó, Spain
	VIBAC6Unid		AJ240010	526	Lake Vilar, Spain
	Vilar		AJ809890	471	Lake Vilar, Spain
	X9Ba35		AY607177	918	(anoxic rice field soil)

[a] picked manually from enrichment culture

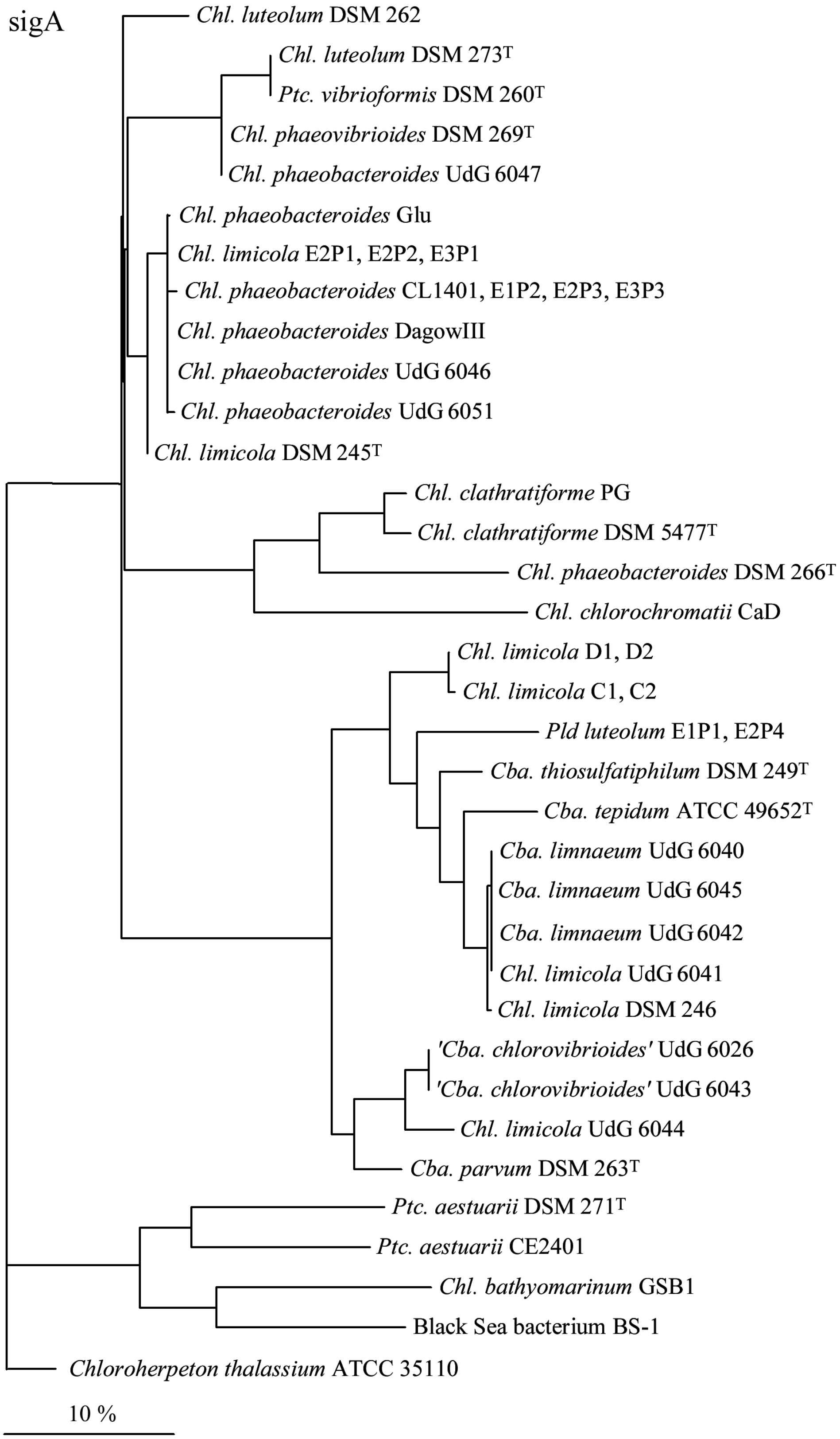
[b] Glaeser & Overmann, 2004

Supplemental Figure 1. 16S rDNA phylogenetic tree of GSB. The tree was constructed based on sequences longer than 1100 bp in ARB, using the Fast_DNAML algorithm (thick lines). Thin lines indicate branches of environmental sequences, which were inserted with PARSIMONY INTERACTIVE. Stable clusters are tagged by bootstrap values, which were calculated in PHYLIP using three different algorithms: DNAPARS (upper/left), NJ (middle), DNAML (lower/right). Boxed species were used in Figure 2, as reference for the concatenated tree. Bar denotes fixed substitutions per nucleotide.

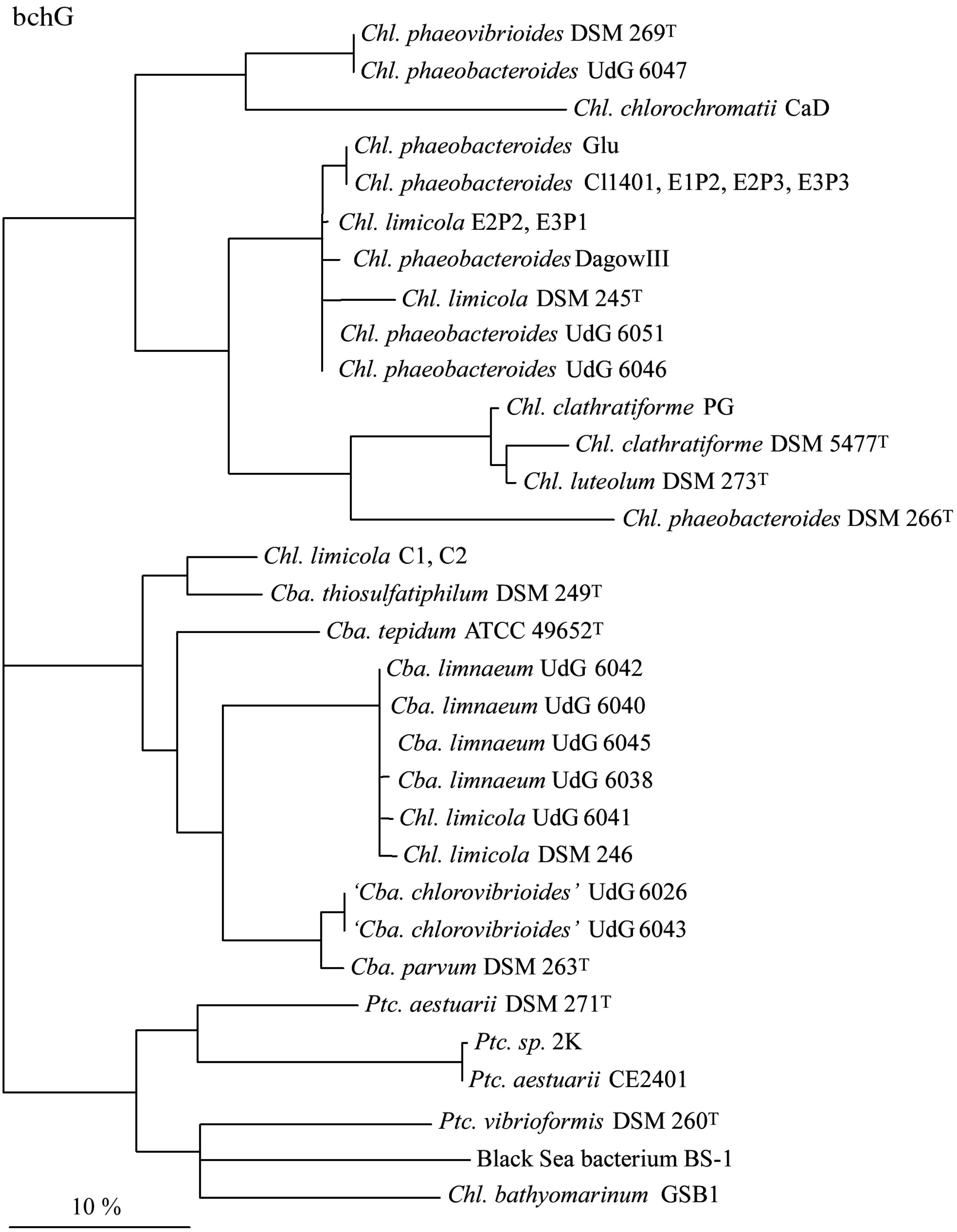
See appendix for bigger sized tree.



Supplemental Figure 2. Phylogenetic tree of the 16S-23S rRNA intergenic spacer (ITS) region of selected GSB species. The alignment was done with CLUSTAL-X followed by manual corrections using GeneDoc. The tree was contructed in PHYLIP, using DNAML. Bar denotes fixed substitutions per nucleotide.



Supplemental Figure 3. Phylogenetic tree of the partial sigma-A gene of selected GSB species. The alignment was done with CLUSTAL-X followed by manual corrections using GeneDoc. The tree was contructed in PHYLIP, using DNAML. Bar denotes fixed substitutions per nucleotide.



Supplemental Figure 4. Phylogenetic tree of *bchG* gene sequences of selected GSB species. The alignment was done with CLUSTAL-X followed by manual corrections using GeneDoc. The tree was contructed in PHYLIP, using DNAML. Bar denotes fixed substitutions per nucleotide.

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Vielen Dank an alle, die mich während der Doktorarbeit begleitet haben und mir immer mit Rat und Tat und Unterstützung zur Seite standen!

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LEBENSLAUF

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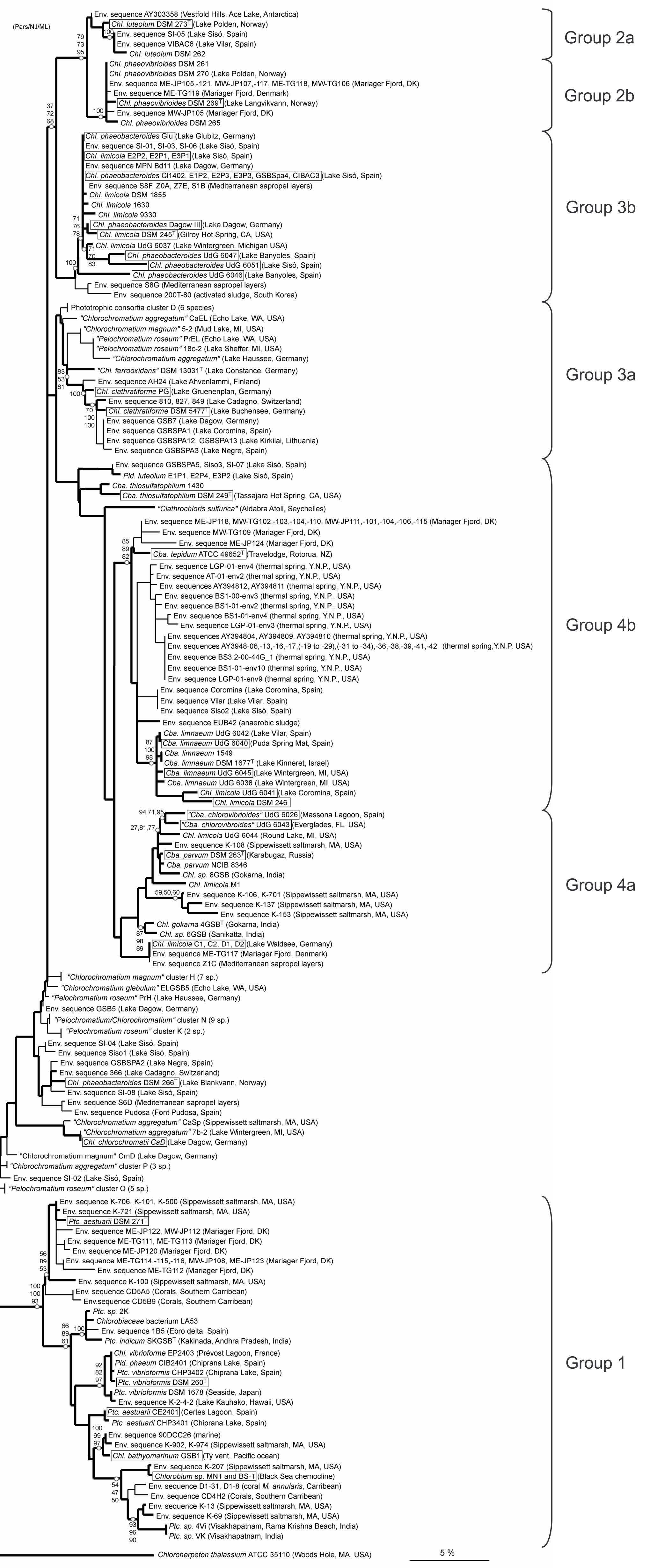
Berufliche Tätigkeiten

Seit Okt. 2005 Pharma-Außendienst bei INNOVEX GmbH, Mannheim

ANHANG


(Pars/NJ/ML)
Env. sequence AY303358 (Vestfold Hills, Ace Lake, Antarctica)
Chl. luteolum DSM 273^T (Lake Polden, Norway)
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Env. sequence SI-05 (Lake Sisó, Spain)
Env. sequence VIBAC6 (Lake Vilar, Spain)
79 73 95
Chl. luteolum DSM 262
Chl. phaeovibrioides DSM 261
Chl. phaeovibrioides DSM 270 (Lake Polden, Norway)
Env. sequence ME-JP105,-121, MW-JP107,-117, ME-TG118, MW-TG106 (Mariager Fjord, DK)
Env. sequence ME-TG119 (Mariager Fjord, Denmark)
Chl. phaeovibrioides DSM 269^T (Lake Langvikvann, Norway)
37 72 68
100
Env. sequence MW-JP105 (Mariager Fjord, DK)
Chl. phaeovibrioides DSM 265
Chl. phaeobacteroides Glu (Lake Glubitz, Germany)
Env. sequence SI-01, SI-03, SI-06 (Lake Sisó, Spain)
Chl. limicola E2P2, E2P1, E3P1 (Lake Sisó, Spain)
Env. sequence MPN Bd11 (Lake Dagow, Germany)
Chl. phaeobacteroides Cl1402, E1P2, E2P3, E3P3, GSBSpa4, CIBAC3 (Lake Sisó, Spain)
Env. sequence S8F, Z0A, Z7E, S1B (Mediterranean sapropel layers)
Chl. limicola DSM 1855
Chl. limicola 1630
Chl. limicola 9330
71 76 78
Chl. phaeobacteroides Dagow III (Lake Dagow, Germany)
Chl. limicola DSM 245^T (Gilroy Hot Spring, CA, USA)
Chl. limicola UdG 6037 (Lake Wintergreen, Michigan USA)
71 70 83
Chl. phaeobacteroides UdG 6047 (Lake Banyoles, Spain)
100
Chl. phaeobacteroides UdG 6051 (Lake Sisó, Spain)
Chl. phaeobacteroides UdG 6046 (Lake Banyoles, Spain)
Env. sequence S8G (Mediterranean sapropel layers)
Env. sequence 200T-80 (activated sludge, South Korea)
Phototrophic consortia cluster D (6 species)
"Chlorochromatium aggregatum" CaEL (Echo Lake, WA, USA)
"Chlorochromatium magnum" 5-2 (Mud Lake, MI, USA)
"Pelochromatium roseum" PrEL (Echo Lake, WA, USA)
"Pelochromatium roseum" 18c-2 (Lake Sheffer, MI, USA)
"Chlorochromatium aggregatum" (Lake Haussee, Germany)
83 53 81
"Chl. ferrooxidans" DSM 13031^T (Lake Constance, Germany)
Env. sequence AH24 (Lake Ahvenlammi, Finland)
100
Chl. clathratiforme PG (Lake Gruenenplan, Germany)
Env. sequence 810, 827, 849 (Lake Cadagno, Switzerland)
70 100 100
Chl. clathratiforme DSM 5477^T (Lake Buchensee, Germany)
Env. sequence GSB7 (Lake Dagow, Germany)
Env. sequence GSBSPA1 (Lake Coromina, Spain)
Env. sequence GSBSPA12, GSBSPA13 (Lake Kirkilai, Lithuania)
Env. sequence GSBSPA3 (Lake Negre, Spain)
Env. sequence GSBSPA5, Siso3, SI-07 (Lake Sisó, Spain)
Pld. luteolum E1P1, E2P4, E3P2 (Lake Sisó, Spain)
Cba. thiosulfatophilum 1430
Cba. thiosulfatophilum DSM 249^T (Tassajara Hot Spring, CA, USA)
"Clathrochloris sulfurica" (Aldabra Atoll, Seychelles)
Env. sequence ME-JP118, MW-TG102,-103,-104,-110, MW-JP111,-101,-104,-106,-115 (Mariager Fjord, DK)
Env. sequence MW-TG109 (Mariager Fjord, DK)
85 89 82
Env. sequence ME-JP124 (Mariager Fjord, DK)
Cba. tepidum ATCC 49652^T (Travelodge, Rotorua, NZ)
Env. sequence LGP-01-env4 (thermal spring, Y.N.P., USA)
Env. sequence AT-01-env2 (thermal spring, Y.N.P., USA)
Env. sequences AY394812, AY394811 (thermal spring, Y.N.P., USA)
Env. sequence BS1-00-env3 (thermal spring, Y.N.P., USA)
Env. sequence BS1-01-env2 (thermal spring, Y.N.P., USA)
Env. sequence BS1-01-env4 (thermal spring, Y.N.P., USA)
Env. sequence LGP-01-env3 (thermal spring, Y.N.P., USA)
Env. sequences AY394804, AY394809, AY394810 (thermal spring, Y.N.P., USA)
Env. sequences AY3948-06,-13,-16,-17,(-19 to -29),(-31 to -34),-36,-38,-39,-41,-42 (thermal spring,Y.N.P, USA)
Env. sequence BS3.2-00-44G_1 (thermal spring, Y.N.P., USA)
Env. sequence BS1-01-env10 (thermal spring, Y.N.P., USA)
Env. sequence LGP-01-env9 (thermal spring, Y.N.P., USA)
Env. sequence Coromina (Lake Coromina, Spain)
Env. sequence Vilar (Lake Vilar, Spain)
Env. sequence Siso2 (Lake Sisó, Spain)
Env. sequence EUB42 (anaerobic sludge)
Cba. limnaeum UdG 6042 (Lake Vilar, Spain)
87 100 98
Cba. limnaeum UdG 6040 (Puda Spring Mat, Spain)
Cba. limnaeum 1549
Cba. limnaeum DSM 1677^T (Lake Kinneret, Israel)
Cba. limnaeum UdG 6045 (Lake Wintergreen, MI, USA)
Cba. limnaeum UdG 6038 (Lake Wintergreen, MI, USA)
Chl. limicola UdG 6041 (Lake Coromina, Spain)
Chl. limicola DSM 246
94,71,95
"Cba. chlorovibrioides" UdG 6026 (Massona Lagoon, Spain)
27,81,77
"Cba. chlorovibroides" UdG 6043 (Everglades, FL, USA)
Chl. limicola UdG 6044 (Round Lake, MI, USA)
Env. sequence K-108 (Sippewissett saltmarsh, MA, USA)
Cba. parvum DSM 263^T (Karabugaz, Russia)
Cba. parvum NCIB 8346
Chl. sp. 8GSB (Gokarna, India)
Chl. limicola M1
59,50,60
Env. sequence K-106, K-701 (Sippewissett saltmarsh, MA, USA)
Env. sequence K-137 (Sippewissett saltmarsh, MA, USA)
Env. sequence K-153 (Sippewissett saltmarsh, MA, USA)
Chl. gokarna 4GSB^T (Gokarna, India)
87 98 89
Chl. sp. 6GSB (Sanikatta, India)
Chl. limicola C1, C2, D1, D2 (Lake Waldsee, Germany)
Env. sequence ME-TG117 (Mariager Fjord, Denmark)
Env. sequence Z1C (Mediterranean sapropel layers)
"Chlorochromatium magnum" cluster H (7 sp.)
"Chlorochromatium glebulum" ELGSB5 (Echo Lake, WA, USA)
"Pelochromatium roseum" PrH (Lake Haussee, Germany)
Env. sequence GSB5 (Lake Dagow, Germany)
"Pelochromatium/Chlorochromatium" cluster N (9 sp.)
"Pelochromatium roseum" cluster K (2 sp.)
Env. sequence SI-04 (Lake Sisó, Spain)
Env. sequence Siso1 (Lake Sisó, Spain)
Env. sequence GSBSPA2 (Lake Negre, Spain)
Env. sequence 366 (Lake Cadagno, Switzerland)
Chl. phaeobacteroides DSM 266^T (Lake Blankvann, Norway)
Env. sequence SI-08 (Lake Sisó, Spain)
Env. sequence S6D (Mediterranean sapropel layers)
Env. sequence Pudosa (Font Pudosa, Spain)
"Chlorochromatium aggregatum" CaSp (Sippewissett saltmarsh, MA, USA)
"Chlorochromatium aggregatum" 7b-2 (Lake Wintergreen, MI, USA)
Chl. chlorochromatii CaD (Lake Dagow, Germany)
"Chlorochromatium magnum" CmD (Lake Dagow, Germany)
"Chlorochromatium aggregatum" cluster P (3 sp.)
Env. sequence SI-02 (Lake Sisó, Spain)
"Pelochromatium roseum" cluster O (5 sp.)
Env. sequence K-706, K-101, K-500 (Sippewissett saltmarsh, MA, USA)
Env. sequence K-721 (Sippewissett saltmarsh, MA, USA)
Ptc. aestuarii DSM 271^T
Env. sequence ME-JP122, MW-JP112 (Mariager Fjord, DK)
Env. sequence ME-TG111, ME-TG113 (Mariager Fjord, DK)
Env. sequence ME-JP120 (Mariager Fjord, DK)
56 89 53
Env. sequence ME-TG114,-115,-116, MW-JP108, ME-JP123 (Mariager Fjord, DK)
Env. sequence ME-TG112 (Mariager Fjord, DK)
Env. sequence K-100 (Sippewissett saltmarsh, MA, USA)
100 100 93
Env. sequence CD5A5 (Corals, Southern Carribean)
Env.sequence CD5B9 (Corals, Southern Carribean)
Ptc. sp. 2K
Chlorobiaceae bacterium LA53
66 89 61
100
Env. sequence 1B5 (Ebro delta, Spain)
Ptc. indicum SKGSB^T (Kakinada, Andhra Pradesh, India)
Chl. vibrioforme EP2403 (Prévost Lagoon, France)
Pld. phaeum CIB2401 (Chiprana Lake, Spain)
92 82 97
Ptc. vibrioformis CHP3402 (Chiprana Lake, Spain)
Ptc. vibrioformis DSM 260^T
Ptc. vibrioformis DSM 1678 (Seaside, Japan)
Env. sequence K-2-4-2 (Lake Kauhako, Hawaii, USA)
Ptc. aestuarii CE2401 (Certes Lagoon, Spain)
Ptc. aestuarii CHP3401 (Chiprana Lake, Spain)
100 99 97
Env. sequence 90DCC26 (marine)
Env. sequence K-902, K-974 (Sippewissett saltmarsh, MA, USA)
Chl. bathyomarinum GSB1 (Ty vent, Pacific ocean)
Env. sequence K-207 (Sippewissett saltmarsh, MA, USA)
Chlorobium sp. MN1 and BS-1 (Black Sea chemocline)
54 47 50
Env. sequence D1-31, D1-8 (coral M. annularis, Carribean)
Env. sequence CD4H2 (Corals, Southern Carribean)
Env. sequence K-13 (Sippewissett saltmarsh, MA, USA)
Env. sequence K-69 (Sippewissett saltmarsh, MA, USA)
93 96 90
Ptc. sp. 4Vi (Visakhapatnam, Rama Krishna Beach, India)
Ptc. sp. VK (Visakhapatnam, India)
Chloroherpeton thalassium ATCC 35110 (Woods Hole, MA, USA)
5 %
Group 2a
Group 2b
Group 3b
Group 3a
Group 4b
Group 4a
Group 1