In situ Metabolism and Biogeography of Phototrophic Consortia

DISSERTATION
DER FAKULTÄT FÜR BIOLOGIE
DER LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

Vorgelegt
von
Jens Glaeser

---

im Oktober 2003

1. Gutachter: Prof. Dr. J. Overmann

2. Gutachter: Prof. Dr. A. Hartmann

Tag der mündlichen Prüfung: 12. Februar 2004

Dedicated to my parents

PUBLISHED MANUSCRIPTS

Chapter 2:

Glaeser J., Bañeras L., Rütters H. and Overmann J. (2001) Novel bacteriochlorophyll *e* structures and species-specific variability of pigment composition in green sulfur bacteria. Arch. Microbiol. **177:** 476-485

Chapter 3:

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

Chapter 4:

Glaeser J. and Overmann J. (2003) The significance of organic carbon compounds for *in situ* metabolism and chemotaxis of phototrophic consortia. Environ. Microbiol. (in press)

SUBMITTED MANUSCRIPTS

Chapter 5:

Glaeser J. and Overmann J. (2003) Diversity and biogeography of phototrophic consortia. Appl. Environ. Microbiol.

CONTRIBUTIONS BY THE COAUTHORS

Chapter 2

Dr. L. Bañeras has contributed the NMR studies on isolated BChl *e* homologs (Fig. 4) and Dr. H. Rütters was in charge of the HPLC-MS facility and provided useful support for the interpretation of the obtained mass spectra.

Chapter 3

Prof. Dr. J. Overmann has contributed the data for CO_2 assimilation (Fig. 2F).

Chapter 4

Prof. Dr. J. Overmann has contributed the data for the heterotrophic potential (Fig 4).

Chapter 5

Prof. Dr. J. Overmann has contributed the data for the chemotaxis of "*C. aggregatum*" from Lake Sisó (Fig 7, lower panel).

I hereby confirm the above statements.

Jens Glaeser Prof. Jörg Overmann

TABLE OF CONTENTS

FIGURES

(Titles abbreviated)

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

TABLES

(Titles abbreviated)

Chapter 1

Chapter 2

Chapter 3

Chapter 5

ABBREVIATIONS AND UNITS

°C	degree Celsius
$\delta^{13}C$	stable carbon isotope ratio
$\Delta\delta^{13}C$	stable carbon isotope ratio relative to PeeDee belimnite
A	Adenin
Å	Ångström
APCI	ATMOSPHERIC PRESSURE CHEMICAL IONIZATION
ATP	Adenosintriphosphate
ATCC	American type culture collection
BChl	Bacteriochlorophyll
BChl *a*	Bacteriochlorophyll *a* (analogously: BChl *b*, *c*, *d*, *e* and *g*)
bp	Base pairs
BPh	Bacteriophaeophytin
C	Cytosin
C.	*Chlorochromatium*
Chl *a*	Chlorophyll *a*
Chl.	*Chlorobium*
COSY	Correlated Spectroscopy
CTC	5-cyano-2,3-di-4-tolyl-tetrazolium chloride
DAD	Diode array detection
DAPI	4',6-diamidino-2-phenylindol
DGGE	Denaturing gradient gel electrophoresis
DIC	Dissolved inorganic carbon
DNA	Desoxyribonucleic acid
DOC	Dissolved organic carbon
DSMZ	Deutsche Stammsammlung für Mikroorganismen und Zellkulturen
[E,E] BChl e_F	8, 12-diethyl BChl e esterified with farnesol (analogously: [M], methyl, [I], isobutyl, [Pr], propyl, [N], neopentyl
EDTA	Ethylendiaminotetraacetic acid
FMO	Fenna-Matthew-Olsen
G	Guanin
g	gramm
GBq	Gigabecquerel
GC	Gas-chromatography

GSB	Green sulfur bacteria
FID	Flame injection detetctor
HPLC	High performance liquid chromatography
irm	isotope ratio monitoring
l	liter (analogously (ml and µl)
M	$mol \cdot l^{-1}$ (analogously: mM, µM, nM)
m	meter (analogoouly: mm, µm, nm)
µ	micro
$[M+H]^+$	quasi-molecular ion
MHz	Megaherz
MS	Mass-spectroscopy
m/z	mass per charge
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser Enhancement Spectroscopy
P.	*Pelochromatium*
PCR	Polymerase chain reaction
PDB	PeeDee belemnite
Pld.	*Pelodicyton*
POC	Particulate organic carbon
PSB	Purple sulfur bacteria
PVC	Poly vinyle chloride
rRNA	ribosomal Ribonucleic acid
SD	Standard deviation
T	Thymin
TCA	Tricarboxylic acid
TCN	Total cell count
TIC	Total ion chromatogram
TMS	Trimethylsilyl
U	Uracil
UdG	Universitat de Girona
UV	Ultra violet
v/v	volume / volume
w/v	weight / volume
VIS	Visible
xg	x-times gravity force

Chapter 1

Introduction

ORIGIN AND SIGNIFICANCE OF PHOTOSYNTHESIS

Photosynthesis is the conversion of light energy into chemical energy and represents the most important process for the formation of biomass on earth (74). Light is used as source of energy in all three domains of life. Chloroplasts of eukaryotic organisms represent organelles, which have developed from ancestral cyanobacteria. Chlorophyll based photosynthesis has evolved in the domain Bacteria, where a total of 5 phyla contain phototrophic organisms (Fig. 1). In contrast, the ability to use light energy in the Archaea is not chlorophyll mediated and based on a fundamentally different process (see below). Because of its complexity it has been argued that bacterial photosynthesis has not evolved more than once (109). The comparison of multiple photosynthesis genes traced the origin of photosynthesis back to the α-*Proteobacteria* (111, 112, 113) and recent evidence suggests that lateral gene transfer has spread the capacity for photosynthetic growth across the domain Bacteria (11, 62, 63, 85, 86).

Phototrophic bacteria can be separated into two physiological groups, the oxygenic phototrophic bacteria and the anoxygenic phototrophic bacteria. On the primordial earth, anoxygenic photosynthesis was responsible for the entire photosynthetic carbon fixation. Presently, the global primary production on earth is mainly achieved by oxygenic phototrophs: terrestrial plants and marine phytoplankton (74). Plants contribute most of the photosynthesis in temperate terrestrial environments. In contrast, cyanobacteria accomplish most of the marine primary production (16, 35, 57, 107) and contribute considerably to the

phototrophic biomass in extreme terrestrial environments (31). The fraction of anoxygenic photosynthesis is very limited today and represents much less than 1% of the global primary production (74). In fact, a true primary production by anoxygenic photosynthesis occurs only in sulfidic hot springs where electron donors are of abiotic origin. In all other environments "primary production" is secondary, because electron donors for photosynthesis originate from the decay of organic material, which has previously been fixed by oxygenic phototrophs (74). However, in some anoxic pelagic environments anoxygenic phototrophic bacteria can amount up to 84% of the photosynthetic carbon dioxide fixation, if allochtonous organic matter funnels the production of hydrogen sulfide by sulfate reducing bacteria (68).

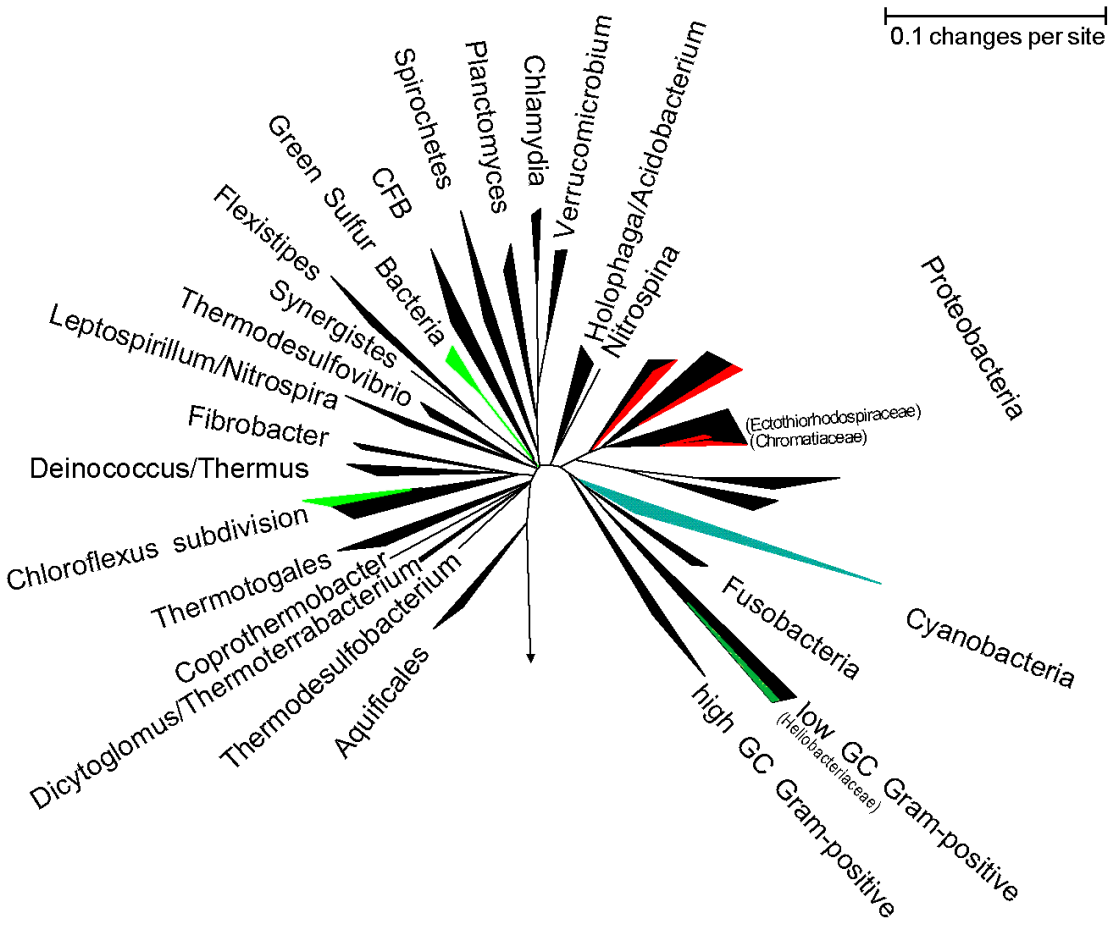


Figure 1. Phylogenetic tree based on 16S rRNA gene sequences (74). Only bacterial divisions with culturable representatives were included in the analyses. Colors indicate phyla containing phototrophic bacteria: **light green**, Bacteria containing chlorosomes as light-harvesting antenna; **red**, Bacteria containing antenna complexes within the cytoplasmic membrane and quinone/pheophytin-type reaction centers; **medium green**, Gram-positive phototrophic bacteria with FeS-type reaction centers; **blue-green**, Bacteria containing the two types of reaction centers. Width of colored wedges indicates the phylogenetic divergence. As an outgroup, the Archaeon *Methanopyrus kandleri* DSM 6324 was used.

ESSENTIAL PROCESSES AND STRUCTURAL BASIS OF PHOTOSYNTHESIS

The process of photosynthesis can be divided into (i) the light reaction and ATP-synthesis and (ii) the dark reactions involving carbon dioxide fixation (103). Two functionally different mechanisms for the photosynthetic light reaction are recognized today and many metabolic pathways have been developed for carbon dioxide fixation (74).

In the bacterial lineages, the light reaction is driven by the photosynthetic apparatus (see below), which contains either chlorophyll or bacteriochlorophyll and, in addition, carotenoids. In contrast, phototrophic species among the Archaea use the retinal-based bacteriorhodopsin system (65, 66). The bacterial photosynthetic apparatus consists of many protein subunits forming the reaction center and specialized light-harvesting antenna, which harbor the photosynthetic pigments. The light energy is harvested by the antenna and funneled to the closely located reaction centers where charge separation takes place (74). A cytochrome complex is involved in subsequent redox reactions and protons are translocated across the membrane into the extracellular space (anoxygenic phototrophs) or into the thylakoid lumen (cyanobacteria). In contrast, the retinal-based bacteriorhodopsin system in Archaea is lacking antenna and a proton gradient is directly established by the bacteriorhodopsin cycle (66). The prerequisite for the generation of a proton motive force is a membrane impermeable to protons, which separates cell compartments. Finally, the ATP-synthase converts the proton motive force into chemical energy, hence, ATP (74).

In most phototrophic bacteria specialized intracellular membrane systems harbor the photosynthetic apparatus (12, 20), but in some lineages the photosynthetic apparatus is located in the cytoplasmatic membrane only (58, 74). Usually, photosynthetic pigments are bound non-covalently to the protein subunits of the photosynthetic apparatus (74). However, the light harvesting antenna of green sulfur bacteria and *Chloroflexaceae*, the chlorosomes, are fundamentally different from all other light-harvesting antenna with respect to the organization of the photosynthetic pigments. Chlorosomes are ovoid structures containing bacteriochlorophylls arranged in paracristalline rod-likes structures and located below the cytoplasmatic membrane (69). In green sulfur bacteria, the chlorosome envelope consists of a one-unit membrane of monogalactosyl diglyceride (69) containing 10 different polypeptides (18). The reaction centers are connected to the chlorosomes via the bacteriochlorophyll *a* containing FMO (Fenna-Mathews-Olsen) protein, which passes the excitation energy to the reaction centers (69). However, FMO is lacking in *Chloroflexaceae* (40).

Two types of reaction centers are found in phototrophic bacteria and can be differentiated by their molecular architecture and early electron acceptors (11). The primary

electron acceptor in type II reaction centers is phaeophytin, which subsequently reduces a quinone (74, 87). The primary electron acceptor in type I reaction centers is a four iron/ four sulfur (Fe_4S_4) cluster, which subsequently reduce a soluble ferredoxin (41).

In the so-called dark-reactions carbon dioxide is reduced by ATP and reducing equivalents, which are generated by the light reactions. Carbon dioxide is the principal source of carbon for phototrophic prokaryotes in most of the environments (59, 90, 96) and the Calvin cycle is used for fixation of carbon dioxide by most of the phototrophic bacteria. The exceptions are again represented by the green sulfur bacteria and *Chloroflexus aurantiacus*, which use the reductive tricarboxylic acid cycle (29, 91) and the hydroxypropionate cycle, respectively (95).

PHYLOGENY OF PHOTOTROPHIC BACTERIA

Phototrophic bacteria do not form a phylogenetically homogeneous and closely related group of organisms, but belong to distantly related bacterial lineages (Fig. 1). Oxygenic phototrophic bacteria are exclusively found in the cyanobacteria, which represent a monophyletic and very diverse group of phototrophic prokaryotes well separated from their closest relatives (30, 33, 98, 108). In contrast, anoxygenic phototrophic bacteria are only distantly related to each other (93) and belong to four of the >36 bacterial lineages inferred from 16S rRNA gene phylogeny known to date (44, Fig. 1). Anoxygenic phototrophic bacteria are found in the *Chlorobiaceae* (green sulfur bacteria), the *Chloroflexus*-subdivision, the *Proteobacteria* and the *Heliobacteriaceae* (93). In the Archaea only one group, the *Halobacteriales*, contain species which utilize light energy (67).

Green sulfur bacteria represent an isolated, phylogenetically remarkably homogenous phylum. The similarity of all green sulfur bacteria, except for *Chloroherpeton thalassium*, is larger 90.1% with respect to the gene sequence of the 16S rRNA gene (48, 69, 77). As a common feature, all green sulfur bacteria known to date are obligate photoautotrophs and depend on reduced sulfur compounds as electron donors (70). The *Chloroflexus*-subgroup contains only five genera and represents a rather diverse group of phototrophic prokaryotes closely related to non-phototrophic bacteria. Interestingly, *Chloroflexus aurantiacus* shares chlorosomes as light harvesting antenna with green sulfur bacteria, but contain a type II reaction center. Phototrophic bacteria of α- and β-*Proteobacteria* are also named "purple non sulfur bacteria" due to the inability of many species to oxidize sulfide. Phototrophic α- and β-*Proteobacteria* are phylogenetically highly diverse and some genera are more closely related to non-phototrophic than to phototrophic genera (46, 47), which has been interpreted

as a repeated loss of the ability to perform photosynthesis (74). Most phototrophic γ-*Proteobacteria* (purple sulfur bacteria) are restricted to reduced sulfur compounds for anoxygenic photosynthesis as the green sulfur bacteria. They are divided into two families, the *Chromatiaceae* and *Ectothiorhodospiraceae* (45, 84). Heliobacteria belong to the low G+C Gram-positive bacteria (109). They are closely related to the endosporulating rod-shaped bacteria *Bacillus* and *Clostridium* and form, as green sulfur bacteria, a rather tight phylogenetic cluster. In contrast to the green sulfur bacteria they are obligate photoorganotrophs (58, 110).

HABITATS OF PHOTOTROPHIC BACTERIA

Cyanobacteria are the most versatile group of phototrophic bacteria and thrive in all environments as long as light and water is available at least transiently. They are the main photosynthetic organisms in the open oceans (16, 35, 57, 107), in hot springs, hot and dry terrestrial environments as desert crusts (31) and cold environments (24, 25).

Anoxygenic phototrophic prokaryotes are restricted to the anoxic zones of aquatic pelagic environments, littoral sediments, hot springs and soils (74, 102). A prerequisite for the development of anoxic zones is the physical structure of the environment (102). The physical stratification of the water column in holomictic freshwater lakes is established by temperature gradients during summer, whereas meromictic lakes, saline lagoons and the Black Sea are stratified by differences in salinity between surface and bottom water layers. Microbial mats are prevented from mixing by the rigid sediment matrix (102). As a consequence of oxygen consumption, anoxygenic zones develop in the stagnant parts of water bodies and in sediments, which is facilitated by the limited solubility and slow diffusion of oxygen in water (49).

The chemistry of oxic and anoxic water layers differs substantially. Major differences are the composition of dissolved compounds and their abundance. The greatest vertical change in water chemistry usually occurs in the transition zone between oxic and anoxic water bodies and is referred to as the chemocline (5). Often, only the upper layers of anoxic zones are illuminated, which are in consequence colonized by anoxygenic phototrophic bacteria.

Characteristically, multilayered ecosystems are formed in pelagic and littoral environments by the groups of phototrophic prokaryotes. Several features distinguish the pelagic communities of anoxygenic phototrophic bacteria from those in littoral microbial mats. Firstly, chemocline communities of anoxygenic phototrophic bacteria in lakes and

lagoons have a vertical extension of 0.5 to 2 m and occur in depth of 2 to 20 m. In contrast to the relatively large vertical distribution of anoxygenic phototrophic bacteria in pelagic environments the layers occurring in microbial mats are in the range of 1.5 to 5 mm (74, 102). Secondly, gradients of light intensity, solute compounds and gases in pelagic anoxygenic phototrophic communities are less steep than in those of microbial mats. Because of the rigid matrix, diffusion is the only way of mass transport in microbial mats. Thirdly, the biomass of phototrophic bacteria as observed from bacteriochlorophyll concentrations, is much higher in microbial mats. Up to 900 mg BChl·dm^{-3} are found in microbial mats whereas maximum concentrations in pelagic environments are as high as 28 mg BChl·l^{-1} (72, 101). Finally, the spectral composition of light energy available for pelagic anoxygenic phototrophic communities usually is very different compared to those in littoral sediments. Blue to yellow-green wavelength bands are characteristic for the deep anoxic layers of pelagic environments, because of the absorption characteristics of water and the absorption of surface blooms of oxygenic phototrophs (80, 81). The anoxic layers of littoral sediments receive mainly infrared light because of the backscatter and absorption of shorter wavelength bands by quartz sand, overlaying particulate organic material and dense accumulations of oxygenic phototrophs (50, 51, 54).

COMPETITION BETWEEN PHOTOTROPHIC SULFUR BACTERIA

Sediment layers and water samples are green, brown, yellowish green or pink to purple-red if mass accumulations (blooms) of green sulfur bacteria and *Chromatiaceae* are present (102). Other anoxygenic phototrophic bacteria occur rarely at high numbers under natural conditions or form blooms only in hot springs or hypersaline environments as *Chloroflexus aurantiacus* and the *Ectothiorhodospiraceae*, respectively (40, 45). The enrichment of phototrophic sulfur bacteria was also successful with samples from locations lacking blooms of phototrophic bacteria, as long as sulfide was present in the environment. This clearly indicates the broad distribution of phototrophic sulfur bacteria in nature (74).

Typically, populations of green sulfur bacteria are found below layers of purple sulfur bacteria reflecting their adaptation to low light intensities (61), but in the chemoclines of many lakes, green sulfur bacteria and *Chromatiaceae* occur in the same water layers (74, 102). Photosynthesis of phototrophic sulfur bacteria is restricted to the simultaneous occurrence of light and reduced sulfur compounds and the amount of anoxygenic photosynthesis is correlated to the light intensity (102). Because light is the most important selective pressure between anoxygenic phototrophic bacteria under natural conditions (102)

Table 1. Major absorption maxima of chlorins in whole cells of phototropic bacteria (adapted from 74)

Groups of organisms	**Chlorin**	***In vivo* absorption maxima**
Cyanobacteria	Chl *a*	670-675
α-, β- and γ-Proteobacteria	BChl *a*	375, 590, 805, 830-911
β-Proteobacteria	BChl *b*	400, 605, 835-850, 986-1035
green-colored *Chlorobiaceae* and *Chloroflexus* sp.	BChl *c*	457-460, 745-755
green-colored *Chlorobiaceae*	BChl *d*	450, 715-745
brown-colored *Chlorobiaceae*	BChl *e*	460-462, 710-725
Heliobacteriaceae	BChl *g*	375, 419, 575, 788

the intensity and quality of light determines the community composition of anoxygenic phototrophic bacteria (80, 81). In consequence, the potential of phototrophic prokaryotes to colonize a certain habitat is directly linked to the light absorption features of their photosynthetic apparatus (Tab. 1).

Green sulfur bacteria dominate the natural communities of phototrophic sulfur bacteria under low light conditions. Compared to *Chromatiaceae*, green sulfur bacteria need only one fourth of the light intensity to reach similar growth rates under laboratory conditions (10), but are saturated at light intensities of 5 to 10 µmol quanta·m^{-2}·s^{-1} (9, 73, 75). This selective advantage is based on the large light harvesting antenna of green sulfur bacteria and the ratio of antenna bacteriochlorophyll molecules to reaction centers. A chlorosome contains about 10,000 bacteriochlorophyll molecules and is linked to 5 to 10 reaction centers (1). In contrast, light harvesting antenna of *Chromatiaceae* contain 10 times less bacteriochlorophylls per reaction center. Proteins are not involved as ligands for bacteriochlorophylls in the chlorosomes; therefore less ATP is consumed for the synthesis of the light-harvesting antenna in green sulfur bacteria compared to *Chromatiaceae*. Also, the quantum requirement for photosynthetic CO_2 fixation in green sulfur bacteria is lower that in *Chromatiaceae*, because less ATP is used in the reverse tricarboxylic acid cycle compared to the Calvin cycle (74). Because the major source for carbon in most of the environments is CO_2 (13, 34), the energy efficient light harvesting system and the energy efficient reverse tricarboxylic acid pathway are major reasons for the dominance of green sulfur bacteria over *Chromatiaceae* under low light conditions.

The absorption of light by the overlaying water column determines the quality of light available for photosynthesis in the anoxic zones. In eutrophic lakes and in very deep anoxic layers of oligotrophic lakes, brown *Chlorobiaceae* outcompete their green colored

counterparts due to their ability to harvest blue-green light. In dystrophic lakes, yellow colored allochtonous humic material (gilvin) absorbs large fractions of blue and green light; as a consequence red shifted wavelength bands prevail in the environment (105). Therefore, green colored *Chlorobiaceae* dominate the community and outcompete their brown colored counterparts and purple sulfur bacteria (61, 80). Competition for light with respect to its intensity is presumably most intense between species with similar absorption characteristics, e.g. two species of green sulfur bacteria containing BChl *e*, which have the same *in vivo* absorption spectrum (74).

At light intensities greater than 5 to 10 µmol quanta·m^{-2}·s^{-1} purple sulfur bacteria dominate the chemocline of stratified lakes. Mostly okenone containing *Chromatiaceae* are present, which absorb similar wavelength bands as green sulfur bacteria. Carotenoids can contribute significant amounts of light harvesting in okenone containing purple sulfur bacteria (52, 82). In contrast, recent results suggest that carotenoids in green sulfur bacteria mainly protect the reaction center bacteriochlorophylls but do not contribute significantly in light harvesting (3, 4)

A further important selective factor is the availability and affinity to hydrogen sulfide, because concentrations of sulfide in the layers colonized phototrophic sulfur bacteria are usually in the micromolar range (102). Green sulfur bacteria have a 10 times higher affinity to hydrogen sulfide compared to *Chromatiaceae* and therefore an advantage in the scavenging of electron donor (102).

When light and electron donors are limiting, competition for organic substrates becomes important. Green sulfur bacteria are capable of photoassimilating a limited number of organic substrates. Acetate is used by all strains and pyruvate and propionate are used by about the half of all strains of green sulfur bacteria isolated so far (70). Under limitation of electron donors the growth yield of green sulfur bacteria increases three times per mol sulfide oxidized when acetate is added to the medium (9), but the affinity of purple sulfur bacteria to acetate is 30 times higher than in green sulfur bacteria (104). Therefore, they would outcompete green sulfur bacteria under conditions of limited supply with electron donors, but excess organic substrate. In contrast to green sulfur bacteria, many *Chromatiaceae* are able to switch between phototrophic growth with sulfide in the light and lithotrophy with oxygen in the dark or heterotrophy with organic substrates and oxygen in the dark (84). This physiological versatility is mainly found in *Chromatiaceae* species from littoral microbial mats. *Chromatiaceae* isolated from the chemoclines of freshwater lakes are mostly, as green sulfur bacteria, obligate photoautotrophs and photoassimilate only a narrow range of organic

substrates. In contrast, green sulfur bacteria need permanently reducing conditions, because energy transfer from chlorosomes to the reaction center decreases significantly in the presence of oxygen. Under the presence of oxygen Chlorobium-quinone is quenching the energy transfer to decrease photooxidative damage (26), which decreases the growth rate significantly and therefore restricts green sulfur bacteria to oxygen free environments. In comparision, the physiological versatility of *Chromatiaceae* is reflected by the colonization of environments with great fluctuations in oxygen concentrations as microbial mats in marine intertidal environments by e.g. *Thiocapsa roseopersicia* (102).

BACTERIAL ENDEMISM AND BIOGEOGRAPHY

Numerous examples for the biogeography of plants and animals are known and cited in standard textbooks of biogeography (e.g. 19). In the background of biogeography, an endemic species is restricted to a particular region and has originated from an ancestral population by spatial and temporal isolation. Its restricted distribution indicates either the presence of barriers to dispersal or insufficient time for range expansion.

In contrast to the obvious evidence for endemism of higher organisms, the investigation of bacterial endemism has received little attention. The lack of investigation on bacterial endemism is based on two reasons. Firstly, bacteria were thought to be cosmopolitan (6, 8). This assumption has been accepted as a dogma in microbiology and was supported by the ease with which very similar free-living heterotrophic bacteria from soil and water samples of remote locations were isolated. Secondly, the lack of a bacterial systematic taxonomy based on molecular data as the 16S rDNA sequence was not available until the last decade of the 20th century. Basically, the biochemical data and morphological features used at that time for the bacterial taxonomy were insufficient to detect bacterial endemism before the onset of the phylogenetically based bacterial taxonomy.

Originally, Baas-Becking and Beijerinck made reference to free-living, non-pathogenic bacteria in their early publications on prokaryote distribution (6, 8). It has been observed early, that some pathogenic bacteria indeed are endemic. Prominent examples of endemic populations of human pathogens are strains of *Yersinia pestis*, *Vibrio cholera* and *Neisseria meningitidis* (60). Many more examples of endemism have been found for animal and fish pathogens and symbiotic bacteria of higher organisms (21, 38, 64). In contrast, bacteria from freshwater and marine habitats follow in several cases the ubiquitous distribution claimed by Baas-Becking and Beijerinck (e.g. 7, 22, 23, 114) and only soil bacteria showed a limited geographical distribution (17). However, cosmopolitans were not observed among sea ice

bacteria obtained from Artic and Antarctic samples (94) and the analysis of different populations of *Achromatium oxaliferum* suggested a geographical separation (37). However, information on the distribution of bacterial species is usually not available.

SIGNIFICANCE OF PHOTOTROPHIC CONSORTIA

Phototrophic consortia were discovered early in the 20th century. Lauterborn described in 1906 "*Chlorochromatium aggregatum*" (55), a highly structured, small aggregate containing green colored cells, which thrive between decaying organic material in the sediments of small ponds. Independently, Buder described the same aggregates in 1913 using the name "*Chloronium mirabile*" (14). "*Pelochromatium roseum*", a very similar but brown-colored consortium was described shortly after (56). Buder recognized that "*C. aggregatum*" contained not only green colored cells but also a motile colorless bacterium in the center of the aggregates. From his observations he concluded that a symbiotic relationship with tight physiological interaction might exist between the two morphologically distinct cells in the aggregates. To describe this association he introduced the term *consortium* (from the Latin verb *consere*: to assemble, twist together, compose). The term consortium as used by Buder has been defined later more precisely and may further be used only for highly structured associations of two or more microorganisms which maintain a permanent cell-to-cell contract (42, 88, 97). All binary names of phototrophic consortia are without standing in nomenclature since the consortia consist of two bacteria. Therefore names of phototrophic consortia are given in quotation marks.

Eight motile phototrophic consortia have been described so far, which can be clearly distinguished according to their size, the color and shape of the epibionts and the presence of gas vacuoles (Tab. 2). "*C. aggregatum*" and "*P. roseum*" are barrel shaped and represent the smallest and most frequently observed phototrophic consortia in the chemoclines of freshwater lakes (78, Fig. 2). Both morphotypes contain only one layer of epibionts around a spindle shaped, straight central bacterium. In enrichments, "*C. aggregatum*" contained on average 13 epibionts, however, under natural conditions the number of epibionts may be slightly higher. In comparison, the morphologically similar "*P. roseum*" contained an average of 19 epibionts in the chemocline of a small mesotrophic lake (Lake Dagow, 78). All other consortia exceed these numbers by a factor of two and contain more than one layer of epibionts (Tab. 2). Among these the type of "*C. glebulum*" is easily distinguished from other phototrophic consortia by its bent shape. "*C. magnum*" and the recently discovered "*P. latum*" resemble a sphere and contain, as "*C. glebulum*", numerous epibionts (Tab. 2). An outer green

layer and an inner brown layer of epibionts distinguishes "*P. roseo-viride*" from all other phototrohic consortia, which contain epibionts of only one color. "*C. lunatum*" and "*P. selenoides*" resemble a pine-cone shape type and contain half-moon shaped epibionts (Tab. 2). Gas vesicles are common in those phototrophic consortia with a large number of epibionts, whereas none were observed in the small consortia (Tab. 2)

The presence of chlorosomes and the color of the epibiotic cells have early been used as evidence for their taxonomic position in the phylum of the green sulfur bacteria (15, 83). The application of modern molecular tools has recently revealed that the epibionts are truly green sulfur bacteria, but phylogenetically distinct from previously known species (28, 99, Fig. 2B). From comparisons with syntrophic continuous co-cultures of green sulfur bacteria with sulfur and sulfate reducing bacteria, a syntrophic sulfur cycle was suggested to occur inside the phototrophic consortia (83, 88). In these cultures, sulfide is oxidized by the green sulfur bacterium to zero-valent sulfur or to sulfate. In turn, the chemotrophic partner reduces the zero-valent sulfur (sulfur reducing bacterium) or the sulfate (sulfate reducing bacterium) by the simultaneous oxidation of an organic substrate, e.g. acetate (10, 83, 106). Interestingly, sulfide concentrations as low as 190 µM to 220 µM were sufficient to establish a sulfur cycle in syntrophic co-cultures between *Chlorobium limicola* and *Desulfovibrio desulfuricans* (10).

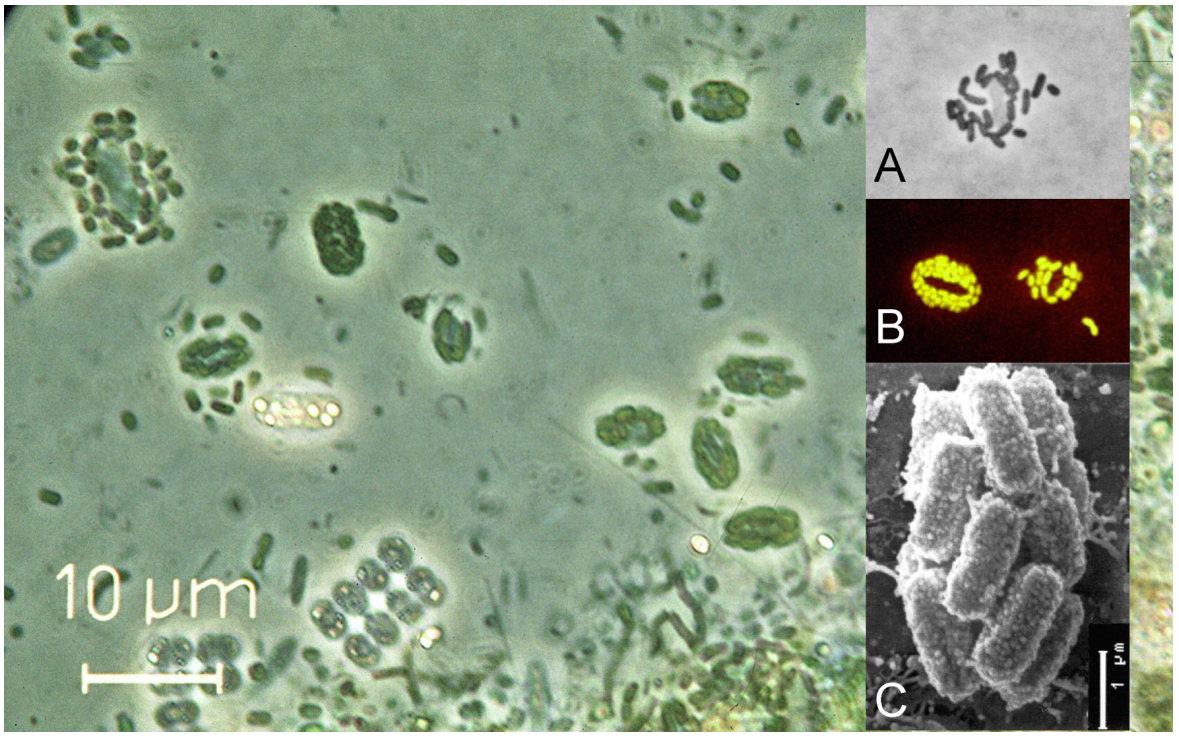
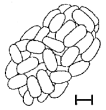
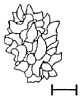
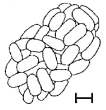
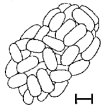


Figure 2. Phase contrast photomicrograph of bacteria from the chemocline of Lake Dagow (Brandenburg, Germany). The aggregates represent "*P. roseum*" consortia (taken from 79) **A.** Disaggregated single "*P. roseum*" consortium. Note the colorless rod-shaped bacterium in the center. **B.** Fluorescent *in situ* hybridization of "*P. roseum*" epibionts with green sulfur bacteria specific oligonucleotide probe GSB-532 (99). **C.** Visualization of the regular arrangement of the epibionts in "*P. roseum*" by scanning electron microsopy.

Table 2. Overview of the motile phototrophic consortia described so far. A flagellum is depicted only for "*C. aggregatum*" Bars represent 1 µm (modified from 28 and 79)

Type of Consortium	Shape of whole consortium	Color	No. of epitionts	Shape of epibionts	Gas vesicles	References
"*Chlorochromatium aggregatum*"	 Barrel	Green	12.9±4.5	Rod	-	55, 28
"*C. glebulum*"	 U-shaped	Green	7-40	Rod	+	92, 28
"*C. magnum*"	 Spherical	Green	36±4.4	Rod	+	28
"*C. lunatum*"	 Pine-cone	Green	68.6±2.5	Half-moon	+	1
"*Pelochromatium roseum*"	 Barrel	Brown	19.4±4.4	Rod	-	56, 99
"*P. latum*"	 Spherical	Brown	n.d	Rod	?	This study (Chapter 5)
"*P. selenoides*"	 Pine-cone	Brown	44.5±3.5	Half-moon	+	1
"*P. roseo-viride*"	 Barrel	Green outer layer and brown inner layer	n.d	Rod	+	36

n.d. Not determined

In the case of a syntrophic interaction with a closed sulfur cycle, the epibiont would photooxidize hydrogen sulfide to sulfur or sulfate, which in turn would serve as electron acceptor for the central bacterium. Unexpectedly, the central bacteria of "*C. aggregatum*" and "*C. magnum*" have been identified as β-proteobacteria, hence, not typical sulfur or sulfate reducing bacteria (28). Therefore, a closed sulfur cycle as a physiological basis of the interaction seems to be unlikely.

Phototrophic consortia are highly structured aggregates (Fig. 2C) and the growth and physiology of epibionts and central bacterium are assumed to be highly coordinated (27). A constant ratio of epibionts to central bacterium is maintained and cell division of epibionts and central bacterium is coordinated in the phototrophic consortia "*C. aggreagatum*" and "*P. roseum*" (78, 27). The maintenance of a highly structured arrangement between different bacterial species indicates a signal exchange between epibionts and central bacterium. This finding is supported by the scotophobic response of "*C. aggregatum*" and its accumulation in a continuous light spectrum at 740 nm, the *in vivo* spectrum of the epibiont (27). Because only the central bacterium is motile (27), the light harvesting antenna of the epibiont are presumably the photoreceptor for the scotophobic response of "*C. aggregatum*" (27). In consequence, the signal has to be transduced from the epibiont to the central bacterium. Further evidence for a specific signal transduction between epibionts and central bacterium derived from physiological experiments with "*C. aggregatum*". The simultaneous reduction of the alternative electron acceptor CTC (carbonyl cyanide *m*-chlorophenyl hydrazone) by the central bacterium and the epibionts occurred only in the presence of sulfide, 2-oxoglutarate and light (27). Specific structures elucidated by electron microscopy were found on the cytoplasmatic side at the contact points of both, the epibiont and the central bacterium. Presumably, these structures are involved in the specific signal transduction between the two symbiotic partners (Fig. 3, 89).

Sharply stratified mass accumulations of phototrophic consortia have only been reported recently (78, 105) and may contribute up to two-thirds of the total bacterial biomass (32). Although the occurrence of phototrophic bacteria has been reported from lakes in various locations worldwide (see references in 78), their ecology has not investigated in detail so far. Due to their high abundance, phototrophic consortia are presumably significantly involved in the biogeochemical cycles of carbon and sulfur in the chemoclines of freshwater lakes.

The factors selecting for the dominance of epibionts of phototrophic consortia over free-living green sulfur bacteria are not known to a sufficient extent to date, but the symbiotic

interaction with the central β-proteobacterium provides a likely explanation. The motility gained by epibiotic green sulfur bacteria by the tight association with the colorless β-proteobacterium is presumably a selective advantage in the chemocline. Environmental signals as light intensity or sulfide concentrations are likely to be sensed by the epibiont. A subsequent signal transduction to the motile central bacterium may explain the sharp peak of phototrophic consortia numbers in the chemoclines of freshwater lakes (78). Phototrophic consortia have a significantly higher specific density than the surrounding water. Therefore, the motility of the central rod is essential to prevent loss of consortia from the chemocline community by sedimentation (78). In comparison, green sulfur bacteria like *Pelodictyon clathratiforme* use gas vesicles to maintain their position in the chemocline and gas-vesicle deficient green sulfur bacteria as e.g. *Chlorobium phaeobacteroides* should have a higher rate of sedimentation. A competitive advantage of epibionts of phototrohpic consortia over gas vesicle containing green sulfur bacteria does only occur, when a horizontal

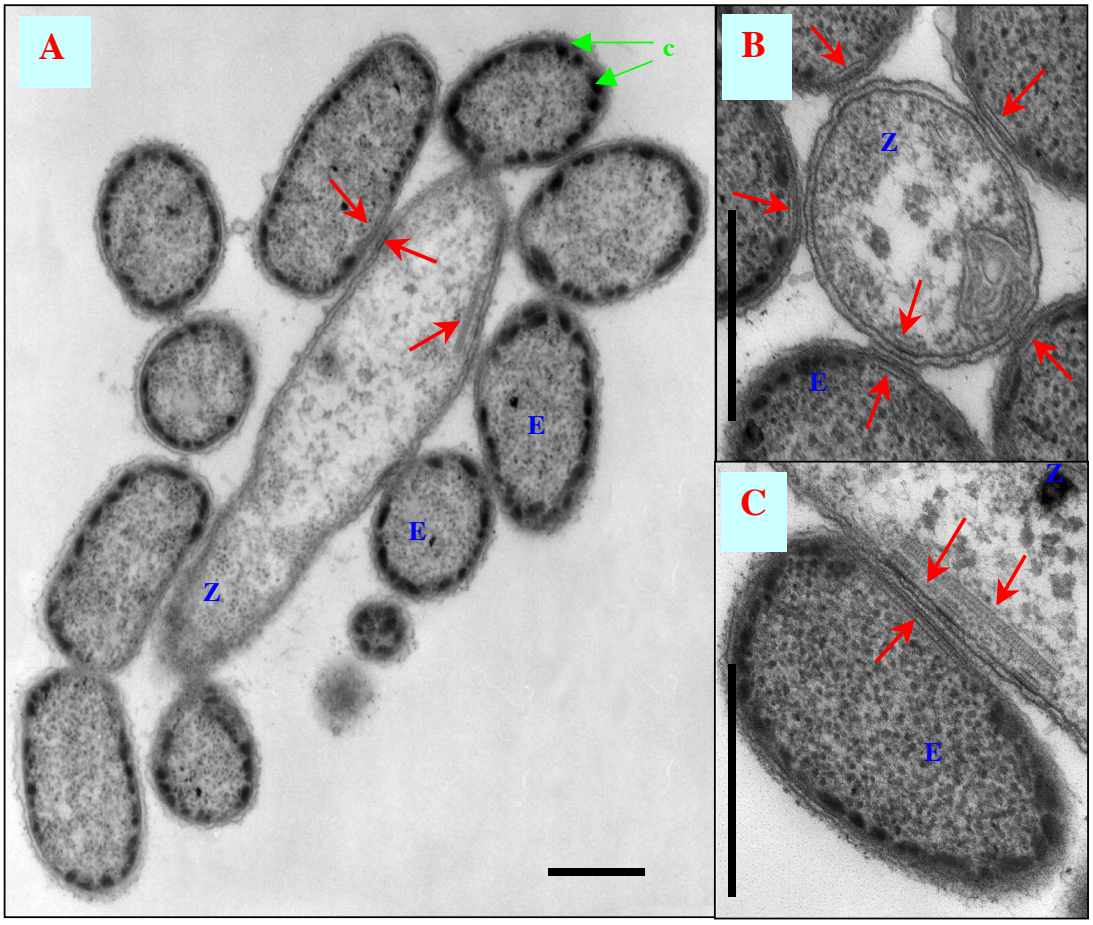


Figure 3. Transmission electron micrographs of thin sections of "*C. aggregatum*" (89). E: epibiont, Z: central bacterium, c: chlorosomes. Red arrows point towards a 3-layered electron dense struktur occurring in both, the epibiont and the central bacterium. Bars represent 0.5 µm **A.** Longitutal thin section of a consortium. **B**. Cross section of a consortium. **C**. Enlargement of a connection site between epibiont and central bacterium.

patchiness of the distribution of organic material and sulfate and sulfur reducing bacteria is assumed for the chemocline.

In the competition for light, epibionts of phototrophic consortia are unlikely to have a selective advantage over free-living green sulfur bacteria. Because the same pigments are used for light harvesting, light quality may not play a role for brown- or green-colored epibionts over their brown- or green-colored free-living counterparts. However, the composition of the bacteriochlorophyll homologs in the chlorosomes affect the *in vivo* absorption of the cell and it has not yet been elucidated if also the efficiency of energy transfer is affected as well.

Many interacting bacteria keep a close spatial proximity, because the diffusion of soluble compounds decreases sharply with distance. If interacting bacteria have a spatial distance of 10 µm the efficiency of diffusive transport of soluble compounds decreases to 0.01% compared with a distance of 1 µm (76). Clearly, a selective pressure drives those bacteria that interact by the exchange of soluble compounds to develop mechanisms to keep close contact with their interacting partner. Consortia represent the most developed state of interaction in which a cell-to-cell contact is maintained. Other non-related microorganisms keep a close spatial proximity in the natural environment by their high occurrence in aggregates, biofilms and microbial mats. Also in aquatic environments bacteria seem to occur in patches with distances of 10 to 100 µm (53). The exchange of soluble compounds is most efficient when cells maintain close contact with each other as in phototrophic consortia. Therefore, the advantage of bacterial consortia in terms of exchange of signals and soluble compounds is obvious compared to free living bacteria that only transiently reach a close proximity to possible interacting partners (71, 76) and may represent a major advantage in the competition with other bacteria under natural conditions.

Bacterial consortia are presumably the most developed bacterial interactions known so far and represent a model system for the co-evolution of a symbiosis between non-related bacterial species (76). However, the bacteria involved in the almost all consortia known today were not cultivated in pure culture yet (71). In most cases the phylogenetic affiliation of the bacteria, their physiological interaction and signal exchange as well as possible ways of cell-to-cell recognition remained unclear. Only recently, stable enrichments became available from the phototrophic consortium "*C. aggregatum*" (27) and the epibiont of "*C. aggregatum*" was cultivated in pure cultures (K. Vogl, J. Glaeser, J. Overmann, unpublished results). Therefore, the phototrophic consortia may function as a model system for cell-cell recognition and interaction between non-related bacterial species in the near future**.**

SCOPE OF THE PRESENT WORK

Phototrophic consortia are known since the beginning of the 20th century (55) and were discovered in the chemoclines of freshwater lakes worldwide (78). Although mass accumulations of phototrophic consortia have been reported (32), aspects concerning the ecological significance, the physiology and the selective advantage of phototrophic consortia in the natural environment remained obscure.

In consequence, the first aim of the present study was to gain a deeper understanding of the physicochemical and biological factors providing a selective advantage of "*P. roseum*" under natural conditions. This approach was based on the characterization of "*P. roseum*" population in the chemocline of Lake Dagow (78). The rather small, mesophilic Lake Dagow is located approximately 100 km in the north of Berlin (Germany) and develop a stratified water column each summer. Subsequently a large population of "*P. roseum*" occurs in the anoxic hypolimnion. Depending on year and seasonal development the consortia are found between a depths of 6.6 to 7.4 m (78, Chapter 3). From microscopic studies of chemocline samples it became clear that a uniform community of phototrophic bacteria, mainly consisting of "*P. roseum*", dominated the chemocline of Lake Dagow (78). Although the ecological niche of "*P. roseum*" was inferred from a two year study of the chemocline and literature data (78), the vertical distribution, relative abundance and diversity of epibiotic green sulfur bacteria in Lake Dagow remained obscure. Our approach involved the analysis of the numerical significance and phylogenetic diversity of epibiotic and free-living green sulfur bacteria in the chemocline of Lake Dagow by applying specific fluorescent oligonucleotide probes (99) and 16S rRNA gene specific primers for green sulfur bacteria (77).

The dominance of "*P. roseum*" over other phototrophic bacteria made the chemocline of Lake Dagow an ideal model system to study "*P. roseum*" under natural conditions. Therefore, the second aim focused on the *in situ* physiology of "*P. roseum*" epibionts with respect for their carbon substrates. Although green sulfur bacteria are obligate photoautotrophs, organic substrates may play an important role for the growth of green sulfur bacteria under conditions when electron donors become limiting (9). Clearly, sulfide concentrations in the chemocline and the flux of sulfide from the hypolimnion into the chemocline were not sufficient to explain the large biomass accumulation of "*P. roseum*" in Lake Dagow (78). Because a syntrophic interaction based on a closed sulfur cycle became unlikely due to the phylogenetic position of the central bacterium (28) electron donors are presumably a limiting factor in the chemocline and organic substrates may be important for the growth of the epibionts of "*P. roseum*" *in situ*. Biomarkers from green sulfur bacteria differ significantly in the

composition of stable carbon isotopes (91, 100) and can therefore be used to determine the *in situ* carbon substrate. For this investigation, the community of green sulfur bacteria had to consist of almost only epibionts of "*P. roseum*". The obstacle of this study was the absence of the typical biomarker for brown colored *Chlorobiaceae*, isorenieratene. Therefore, a search for new biomarkers was necessary to be able to investigate the *in situ* physiology of "*P. roseum*" epibionts. Because of their high abundance in green sulfur bacteria, derivatives of bacteriochlorophylls have been used as biomarkers previously (39, 43). The esterifying alcohols of bacteriochlorophyll *e* provide useful biomarkers for stable carbon isotope analysis and the investigation of the *in situ* physiology of brown-colored *Chlorobiaceae*, but have not been investigated to a sufficient extent before. The detailed investigation of biomarkers derived from photosynthetic pigment of green sulfur bacteria in general and "*P. roseum*" epibionts in particular was followed by the *in situ* study of stable carbon isotope fractionation in the esterifying alcohols of bacteriochlorophylls and fractions of particulate and dissolved organic and dissolved inorganic carbon. ^{14}C-labled bicarbonate was incubated with chemocline samples to assess the extent of photosynthetic activity in the bacterial community of the chemocline and, hence, the photosynthetic activity of the epibiotic green sulfur bacteria in Lake Dagow.

Enrichments of "*C. aggregatum*" could only be established in the presence of 2-oxoglutarate (27). Therefore, it remained an open question whether 2-oxoglutarate is important for other consortia, as "*P. roseum*" and if organic substrates are important for the growth of phototrophic consortia and especially for the central bacterium in nature. Consequently, the third aim of this study was to investigate the effect of environmental stimuli like organic substrates or electron donors on the chemotaxis of "*P. roseum*". Because the attraction of "*C. aggregatum*" by sulfide and 2-oxoglutarte was known from studies with enrichment cultures it was important to test whether substrates like 2-oxoglutate function as attractant of "*P. roseum*" and substrate of the central bacterium under *in situ* conditions. Initial experiments with enrichments of "*C. aggregatum*" suggested a strong physiological interaction and a rapid signal transfer between epibionts and central bacterium, which presumably act as a selective factor under natural conditions (27). The chemotaxis towards sulfide and 2-oxoglutarate and the scotophobic response as observed in "*C. aggregatum*" (27) might represent a reaction to stimuli encountered in nature that may help to gain and maintain the optimum position in the chemocline. *In situ* experiments would provide valuable information on the importance and nature of interaction for the search of substrates under natural conditions. Therefore, chemotaxis assays were performed *in situ* and the uptake of

^{14}C-labled 2-oxoglutarate by "*P. roseum*" was analyzed by microautoradiography in chemocline samples.

The phylogenetic position of the epibionts from a limited number of phototrophic consortia was analyzed previously and determined as unique phylotypes within the radiation of green sulfur bacteria (28, 99). Because morphologically identical phototrophic consortia from different lakes represented unique phylotypes a non-ubiquitous distribution of phototrophic consortia was suggested (28). The major focus of the fourth aim of this study was the detailed analysis of the phylogenetic position of the epibiotic green sulfur bacteria from most of the morphologically described phototrophic consortia. The obtained data would also provide the basis to elucidate the global diversity among these morphotypes. Therefore, chemocline samples containing phototrophic consortia from 14 Lakes of 6 geographical regions were studied. The assessment of the global diversity of epibiotic green sulfur bacteria will furthermore provide (i) answers to the question if this type of symbiosis has developed only once in the evolution of green sulfur bacteria or arose convergently among various phylogenetic lines of green sulfur bacteria; (ii) data to investigate if competitive exclusion exists between different morphological types of phototrophic consortia and (iii) insight into the question if endemic populations exists among epibiont phylotypes. Again, a culture independent approach was chosen to determine the diversity of epibionts from chemocline samples. Phototrophic consortia were separated by micromanipulation and subsequently 16S rRNA gene fragments were amplified with oligonucleotide primers specific for green sulfur bacteria (77). The amplified 16S rRNA gene fragments were analyzed by denaturing gradient gel electrophoresis and subsequently sequenced. The phylogenetic analysis of the partial 16S rRNA gene sequences provided the information to answer the questions asked previously.

REFERENCES

1. **Abella C.A., Cristina X.P., Martinez A., Pibernat I. and Vila X.** (1998) Two new motile phototrophic consortia: "*Chlorochromatium lunatum*" and "*Pelochromatium selenoides*". Arch. Microbiol. **169:**452-459
2. **Amesz J.** (1991) Green photosynthetic bacteria and heliobacteria, pp. 99-119. *In*: Barton, L.L. and Shively J.M. (eds.), Variations in Autotrophic Life. Academic Press London, London, United Kingdom
3. **Arellano J.B., Melø T.B., Borrego C.M., Garcia-Gil L.J. and Naqvi K.R.** (2000) Nanosecond laser photolysis studies of chlorosomes and artificial aggregates containing bacteriochlorophyll *e*: Evidence for the proximity of carotenoids and bacteriochlorophyll *a* in chlorosomes from *Chlorobium phaeobacteroides* CL1401. Photochem. Photobiol. **72:**669-675
4. **Arellano J.B., Borrego C.M., Martínez-Planells A. and Garcia-Gil L.J.** (2001) Effect of carotenoid deficiency on cells and chlorosomes of *Chlorobium phaeobacteroides*. Arch. Microbiol. **175:**226-233
5. **Atlas R.M. and Bartha R.** (1998) Microbial Ecology - Fundamentals and Applications. 4th edition, Benjamin/Cummings Science Publishing, Menlo Park, California, USA
6. **Baas-Becking L.G.M.** (1934) Geobiologie of Inleiding Tot de Milieukunde. W.P. van Stockum & Zoon N.V., The Hague, The Netherlands
7. **Beeder J., Nilsen R.K., Rosnes J.T., Torsvik T. and Lien T.** (1994) *Archaeoglobus fulgidus* isolated from hot North Sea oil field waters. Appl. Environ. Microbiol. **60:**1227-1231
8. **Beijerinck M.W.** (1913) De infusies en de ontdekking der backteriën, Jaarboek van de Koninklijke Akademie v. Wetenschappen. Müller, Amsterdam, The Netherlands
9. **Bergstein T., Henis Y. and Cavari B.Z.** (1979) Investigations on the photosynthetic sulfur bacterium *Chlorobium phaeobacteroides* causing seasonal blooms in Lake Kinneret. Can. J. Microbiol. **25:**999-1007
10. **Biebl H. and Pfennig N.** (1978) Growth yields of green sulfur bacteria in mixed cultures with sulfur and sulfate reducing bacteria. Arch. Microbiol. **117:**9-16
11. **Blankenship R.E.** (1992) Origin and early evolution of photosynthesis. Photosynth. Res. **33:**91-111
12. **Bowyer J.R., Hunter C.N., Ohnishi T. and Niederman R.A.** (1985) Photosynthetic membrane development in *Rhodopseudomonas sphaeroides*. J. Biol. Chem. **260:**3295-3304

13. **Brune D.C.** (1989) Sulfur oxidation by phototrophic bacteria. Biochim. Biophys. Acta. **975:**189-221
14. **Buder J.** (1914) Chloronium mirabile. Berichte Dtsch. Bot. Ges. **31:**80-97
15. **Caldwell D.E. and Tiedje J.M.** (1975) The structure of anaerobic bacterial communities in the hypolimnia of several Michigan lakes. Can. J. Microbiol. **21:**377-385
16. **Campbell L., Nolla H.A. and Vaulot D.** (1994) The importance of *Prochlorococcus* to community structure in the central North Pacific Ocean. Limnol. Oceanogr. **39:**954-961
17. **Cho J.-C. and Tiedje J.M.** (2000) Biogeography and degree of endemicity of fluorescent *Pseudomonas* strains in soil. Appl. Environ. Microbiol. **66:** 5448-5456
18. **Chung S., Frank G., Zuber H. and Bryant D.A.** (1994) Genes encoding two chlorosome components from the green sulfur bacteria *Chlorobium vibrioforme* strain 8327D and *Chlorobium tepidum*. Photosynth. Res. **41:**261-275
19. **Cox C.B. and Moore P.D.** (1993) Biogeography. An ecological and evolutionary approach. Blackwell Scientific Publications, London, United Kingdom
20. **Drews G. and Golecki J.R.** (1995) Structure, molecular organization, and biosynthesis of membranes of purple bacteria, pp. 231-257. *In*: Blankenship R.E., Madigan M.T. and Bauer C.E. (eds.), Anoxygenic Photosynthetic Bacteria. Kluwer Academic Publishers Dordrecht, The Netherlands
21. **Eldar A., Goria M., Ghittino C., Zlotkin A. and Bercovier H.** (1999) Biodiversity of *Lactococcus garvieae* Strains Isolated from Fish in Europe, Asia and Australia. Appl. Environm. Microbiol. **65:**1005-1008
22. **Field K.G., Gordon D., Wright T., Rappé M., Urbach E., Vergin K. and Giovannonni S.J.** (1997) Diversity and depth-specific distribution of SAR11 cluster rRNA genes from marine planktonic bacteria. Appl. Environ. Microbiol. **63:**63-70
23. **Finlay B.J.** (2002) Global dispersal of free-living microbial eukaryote species. Science **296:**1061-1063
24. **Friedmann E.I.** (1982) Endolithic microorganims in the Antactc Cold Desert. Science. **215:**1045-1053
25. **Friedmann E.I. and Ocampo R.** (1976) Endolithic blue-green algae in the Dry valleys: primary producers in the Anctarctic Desert Ecosystem. Science. **193:**1247-1249
26. **Frigaard N.-U., Takaichi S., Hirota M., Shimada K., and Matsuura K.** (1997) Quinones in chlorosomes of green sulfur bacteria and their role in the redox-dependent

fluorescence studied in chlorosome-like bacteriochlorophyll *c* aggregates Arch. Microbiol. **167:**343-349

27. **Fröstl J.M. and Overmann J.** (1998) Physiology and tactic response of the phototrophic consortium "*Chlorochromatium aggregatum*". Arch. Microbiol. **169:**129-135
28. **Fröstl J.M. and Overmann J.** (2000) Phylogenetic affiliation of the bacteria that constitute phototrophic consortia. Arch. Microbiol. **174:** 50-58
29. **Fuchs G., Stupperich E. and Jaenchen R.** (1980) Autotrophic CO_2 fixation in *Chlorobium limicola*: Evidence for the operation of a reductive tricarboxylic acid cycle in growing cells. Arch. Microbiol. **128:**64-72
30. **Garcia-Pichel F.** (1999) Cyanobacteria. *In*: Lederberg J. (ed.), Encyclopedia of Microbiology. Academic Press, San Diego, USA
31. **Garcia-Pichel F., Belnap J., Neuer S. and Schanz F.** (2003) Estimates of global cyanobacterial biomass and its distribution. Algological Studies. **109:**213-228
32. **Gasol J.M., Jürgens K., Massana R., Calderón-Paz J.I. and Pedrós-Alió C.** (1995) Mass development of *Daphnia pulex* in a sulfide-rich pond (Lake Cisó). Arch. Microbiol. **132:**279-296
33. **Giovanonni S.J., Turner S., Olsen G.J., Barns S., Lane D.J. and Pace N.R.** (1988) Evolutionary relatiosnhips among cyanobacteria and green chloroplasts. J. Bacteriol. **170:**3548-3592
34. **Göbel F.** (1978) Quantum efficiencies of growth, pp. 907-925. *In*: Clayton R.K., Sistrom W.R. (eds.), The photosynthetic bacteria. Plenum Press, New York, USA
35. **Goericke R. and Welschmeyer N.A.** (1993) The marine prochlorophyte *Prochlorococcus* contributes significantly to phytoplankton biomass and primary production in the Sargasso Sea. Deep-Sea Res. **40:**2283-2294
36. **Gorlenko V.M. and Kuznetsov S.I.** (1971) Vertical distribution of photosynthetic bacteria in the Kononier lake of the Mari ASSR. Mikrobiologiia. **40:**651-652
37. **Gray N.D., Howarth R., Rowan A., Pickup R.W., Jones J.G. and Head I.M.** (1999) Natural communities of *Achromatium oxaliferum* comprise genetically, morphologically, and ecologically distinct subpopulations. Appl. Environ. Microbiol. **65:**5089-5099
38. **Grayson T.H., Cooper L.F., Atienzar F.A., Knowles M.R. and Gilpin M.L.** (1999) Molecular Differentiation of Renibacteium salmoninarum Isolates from Worldwide Locations. Appl. Environ. Microbiol. **65:**961-968

39. **Grice K., Gibbison R., Atkinson J.E., Schwark L., Eckhardt C.B. and Maxwell J.R.** (1996) Maleimides (1*H*-pyrolle-2,5-diones) as molecular indicators of anoxygenic photosynthesis in ancient water columns. Geochim. Cosmochim. Acta **60:**3913-3924
40. **Hanada S. and Pierson B.Y.** (2002). The family *Chloroflexaceae*. *In*: Dworkin M. et al. (eds.), The prokaryotes: and evolving electronic resource for the microbiological community, 3rd ed. (Online) Springer, New York, N.Y., USA (latest update release. 3.11, September 2002, http://ep.springer-ny.com:6336/contents/)
41. **Heathcote P., Jones M.R. and Fyfe P.K.** (2002) Type I photosynthetic reaction centres: structure and function. Phil. Trans. R. Soc. Lond. B. **358:**231-243
42. **Hirsch P.** (1984) Microcolony formation and consortia, pp. 373-393 *In*: Marshal K.C. (ed.), Microbial adhesion and aggregation. Springer, Berlin, Heidelberg, New York
43. **Huang Y., Freeman K.H., Wilkin R.T., Arthur M.A. and Jones A.D.** (2000) Black Sea chemocline oscillations during the Holocene: molecular and isotopic studies of marginal sediments. Org. Geochem. **31:**1525-1531
44. **Hugenholtz P., Pitulle C., Hershberger K.L. and Pace N.R.** (1998). Novel division level bacterial diversity in a Yellowstone hot spring. J. Bacteriol. **180:**366-376
45. **Imhoff J.F.** (2000) The family *Ectothiorhodospiraceae*. *In*: Dworkin M. et al. (eds.), The prokaryotes: and evolving electronic resource for the microbiological community, 3rd ed. (Online) Springer, New York, N.Y., USA (latest update release. 3.11, September 2002, http://ep.springer-ny.com:6336/contents/)
46. **Imhoff J.F.** (2001a) The phototrophic alpha-Proteobacteria. *In*: Dworkin M. et al. (eds.), The prokaryotes: and evolving electronic resource for the microbiological community, 3rd ed. (Online) Springer, New York, N.Y., USA (latest update release. 3.11, September 2002, http://ep.springer-ny.com:6336/contents/)
47. **Imhoff J.F.** (2001b) The phototrophic beta-Proteobacteria. *In*: Dworkin M. et al. (eds.), The prokaryotes: and evolving electronic resource for the microbiological community, 3rd ed. (Online) Springer, New York, N.Y., USA (latest update release. 3.11, September 2002, http://ep.springer-ny.com:6336/contents/)
48. **Imhoff J.F.** (2003) Phylogenetic taxonomy of the family *Chlorobiaceae* on the basis of 16S rRNA and fmo (Fenna-Metthews-Olson protein) gene sequences. Int. J. Syst. Bacteriol. **53:**941-951
49. **Jørgensen B.B.** (1995) Die Mikrowelt der Meerresbakterien. Naturwissenschaften **82:** 269-278

50. **Jørgensen B.B. and Des Marais D.J.** (1986) A simple fiber-optic microprobe for high resolution light measurements: applications in marinesediments. Limnol. Oceanogr. **31:**1374-1383
51. **Jørgensen B.B. and Des Marais D.J.** (1988) Optical properties of benthic photosynthetic communities: fiber-optic studies of cyanobacterial mats. Limnol. Ocean. **33:**99-113
52. **König M.** (2000) Anpassungsmechanismen von Schwefelpurpurbakterien. Diplom-arbeit at the Carl von Ossietzky Universität, Oldenburg, Germany
53. **Krembs C., Juhl A.R., Long R.A. and Azam F.** (1998) Nanoscale patchiness of bacteria in lake water studied with the spatial information preservation method. Limnol. Oceanogr. **43:**307-314
54. **Kühl M. and Jørgensen B.B.** (1992) Spectral light measurements in microbenthic phototrophic communities with a fiber-optic microprobe coupled to a sensitive diode array detector. Limnol. Oceanogr. 37:1813-1823
55. **Lauterborn R.** (1906) Zur Kenntnis der sapropelischen Flora. Allg. Bot.Z. **12:**196-197
56. **Lauterborn R.** (1915) Die sapropelische Lebewelt. Verhandlungen der naturhistorisch-medizinischen Vereinigung zu Heidelberg. **13:**395-481
57. **Liu H., Nolla H.A. and Campbell L.** (1997) *Prochlorococcus* growth rate and contribution to primary production in the equatorial and subtropical North Pacific Ocean. Aquat. Microb. Ecol. **12:**39-47
58. **Madigan M.T.** (2001) The family *Heliobacteriacea*. *In*: Dworkin M. et al. (eds.), The prokaryotes: and evolving electronic resource for the microbiological community, 3rd ed. (Online) Springer, New York, N.Y., USA (latest update release. 3.11, September 2002, http://ep.springer-ny.com:6336/contents/)
59. **Madigan M.T., Takigiku R., Lee R.G., Gest H. and Hayes J.M.** (1989) Carbon isotope fractionation by thermophilic phototrophic sulfur bacteria: Evidence for autotrophic growth in natural populations. Appl. Environ. Microbiol. **55:**639-644
60. **Maynard-Smith J., Smith N.H., O´Rourke M. and Spratt B.G.** (1993). How clonal are bacteria? Proc. Natl. Acad. Sci. **90:**4384-4388
61. **Montesinos E.R., Guerrero R., Abella C. and 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

62. **Nagashima K.V.P., Shimada K. and Matsuura K.** (1993) Phylogenetic analysis of photosynthesis genes of *Rhodocyclus gelatinosus*: possibility of horizontal gene transfer in purple bacteria. Photosynth. Res. **36:**185-191

63. **Nagashima K.V.P., Hiraishi A., Shimada K. and Matsuura K.** (1997) Horizontal transfer of genes coding for the photosynthetic reaction centers of purple bacteria. J. Mol. Evol. **45:**131-136

64. **Nilsson M., Gergman B. and Rasmussen U.** (2000). Cyanobacterial diversity in geographically related and distant host plants of the genus *Gunnera*. Arch. Microbiol. 173:97-102

65. **Oesterhelt D. and Krippahl G.** (1983). Phototropic growth of halobacteria and its use for isolation of photosynthetically-deficient mutants. Ann. Microbiol. (Inst. Pasteur). **134 B:**137-150

66. **Oesterhelt D. and Stoeckenius W.** (1973) Function of a new photoreceptor membrane. Proc. Natl. Acad. Sci. USA **70:**2853-2857

67. **Oren A.** (2001) The order *Haliobacteriales*. *In*: Dworkin M. et al. (eds.), The prokaryotes: and evolving electronic resource for the microbiological community, 3rd ed. (Online) Springer, New York, N.Y. (latest update release. 3.11, September 2002, http://ep.springer-ny.com:6336/contents/)

68. **Overmann J.** (1997) Mahoney Lake: A case study of the ecological significance of phototrophic sulfur bacteria, pp.251-288. *In*: Jones J.G. (eds.), Advances in Microbial Ecology, Vol. 15. Plenum Press New York, New York, USA

69. **Overmann J.** (2001a) The family *Chlorobiaceae***.** *In*: Dworkin M. et al. (eds.), The prokaryotes: and evolving electronic resource for the microbiological community, 3rd ed. (Online) Springer, New York, N.Y., USA (latest update release. 3.11, September 2002, http://ep.springer-ny.com:6336/contents/)

70. **Overmann J.** (2001b) Green sulfur bacteria, pp. 601-605. *In*: Boone D.R., Castenholz R.W. and Garrity G.M. (eds.), Bergey´s Manual of Systematic Bacteriology, 2nd edn. volume 1. Springer, New York, N. Y., USA

71. **Overmann J.** (2001c) Phototrophic consorita: a tight cooperation between non-related eubacteria, pp. 239-255. *In*: Seckbach, J. (ed.), Symbiosis. Mechanisms and model systems. Kluwer, Dordrecht, The Netherlands

72. **Overmann J., Beatty J.T. and Hall K.J.** (1994). Photosynthetic activity and population dynamics of *Amoebobacter purpureus* in a meromictic saline lake. FEMS Microbiol. Ecol. **15:**309-320

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

74. **Overmann J. and Garcia-Pichel F.** (2001) The phototrophic way of life. *In*: Dworkin M. et al. (eds.), The prokaryotes: and evolving electronic resource for the microbiological community, 3rd ed. (Online) Springer, New York, N.Y., USA (latest update release. 3.11, September 2002, http://ep.springer-ny.com:6336/contents/)

75. **Overmann J., Lehmann S. and Pfennig N.** (1991) Gas vesicle formation and buoyancy regulation in *Pelodictyon phaeoclathratiforme* (green sulfur bacteria). Arch. Microbiol. **157:**29-37

76. **Overmann J. and Schubert K.** (2002) Phototrophic consortia: model systems for symbiotic interrelations between prokaryotes. Arch. Microbiol. **177:** 201-208

77. **Overmann J. and Tuschak C.** (1997) Phylogeny and molecular fingerprinting of green sulfur bacteria. Arch. Microbiol. **167:** 302-309

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

79. **Overmann J. and van Gemerden H.** (2000). Microbial interactions involving sulfur bacteria: implications for the ecology and evolution of bacterial communities. FEMS Microbiol. Rev. **24:**591-599

80. **Parkin T.B. and Brock T.D.** (1980a) The effects of light quality on the growth of phototrophic bacteria in lakes. Arch. Microbiol. **125:**19-27

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

82. **Permentier H.** (2001) Light-harvesting and core complexes of anoxygenic phototrophic bacteria. Dissertation at the University of Leiden, The Netherlands

83. **Pfennig N.** (1980) Syntrophic mixed cultures and symbiotic consortia with phototrophic bacteria: A review, pp. 127-131. *In* Gottschalk G., Pfennig N. and Werner H. (eds.), Anaerobes and Anaerobic Infections. Gustav Fischer Verlag, Stuttgart, Germany

84. **Pfennig N. and Trüper H.G.** (1999) The family *Chromatiaceae*. *In*: Dworkin M. et al. (eds.), The prokaryotes: and evolving electronic resource for the microbiological community, 3rd ed. (Online) Springer, New York, N.Y., USA (latest update release. 3.11, September 2002, http://ep.springer-ny.com:6336/contents/)

85. **Raymond J., Zhaxybayeva O., Gogarten J.P. and Blankenship R.E.** (2002) Whole genome analysis of photosynthetic prokaryotes. Science. **298:**1616-1620

86. **Raymond J., Zhaxybayeva O., Gogarten J.P. and Blankenship R.E.** (2003) Evolution of photosynthetic prokaryotes: a maximum likelihood mapping approach. Phil. Trans. of the Royal. Soc. B. **358:**223-230

87. **Rutherford A.W. and Faller P.** (2002) Photosystem II: evolutionary perspectives. Phil. Trans. R.Soc. Lond. B. **358:**245-253

88. **Schink B.** (1991) Syntrophism among prokaryotes. *In*: Balows A., Trüper H.G., Dworkin M., Harder W. and Schleifer K.-H. (eds.), The Prokaryotes. Springer Verlag, New York, Berlin, Heidelberg

89. **Schlickenrieder M.** (2002) Wachstum, Aktivität und Zell-Zell-Interaktion von "*Chlorochromatium aggregatum*". Diplomarbeit at the Ludwig-Maximilians-Universität, München, Germany

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

91. **Sirevåg, R.** (1995) Carbon metabolism in green bacteria, pp. 871-883. *In*: Blankenship R.E., Madigan M.T. and Bauer C.E. (eds.), Anoxygenic Photosynthetic Bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands

92. **Skuja H.** (1957) Taxonomische und biologische Studien über das Phytoplankton schwedischeer Binnengewässer. Nova Acta Reg. Soc. Sci. Uppsala, Ser. IV. **16:**1-404

93. **Stackebrandt E., Rainey F.A. and Ward-Rainey N.** (1996) Anoxygenic phototrophy across the phylogenetic spectrum: current understanding and future perspectives. Arch. Microbiol. **166:**211-223

94. **Staley J.T. and Gosink J.J.** (1999) Poles apart: Biodiversity and biogeography of sea ice bacteria. Annu. Rev. Microbiol. **53:**189-215

95. **Strauss G. and Fuchs G.** (1993) Enzymes of a novel autotrophic CO_2 fixation pathway in the phototrophic bacterium *Chloroflexus aurantiacus*, the 3-hydroxypropionate cycle. FEBS Eur. J. Biochem. **215:**633-643

96. **Takahashi K., Wada E. and Sakamoto M.** (1990). Carbon isotope discrimination by phytoplankton and photosynthetic bacteria in monomictic Lake Fukami-ike. Arch. Hydrobiol. **120:**197-210

97. **Trüper H.G. and Pfennig N.** (1971). Family of phototrophic Green Sulfur Bacteria: *Chlorobiaceae* Copeland, the correct family name; rejection of Chlorobacterium Lauterborn; and the taxonomic situation of the consortium-forming species. Int. J. Syst. Bacteriol. **21:**8-10

98. **Turner S.** (1987) Molecular systematics of oxygenic photosynthetic bacteria. Plant Syst Evol. **11:**13-52

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

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

101. **van Gemerden H., Tughan C.S., de Wit R. and Herbert R.A.** (1989) Laminated microbial ecosystems on sheltered beaches in Scapa Flow, Orkney Islands. FEMS Microbiol. Ecol. **62:**87-102

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

103. **van Niel C.B.** (1931) On the morphology and physiology of the purple and green sulphur bacteria. Archiv für Mikrobiologie **3:**1-112

104. **Veldhuis M.J.W. and van Gemerden H.** (1986) Competition between purple and brown phototrophic bacteria in stratified lakes: sulfide, acetate, and light as limiting factors. FEMS Microbiol. Ecol. **38:**31-38

105. **Vila X., Abella C.A., Figueras J.B., Hurley J.P.** (1998) Vertical models of phototrophic bacterial distribution in metalimnetic microbial communities of several freshwater North-American kettle lakes. FEMS Microb. Ecol. **25:**287-299

106. **Warthmann R., Cypionka H. and Pfennig N.** (1992) Photoproduction of H_2 and acetate by syntrophic cocultures of green sulfur bacteria and sulfur-reducing bacteria. Arch. Microbiol. **157:**343-348

107. **Waterbury J.B., Watson S.W., Valois F.W. and Franks D.G.** (1986) Biological and ecological characterization of the marine unicellular cyanobacterium *Synechococcus*, pp. 71-120. *In*: Platt T. and Li W.K.W. (eds.), Photosynthetic Picoplankton. Can. Bull. Fish Aquat. Sci. 214

108. **Wilmotte A. and Herdman M.** (2001) Phylogenetic relationships among the Cyanobacteria based on 16S rRNA sequences pp. 487-493. *In*: Boone D.R., Castenholz R.W. and Garrity G. (eds.), Bergey's Manual of Systematic Bacteriology. Volume 1: The Archaea and the Deeply Branching and Phototropic Bacteria. Second Edition. Springer-Verlag, New York, USA

109. **Woese C.E.** (1987) Bacterial evolution. Microbiol. Rev. **51:**221-271

110. **Woese C.E., Stackebrandt E., Macke T.J. and Fox G.E.** (1985) The phylogenetic definition of the major eubacterial taxa. Syst. Appl. Microbiol. **6:**143-151

111. **Xiong J., Inoue K., Nakahara M. and Bauer C.E.** (2000) Molecular evidence for the early evolution of photosynthesis. Science. **289:**724-1730

112. **Xiong J. and Bauer C.E.** (2002a) Complex evolution of photosynthesis. Ann Rev. Plant Physiol. **53:**503-521

113. **Xiong J. and Bauer C.E.** (2002b) A cytochrome b origin of photosynthetic reaction centers: an evolutionary link between respriation and photosynthesis. J. Mol. Biol. 322:1025-1037

114. **Zwart G., Hiorns W.D., Methé B.A., van Agterveld M.P., Huismans R., Nold S.C., Zehr J.P. and Laanbroek H.J.** (1998) Nearly identical 16S rRNA sequences recovered from lakes in North America and Europe indicate the existence of clades of globally distributed freshwater bacteria. System. Appl. Microbiol. **21:** 546-556

Chapter 2

Novel bacteriochlorophyll *e* structures and species-specific variability of pigment composition in green sulfur bacteria

INTRODUCTION

Green sulfur bacteria are obligate photoautotrophs and thrive where light reaches sulfidic water layers or sediments. Typical habitats comprise stagnant lakes, ponds, and microbial mats in hot springs or shallow sandy sediments (42).

The photosynthetic apparatus of green sulfur bacteria contains exceptionally large photosynthetic antennae (~1,500 BChl molecules per reaction center, 13). As a consequence, these bacteria are capable of growing at significantly lower light intensities than any other known phototrophic organism (32). The antenna structures, so called chlorosomes, are oblong bodies with 70 - 260 nm in length and 30 - 100 nm in width, which are localized at the inner surface of the cytoplasmatic membrane. Each chlorosome consists of a glycolipid monolayer envelope which encloses aggregates of antenna bacteriochlorophylls (BChl) *c*, *d* or *e*. Chlorosomes are attached to reaction centers in the cytoplasmic membrane through Fenna-Matthew-Olson (FMO) protein complexes. The portion of the envelope which is in contact with FMO complexes (the so-called baseplate) contains low amounts of BChl *a* associated with proteins (35). Besides bacteriochlorophylls, chlorosomes contain carotenoids and quinones (15, 36). Recent studies of *Chlorobium phaeobacteroides* strain CL 1401 suggest that the carotenoids are located near the baseplate of the chlorosomes and, rather than serving as antenna pigments, are important for chlorosome structure and photoprotection (2, 3).

Based on electron microscopical observations, chlorosomes are filled with 10-30 rod-shaped elements which are oriented parallel to the long axis of the chlorosomes and have a diameter of 10 nm. According to the present model, each rod consists of a tubular bilayer formed by self organization of the antenna bacteriochlorophyll molecules (5, 28, 40, 43). The pigment organization of chlorosomes differs fundamentally from that of all other known photosynthetic antenna in that it is based on pigment-pigment interactions rather than binding of pigments to proteins. Consequently, a detailed knowledge of the molecular structure of BChl and its effect on self-organization are essential to a better understanding of chlorosome architecture and function, and the unusual low-light adaptation of green sulfur bacteria.

Each of the bacteriochlorophylls *c*, *d*, or *e* consists of porphyrin ring systems that bear up to 5 different combinations of substituents, and are esterified to farnesol or other long-chain alcohols. As a consequence, one bacteriochlorophyll may actually consist of up to 15 structurally related molecules (7, 9). These different homologs exhibit identical absorption properties in solution (7, 11, 30), but differ in the stereochemistry of the hydroxyethyl group at position C-3, the degree of alkylation of the tetrapyrrol carbon atoms at positions C-8 and C-12 and the esterifying alcohol at position C-17[3]; the alcohol esterified to the third carbon of the side chain at C-17 of porphyrin ring IV (Fig. 1) (10, 11, 19, 25, 29). Whereas the

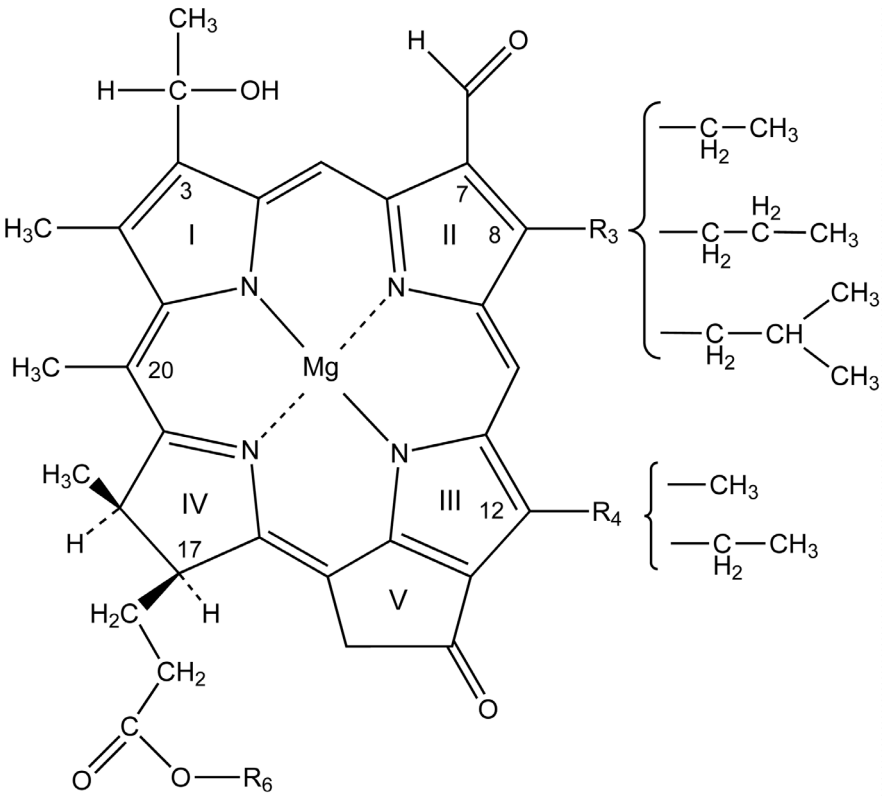


Figure 1. Molecular structure of BChl *e*. Ring systems of the macrocycle are numbered I-V according to Olson (1998). Variable length of alkyl side chains R_3 and R_4 at positions C-8 and C-12, and the position of the esterified alcohol residue R6 at C-17[3] are indicated

quantitatively dominating (primary) series of homologs has been identified as farnesyl esters, the molecular structures of the minor (secondary) homologs series has remained tentative (7).

Aside from being of significance for the understanding of the molecular events of photosynthesis, the particular chemical structure of BChl *e* homologs is of fundamental importance for organic geochemistry. BChl *e* and its degradation products have been employed as molecular markers for green sulfur bacteria and thus permitted to detect past anoxic events in the paleoceans (17, 22).

In the present study, the molecular structure and composition of BChl *e* homologs from five strains of green sulfur bacteria were investigated. Brown-colored members of the green sulfur bacteria were chosen since they dominate in extreme low-light environments (26, 32, 33), contain higher relative amounts of secondary homologs (14), and since less information on homologous structures of BChl *e* is available as compared to the BChl *c* and *d* present in green-colored counterparts. Although some homologs of BChl *e* have already been identified by MS and NMR spectroscopy (10, 30, 38), more recent results indicate the existence of unknown molecular structures (7, 8, 14).

MATERIAL AND METHODS

Origin and cultivation of bacterial strains

Pelodictyon phaeoclathratiforme DSMZ 5477^T, *Chl. phaeobacteroides* strain Dagow III, and *Chlorobium* sp. strain Glubitzsee were grown in the basal medium described by Overmann and Pfennig (31) containing 2.5 mM sulfide and 3 mM acetate as organic carbon source. The two latter strains had been isolated from Lake Dagow and Lake Glubitz located in Eastern Germany (33), respectively. The growth medium for the marine strain *Chl. phaeovibrioides* DSMZ 269^T was amended with 240 mM NaCl and 13.8 mM $MgCl_2$. An enrichment of the phototrophic consortium "*Pelochromatium roseum*" was grown in synthetic freshwater medium (4). Batch cultures of bacteria were incubated at 20°C in continuous light at a light intensity of 20 µmol quanta·m^{-2}·s^{-1} as determined with a LiCor LI-250 quantum meter equipped with a LI-200 SA pyranometer sensor (LI Cor, Lincoln, Neb., USA). Daylight fluorescent tubes (Osram daylight 5000 de luxe, 18 W) served as light source. The enrichment culture of "*Pelochromatium roseum*" was incubated at 15°C in a 12-h light / 12-h dark cycle and a light intensity of 3 µmol quanta·m^{-2}·s^{-1}, because intact consortia did not grow at higher light intensities and under continuous light. Cells were harvested after reaching the stationary growth phase. Additional cultures of *Chl. phaeobacteroides* strain Dagow III were grown at

50 µmol quanta·m^{-2}·s^{-1} (high light conditions) and at 0.5 µmol quanta·m^{-2}·s^{-1} (low light conditions) and harvested during the exponential growth phase.

Pigment extraction

Bacterial cells were harvested by centrifugation for 15 min at 9,500x*g* and at 4°C using Pyrex glass centrifuge tubes. The pellets were lyophilized and stored at -80°C until extraction. Three consecutive extractions of photosynthetic pigments were carried out with HPLC grade acetone/methanol (7/2, v/v; 15 min at 4°C in a sonication bath) and the extracts clarified by centrifugation for 15 min at 9,500x*g*. Since initial experiments revealed that pigments in crude extracts are highly sensitive to light, but not to a short exposure to an oxygen atmosphere, all extraction steps were performed in extremely dim light. The supernatants were combined and concentrated in a rotary evaporator in the dark. Finally, the extracts were transferred to brown glass tubes, dried at room temperature in a stream of nitrogen gas and stored under a nitrogen atmosphere at -80°C until further analysis.

HPLC-DAD and HPLC-MS/MS analysis

Prior to HPLC-MS/MS measurements, the composition of pigment extracts was analyzed with an HPLC system (Sykam, Fürstenfeldbruck, Germany). Pigments were separated by a modification of the method of Borrego and Garcia-Gil (7) using a Nova-Pack C_{18} (4.6x250 mm, 4-µm mesh) HPLC column (Waters, Milford, Mass., USA) and starting with 35% of the methanol/ethylacetate/acetonitrile (50/30/20) solvent. Pigment absorption spectra were recorded between 300 and 800 nm using a diode array spectrophotometer (TIDAS NMC 301, J&M, Aalen, Germany). Samples of bacterial pigments were dissolved in 1 ml acetone/ methanol (7/2, v/v), and 100 µl of 1 M aqueous ammonium acetate solution was added 15 min before injection. Ammonium acetate served as ion-pairing and ionization reagent for HPLC-DAD (diode array detection) and HPLC-MS/MS measurements; its concentration in the HPLC solvents was 10 mM. Bacteriochlorophyll *e* concentrations were quantified employing the molar extinction coefficient determined recently (9).

Mass spectra of photosynthetic pigments were determined employing a second HPLC system (Thermo Separation Products, San Jose, Calif., USA), which was coupled via a flow splitter (split ratio 1:4, with the larger flow feeding the mass spectrometer) to: (1) an ion trap mass spectrometer (Finnigan LCQ, Thermoquest-Finnigan, San Jose, Calif., USA) equipped with an APCI source, and (2) to a UV2000 UV/VIS detector (Thermo Separation Products). Mass spectra were measured in the positive ion mode. The following APCI conditions were chosen: source current, 5 µA; vaporizer temperature, 450°C; temperature of transfer capillary,

250°C. MS/MS experiments were done in the dependent scan mode. For these experiments, helium was used as a collision gas (relative collision energy: 35%). The UV/VIS detector was operated at a fixed wavelength of 450 nm.

Isolation of BChl* e$_F$ *homologs and* ^{1}*H-NMR determination

For the isolation of bacteriochlorophylls, carotenoids were first removed from the crude extract by extraction with HPLC-grade hexane in a separatory funnel. The resulting BChl fraction was dried and redissolved in absolute methanol (HPLC-grade). BChls were separated with a Spherisorb ODS2 semi preparative HPLC column (10x250 mm, 10-µm mesh) as described elsewhere (9). The four farnesyl-esterified BChl *e* homologs (BChl e_F) appeared at a retention time of 27.1, 28.4, 29.5 and 30.5 min and were collected separately. Corresponding fractions of several runs were pooled and dried immediately. Pigments were redissolved in 1 ml of methanol and purified with the Spherisorb column. Finally, aliquots of the purified pigments were analyzed by an analytical HPLC column (Waters Nova-Pak C_{18}, 4–µm mesh, 4.6x250 mm) and DAD to check for contamination by degradation products or traces of other BChl *e* homologs.

For NMR analysis, the purified pigment samples were redissolved in 0.5 ml of CD_3OD. ^{1}H-NMR spectra were obtained at 500 MHz in a Bruker 500 instrument (Bruker, Karsruhe, Germany). Nondeuterated methanol (3.30 ppm) was used as the internal standard. Spectral assignment of proton signals was achieved based on two-dimensional Correlated Spectroscopy (COSY) ^{1}H-NMR experiments. The nature of side chains at positions C-8 and C-12 of the porphyrin ring system were confirmed with Nuclear Overhauser Enhancement Spectroscopy (NOESY) experiments over the –CHO and methine protons at positions 7^1 and 10 (Fig. 1), respectively.

Gas chromatography-mass spectrometry analysis of esterifying alcohols

Bacterial pigments were hydrolyzed in a 1 M KOH solution in HPLC-grade methanol by reflux for 1 h at 65°C under a nitrogen atmosphere. Hydrophobic compounds were extracted in a separatory funnel after addition of one volume of double-distilled water followed by repeated addition (3x) of HPLC-grade dichloromethane. The organic extract was concentrated in a rotary evaporator and dried in a stream of nitrogen gas. Polar compounds were separated from apolar ones with silica-gel columns (silica-gel 100, 60- to 100-Å mesh; Merck, Darmstadt, Germany). Three fractions were obtained with HPLC-grade hexane (10 volumes), hexane/dichloromethane (8/2, v/v; 10 volumes), and 5 volumes ethylacetate followed by 5 volumes methanol.

After evaporation of the solvent, aliquots of samples were dissolved in dichloromethane and transformed into their trimethylsilyl (TMS) ether derivatives using *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (CS Chromatographie Service, Langerwehe, Germany) and an incubation at 60°C for 1 hour. The TMS ethers of fatty alcohols were analyzed by GC-FID (Hewlett Packard HP 5890 Series II gas chromatograph, Hewlett Packard, Waldbronn, Germany) on a DB-5HT column (30 m x 0.25 mm, 0.1-µm film thickness, J&W, Folsom, Calif., USA) and by gas chromatography (GC-MS) using a Finnigan MAT SSQ 710B mass spectrometer.

RESULTS

Separation and detection of BChl e *homologs*

Photosynthetic pigments of brown-colored green sulfur bacteria were separated by reversed-phase HPLC and identified as homologs of BChl *e* based on their characteristic UV/VIS absorption. Depending on the strain, 12 to 16 different peaks of BChl *e* could be distinguished (Fig. 2A). Typically, BChl *e* homologs eluted between 27.1 min and 40 min under the conditions chosen. The last two UV/VIS absorption peaks in the chromatogram were formed by small amounts of bacteriopheophytin (BPh) *e* as identified by the red shift of the long-wavelength (Q_y) absorption band compared to BChl *e*. BChl *a* was identified by its Q_y absorption band and coeluted with BChl *e* homologs in peak 14 at 38.7 min. Peaks of the *trans* and *cis* isomers of isorenieratene were detected at 47.7 min and 48.6 min and those of β-isorenieratene at 50.7 min and 52 min, respectively (not shown in Fig. 2A).

Detection of the total ion current (TIC) by HPLC-MS yielded an elution pattern of BChl *e* homologs very similar to that obtained by HPLC-DAD. Due to the different mode of detection, compounds without UV/VIS absorption could be recognized (Fig. 2B). TIC peaks at 39.3, 40.7, 41.1 and 42 min (Fig. 2B, *triangles*) represented a contamination from the HPLC system since these peaks were also observed in blank runs. Five additional peaks were observed in the TIC (Fig. 2B, *asterisks*) and were present only in extract runs but not in blank runs. These latter peaks were not further characterized. In our study, a total number of 23 BChl *e* homologs were identified based on the masses of their quasi-molecular ions $[M+H]^+$ and their retention times (Tab. 1). Five of the 16 HPLC peaks contained more than one BChl *e* homolog (Tab. 1).

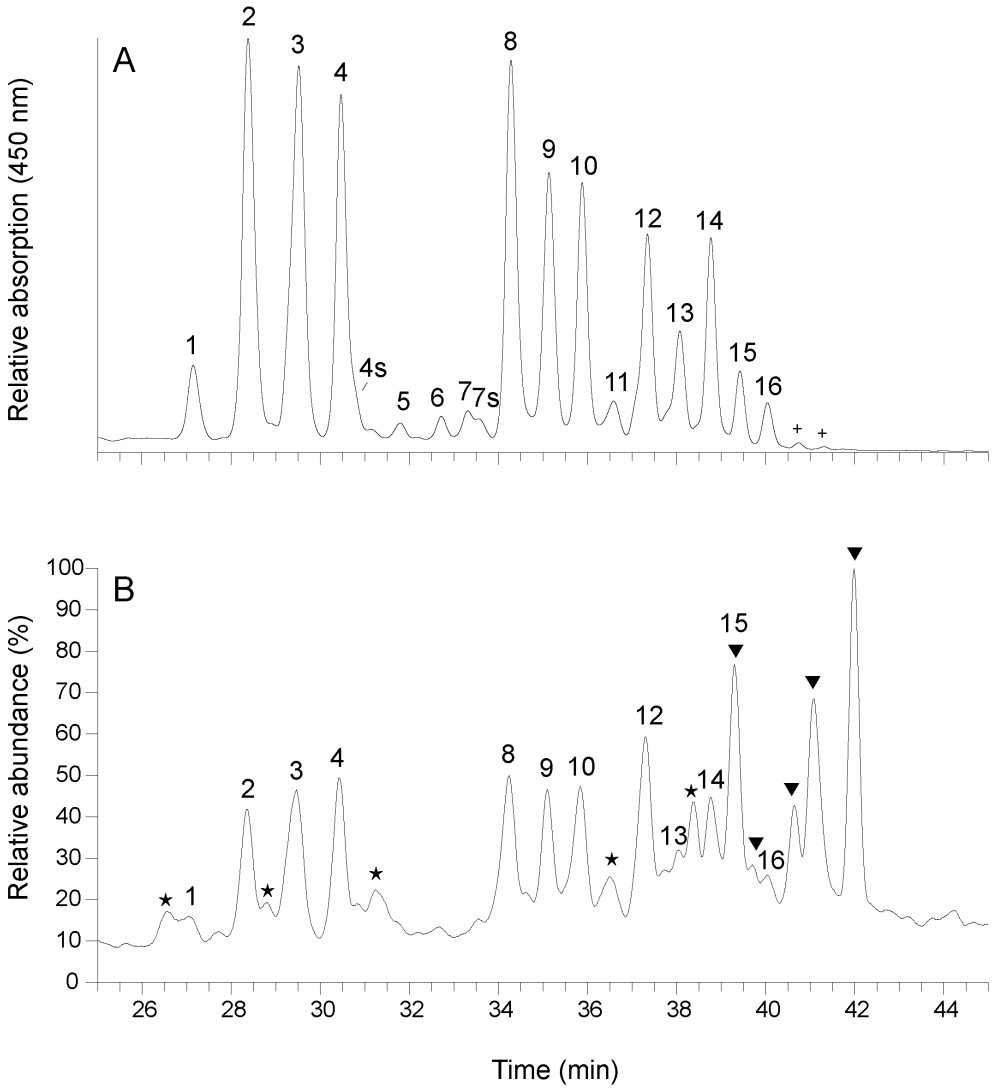


Figure 2. HPLC separation of BChl *e* homologs from *Chl. phaeobacteroides* strain Dagow III. **A.** UV/VIS absorption peaks detected at 450 nm by HPLC-DAD. For exact retention time and peak numbers see Tab. 1. BChl *a* is not visible due to its absorption characteristics and low abundance. **B.** HPLC-MS, detection of total ion current, *m/z* 50-2000, 100% = 5.15 · 10^7 counts. ▼: unidentified compounds derived from HPLC system, ★: unidentified compounds from pigment extracts, + bacteriophaeophytin *e*

MS/MS experiments with BChl e *homologs*

In order to obtain further information on the structure of the BChl *e* homologs, HPLC-MS/MS measurements were carried out. Quasi-molecular ions of the BChl *e* homologs esterified to isoprenoid alcohols generally produced fragments of a high abundance whereas only a small proportion of the quasi-molecular ion remained detectable. As an example, Fig. 3A shows the MS/MS spectrum obtained for peak 1 (compare Fig. 2) which consists of a BChl *e* homolog with farnesol as the esterifying alcohol at position C-17[3]. The quasi-molecular ion of this peak is at *m/z* 807. From this quasi-molecular ion, three fragments with *m/z* 789, 603 and 585 were generated in the MS/MS experiment. The formation of the fragment with *m/z* 789 is caused by the loss of water from the α-hydroxyethyl group at position C-3 of the quasi-molecular ion

Table 1. Identification of BChl *e* homologs and esterified alcohols by MS/MS fragmentation. Novel molecules are highlited. *[M+H]*$^+$; quasi-molecular ion; *M*, Methyl; *E*, Ethyl; *P*, *n*-Propyl; *I*, Isobutyl; *s*, shoulder; *n.d.* = not detected

HPLC peak	Retention (min)	Mass (*m/z*)		Identification		
		$[M+H]^+$	Fragments	[8, 12] BChl *e* homolog	Esterifying alcohol (*m/z*)	
1	**27.1**	**807**	**789, 603, 585**	**[E, M] BChl e_F**	**Farnesol**	**(222)**
2	28.4	821	803, 617, 599	[E, E] BChl e_F	Farnesol	(222)
3	29.5	835	817, 631, 613	[P, E] BChl e_F	Farnesol	(222)
4	30.5	849	831, 645, 627	[I, E] BChl e_F	Farnesol	(222)
4s	**30.8**	**785**	**767, 617, 599**	**[E, E] BChl e_D**	**Dodecanol**	**(186)**
5	**31.8**	**799**	**781, 613**	**[P, E] BChl e_D**	**Dodecanol**	**(186)**
6	**32.6**	**813**	**795, 627**	**[I, E] BChl e_D**	**Dodecanol**	**(186)**
6	**32.7**	**825**	**807, 599**	**[E, E] BChl e_{Pen}**	**Pentadecenol**	**(226)**
7s	**33.6**	**839**	**821, 631, 613**	**[P, E] BChl e_{Pen}**	**Pentadecenol**	**(226)**
7	**33.3**	**825**	**807, 603, 585**	**[E, M] BChl e_{Hen}**	**Hexadecenol**	**(240)**
8	34.3	839	821, 617, 599	[E, E] BChl e_{Hen}	Hexadecenol	(240)
9	35.1	853	835. 631, 613	[P, E] BChl e_{Hen}	Hexadecenol	(240)
10	35.9	867	849, 645, 627	[I, E] BChl e_{Hen}	Hexadecenol	(240)
8	**34.2**	**813**	**795, 599**	**[E, E] BChl e_T**	**Tetradecanol**	**(214)**
9	**35.1**	**827**	**809, 613**	**[P, E] BChl e_T**	**Tetradecanol**	**(214)**
10	**35.8**	**841**	**823, 627**	**[I, E] BChl e_T**	**Tetradecanol**	**(214)**
11	**36.6**	**881**	**n.d.**	**[P, E] BChl e_{Oen}**	**Octadecenol**	**n.d.**
12	37.3	841	823, 617, 599	[E, E] BChl e_{Han}	Hexadecanol	(242)
13	**38.1**	**855**	**837, 631, 613**	**[P, E] BChl e_{Han}**	**Hexadecanol**	**(242)**
14	**38.7**	**869**	**851, 645, 627**	**[I, E] BChl e_{Han}**	**Hexadecanol**	**(242)**
14	**38.7**	**895**	**877, 617, 599**	**[E, E] BChl e_P**	**Phytol**	**(296)**
15	**39.4**	**909**	**891, 613**	**[P, E] BChl e_P**	**Phytol**	**(296)**
16	**40.0**	**923**	**905, 645, 627**	**[I, E] BChl e_P**	**Phytol**	**(296)**

(compare Figs. 1 and 4A). The fragment with *m/z* 603 is most likely formed by a loss of the farnesyl residue at position C-17^3, whereas the fragment with *m/z* of 585 probably represents an additional loss of water from the α-hydroxethyl group.

The remaining other three primary BChl *e* homologs yielded similar MS/MS spectra. The quasi-molecular ions and fragments from peaks 2, 3 and 4 (Fig. 2) differed from those in the spectrum of peak 1 by an *m/z* of 14, 28 and 42, respectively (Tab. 1). The difference in *m/z* between the quasi-molecular ion and fragments arising from the loss of the residue at position C-17^3 was 204 mass units in all four MS/MS spectra. This result confirms the presence of farnesol as the common esterifying alcohol in the four homologous molecules.

The MS/MS spectrum of a BChl *e* homologue esterified to hexadecanol (peak 12, Fig. 2) showed six characteristic fragments with *m/z* 823, 812, 797, 769, 617 and 599 formed from the quasi-molecular ion with *m/z* 841 (Fig. 3B). The fragments with *m/z* 617 and 599 are generated by the loss of hexadecene at position C-17^3 and, in the second case, the additional loss of water. The fragment with *m/z* 812 probably has lost the formyl group at position C-7 and the fragment with *m/z* 797 is probably formed by the loss of the α-hydroxyethyl group at

position C-3. In the fragment with *m/z* 769 both groups at positions C-3 and C-7 were eliminated. A similar fragmentation pattern was observed for BChl *e* homologs with quasi-molecular ions at *m/z* 855 and 869 (peaks 13 and 14). MS/MS spectra similar to those generated from hexadecanyl-esterified BChl *e* were obtained for all homologs esterified to saturated and monounsaturated fatty alcohols.

To distinguish between homologs esterified with different alcohols we used the difference between the *m/z* values of the quasi-molecular ion and those of the fragments devoid of the residue at position C-17[3]. In this way, we determined the mass of the esterifying

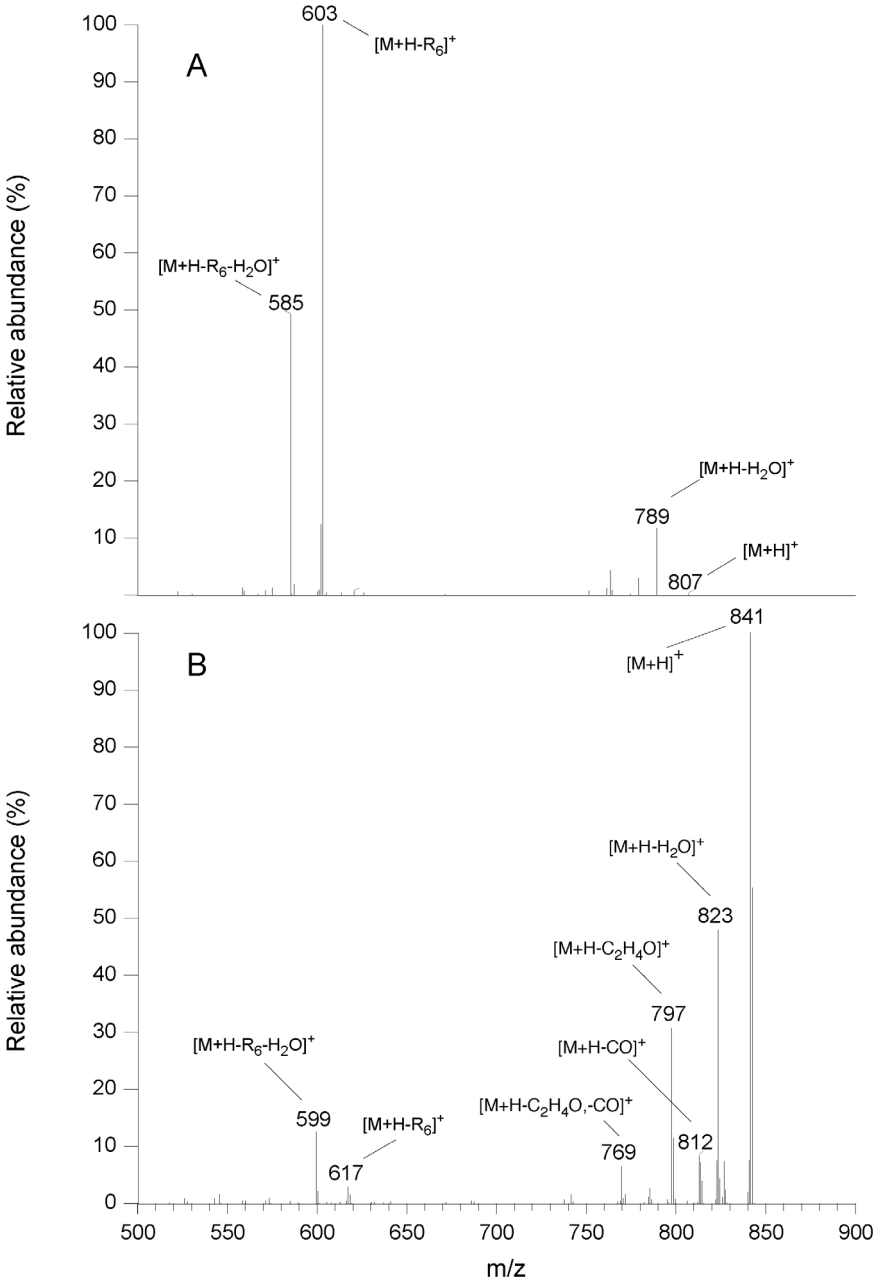


Figure 3. A. MS/MS spectrum of HPLC peak 1 containing [E,M] BChl e_F. R_6 refers to farnesol. **B.** MS/MS spectrum of spectrum of HPLC peak 12 containing [E,E] BChl e_{Han}. R_6 refers to hexdecanol

alcohol as well as the mass of the chlorin ring. The latter also gave information about the length of the alkyl chains at positions C-8 and C-12.

Only the farnesyl- and hexadecenyl-esterified BChl *e* homolog series showed four peaks in the HPLC chromatogram (Fig. 2, Tab. 1). The first HPLC peak of this series of homologs had the lowest abundance within each series (Peaks 1 and 7, Fig. 2). Fragments at *m/z* 585 and 603 in the mass spectra of HPLC peaks 1 and 7 suggested the presence of so far undescribed ethyl, methyl [E,M] homologs of BChl *e*. Because the position of the methyl group remained unclear from the MS/MS experiments, NMR studies were performed (see below).

***Esterifying alcohols of BChl* e**

MS/MS spectra of BChl *e* homologs from the HPLC separation (Fig. 2) revealed a total of 8 different esterifying alcohols. Primary homologs were esterified with farnesol in all strains tested. Similar to the series of the four BChl e_F homologs, all other homolog series eluted in the order of decreasing polarity from the HPLC column (Fig. 2, Tab. 1).

The most abundant compound of the secondary BChl *e* homologs were esterified with hexadecenol, hexadecanol and phytol (peaks 8 to 10 and 12 to 16; Fig. 2, Tab. 1). Minor peaks of secondary BChl *e* homologs were esterified with dodecanol, tetradecanol, pentadecenol and octadecenol (peaks 8 to 10, 4s to 6, 7s and 11; Fig. 2, Tab. 1). Hexadecenol- and tetradecenol-esterified homologs coeluted in peaks 8 to 10. Based on the relative intensities of their quasi-molecular ions, the tetradecenol-esterified homologs represented a minor fraction in those BChl *e* peaks. When the mixture of pigments was hydrolyzed, and the TMS derivatives analyzed by GC-MS, the presence of all esterifying alcohols (with the exception of octadecenol) could be verified (data not shown).

***^{1}H- NMR analysis of Bchl* e**

The four farnesyl-esterified BChl *e* homologs (BChl e_F)were analyzed by NMR to determine the position of the methyl group indicated by the fragments with *m/z* 585 and 603. One-dimensional and two-dimensional COSY ^{1}H-NMR spectra were obtained from HPLC peaks 1 to 4 (Fig. 2) for the structural determination of monomeric BChl *e*. Assignments of proton resonance peaks were made in part by comparison of the spectra with those of BChl *c,* which is chemically similar to BChl *e* (27). In contrast to BChl *c*, BChl *e* contains a formyl group, instead of a methyl group, at position C-7, and has alkyl side chains of variable length in position C-8 (Fig. 1). The substitution at position C-7 was confirmed by the presence of an additional proton signal between 11.31 to 11.17 ppm and the disappearance of the corresponding methyl proton signal of BChl *c* around 3.1 ppm.

However, no data are available in the literature for the specific chemical interactions between C-7[1] –CHO and C-8[1] $–CH_2$. Therefore, a 2D-NOESY experiment using [E,E] BChl e_F (peak 2, Fig. 2), was performed to unambiguously assign the chemical shifts of alkyl substituents in the spectra. From the observed chemical interactions, we conclude that the quartet signals at 4.23 and 4.07 ppm were due to the C-8[1] and C-12[1] groups, respectively (Fig. 4). Both signals showed chemical interaction with the corresponding triplets at 1.84 (C-8[2]) and 1.92 ppm (C-12[2]) (data not shown), thus confirming the presence of two ethyl substituents at positions C-8 and C-12 of the compound represented by peak 2. Proton signals of C-8[1] and C-8[2] were shifted by about 0.43 and 0.10 ppm, respectively, compared to previously reported data from the corresponding BChl c_F homolog (27).

The major differences in the NMR spectra of the compounds corresponding to peaks 1, 2, 3 and 4 of the HPLC chromatogram (Fig. 2) were observed in the 4.3 to 3.5 ppm region of the spectra, indicating a different length of the alkyl side chain at positions C-8 and C-12 (Tab. 2). The ^{1}H-NMR spectrum of peak 3 differed from that of peak 2 with respect to the chemical shifts and multiplicities of C-8[1] and C-8[1], whereas peak 4 produced extra signals at 1.29 and 1.38 ppm (Tab. 2). Chemical coupling between adjacent carbon atoms determined

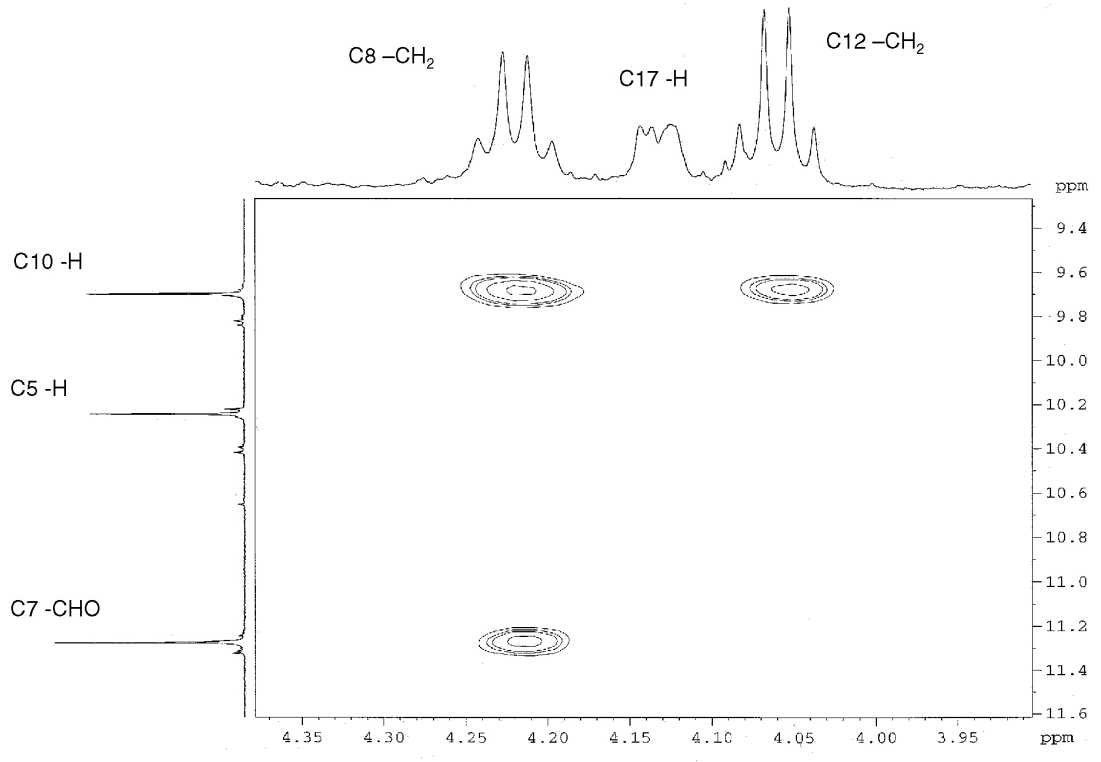


Figure 4. 500 MHz ^{1}H-NMR NOESY spectrum of peak 2 in CD_3OD. Selected ranges are displayed to specifically show the correlations between C-8 $–CH_2$ and C-10 methine, C-8 $–CH_2$ and C-7[1] - CHO, and C-12 $–CH_2$ and C-10 methine groups

Table 2. ^{1}H-NMR signals and assignments of substituents at positions C-8 and C-12 of the macrocycle of BChle_F homologs (peaks **1**, **2**, **3** and **4**. *S* singlet, *d* doublet, *t* triplet, *q* quartet, *m* multiplet)

Peak **1**			Peak **2**			Peak **3**			Peak **4**		
Proton	(ppm)	mult.	proton	(ppm)	mult.	proton	(ppm)	mult.	proton	(ppm)	mult.
$C8^1$ –CH_2	4.21	q	$C8^1$ –CH_2	4.23	q	$C8^1$ –CH_2	4.17	t	$C8^1$ –CH_2	4.11	d
$C8^2$ –CH_3	1.85	t	$C8^2$ –CH_3	1.84	t	$C8^2$ –CH_3	2.25	m	$C8^2$ –CH_3	2.54	m
						$C8^3$ –CH_3	1.29	t	$C8^3$ –2(CH_3)	1.38	d
$C12^1$ –CH_3	3.61	s	$C12^1$ –CH_2	4.07	q	$C12^1$ –CH_2	4.05	q	$C12^1$ –CH_2	4.02	q
			$C12^2$ –CH_3	1.92	t	$C12^2$ –CH_3	1.91	t	$C12^2$ –CH_3	1.9	t

on the basis of 2D-COSY experiments confirmed the presence of *n*-propyl and *iso*-butyl substituents at C-8 (data not shown). Chemical shifts and multiplicities of the C-12^1 and C-12^2 signals were fairly similar for peaks 2, 3 and 4, indicating the presence of an ethyl group in all molecules.

The structure at the C-12 position of peak 1 was shown to be different from the rest of the analyzed molecules as revealed from the absence of C-12^1 and C-12^1 signals at positions 4.07 and 1.92, respectively. Instead, an extra signal was present at 3.61 ppm (singlet) indicating the presence of a methyl group at this position. The changes in the NMR spectra and the molecular masses calculated from the mass spectra of the four fractions confirmed the presence of ethyl, methyl [E,M] BChl e_F, ethyl, ethyl [E,E] BChl e_F, *n*-propyl, ethyl [P,E] BChl e_F and isobutyl, ethyl [I,E] BChl e_F in the HPLC peaks 1 to 4 (Fig. 2), respectively.

BChl* e *homologs and carotenoids in different bacterial strains

In the stationary phase, the five strains of brown-colored green sulfur bacteria exhibited clear differences in the composition of BChl *e* homologs. Homologs esterified to phytol were not detected in *Pelodictyon phaeoclathratiforme* DSMZ 5477^T and in *Chl. phaeovibrioides* DSMZ 269^T. Instead these two strains were the only ones containing homologs esterified to octadecanol (Tab. 3). [E,M] BChl *e* homologs were absent from *Pld. phaeoclatrathiforme* DSMZ 5477^T and from low-light exponential cultures of *Chl. phaeobacteroides* strain Dagow III (Tab. 3).

The amount of primary homologs in stationary phase cultures of all tested strains ranged from 67% to 25.6% (Tab. 3). Whereas 35% of the BChl *e* molecules were esterified to farnesol in stationary phase cultures of *Chl. phaeobacteroides* strain Dagow III, more than 85% of the BChl *e* molecules were esterified to farnesol in exponential cultures irrespective of high- or low-light incubation. Additional differences between exponential and stationary

Table 3. Composition of BChl *e* homologs of five strains of green sulfur bacteria. Only homologs varying between the strains are given. + present; - not detected, *n.a.* not applicable, *Iso* isorenieratene

Type of culture	Exponential cultures		Stationary cultures				
Incubation light intensity (μmol quanta·m^{-2}·s^{-1})	0.5	50	20				3
BChl *e* homolog	*Chorobium phaeobacteroides* strain Dagow III			*Chlorobium sp.* strain Glubitzsee	*Pelodictyon phaeoclathratiforme* DSMZ 5477^{T}	*Chl. phaeovibrioides* DSMZ 269^{T}	"*P. roseum*" Echo Lake 9
[E,M] BChl e_F	-	+	+	+	-	+	+
[E,E] BChl e_D	-	+	+	+	+	+	+
[P,E] BChl e_D	-	+	+	+	+	+	+
[I,E] BChl e_D	-	+	+	+	+	+	+
[E,E] BChl e_{Pen}	-	+	+	+	+	+	+
[P,E] BChl e_{Pen}	-	+	+	+	+	+	+
[E,M] BChl e_{Hen}	-	+	-	+	-	+	+
[P,E] BChl e_{Oen}	-	-	-	-	+	+	-
[E,E] BChl e_P	+	+	+	+	-	-	+
[P,E] BChl e_P	+	+	+	+	-	-	+
[I,E] BChl e_P	+	+	+	+	-	-	+
BChl e_F (% of total BChl *e*)	87.6	85.1	35.0	67.0	40.7	25.6	63.5
Carotenoid/BChl *e* [a]	0.165	0.206	0.341	0.176	0.339	0.377	0.025
(Iso+β-Iso)/BChl *e* [a]	0.141	0.156	0.180	0.147	0.267	0.366	0[b]
Iso/β-Iso	3.94	2.18	2.72	4.93	0.58	12.6	n.a.

[a] Molar ratios

[b] Isorenieratene and β-isorenieratene were not detected in "*P. roseum*"

growth phase cultures of this strain became apparent with respect to the occurrence of minor esterifying alcohols, since homologs esterified to dodecanol and pentadecenol were not detected in cultures grown under low-light conditions (Tab. 3).

Besides the *cis*- and *trans*-isomers of isorenieratene and β-isorenieratene, also *cis*- and *trans*- β-carotene were identified based on their retention times and absorption characteristics. Minor amounts of other, unidentified carotenoids were quantified using a mean of the absorption coefficients of isorenieratene and β-carotene. However, β-carotene represented only a minor fraction of total carotenoids in the free-living strains of green sulfur bacteria. Unexpectedly, the epibiont of the phototrophic consortium "*Pelochromatium roseum*" did not contain detectable amounts of isorenieratene and β-isorenieratene and had a very low overall total carotenoid content.

For all free-living strains of brown-colored green sulfur bacteria, the amount of primary BChl *e* homologs was inversely correlated with the ratio of total carotenoids to BChl *e* ($r^2 = 0.846$, $p < 0.01$).

DISCUSSION

Porphyrin ring structures of BChl e

The light-harvesting bacteriochlorophylls *c*, *d* and *e* of green sulfur bacteria are chlorins which contain only one reduced pyrrole ring and therefore exhibit spectral properties similar to chlorophylls *a* and *b* but different from BChl *a*, *b* and *g* (37). BChl *c*, *d* and *e* occur as mixtures of homologs which differ with regard to their alkyl substituents at positions C-8 and C-12 of the porphyrin ring system. At position C-8, an ethyl, propyl or isobutyl group is present in BChl *c*, *d* and *e* (37). Additionally, a neopentyl group was described for position C-8 of BChl *d* (39). In BChl *c* and *d*, position C-12 carries either a methyl or an ethyl group whereas all BChl *e* homologs identified so far contain an ethyl group (37,38). Besides the three known homologs of BChl *e*, a fourth substitution pattern of the porphyrin ring system has been detected and described as neopentyl, ethyl [N,E] BChl *e* (9, 30). Our results question this earlier assignment since neither our NMR nor our MS/MS data supported the existence of the [N,E] BChl *e* homolog. Instead, a methyl group could be unambiguously assigned to position C-12.

Esterifying alcohols

Besides farnesol as the major esterifying alcohol of bacteriochlorophylls *c*, *d*, or *e*, decanol, 4-undecyl-2-furanmethanol, *cis*-9-hexadecen-1-ol, geranylgeraniol, and phytol have been

found in BChls *c* or *d* (11, 30), whereas 4-undecyl-2-furanmethanol, hexadecanol and 9-octadecenol were described for BChl *e* (30). Recently, BChl *c* homologs from a microbial mat were found to be esterified with an unidentified C_{14} alcohol (1), indicating that additional esterifying alcohols may be present in the chlorosome antenna bacteriochlorophylls. Indeed, our results indicate that several previously unknown alcohols occur in BChl *e*. Of the eight esterifying alcohols identified in the present study, five (dodecanol, hexadecenol, pentadecenol, tetradecanol and phytol) have not been described previously as constituents of BChl *e*. Moreover, dodecanol, pentadecenol and tetradecenol even have never been detected before in bacteriochlorophylls of green sulfur bacteria or of *Chloroflexus aurantiacus*. In contrast, we did not find evidence for the presence of 4-undecyl-2-furanmethanol, which was previously reported to occur in *Chl. phaeovibrioides* (30).

The composition of BChl *c* homologs of *Chloroflexus aurantiacus* and *Chl. tepidum* can be changed experimentally by adding long-chain aliphatic alcohols like hexadecanol, 9-octadecenol, or phytol to growing cultures. These exogenous alcohols are incorporated into BChl and as a result can then constitute up to 66% of the total esterifying alcohols of BChl *c* (24, 41). Even novel BChl homologs (e.g. esterified with decanol) can be obtained in this way. In the present study, however, cultures were grown in the absence of alcohols. Consequently, our data indicate that the novel esterifying alcohols identified in the present study must be synthesized by the cells *de novo*. In this respect, the lack of phytol-esterified BChl *e* homologs in *Pelodictyon phaeoclathratiforme* DSMZ 5477^T and in *Chl. phaeovibrioides* DSMZ 269^T is of particular interest, since both strains contain phytol-esterified BChl *a*. Possibly, different esterases are involved in synthesis of BChl *a* and BChl *e*. The composition of secondary homologs clearly differed between the five strains investigated and in *Chl. phaeobacteroides* strain Dagow III varied with incubation light intensity. These results question the belief that secondary BChl homologs are simply endproducts of aberrant pathways in the pigment biosynthesis by senescent cells (11).

Theoretically, the combination of four different porphyrin ring systems with eight different esterifying alcohols would yield 32 different homologs. Of these, only 23 were detected. It remains to be elucidated whether the remaining 9 homologs cannot be synthesized or whether they occur at concentrations below the detection limit of our method.

Implications for physiology, ecology and biogeochemistry of green sulfur bacteria

Previous work has demonstrated that the composition of farnesyl homologs in cells of *Chl. phaeobacteroides* UdG 6030 and *Chl. phaeovibrioides* UdG 6035 changes with incubation light intensity (8). [E,M] BChl e_F was lost under extreme light limitation (light intensities of

0.5 µmol quanta·m^{-2}·s^{-1}), whereas [I,E] BChl e_F increased concomitantly. In the present study, a similar response for *Chl. phaeobacteroides* strain Dagow III was observed. In addition, our data show that the synthesis of three homologs esterified to dodecanol, two esterified to pentadecanol, and one esterified to hexadecenol also appear to be regulated by light intensity.

Besides light intensity, the physiological state of the organism influences pigment composition. At saturating light intensities (>10 µmol quanta·m^{-2}·s^{-1}, 33), the fraction of total BChl *e* esterified to farnesol decreased significantly in strain Dagow III during the transition to the stationary phase. Similar results have been reported for *Chlorobium limicola* DSM 249 (11). According to the recently proposed structural model of BChl rods (41), bacteriochlorophylls are arranged in a bilayer, with the alcohol tails of bacteriochlorophylls in the inner layer oriented towards the central cavity of the rod. Because of spatial constraints, the structure of esterifying alcohols may therefore influence the stability of the rod structure and, may be of relevance to chlorosome stability especially in non-growing cells.

The side chain at C-12 of porphyrin ring III is directly involved in the aggregation of BChl molecules (43). At decreasing light intensity the abundance of BChl homologs with a higher degree of alkylation increases and the Q_y absorption maximum *in vivo* is shifted by 7-11 nm towards longer wavelengths (6, 8, 21). Possibly, the variation of the alkyl side chains represent a mechanism of low-light adaptation since BChl homologs with red-shifted Q_y bands may facilitate the channeling of excitation energy toward the reaction center, thereby increasing the energy transfer efficiency of the chlorosome (7, 8). The differences in BChl *e* homolog distribution between our strains of green sulfur bacteria may thus reflect differences in the capability of low-light adaptation. In fact, *Pelodicyton phaeoclathratiforme* which is able to grow in dense blooms at very low light intensities (31), did not contain the BChl *e* homolog with the lowest degree of alkylation ([E,M] BChl e_F).

Initially, the carotenoids of brown-colored green sulfur bacteria were thought to function as light-harvesting antenna pigments and thereby provide a selective advantage under low-light conditions and in green underwater light, which prevails in their natural habitats (26, 32). More recent evidence for an inefficient transfer of singlet energy from carotenoids to BChl (12) suggests, however, that BChl *e* itself functions as the main light-harvesting pigment also under natural conditions. Our newly isolated brown *Chlorobium* strains turned out to contain significantly less carotenoids than the other strains investigated so far. The symbiotic green sulfur bacterial epibionts of "*Pelochromatium roseum*" yielded the most unexpected experimental result. Despite the adaptation of "*Pelochromatium roseum*" to low-light conditions and the dominance of these bacterial associations in various natural low-light

environments (33), isorenieratene and β-isorenieratene could not be detected, and the cells contained only very few carotenoids per BChl *e*. Obviously, carotenoids cannot be significant for light-harvesting by the epibionts of these phototrophic consortia.

Carotenoids provide protection against photobleaching of bacteriochlorophylls by quenching the BChl triplet states in chlorosomes. Experimental evidence indicates that carotenoids mainly interact with BChl *a* in the chlorosome baseplate (2). The molar ratio of carotenoids to BChl *e* in chlorosomes (0.2 in whole cells; 9) appears to be higher than required for the quenching of BChl *a* triplets in the baseplate; laboratory strains of *Chl. phaeobacteroides* in which carotenoid synthesis is inhibited by 2-hydroxybiphenyl contain 0.065 carotenoids per BChl *e* (2). Epibionts of "*Pelochromatium roseum*" contain significantly less carotenoids per BChl *e* (0.025). Therefore, the epibionts of "*Pelochromatium roseum*" represent excellent model systems for biophysical studies of the effect of low carotenoid content on chlorosome function without the undesirable side effects caused by the inhibitor (3).

Because of the nonrandom distribution in chlorosomes it has been suggested that carotenoids increase the stability of the chlorosome baseplate (2, 3). Since cells with a low carotenoid content contained a high fraction of BChl e_F homologs, it may be speculated that chlorosome stability also depends on BChl *e* homolog composition. Again, epibionts of "*Pelochromatium roseum*" with their unusual pigment composition represent an exception to this rule; future studies of chlorosome structure and function should focus on this type of green sulfur bacteria.

Biomarkers of green sulfur bacteria, especially those of the brown-colored forms, have been used to detect anoxic states of ancient water bodies, like the Pliocene Eastern Mediterranean Sea (23, 34). Due to the high specific pigment content of green sulfur bacteria, their bacteriochlorophylls are abundant in illuminated chemoclines of freshwater lakes and sediments. Besides the carotenoids of green sulfur bacteria (18, 23, 34), BChl *c*, *d* and *e*, and oxidation products of their porphyrin ring systems, namely methyl *iso*-butyl maleimide and methyl *n*-propyl maleimide, have been employed as paleoindicators (16, 17). So far, however, the esterifying alcohols of BChl *c*, *d* and *e*, have not been used extensively in geochemical studies. These long-chain alcohols are not specific for green sulfur bacteria but can also originate from other organisms. While stable carbon isotope analyses indicate that all farnesane (derived from farnesol by catalytic hydrogenation with PtO_2) which is present in marginal sediments of the Black Sea originated from green sulfur bacteria (20), these bacteria contributed only a minor fraction of the entire farnesane pool in Permian Kupferschiefer,

which was deposited under anoxic conditions (17). Identification of long-chain alcohols in natural samples thus has to be complemented by analyses of their stable carbon isotope signatures. In this respect, the fatty alcohols of secondary bacteriochlorophyll homologs of green sulfur bacteria represent additional biomarkers, which can serve as valuable controls. The identification of BChl *e* homologs in the present study provides a more solid basis to deduce the presence of green sulfur bacteria in paleoceans and to investigate their *in situ* physiology in extant ecosystems.

REFERENCES

1. **Airs R.L., Atkinson J.E. and Keely B.J.** (2001) Development and application of a high resolution liquid chromatographic method for the analysis of complex pigment distributions. J. Chrom. A **917:**167-177
2. **Arellano J.B., Melø T.B., Borrego C.M., Garcia-Gil L.J. and Naqvi K.R.** (2000) Nanosecond laser photolysis studies of chlorosomes and artificial aggregates containing bacteriochlorophyll *e*: Evidence for the proximity of carotenoids and bacteriochlorophyll *a* in chlorosomes from *Chlorobium phaeobacteroides* CL1401. Photochem. Photobiol. **72:**669-675
3. **Arellano J.B., Borrego C.M., Martínez-Planells A. and Garcia-Gil L.J.** (2001) Effect of carotenoid deficiency on cells and chlorosomes of *Chlorobium phaeobacteroides*. Arch. Microbiol. **175:**226-233
4. **Bartscht K., Cypionka H. and Overmann J.** (1999) Evaluation of cell activity and methods for the cultivation of bacteria from a natural lake community. FEMS Microbiol. Ecol. **28:**249-259
5. **Blankenship R.E., Olson J.M. and Miller M.** (1995) Antenna complexes from green photosynthetic bacteria, pp. 399-435. *In*: Blankenship R.E., Madigan M.T. and Bauer C.E. (eds.), Anoxygenic photosynthetic bacteria, Advances in Photosynthesis, Vol. II. Kluwer Academic Publishers, Dordrecht, The Netherlands
6. **Bobe F.W., Pfennig N., Swansson K.L. and Smith K.M.** (1990) Red shift of the absorption maxima in Chlorobiineae through enzymic methylation of their antenna bacteriochlroophylls. Biochemistry **29:**4340-4348
7. **Borrego C.M. and García-Gil L.J.** (1994) Separation of bacteriochlorophyll homologs from green phototrophic sulfur bacteria by reversed-phase HPLC. Photosynth. Res. **41:**157-163
8. **Borrego C.M. and Garcia-Gil L.J**. (1995) Rearrangement of light harvesting bacteriochlorophyll homologues as a response of green sulfur bacteria to low light intensities. Photosynth. Res. **41:**53-65
9. **Borrego C.M., Arellano J.B., Abella C.A., Gillbro T. and Garcia-Gil L.J.** (1999) The molar extinction coefficient of bacteriochlorophyll *e* and the pigment stoichiometry in *Chlorobium phaeobacteroides*. Photosynth. Res. **60:**257-264
10. **Brockmann Jr. H.** (1976) Bacteriochlorophyll *e*: structure and stereochemistry of a new type of chlorophyll from Chlorobiaceae. Phil. Trans. R. Soc. Lond. B **273:**277-285

11. **Caple B.C., Chow H. and Strouse C.E.** (1978) Photosynthetic pigments of green sulfur bacteria. J. Biol. Chem. **253:**6730-6737
12. **Cox R.P., Miller M., Aschenbrücker J., Ma Y.Z. and Gillbro T.** (1998) The role of bacteriochlorophyll *e* and carotenoids in light harvesting in brown-colored green sulfur bacteria, pp. 149-152. *In*: Garab G. (ed.), Photosynthesis: mechanism and effects, vol. I. Kluwer, Dordrecht, The Netherlands
13. **Fowler C.F., Nugent N.A. and Fuller R.C.** (1971) The isolation and characterization of a photochemically active complex from *Chloropseudomonas ethylica*. Proc. Natl. Acad. Sci. USA **68:**2278-2282
14. **Francke C. and Amesz J.** (1997) Isolation and pigment composition of the antenna system of four species of green sulfur bacteria. Photosynth. Res. **52:**137-146
15. **Frigaard N.U., Takaichi S., Hirota M., Shimada K. and Matsuura K.** (1997) Quinones in chlorosomes of green sulfur bacteria and their role in the redox-dependent fluorescence studied in chlorosome-like bacteriochlorophyll *c* aggregates. Arch. Microbiol. **167:**343-349
16. **Grice K., Gibbison R., Atkinson J.E., Schwark L., Eckhardt C.B. and Maxwell J.R.** (1996) Maleimides (1*H*-pyrolle-2,5-diones) as molecular indicators of anoxygenic photosynthesis in ancient water columns. Geochim. Cosmochim. Acta **60:**3913-3924
17. **Grice K., Schaeffer P., Schwark L. and Maxwell J.R.** (1997) Changes in palaeoenvironmental conditions during deposition of the Permian Kupferschiefer (Lower Rhine Basin, northwest Germany) inferred from molecular and isotopic compositions of biomarker components. Org. Geochem. **26:**677-690
18. **Hartgers W.A., Sinninghe Damsté J.S., Requejo A.G., Allan J., Hyes J.M. and de Leeuw J.W.** (1994) Evidence for only minor contributions from bacteria to sedimentary organic carbon. Nature **369:**224-226
19. **Holt A.S., Purdie J.W. and Wasley J.W.F.** (1966) Structures of *Chlorobium* chlorophylls (66). Can. J. Chem. **44:**88-93
20. **Huang Y., Freeman K.H., Wilkin R.T., Arthur M.A. and Jones A.D.** (2000) Black Sea chemocline oscillations during the Holocene: molecular and isotopic studies of marginal sediments. Org. Geochem. **31:**1525-1531
21. **Huster M. and Smith K.M.** (1990) Biosynthetic studies of substituents homologation in Bacteriochlorophylls *c* and *d*. Biochemistry **29:**4348-4355

22. **Keely B.J., Blake S.R., Schaeffer P. and Maxwell J.R.** (1995) Distributions of pigments in the organic matter of marls from the Vena del Gesso evaporitic sequence. Org Geochem **23:** 527-539

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

24. **Larsen K.L., Miller M. and Cox R.P.** (1995) Incorporation of exogenous long-chain alcohols into bacteriochlorophyll *c* homologs by *Chloroflexus aurantiacus*. Arch. Microbiol. **163:**119-123

25. **Matthews B.W., Fenna R.E., Bolognesi M.C., Schmid M.F. and Olson J.M.** (1979) Structure of a bacteriochlorophyll *a*-protein from the green photosynthetic bacterium *Prosthecochloris aestuarii*. J. Mol. Biol. **131:**259-285

26. **Montesinos E., Guerrero R., Abella C. and 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

27. **Nozawa T., Ohtomo K., Suzuki M., Morishita Y. and Madigan M.T.** (1991) Structures of bacteriochlorophylls *c* in chlorosomes from a new thermophilic bacterium *Chlorobium tepidum*. Chemistry Letters, pp. 1763-1766

28. **Oelze J. and Golecki J.R.** (1995) Membranes and chlorosomes of green bacteria: structure, composition and development, pp. 231-257. *In*: Blankenship R.E., Madigan M.T. and Bauer C.E. (eds.), Anoxygenic photosynthetic bacteria, Advances in Photosynthesis, Vol. II. Kluwer Academic Publishers, Dordrecht, The Netherlands

29. **Olson J.M.** (1998) Chlorosome organization and function in green photosynthetic bacteria. Photochem. Photobiol. **67:**61-75

30. **Otte S.C.M., van de Meen E.J., van Veelen P.A., Pundsnes A.S. and Amesz J.** (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

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

32. **Overmann J., Cypionka H. and Pfennig N.** (1992) An extremely low light adapted phototrophic sulfur bacterium from the Black Sea. Limnol. Oceanog. **32:**150-155

33. **Overmann J., Tuschak C., Fröstl J.M. and Sass H.** (1998) The ecological niche of the consortium "*Pelochromatium roseum*". Arch. Microbiol. **169:**120-128
34. **Passier H.F., Bosch H.-J., Nijenhuis L.J.L., Böttcher M.E., Leenders A., Sinninghe Damste J.S., de Lange G.J. and de Leeuw J.W.** (1999) Sulphidic mediterranean surface waters during pliocene sapropel formation. Nature **397:**146-149
35. **Sakuragi Y., Frigard N.-U., Shimada K. and Matsuura K.** (1999) Association of bacteriochlorophyll *a* with the CsmA protein in chlorosomes of the photosynthetic green filamentous bacterium *Chloroflexus aurantiacus*. Biochim. Biophys. Acta **1413:**172-180
36. **Schmidt K.** (1980) A comparative study on the composition of chlorosomes (*Chlorobium* vesicles) and cytoplasmatic membranes from *Chloroflexus aurantiacus* strain Ok-70-fl and *Chlorobium limicola* f. *thiosulfatophilum* strain 6230. Arch. Microbiol. **124:**21-31
37. **Senge M.O. and Smith K.M.** (1995) Biosynthesis and structures of the bacteriochlorophylls, pp. 137-151. *In*: Blankenship R.E., Madigan M.T. and Bauer C.E. (eds.), Anoxygenic photosynthetic bacteria, Advances in Photosynthesis, Vol. II. Kluwer Academic Publishers, Dordrecht, The Netherlands
38. **Simpson D.J. and Smith K.M.** (1988) Structures and transformation of the bacteriochlorophylls *e* and their bacteriopheophorbides. J. Am. Chem. Soc. **110:**1753-1758
39. **Smith K.M., Goff D.A., Fajer J. and Barkigia K.M.** (1983) Isolation and characterization of two new bacteriochlorophyll *d* bearing neopentyl substituents. J. Am. Chem. Soc. **105:**1674-1676
40. **Steensgaard D.B., Cox R.P. and Miller M.** (1996) Manipulation of the bacteriochlorophyll *c* homolog distribution in the green sulfur bacterium *Chlorobium tepidum*. Photosynth. Res. **38:**385-393
41. **Steensgaard D.B., Wackerbarth H., Hildebrandt P., Holzwarth A.R.** (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
42. **van Gemerden H. and Mas J.** (1995) Ecology of phototrophic sulfur bacteria, pp. 49-85. *In*: Blankenship R.E., Madigan M.T. and Bauer C.E. (eds.), Anoxygenic photosynthetic bacteria, Advances in Photosynthesis, Vol. II. Kluwer Academic Publishers, Dordrecht, The Netherlands
43. **van Rossum B.-J., Steensgard D.B., Mulder F.M., Boender G.J., Schaffner K., Holzwarth A.R. and de Groot H.J.M.** (2001) A refinded 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 corelation spectroscopy. Biochemistry **40:**1587-1595

Chapter 3

Characterization and *in situ* carbon metabolism of phototrophic consortia

INTRODUCTION

Phototrophic consortia are unique, highly structured aggregates consisting of colorless bacteria and green sulfur bacteria (34, 37, 41, 44, 52). In motile phototrophic consortia, numerous green sulfur bacteria (so-called epibionts) are attached around a single, flagellated, colorless cell of a member of the β subclass of the Proteobacteria, thereby forming a barrel-shaped highly regular structure (14, 15, 37). Motile phototrophic consortia were first described in the early 20th century (7, 27) and since then have been detected in numerous stratified freshwater lakes and ponds worldwide (40). To date, seven different types of motile phototrophic consortia have been distinguished on the basis of color, size, and shape and on the basis of the number and morphology of the epibionts (41).

Phototrophic consortia can contribute up to two-thirds of the total bacterial biomass in the chemoclines of stagnant water bodies (17) and therefore are significant for the biogeochemical cycles in these environments (40). However, epibionts of motile phototrophic consortia represent unique phylotypes among the green sulfur bacteria (15) and therefore may differ in physiology from their free-living relatives. Intact consortia of the "*Chlorochromatium aggregatum*" type depend on 2-oxoglutarate for growth and exhibit chemotaxis towards this compound (14). The epibionts are capable of using hydrogen sulfide as an electron donor in the light, as shown by reduction of the alternative electron acceptor

carbonyl cyanide *m*-chloro-phenylhydrazone, and therefore are likely to grow lithotrophically within the association (14).

However, *in situ* measurements for a natural community of the phototrophic consortium "*Pelochromatium roseum*" revealed that the vertical diffusive flux of hydrogen sulfide was not sufficient to explain the large biomass of consortia present in the chemocline (40). Sulfide could be produced within the phototrophic consortium population, either by the central bacterium in each consortium or by accompanying bacteria outside the consortium (40). However, since the cell yield of green sulfur bacteria is significantly increased by assimilation of organic compounds (36), it is also possible that the epibionts assimilate naturally occurring organic carbon substrates and hence grow mixotrophically *in situ*. As a prerequisite for understanding the physiological basis of cell-cell interactions and the biogeochemical significance of phototrophic consortia, the carbon metabolism of the epibionts therefore needs to be elucidated.

All known green sulfur bacteria are obligate photolithoautotrophs and employ the reversed citric acid cycle for CO_2 fixation (58). Because different enzymes are involved in the primary steps of CO_2 fixation, the fractionation of stable carbon isotopes in the cell material of green sulfur bacteria differs significantly from that in other autotrophs, which employ the Calvin cycle (47, 59). In general, the *in situ* physiology of free-living green sulfur bacteria in terms of carbon metabolism has been studied only once by using the stable carbon isotope approach (16); however, mixed microbial communities were used in this study. In the present study, we selected an ecosystem dominated by the phototrophic consortium "*P. roseum*" and investigated its *in situ* carbon metabolism by identifying suitable biomarkers specific for the green sulfur bacterial epibionts and determining their stable carbon isotope ratios ($\delta^{13}C$).

MATERIAL AND METHODS

Study site and sampling procedures

Lake Dagow (area, 0.24 km^2 ; maximum depth, 9.5 m), is a eutrophic lake located approximately 100 km north of Berlin, Germany (9). Each year, an anoxic, sulfide-containing hypolimnion develops after the onset of summer stratification (May until September). The lake was visited on 15 July 1994, 2 to 9 August 1995, 4 to 15 August 1997, 2 to 7 July 1998, and 19 and 21 July 1999. On these dates, communities of anoxygenic phototrophic bacteria had developed at the oxic-anoxic boundary layer (the chemocline).

Water samples were obtained at a location in the deepest part of the lake by using a bilge pump connected to gas-tight isoversinic tubing. The inlet consisted of two polyvinyl

chloride cones that were 1 cm apart (23). This device allowed reproducible sampling of different water layers in 5-cm intervals.

Physical and chemical parameters

Underwater irradiance was determined with an LI-1000 spherical quantum sensor (wavelength range, 400 to 700 nm; Li Cor, Lincoln, Nebr., USA) connected to a data logger. Conductivity and temperature were measured with an LF 196 conductivity meter (WTW, Weilheim, Germany), pH was measured with a pH/T electrode connected to a 196T microprocessor pH meter (WTW), and oxygen concentrations were measured with an Oxi 196 oximeter (WTW).

Sulfide contents were determined by the methylene blue method (10). Contact with atmospheric oxygen was avoided, and aliquots of lake water were directly pumped into glass vials containing zinc acetate in order to precipitate and preserve sulfide.

For separation of particulate organic carbon (POC), dissolved organic carbon (DOC), and dissolved inorganic carbon (DIC), chemocline water samples were filtered through precombusted (450°C, 5 h) GF/F glass fiber filters (nominal pore size, 0.7 μm; Whatman, Göttingen, Germany) to collect the POC. The filtrate was used for DOC measurement, and it was collected in acid-washed glass bottles and subsequently lyophilized. POC and DOC samples were stored at -20°C until further processing occurred. After removal of carbonates by acidification with 0.1 M hydrochloric acid, organic carbon concentrations in the samples were determined by combustion by using a Ströhlein UCI Coulomat 702 (JUWE, Korschenbroich, Germany).

Glass serum bottles were used for collection of DIC samples. Prior to sampling, the bottles were cleaned with acid, sealed with butyl rubber stoppers, evacuated, and filled with nitrogen gas. Approximately 20 to 30 ml of a water sample was injected into each bottle, and the bottles were stored at -20°C until processing occurred.

Cell counting

Numbers of bacteria were determined by epifluorescence microscopy of black polycarbonate membrane filters (pore size, 0.2 μm; Isopore GTBP membranes; Millipore, Eschborn, Germany) after staining with 4′,6-diamidino-2-phenylindole (DAPI) (45). In order to quantify the epibionts of phototrophic consortia, the consortia had to be disaggregated prior to counting. Therefore, glutardialdehyde fixation of the samples was omitted and natural samples were directly stained with DAPI at a final concentration of 0.1 $\mu g \cdot ml^{-1}$ (63). During deposition on a membrane filter, the phototrophic consortia disassembled, but the epibionts

remained arranged in concentric rings around each central bacterium. For samples containing low numbers of phototrophic consortia, a total of 100 microscopic fields were examined. In these cases, the number of consortia (X) per milliliter and the corresponding standard deviation (SD) were calculated from the ratio (p) of microscopic fields containing phototrophic consortia to the total number of fields by employing a most-probable-number formula (8):

$$X = -\frac{F}{V}\ln(1-p) \quad (1)$$

$$S.D.(X) = \frac{F}{V}\sqrt{\frac{p}{n(1-p)}} \quad (2)$$

where, F is the ratio of the total filter area to the area of the microscopic field, n is the total number of fields and V is the volume of sample filtered (in milliliter).

Fluorescence* in situ *hybridization

In order to determine the ratio of free-living green sulfur bacteria to green sulfur bacterial epibionts of phototrophic consortia, fluorescence in situ hybridization on black polycarbonate filters (pore size, 0.2 μm; Isopore GTBP membranes; Millipore) was used. The oligo-deoxynucleotide probe S-F-GSB-532-a-A-15 (63) labeled with Cy3 (Interactiva Biotechnologie, Ulm, Germany) was employed. Prior to filtration of water samples, each filter was rinsed with 5 ml of acetone (analytical grade) and 5 ml of particle-free distilled water to reduce the background fluorescence. Depending on the density of the phototrophic consortia, 0.4- to 4-ml water samples were filtered, and fluorescence *in situ* hybridization was carried out as described previously (63). Cells were counterstained with DAPI (final concentration, 0.2 $\mu g \cdot ml^{-1}$), and the filters were embedded in antifading mounting fluid (10 ml of glycerol, 10 ml of 10x SET [1.5 M NaCl, 10 mM EDTA, 200 mM Tris-HCl; pH 7.8]) for microscopic examination. Epifluorescence microscopy was performed with a Zeiss Axiolab epifluorescence microscope equipped with a no. 1 filter set for DAPI and an HQ-Cy3 filter set (AHF Analysentechnik, Tübingen, Germany) for Cy3 epifluorescence.

16S rRNA gene sequences of epibionts

Phototrophic consortia were separated from the chemocline microbial community by using a micromanipulator and an inverted microscope (13, 14). Batches of 5 to 40 consortia or 50 ng of genomic DNA extracted from the complete microbial community was used to amplify 16S

rRNA gene fragments; primers GC 357f and GSB 840r and the PCR conditions described previously (32) were used. Water samples were concentrated by centrifugation, and genomic DNA was extracted from pellets as described previously (39). Amplification products were separated on the basis of melting behavior by denaturing gradient gel electrophoresis (DGGE) by using a gradient consisting of 35 to 70% denaturing agents (31). The resulting DNA fragments were excised from the gel, recovered by electroelution, reamplified, and sequenced as described previously (32).

Phylogenetic analysis of 16S rRNA gene sequences of epibionts and environmental sequences was performed by using the ARB phylogeny package (28). The program Fast Aligner V1.03 was used for alignment of all complete 16S rRNA gene sequences of green sulfur bacteria available through the National Center for Biotechnology Information website (1). The sequence of *Chloroherpeton thalassium* ATCC 35110 was used as the outgroup. The alignment was manually corrected based on secondary structure information, and a phylogenetic tree was constructed by using the maximum-likelihood program DNA_ML. Partial 16S rRNA gene sequences of epibionts were then inserted into the phylogenetic tree without changing the tree topology by employing the Parsimony Interactive tool.

Photosynthetic CO_2 assimilation

Photosynthetic CO_2 fixation was measured by using an incubation rack which allowed placement of incubation tubes 5 cm apart (vertical distance) but prevented shading of tubes by other tubes (35). The light and dark incubation tubes were filled with water samples from the corresponding depths. Subsequently, 3-(3,4-dichlorophenyl)-1,1-dimethylurea was added to a final concentration of 20 μM to inhibit oxygenic photosynthesis, and each tube was spiked with 150 kBq of an $NaH^{14}CO_3$ solution. Incubation lasted for 6 h. Immediately after the incubation rack was retrieved, 1 ml of each sample was pipetted into 10 ml of scintillation solution (Aquasafe 300; Canberra-Packard, Dreieich, Germany) for determination of the total radioactivity. After this, samples were filtered through 0.2-μm-pore-size membrane filters (cellulose nitrate; diameter, 25 mm; Schleicher and Schuell, Dassel, Germany), and the filters were placed in glass vials containing 5 drops of 1 M HCl. After 30 min, the vials were opened in a fume hood, the filters were dried in air, and 10 ml of scintillation solution was added to each vial. All samples were counted with a Packard 1600TR scintillation counter. To calculate photosynthetic rates, the CO_2 assimilation values of dark controls were subtracted.

Analysis of photosynthetic pigments

In 1994 to 1998, photosynthetic pigments were quantified after collection of particulate matter on 0.2-µm-pore-size polycarbonate filters and extraction in 99.5% acetone. Bacteriochlorophyll (BChl) *a* was quantified as described by Steenbergen et al. (60). Dichromatic equations (38) were used for quantification of BChl *d* and *e* since some samples also contained chlorophyll (Chl) *a*.

A detailed analysis of the composition of BChl homologs and different carotenoids was performed with chemocline samples obtained in 1999. Pigments were analyzed by high-performance liquid chromatography (HPLC) (Sykam, Fürstenfeldbruck, Germany) after extraction in methanol by using the method of Borrego and Garcia-Gil (6) as modified by Glaeser et al. (18) and a Nova-Pack C_{18} HPLC column (4.6 by 250 mm; 4-µm mesh; Waters, Milford, Mass., USA) together with a C_{18} ODS-2 guard column (4 by 40 mm; 4-µm mesh; Waters). Absorption spectra were recorded between 300 and 800 nm with a diode array spectrophotometer (TIDAS NMC 301; J&M, Aalen, Germany), and BChl *e* concentrations were calculated by using the molar extinction coefficient determined recently (5).

For identification and calibration of the separated pigments, we employed extracts of pure cultures of brown- and green-colored green sulfur bacteria. For identification of different BChl homologs, mass spectra were recorded with an HPLC-mass spectrometry (MS) system (Thermo Separation Products, San Jose, Calif., USA) (18). The HPLC was coupled via a flow splitter (split ratio, 1:4, with the larger flow feeding the mass spectrometer) to an ion trap mass spectrometer (Finnigan LCQ; Thermoquest-Finnigan, San Jose, Calif., USA) equipped with an atmospheric pressure chemical ionization source and to a UV2000 UV/visible light detector (Thermo Separation Products). Mass spectra were measured in the positive ion mode. The following atmospheric pressure chemical ionization conditions were used: source current, 5 µA; vaporizer temperature, 450°C; and temperature of the transfer capillary, 250°C. MS-MS experiments were done in the dependent scan mode. For these experiments, helium was used as the collision gas (relative collision energy, 35%). The UV/VIS detector was operated at a fixed wavelength of 450 nm.

$\delta^{13}C$

Large quantities of photosynthetic pigments were required to determine stable carbon isotope fractionation values of individual BChl homologs or carotenoids. Particulate matter from 20 liters of chemocline water was harvested by centrifugation (15 min at 13,500x*g* at 4°C), and the pellets were pooled, lyophilized and stored at -80°C until extraction. Photosynthetic pigments were recovered by consecutive extraction (15 min at 4°C with sonication) with

methanol, methanol-dichloromethane, and dichloromethane (18). Supernatants were pooled, concentrated with a rotary evaporator, transferred to brown glass tubes, dried at room temperature in a stream of nitrogen gas, and stored at -80°C until they were used. All extraction steps were performed in dim light, and pesticide residue analysis-grade solvents were used.

Prior to analysis, carotenoids in the bulk extracts were completely hydrogenated by a 1-h pretreatment in ethyl acetate by using hydrogen gas as the electron donor and platinum oxide (PtO_2) as the catalyst. A few drops of glacial acetic acid were added to the solvent to facilitate saturation of double bounds. The PtO_2 was removed by centrifugation and subsequent filtration of the solvent through extracted cotton wool.

Solutions of the esterifying alcohols in dichloromethane were then transesterified into their trimethylsilyl (TMS) ester derivatives in dichloromethane after addition of N-methyl-N-trimethylsilyl-trifluoroacetamide (CS Chromatographie Service, Langerwehe, Germany) and incubation at 70°C for 1 h. The TMS esters were first identified by injection of 1- to 2-µl samples into a gas chromatograph (GC)-flame ionization detector system (HP 5890 Series II GC; Hewlett-Packard, Waldbronn, Germany) equipped with a DB-5 fused silica column (30 m by 0.25 mm; film thickness, 0.1 µm; J&W, Folsom, Calif., USA) and a temperature-programmable injector (KAS 3; Gerstel, Mühlheim an der Ruhr, Germany). The temperature of the GC oven was programmed to increase from 60°C (1 min isothermal) to 305°C (50 min isothermal) at a rate of $3°C \cdot min^{-1}$. The final temperature of the GC oven was set at 320°C to measure hydrogenated carotenoids. Helium was used as the carrier gas at a linear flux of $22.7\ cm \cdot s^{-1}$.

GC-MS and GC-isotope ratio monitoring (irm) MS were performed as described above by using a DB-5MS column for GC-MS and a DB-5HT column for GC-irm MS. Mass spectra were obtained with a Finnigan MAT SSQ 710B mass spectrometer (Finnigan-Thermoquest, San Jose, Calif., USA) operated at 70 eV with a scan range of *m/z* 50 to 650 and a scan rate of $1\ scan \cdot s^{-1}$. $\delta^{13}C$ values were determined with an irm MS system equipped with a Cu-Ni-Pt oxidation reactor by using O_2 for in-line oxidation of the alcohols to CO_2 at 940°C, a Cu reduction reactor operated at 600°C, and an irm mass spectrometer (MAT 252; Finnigan-Thermoquest). Water was trapped in line by using a Nafion membrane. Ratios of *m/z* 45, 46, and 47 were monitored, and $\delta^{13}C$ values were calibrated with a CO_2 standard at the beginning and end of each sample. Squalane was used as the internal standard. In all cases duplicate samples were analyzed, and $\delta^{13}C$ values (per mille) were calculated as follows (51):

$$\delta^{13}C[‰] = [\frac{(^{13}C/^{12}C_{Sample})}{(^{13}C/^{12}C_{Standard})} - 1] \cdot 1000 \quad (3)$$

All values are given relative to the $\delta^{13}C$ of PeeDee belemnite (51). The $\delta^{13}C$ values of fatty alcohols and fatty acids were corrected for the isotopic contribution of theTMS group using the following equation (46):

$$\delta^{13}C_{RO-TMS} = x \cdot \delta^{13}C_{ROH} + (1-x) \cdot \delta^{13}C_{TMS} \quad (4)$$

where *x* is the ratio of carbon atoms present in the free alcohol to the number of carbon atoms present in its TMS derivative, $\delta^{13}C_{ROH}$ is the $\delta^{13}C$ of the free alcohol, $\delta^{13}C_{RO\text{-}TMS}$ the $\delta^{13}C$ of the TMS derivative of the alcohol, and $\delta^{13}C_{TMS}$ the $\delta^{13}C$ of the TMS group as determined by the measurement of farnesol contents before and after derivatisation. The $\delta^{13}C_{TMS}$ was -38.9‰.

$\delta^{13}C$ values of DIC ($\delta^{13}C_{DIC}$) were determined from CO_2 generated from water samples in glass serum bottles by acidification with phosphoric acid. Subsamples were retrieved from the headspace with a gas tight syringe and injected directly into the GC-irm-MS. $\delta^{13}C$ values of CO_2 ($\delta^{13}C_{CO2}$) values were calculated from $\delta^{13}C_{DIC}$ values and *in situ* temperatures T_K (in Kelvins) (48, 49) as follows:

$$\delta^{13}C_{CO_2} = \delta^{13}C_{DIC} + 23.644 - (9701.5 / T_K) \quad (5)$$

POC and DOC samples were combusted in a Carlo Erba EA 1108 elementary analysator coupled to the irm-MS system. Organic carbon was combusted in line at 1040°C in a Cr_2O_3-Co_2O_3-Ag oxidation reactor. Inorganic carbonates were removed by acidification with 0.1 M hydrochloric acid prior to measurements and water was trapped using $MgClO_4$.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences determined in the present study have been deposited in the GenBank database under accession numbers AJ006182 to AJ006184 (DGGE bands 2 to 4), AJ272090 (DGGE band 8), AJ 272094 (DGGE band 7), and AY 247957 to AY247959 (DGGE bands 5, 6, and 1).

RESULTS

Physicochemical conditions

Light microscopic investigation of samples from the chemocline of Lake Dagow revealed a dense assemblage of phototrophic bacteria and the numerical dominance of the brown-colored phototrophic consortium designated "*P. roseum*" (Fig. 1). In order to obtain initial information about the potential *in situ* physiology of phototrophic consortia, the physicochemical conditions in the natural habitat were investigated. The vertical gradients of temperature and conductivity indicated that there was stable stratification of the water column below a depth of 4 m in all years (Fig. 2). On all sampling dates, steep opposing vertical gradients of molecular oxygen and sulfide concentrations were found between depths of 5.5 and 8.0 m (Fig. 2B and 3A). In this depth interval, the water temperature ranged from 12 to 7°C and the pH ranged from 7.3 to 7.4, whereas the conductivity remained constant (Fig. 2A). The light intensity was between 0.4 and 1.1 µmol of quanta·m^{-2}·s^{-1}, and molecular oxygen was absent at the depth at which phototrophic consortia were most abundant (Fig. 2B and C and

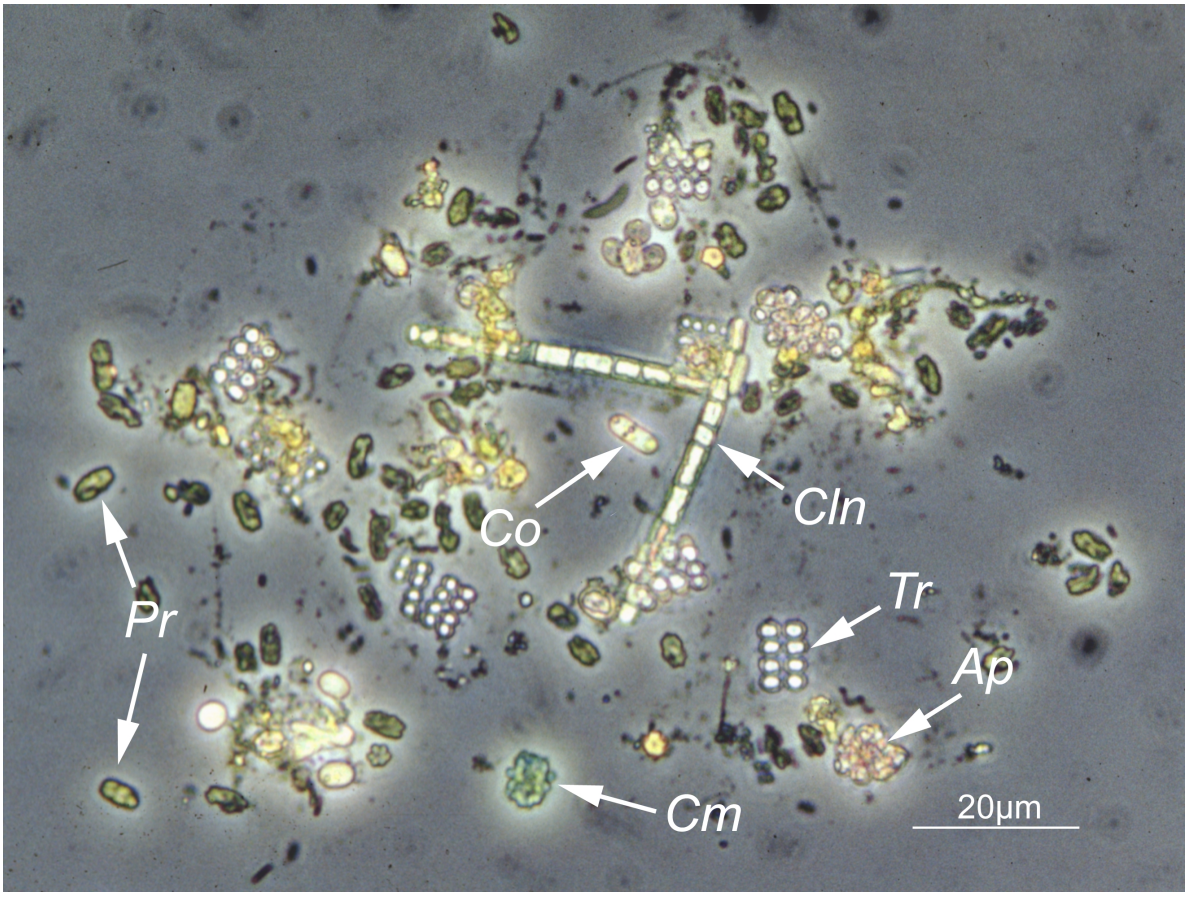


Figure 1. Phase-contrast microphotograph of the bacterial community present at 6.4 m depth in Lake Dagow on 4 August 1998. *Ap*, *Amoebobacter purpureus*-like cells; *Cln*, *Chloronema*-like cells; *Cm*, *"Chlorochromatium magnum"*; *Co*, *Chromatium okenii*-like cells; *Pr*, *"Pelochromatium roseum"*; *Tr*, *Thiopedia rosea*-like cells. With bright-field microscopy, *"P. roseum"* exhibits a distinct brown color.

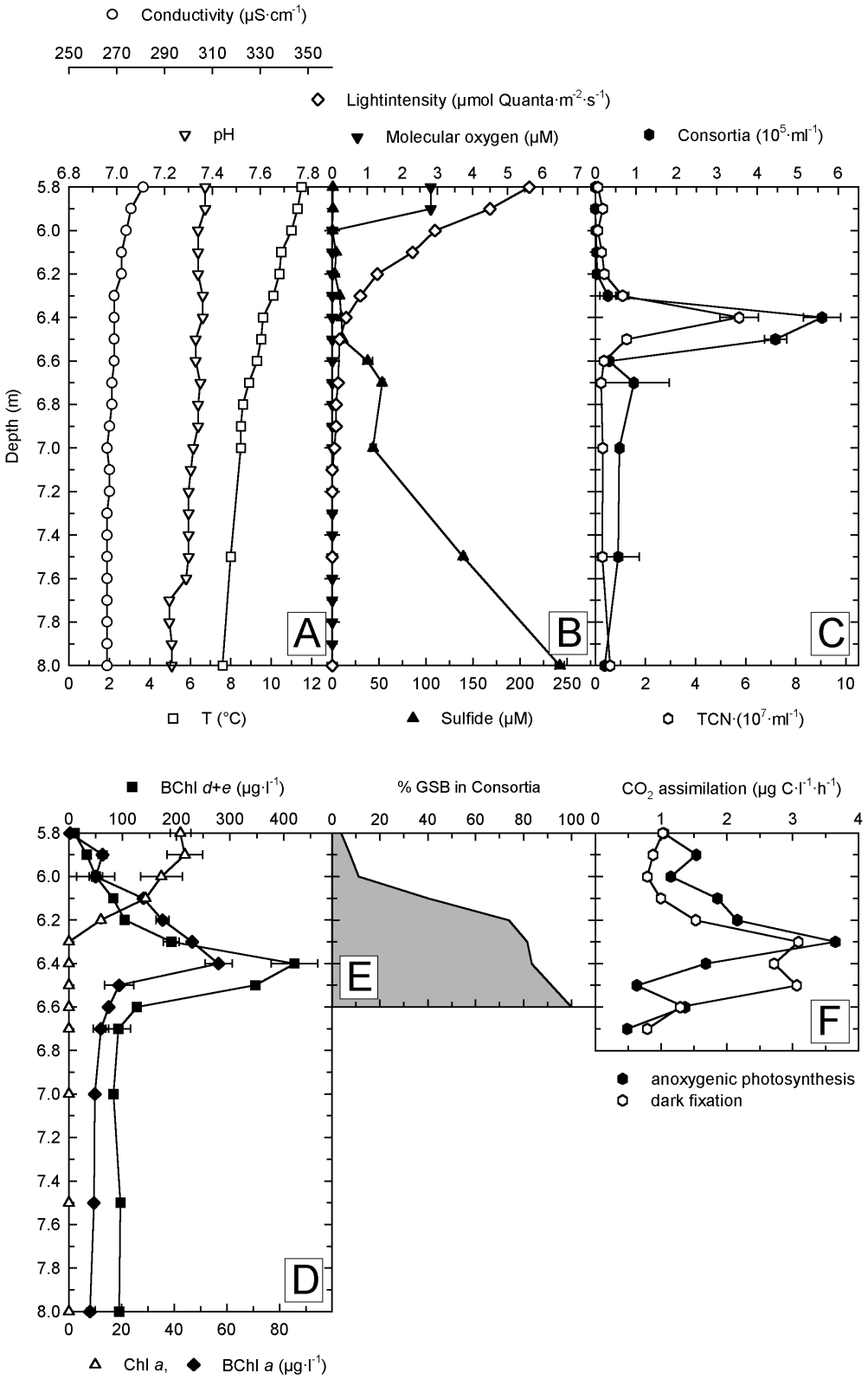


Figure 2. Physical, chemical and biological parameters in the chemocline of Lake Dagow on 4 July 1998. **A.** Conductivity, temperature and pH. **B.** Concentrations of molecular oxygen and sulfide. **C.** Numbers of phototrophic consortia and total cell number (TCN). **D.** Concentrations of BChl *a*, BChl *d* and *e* and Chl *a*. **E.** Fraction of green sulfur bacteria (GSB) associated with phototrophic consortia as determined by fluorescent *in situ* hybridization. **F.** CO_2 assimilation rates as determined by incubation with $NaH^{14}CO_3$.

3A). A comparison of five consecutive years showed that the environmental parameters for the depth at which the maximum abundance of phototrophic consortia occurred were different in the different years that samples were obtained (Tab. 1). However, the sulfide concentrations and light intensities consistently were very low in the layer in which phototrophic consortia occurred.

Pigment composition of the chemocline community of phototrophic bacteria

The maximum numbers of brown-colored phototrophic consortia coincided with the maximum concentrations of BChl *d* and *e* in the chemocline (Fig. 2C and D and 3A and B). Only low numbers of the green-colored "*Chlorochromatium magnum*" (15) and "*C. aggregatum*" consortia were present (Fig. 1). Occasionally, green- and brown-colored cells resembling *Pelodictyon clathratiforme* and *Pelodictyon phaeoclathratiforme*, respectively, were observed (not visible in Fig. 1). In addition, purple-colored cells with intracellular sulfur globules were detected. In terms of cell morphology and size, the capacity to form cell aggregates, and the presence of gas vacuoles, these cells closely resembled *Amoebobacter purpureus*, *Thiopedia rosea*, and *Chromatium okenii* (Fig. 1). In addition, green-colored filaments containing gas vacuoles resembling the green filamentous bacterium *Chloronema* sp. were observed in some years.

BChl *d* and *e* were the predominant photosynthetic pigments in the chemocline in five consecutive years (Tab. 1 and Fig. 2D and 3B to D). On average, the concentration of BChl *d* and *e* exceeded the concentration of BChl *a* by 1 order of magnitude and the concentration of Chl *a* by 2 orders of magnitude (Tab. 1). A more detailed analysis of the photosynthetic pigments in July 1999 by HPLC and HPLC-MS (Tab. 2) revealed the dominance of BChl *e*-containing green sulfur bacteria in the chemocline. BChl *e* was present at concentrations as high as 934 $\mu g \cdot liter^{-1}$ at the depth at which the maximum abundance of phototrophic consortia occurred. The BChl *e* concentrations exceeded the concentrations of BChl *a* and *d* by about 20-fold and the concentrations of Chl *a* by about 100-fold (Fig. 3B to D). BChl *c* was never detected in the chemocline.

The carotenoids isorenieratene and β-isorenieratene are specific for BChl *e*-containing green sulfur bacteria. Even at the maximum biomass of "*P. roseum*", however, the concentrations of both carotenoids were below the detection limit (2 $\mu g \cdot liter^{-1}$). The only carotenoids present at considerable levels in the chemocline were okenone and γ-carotene. The concentration of okenone was as high as 47 $\mu g \cdot liter^{-1}$, and the vertical distribution of this compound was similar to that of BChl *a* (Fig. 3C). The vertical distribution of γ-carotene clearly correlated with the distribution of Chl *a* (Fig. 3D). Traces of additional yellow

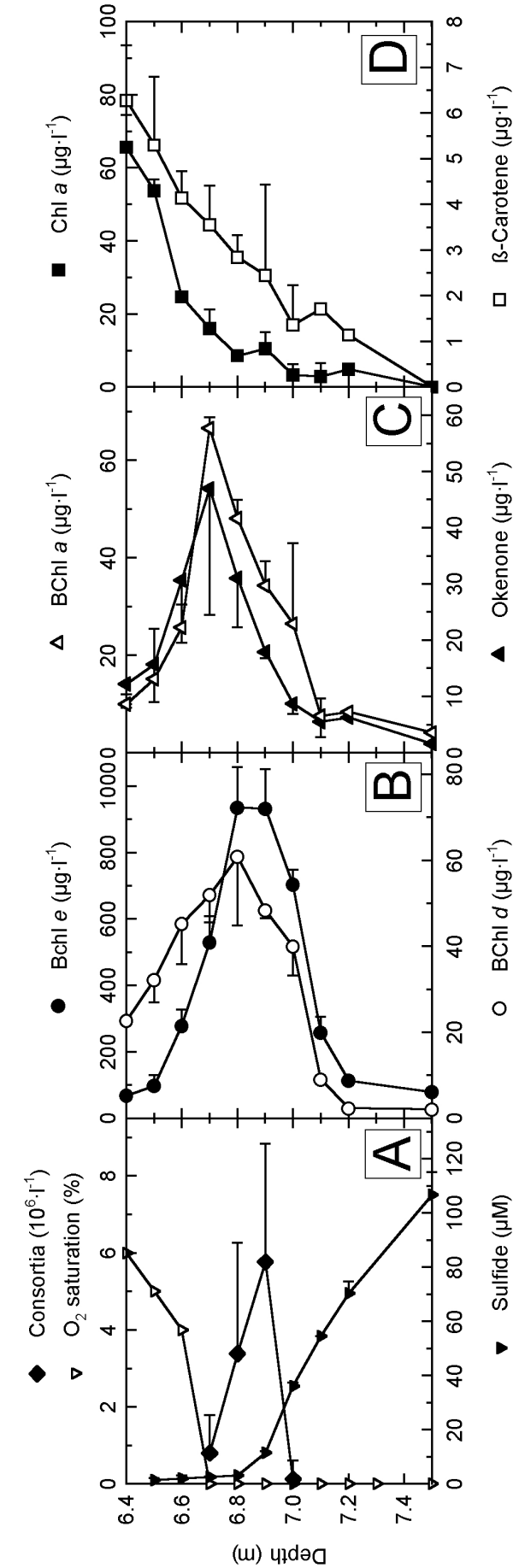


Figure 3. Composition of pigments of phototrophic bacteria in the Lake Dagow chemocline on 19 July 1999 as determined by HPLC analyses. **A.** Numbers of phototrophic consortia, molecular oxygen saturation (expressed as a percentage of air saturation) and concentrations of sulfide. **B.** to **D.** Photosynthetic pigments of green sulfur bacteria (BChl *d* and BChl *e*) (**B**), purple sulfur bacteria (BChl *a* and okenone) (**C**), and cyanobacteria and algae (Chl *a* and ß-carotene) (**D**).

carotenoids were detected in the chemocline, but these compounds were not characterized further.

Phylogenetic composition of green sulfur bacteria in the chemocline

The green sulfur bacteria present in the chemocline of Lake Dagow were characterized further by using a culture-independent molecular approach. A 465-bp fragment of the 16S rRNA gene was amplified from genomic DNA by a PCR method specific for green sulfur bacteria, and the DNA fragments were separated by DGGE on the basis of their different melting behaviors. Up to four different DNA fingerprints were detected in the chemocline microbial community (Fig. 4). Two of the 16S rRNA gene fragments had the same melting behavior as the fragments obtained from intact "*P. roseum*" consortia separated manually by micromanipulation. In contrast, 16S rRNA gene fragments of the green-colored "*C. aggregatum*" and "*C. magnum*" consortia were not detected in the natural community (Fig. 4).

The 16S rRNA gene fragments of the phototrophic consortia and four DNA bands from the natural community that exhibited different melting behaviors (indicated by numbers in

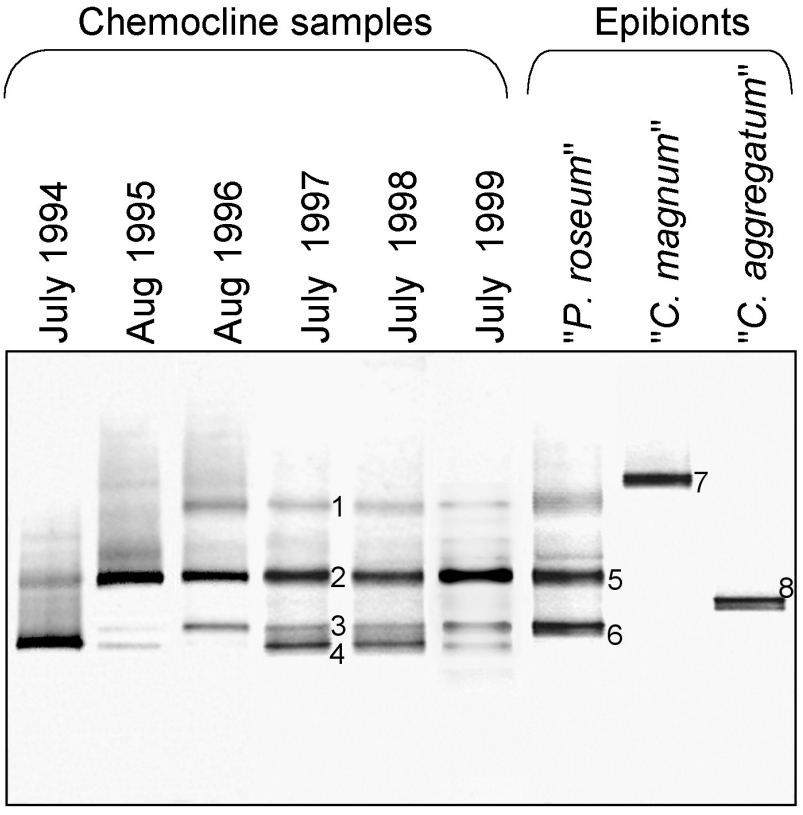


Figure 4. 16S rRNA gene fingerprints of green sulfur bacteria of the chemocline microbial community in Lake Dagow for six consecutive years compared to fingerprints and isolated consortia. The latter were generated from 10 isolated intact consortia (central bacterium plus attached epibionts) of "*C. aggregatum*", 10 consortia of "*P. roseum*", or 5 consortia of "*C. magnum*". A negative image of an ethidium bromide-stained DGGE gel is shown. Numbers of DNA bands correspond to sequences in the phylogenetic tree in Fig. 5.

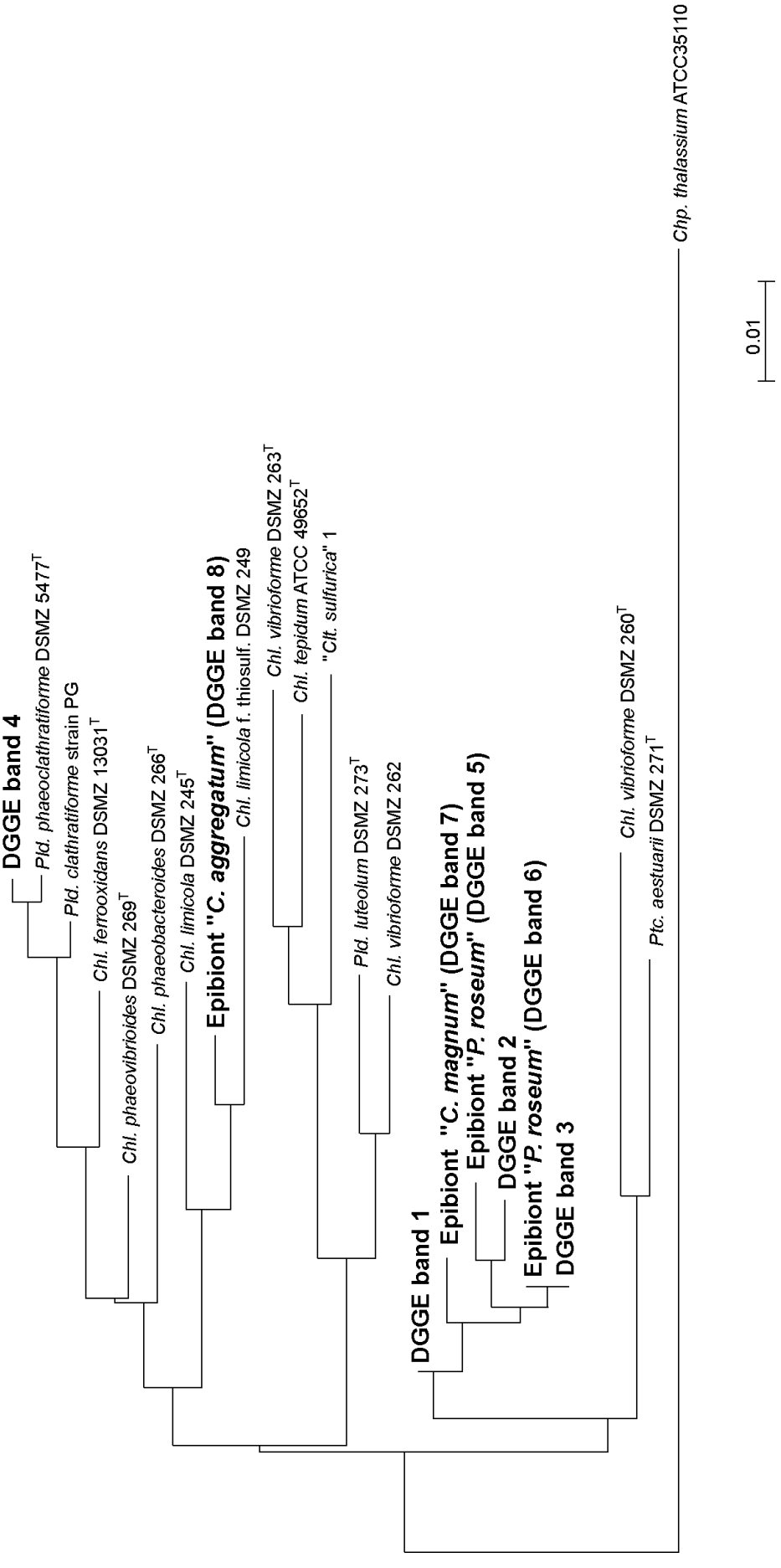


Figure 5. Phylogenetic position of the "*P. roseum*" epibiont in the radiation of green sulfur bacteria. A phylogenetic tree was constructed with the maximum likelihood method from full-length sequences of the most closely related green sulfur bacteria. The partial 16S rRNA gene sequences were then inserted by maximum parsimony tool as implemented in the ARB phylogeny package. The DNA-sequence of the epibiont of "*P. roseum*" band 6 is identical with band 3 from the entire chemocline microbial community (compare Fig. 4). Bar = 0.01 fixed point mutations per base. *Pld. Pelodictyon*; *Chl., Chlorobium*; *Clt., Clathrochloris*; *Ptc., Prosthecochloris*; *Chp., Chloroherpeton*.

Fig. 4) were excised and sequenced. Phylogenetic analysis con firmed that one of the two 16S rRNA gene sequences from the epibionts of "*P. roseum*" (Fig. 4, band 6) was indeed identical to a sequence (band 3) from the natural community (Fig. 5). However, the second fingerprint of "*P. roseum*" (Fig. 4, band 5) and the corresponding DNA band from the natural community (band 2) contained two different 16S rRNA gene sequences (Fig. 5).

The combined evidence from pigment determination and molecular fingerprinting analyses indicated that the consortium "*P. roseum*" dominates the phototrophic community in Lake Dagow. Whole-cell *in situ* hybridization was used to quantify the abundance of "*P. roseum*" epibionts in comparison to the abundance of other green sulfur bacteria in the chemocline.

Numerical dominance of epibiotic green sulfur bacteria

The fraction of green sulfur bacteria associated with phototrophic consortia was determined in 1998 and 1999. Fluorescence *in situ* hybridization with a probe specific for green sulfur bacteria (GSB-532) revealed that in July 1998, 84% of all green sulfur bacteria at the depth at which the maximum biomass occurred (6.4 m) (Fig. 2) was associated with phototrophic consortia (Fig. 2E and Tab. 1). In the following year, 88.5% of all green sulfur bacteria were epibionts (Tab. 1). Evidently, epibionts of "*P. roseum*" are the dominant anoxygenic phototrophs in the chemocline of Lake Dagow.

Based on a value of 20 epibionts per consortium (40), epibionts of "*P. roseum*" represented a significant fraction of the total cell counts and contributed up to 37% of all cells at the depth at which the maximum abundance of phototrophic consortia occurred (Tab. 1).

Photosynthetic CO_2 assimilation

As a first assessment of the *in situ* physiology of the phototrophic epibionts, the vertical distribution of anoxygenic $NaH^{14}CO_3$ assimilation was measured across the chemocline. Oxygenic photosynthesis was inhibited by the addition of 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea. The maximum level of of anoxygenic photosynthesis was 3.7 µg of $C \cdot liter^{-1} \cdot h^{-1}$ and was detected at the top of the layer of phototrophic consortia at a depth of 6.3 m (Fig. 2F) and a light intensity of 0.8 µmol $quanta \cdot m^{-2} \cdot s^{-1}$. The maximum values of dark CO_2 fixation were detected between 6.3 and 6.5 m and were as high as 3.1 µg of $C \cdot liter^{-1} \cdot h^{-1}$ (Fig. 2F). These comparatively high values for dark CO_2 fixation were probably caused by chemolithoautotrophic bacteria or by anaplerotic CO_2 fixation reactions of chemoorgano-heterotrophic bacteria or both.

Table 1. Levels of photosynthetic consortia and environmental conditions in the biomass maximum over 5 consecutive years

Dates	Depth of maximum abundance of "*P. roseum*" (m)	Maximum no. of "*P. roseum*" (cells/ml)	Relative abundance of epibionts (% of total cell no.)	Relative abundance of epibionts (% of green sulfur bacteria)	Light intensity (μmol of quanta $\cdot$ $m^{-2} \cdot s^{-1}$)	Concn ($\mu g \cdot liter^{-1}$) of:			Sulfid concn
						BChl *d* + BChl *e*	BChl *a*	Chl *a*	
2 to 9 August 1995[a]	7.7	$(3.2 \pm 1.5) \times 10^5$	19.1	ND[b]	0.42	342	13.7	0	18
2 to 11 August 1996[a]	7.7	5.8×10^4	6.4	ND	0.06	493	29.4	22.2	219
4 to 15 August 1997	6.6	$(7.8 \pm 0.8) \times 10^4$	1.7	ND	1.1	292	13.7	0	25
2 to 7 July 1998	6.4	$(5.6 \pm 0.5) \times 10^5$	16.4	84	0.4	420	56.9	0	10
19 and 20 July 1999	6.8	$(3.4 \pm 2.9) \times 10^6$	37.2	88	0.7	1,002	50	0	3

[a] Values from a previous study (41).
[b] ND, not determined.

Table 2. Identification of BChls and Chl *a* by MS-MS fragmentation

HPLC peak	Retention time (min)	Mass (*m/z*)		Identification	
		$[M+H]^{+a}$	Fragments	[8, 12] BChl substituents[b]	Esterifying alcohol
1	28.4	821	803, 617, 599	[E, E] BChl e_F	Farnesol (222)[d]
2	29.5	835	817, 631, 613	[P, E] BChl e_F	Farnesol (222)
2s[c]	29.5	793	589	[E, E] BChl d_F	Farnesol (222)
3	30.5	849	831, 645, 627	[I, E] BChl e_F	Farnesol (222)
4	34.2	839	821, 631, 613	[E, E] BChl e_{Hen}	Hexadecenol (240)
4	34.2	813	795, 599	[E, E] BChl e_T	Tetradecanol (214)
5	35.0	853	835, 631, 613	[P, E] BChl e_{Hen}	Hexadecenol (240)
5	35.0	827	809, 613	[P, E] BChl e_T	Tetradecanol (214)
6	36.3	811	589	[E, E] BChl d_{Hen}	Hexadecenol (240)
7	35.8	867	849, 645, 627	[I, E] BChl e_{Hen}	Hexadecenol (240)
7	35.8	841	823, 7	[I, E] BChl e_T	Tetradecanol (214)
9	37.1	841	823, 617, 599	[E, E] BChl e_{Han}	Hexadecanol (242)
10	37.8	855	837, 631, 613	[P, E] BChl e_{Han}	Hexadecanol (242)
11	38.1	813	589	[E, E] BChl d_{Han}	Hexadecanol (242)
12	38.5	869	851, 645, 627	[I, E] BChl e_{Han}	Hexadecanol (242)
12	38.5	911	853, 633	BChl a_P	Phytol (296)
13	38.8	839	589	[E, E] BChl d_{Oen}	Octadecanol (268)
14	39.4	867	589	[E, E] BChl d_p	Phytol (296)
17	43.0	893	615	Chl a_P	Phytol (296)

[a] $[M + H]^+$, quasi-molecular ion.
[b] E, ethyl; I, isobutyl; M, methyl; P, *n*-propyl.
[c] s, shoulder.
[d] The numbers in parentheses are *m/z* values.

Stable carbon isotope fractionation of biomarkers and bulk carbon fractions

The carbon metabolism of the epibionts was investigated further by using the $\delta^{13}C$ values of the biomarkers.The carotenoids isorenieratene and β-isorenieratene occur exclusively in BChl *e*-containing strains of green sulfur bacteria (12) and a few streptomycete species (25) and are therefore the most commonly used biomarkers of green sulfur bacteria. In contrast to all other known brown-colored green sulfur bacteria, however, isorenieratene and β-isorenieratene were absent in "*P. roseum*". Accordingly, we used esterifying alcohols of BChl *e* as specific biomarkers and compared their $\delta^{13}C$ values to those of the esterifying alcohols of other BChls and Chl *a* and to those of fatty acids, carotenoids, POC, DOC, and DIC. Esterifying alcohols of BChls and Chl *a* were first identified by mass spectra derived from HPLC-MS-MS analysis and are listed in Tab. 2. Minor BChl *e* homologs containing dodecanol and pentadecenol were detected but not analyzed further, because the amounts of esterifying alcohols obtained were far too small to permit determination of $\delta^{13}C$ values. Farnesol, hexadecanol, and hexadecenol occurred in BChl d, as well as in BChl *e*, whereas

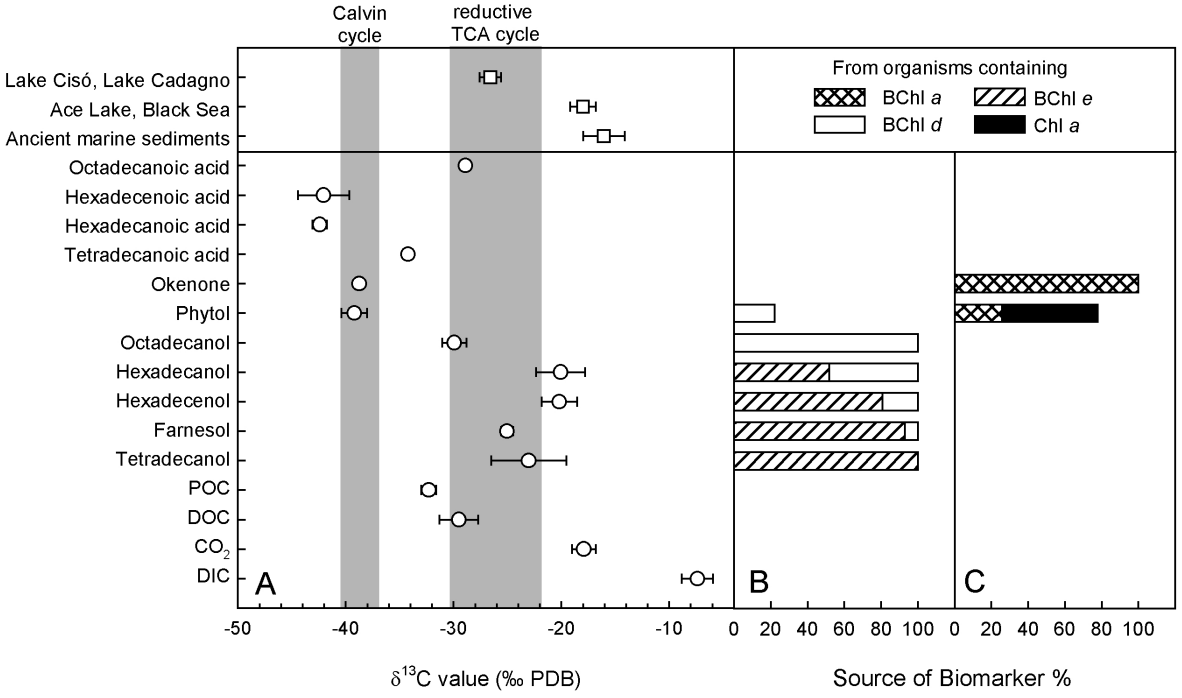


Figure 6. $\delta^{13}C$ values and origin of biomarkers of phototrophic bacteria isolated from 6.8 m depth in Lake Dagow on 19 July 1999. **A.** $\delta^{13}C$ values of specific biomarkers. Symbols: ○, $\delta^{13}C$ values from the chemocline of Lake Dagow; □, average of $\delta^{13}C$ values from previously published papers (for Lake Cisó, Lake Cadagno: isorenieratene from sediments [20, 50]; for Ace Lake and Black Sea, isorenieratene, chlorobactene and farnesane from sediments [22, 55, 57]; for ancient sediments: isorenieratene isolated from rocks [19, 21, 42, 55, 56]). Shaded areas indicate the range of $\Delta\delta^{13}C$ values for the biomass of green and purple sulfur bacteria for the chemocline of Lake Dagow as calculated from previously published data (29, 47, 59). TCA, tricarboxylic acid. **B.** Source of specific biomarkers derived from CO_2 fixation by the reversed citric acid cycle. **C.** Source of specific biomarkers derived from CO_2 fixation via the Calvin cycle.

phytol was found in BChl *a* and BChl *d*, as well as in Chl *a* (Tab. 2). In order to identify suitable biomarkers for the BChl *e*-containing epibionts, the relative abundance of each esterifying alcohol in BChl *a*, *d*, and *e* homologs and Chl *a* was calculated based on the pigment composition determined by HPLC. BChl *e* homologs containing tetradecanol and hexadecenol coeluted. In these cases the relative contributions of different BChls to esterifying alcohols were calculated from the relative intensities of the quasi-molecular ions generated from the corresponding BChl homologs during MS. This calculation revealed that one-third of BChl *e* in the three HPLC peaks was esterified to tetradecanol. According to our calculations, 100% of the tetradecanol, 92.9% of the farnesol, 80.7% of the hexadecenol, and 51.8% of the hexadecanol originated from BChl *e* (Fig. 6B). Consequently, tetradecanol, farnesol, and hexadecenol are suitable biomarkers for brown-colored green sulfur bacteria in Lake Dagow and, due to the strong numerical dominance of phototrophic consortia, also for epibionts of "*P. roseum*". The remaining amounts of the esterifying alcohols indicated above most likely originated from additional green sulfur bacteria present in the chemocline, mostly the epibionts of "*C. magnum*" and the free-living organism *P. clathratiforme*, which contain BChl *d* homologs with the same HPLC elution pattern (6). Both bacteria most likely were also sources of octadecanol in July 1999.

Farnesol, the major esterifying alcohol of BChl *e*, was depleted in ^{13}C compared to CO_2 (difference between $\delta^{13}C$ values [$\Delta\delta^{13}C$] = -7.1‰) and enriched in ^{13}C compared to DOC ($\Delta\delta^{13}C$ = 11.6‰) and POC ($\Delta\delta^{13}C$ = 14.4‰) (Fig. 6A). Compared to farnesol, straight-chain esterifying alcohols of BChl *e* were less depleted in ^{13}C (average $\Delta\delta^{13}C$ = -3.2‰). Okenone originated exclusively from BChl *a*-containing bacteria, and phytol originated from organisms containing BChl *a*, Bchl *d*, and Chl *a* (Fig. 6C). The $\Delta\delta^{13}C$ values of phytol and okenone were -21.3 and -20.8‰, respectively.

In order to account for vertical differences in the stable carbon fractionation of DIC, $\delta^{13}C_{DIC}$ values were determined from 1997 to 1999 for 10-cm intervals across the chemocline and converted to $\delta^{13}C_{CO2}$ values. However, the differences in $\delta^{13}C_{CO2}$ values for different depths were not significant. For example, on 19 July 1999, $\delta^{13}C_{CO2}$ was -17.3‰ ± 1.5‰ at 6.7 m and -18.4‰ ± 1.3‰ at 6.9 m. Similar $\delta^{13}C_{CO2}$ values were obtained for chemocline samples in 1997 and 1998. However, larger differences in $\delta^{13}C_{CO2}$ values were detected within the epilimnion (-12.6‰ ± 0.36‰ at 6.1 m and -15.1‰ ± 0.6‰ at 6.5 m) and between the epilimnion and hypolimnion (-21.2‰ ± 0.6‰ at 7.0 m) in August 1997.

DISCUSSION

Numerical significance and unique characteristics of the epibionts

Combined with the microscopic evidence, our pigment analyses and molecular ecology approaches revealed that epibionts of "*P. roseum*" represent the numerically dominant green sulfur bacteria in the chemocline of Lake Dagow. First, the detailed analysis of photosynthetic pigments demonstrated the predominance of BChl *e*-containing green sulfur bacteria over the green sulfur bacteria containing BChl *d* or other phototrophs containing BChl *a* or Chl *a*. Second, a low diversity of green sulfur bacteria was detected by DGGE fingerprinting in the chemocline of Lake Dagow, and in 1996 to 1999 the 16S rRNA gene sequence of a major band from chemocline samples was identical to one sequence from "*P. roseum*". Third, fluorescence *in situ* hybridization showed that the major fraction (84 to 88%) of green sulfur bacterial cells was associated with intact phototrophic "*P. roseum*" consortia. Because some of the epibionts were dislodged from phototrophic consortia during filtration of the fluorescence *in situ* hybridization samples, this fraction actually represents a minimum estimate. Besides the distinct phylogenetic position of the epibionts and the fact that they are never found in the free-living state, the most obvious physiological characteristic of "*P. roseum*" is the lack of isorenieratene and β-isorenieratene. Previously, these two carotenoids had been assumed to represent the typical biomarkers of brown-colored green sulfur bacteria (12). Our data confirm recent analyses of the pigments of epibionts of "*P. roseum*" obtained from a North American lake (Echo Lake) (18). These epibionts, although clearly phylogenetically different from those in Lake Dagow, also lacked isorenieratene and β-isorenieratene. In addition, the epibionts had a very low overall carotenoid content. The molar ratio of total carotenoids to BChl *e* in Lake Dagow was $5 \cdot 10^{-3}$, and the ratio in the Echo Lake enrichment of "*P. roseum*" was $25 \cdot 10^{-3}$ (18). In contrast, free-living green sulfur bacteria have molar ratios of total carotenoids to BChl *e* of 0.14 to 0.38 depending on the growth conditions (18). Taken together, these data indicate that a low overall carotenoid content and a lack of isorenieratene and β-isorenieratene are common features of brown-colored epibionts of phototrophic consortia, which obviously employ BChl *e* as the only light-harvesting pigment. These features clearly contradict the hypothesis that carotenoids are essential for light harvesting by green sulfur bacteria (30, 33, 62). Correspondingly, cells in laboratory cultures of *Chlorobium phaeobacteroides* CL1401, whose carotenoid content was artificially depleted by 85% by using 2-hydroxybiphenyl as an inhibitor of carotenoid synthesis, did not have an altered growth rate compared to the growth rate of noninhibited cells (2).

In conclusion, epibionts of "*P. roseum*" have distinct characteristics, and therefore, their carbon metabolism potentially could differ from that of their free-living counterparts. Due to their numerical dominance, the consortia in Lake Dagow represent a model population that is well suited for culture-independent physiological studies.

Interpretation of $\delta^{13}C$ values

So far, stable carbon isotope discrimination studies of green sulfur bacteria have been done only with pure cultures (47, 58, 59). In one case, bulk microbial biomass was used to determine $\delta^{13}C$ values (16), but there was not a concomitant determination of $\delta^{13}C_{CO2}$ values, so that the carbon metabolism *in situ* could not be evaluated. Molecular markers of green sulfur bacteria are frequently used in geochemistry for reconstruction of past environmental conditions (19, 21, 24, 42, 43, 53, 55, 57). However, the carbon metabolism of an extant natural community of green sulfur bacteria has never been investigated based on stable carbon isotope fractionation of specific biomarkers.

Theoretically, there could have been unknown farnesol esters other than those of BChls in our samples, and they could have led to false $\delta^{13}C$ values. However, this appeared to be highly unlikely under the specific conditions observed in the chemocline of Lake Dagow. Samples were collected by filtration; hence, the farnesol originated almost exclusively from microbial cells and, to a much lower extent, from particulate matter. Given the high specific BChl *e* content of green sulfur bacteria (100 μg of BChl *e* · mg of protein^{-1} [33]) and the dominance of farnesyl esters among the BChl *e* homologs (18; this study), the cellular content of farnesol is 1% of the cellular dry weight. Even if all accompanying cells were archaea and thus contained especially high amounts of isoprenoid lipids (26), only small amounts of farnesol derived from sources other than BChl *e* would be expected, as demonstrated by the following calculation. Of the total lipids present in archaea (accounting for 2 to 6% of the cellular dry weight [26]), only a small fraction of nonpolar lipids (7 to 30%, corresponding to 0.1 to 1.8% of the cellular dry weight [26]) contains traces of farnesol (≤ 2% of the nonpolar lipids [61]). Consequently, the maximum amount of farnesol which could originate from archaeal lipids is 0.036% of the cellular dry weight. Considering that in the chemocline of Lake Dagow the number of epibionts almost equals the number of all other accompanying prokaryotes, the amount of farnesol derived from BChl *e* even under the unrealistic assumption that archaea are dominant would therefore surpass the amount of archaeal farnesol by a factor of at least 21. We concluded that in the chemocline of Lake Dagow, farnesol is a valid biomarker for green sulfur bacterial epibionts of phototrophic consortia.

Table 3. Biomass $\Delta\delta^{13}C$ values: comparison of values for "*P. roseum*" epibionts and previously published values for green and purple sulfur bacteria

Organisms	$\Delta\delta^{13}C$ (‰ vs PDB)	Origin of sample	Reference(s)
"*P. roseum*" epibionts[a]	$-9.6 \pm 1^{a,b}$	Lake Dagow, Germany	This study
Green sulfur bacteria	$-10.1\ (-12.2)^{b,c}$	Pure cultures	56
Purple sulfur bacteria	−20.3	Lake Dagow, Germany	This study
Purple sulfur bacteria	-19.8 ± 0.6	Pure cultures	29, 47, 56
Thermochromatium tepidum	−21.6	Roland's Well, Yellowstone National Park, Wyoming, United States	29

[a] Assuming a $\Delta\delta^{13}C$ for the isolated isoprenoid biomarker and the total biomass of green sulfur bacteria of 2.5‰ ± 0.5‰ (64).
[b] The $\delta^{13}C_{CO2}$ value was recalculated as described by Rau (48) and Rost et al. (49).
[c] The value in parentheses is the originally published value.

Since the biochemical building block in carotenoids and farnesol is isoprene, very similar $\delta^{13}C$ values are observed for these two biomarkers in cultures of *Chlorobium limicola* (64). Consequently, our $\delta^{13}C$ values for isoprenoid alcohols can be compared to the carbon fractionation values of green sulfur bacterial carotenoids from other environments. The low $\delta^{13}C$ values of the biomarkers of "*P. roseum*" are very similar to those of green sulfur bacteria in sediments of Lake Cadagno (50) and Lake Cisó (20) (Fig. 6). The low $\delta^{13}C$ values have been explained by internal recycling of ^{13}C-depleted bicarbonate (3, 4, 20). Indeed, the $\delta^{13}C_{CO2}$ values in the chemocline of Lake Dagow were very low and compared well with values from other holomictic temperate (66) and meromictic (11) lakes. In contrast, the ^{13}C in isorenieratene isolated from sediments of low-productivity environments like Ace Lake, Antarctica (53), or the oligotrophic Black Sea (22, 57) is much less depleted (Fig. 6A).

The difference between the $\delta^{13}C$ values of biomarkers and the $\delta^{13}C$ values of ambient CO_2 ($\Delta\delta^{13}C$) can be used to elucidate the carbon source used by epibionts of "*P. roseum*". In the chemocline of Lake Dagow, the $\Delta\delta^{13}C$ of farnesol was -7.1‰. In photoautotrophically grown cells of green sulfur bacteria, the 13 C in isoprenoid biomarkers is enriched by 2 to 3‰ compared to the ^{13}C in the total biomass (64). Accounting for this difference, the $\Delta\delta^{13}C$ for the epibiont biomass was -9.6‰ ± 2.7‰ (Tab. 3). An almost identical $\Delta\delta^{13}C$ has been reported for photoautotrophically grown cultures of green sulfur bacteria (-10.1‰ after correction of $\delta^{13}C_{CO2}$ values [equation 5] [57]). In a similar manner, $\Delta\delta^{13}C$ values were calculated for purple sulfur bacteria in the chemocline of Lake Dagow and compared to previously published data (29, 59). The results indicate that bacteria belonging to this group also grow photoautotrophically (Tab. 3). Although $\Delta\delta^{13}C$ values for biomass or biomarkers from nat-ural populations of green sulfur bacteria are not available, photoautotrophic growth of green sulfur bacterial populations in other ecosystems can be inferred from the difference between $\delta^{13}C$ values for isoprenoid biomarkers for green sulfur bacteria (GSB) and purple sulfur bacteria (PSB):

$$\Delta\delta^{13}C(GSB) - \Delta\delta^{13}C(PSB) = \delta^{13}C(isorenieratene, farnesol) - \delta^{13}C(okenone, phytol) \quad (6)$$

Based on our data, green sulfur bacteria, as well as purple sulfur bacteria, grow photoautotrophically in Lake Dagow. The ^{13}C in farnesol in Lake Dagow was enriched by 13.7‰ compared to the ^{13}C in okenone and by 14.1‰ compared to the ^{13}C in phytol (Fig. 6). Similar differences between $\Delta\delta^{13}C$ (GSB) and $\Delta\delta^{13}C$ (PSB) were observed for okenone and isorenieratene in the sediments of Lake Cisó (15‰) (20) and Lake Cadagno (18‰) (50). Together with the fact that the absolute $\Delta\delta^{13}C$ values were also similar in the two lakes

mentioned above and Lake Dagow, these data indicate that photoautotrophic growth of free-living green sulfur bacteria occurs in the three ecosystems.

in situ *physiology of epibionts of "*P. roseum*"*

The major goal of the present study was to gain further insight into the interactions between the partner cells of phototrophic consortia under natural conditions as they occur. The central bacterium of the phototrophic consortia phylogenetically belongs to the β subclass of the Proteobacteria (15) and, in contrast to previous speculation (44, 52), is therefore unlikely to reduce sulfate or sulfur. This conclusion is corroborated by the experimental finding that epibionts of phototrophic consortia exhibit electron transfer only in the presence of external sulfide (14). Since the epibionts do not appear to depend on internal sulfide generation, the selective advantage of consortium formation could be related to carbon metabolism. Because the biomass of green sulfur bacteria increases significantly in the presence of organic carbon substrates (36), it appeared to be feasible that epibionts grow mixotrophically or even heterotrophically by taking advantage of organic carbon substrates excreted by the central chemotrophic bacterium.

However, the $\Delta\delta^{13}C$ values determined in the present study strongly indicate that the epibionts of "*P. roseum*" grow photoautotrophically under *in situ* conditions instead of utilizing significant amounts of organic carbon substrates. Direct assimilation of the organic carbon substrates would be expected to result in lower $\Delta\delta^{13}C$ values for the epibionts, since the ^{13}C in DIC and POC in the chemocline of Lake Dagow was highly depleted (Fig. 6). Although it is unlikely, the only alternative explanation of the observed $\Delta\delta^{13}C$ values is that a major fraction of the cellular carbon of the epibionts is derived from organic carbon substrates which (i) are significantly less depleted in ^{13}C than the bulk DOC, (ii) represent only a small fraction of the total DOC, and (iii) have a $\delta^{13}C$ value which upon assimilation and transformation to lipids results in values precisely like the values in autotrophically grown green sulfur bacteria.

Utilization of organic carbon compounds excreted by the central bacterium also appears to be unlikely, since the stable carbon isotopic signature of organic carbon does not change significantly (i.e., it changes by ~1‰) during chemoorgano-heterotrophic metabolism (16), so that transfer of organic carbon from the central bacterium to the epibionts would yield $\Delta\delta^{13}C$ values lower than those observed. From our results we concluded that assimilation of external or internal organic carbon compounds does not provide a selective advantage to the symbiotic green sulfur bacteria in phototrophic consortia. Since the epibionts of "*P. roseum*" grow photoautotrophically *in situ*, an estimate of their maximum growth rate could be obtained

based on the peak carbon assimilation rate and the corresponding biomass of epibionts. This analysis yielded a growth rate of the epibionts of 0.045 day^{-1}, corresponding to a doubling time of 15.3 days at the *in situ* light flux, 0.8 µmol of quanta·m^{-2}·s^{-1}. For comparison, the growth rates of green sulfur bacteria in pure cultures even at similar low light intensities range from 0.17 to 0.07 day^{-1} (33, 36), and the growth rates of intact "*C. aggregatum*" consortia reach 0.188 day^{-1} (14). Obviously, growth of the epibionts *in situ* is limited by factors other than light intensity. The maximum *in situ* growth rate of free-living phototrophic sulfur bacteria (0.063 day^{-1} [65]) is comparable to that of the epibionts of "*P. roseum*" in Lake Dagow. Therefore, epibionts in the associated state are expected to be able to compete with, but not outcompete, free-living green and purple sulfur bacteria in their natural habitat. Our detailed analysis revealed that the carbon metabolism of epibionts of phototrophic consortia *in situ* is similar to that of their free-living counterparts. Evidently, there is a selective advantage other than internal recycling of carbon or sulfur which maintains epibionts in the associated state and which causes the dominance of green sulfur bacterial epibionts of "*P. roseum*" over free-living green sulfur bacteria in the natural habitat. Whereas green sulfur bacteria (with the exception of the gliding organism *C. thalassium*) are not motile, the epibionts of phototrophic consortia like "*P. roseum*" are carried by the motile central bacterium and hence become motile in the associated state (40). Based on the data obtained in the present study, it appears likely that the acquisition of motility makes epibionts of phototrophic consortia more competitive in heterogeneous aquatic environments, where they can exploit local accumulations of compounds (e.g., sulfide) much faster than their free-living relatives that are not motile.

REFERENCES

1. **Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Miller W. and Lipman D.J.** (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389-3402
2. **Arellano J.B., Borrego C.M., Martínez-Planells A. and Garcia-Gil L.J.** (2001) Effect of carotenoid deficiency on cells and chlorosomes of *Chlorobium phaeobacteroides*. Arch. Microbiol. **175:**226-233
3. **Behrens A., Schaeffer P., Bernasconi S. and Albrecht P.** (2000) Mono- and bicyclic squalene derivatives as potential proxies for anaerobic photosynthesis in lacustrine sulfur-rich sediments. Geochim. Cosmochim. Acta **64(19):**3327-3336
4. **Berry J.A., Throughton J. and Bjørkman O.E.** (1974) Carbon isotope fractionation in open and closed systems. Carnegie Inst. Year Book **73:** 785-790
5. **Borrego C.M., Arellano J.B., Abella C.A., Gillbro T. and Garcia-Gil L.J.** (1999) The molar extinction coefficient of bacteriochlorophyll *e* and the pigment stoichiometry in *Chlorobium phaeobacteroides*. Photosynth. Res. **60:**257-264
6. **Borrego C.M. and Garcia-Gil L.J.** (1994) Separation of bacteriochlorophyll homologs from green phototrophic sulfur bacteria by reversed-phase HPLC. Photosynth. Res. **41:**157-163
7. **Buder J.** (1914) Chlorobium mirabile. Ber. Deut. Bot. Ges. **31:**80-97
8. **Button D.K., Schut F., Quang P., Martin R. and Robertson B.R.** (1993) Viability and isolation of marine bacteria by dilute culture: theory, procedures and initial results. Appl. Environ. Microbiol. **59:**881-891
9. **Caspar S.J.** (1985) (eds.). Lake Stechlin - a temperate oligotrophic lake. Dr. W. Junk Publishers, Dordrecht, The Netherlands
10. **Cline J.D.** (1969) Spectrophotometric determination of hydrogen sulfide in natural water. Limnol. Oceanogr. **14:**454-458
11. **Deevey S.D., Nakai N. and Stuiver M.** (1963) Fractionation of sulfur and carbon isotopes in a meromictic lake. Science **139:**407-408
12. **de Wit R. and Caumette P.** (1995) An overview of the brown-colored isorenieratene containing green sulfur bacteria (*Chlorobiaceae*), pp. 908-909. *In*: Grimalt J.O. and Dorromsoro C. (eds.), Organic Geochemistry: Developments and applications to energy, climate, environment and human history. A.I.G.O.A. Donostia-San Sebastian
13. **Fröhlich J. and König H.** (2000) New techniques for isolation of single prokaryotic cells. FEMS Microbiol. Rev. **24:**567-572

14. **Fröstl J. and Overmann J.** (1998) Physiology and tactic response of "*Chlorochromatium aggregatum*". Arch. Microbiol. **169:**129-135
15. **Fröstl J. and Overmann J.** (2000) Phylogenetic affiliation of the bacteria that constitute phototrophic consortia. Arch. Microbiol. **174:**50-58
16. **Fry B.** (1986) Sources of carbon and sulfur nutrition for consumers in three meromictic lakes of New York State. Linmnol. Oceanogr. **31:**79-88
17. **Gasol J.M., Jürgens K., Massana R., Calderón-Paz J.I. and Pedrós-Alió C.** (1995) Mass development of *Daphnia pulex* in a sulfide-rich pond (Lake Cisó). Arch. Microbiol. **132:**279-296
18. **Glaeser J., Bañeras L., Rütters H. and Overmann J.** (2001) Novel bacteriochlorophyll *e* structures and species-specific variability of pigment composition in green sulfur bacteria. Arch. Microbiol. **177:**476-485
19. **Grice K., Gibbison R., Atkinson J.E., Schwark L., Eckhardt C.B. and Maxwell J.R.** (1996) Maleimides (1*H*-pyrolle-2,5-diones) as molecular indicators of anoxygenic photosynthesis in ancient water columns. Geochim. Cosmochim. Acta **60:**3913-3924
20. **Hartgers W.H., Schouten S., Lopez J.F., Sinninghe Damsté J.S. and Grimalt J.O.** (2000) ^{13}C-contents of sedimentary bacterial lipids in a shallow sulfidic monomictic lake (Lake Cisó, Spain). Org. Geochem. **31:**777-786
21. **Hartgers W.H., Sinninghe Damsté J.S., Requejo A.G., Allan J., Hayes J.M., Ling U., Xie T.-M., Primack J. and de Leeuw J.W.** (1993) A molecular and carbon isotopic study towards the origin and diagenetic fate of diaromatic carotenoids. Org. Geochem. **22:**703-725
22. **Huang Y., Freeman K.H., Wilkin R.T., Arthur M.A. and Jones A.D.** (2000) Black Sea chemocline oscillations during the Holocene: molecular and isotopic studies of marginal sediments. Org. Geochem. **31:**1525-1531
23. **Jørgensen B.B., Kuenen J.G. and Cohen Y.** (1979) Microbial transformation of sulfur compounds in a stratified lake (Solar Lake, Sinai). Limnol. Oceanogr. **24(5):**799-822
24. **Koopmanns M.P., Köster J., van Kaam H.M.E., Kenig F., Schouten S., Hartgers A.H., de Leeuw J.W. and Sinninghe Damsté J.S.** (1996) Diagenetic and catagenetic products of isorenieratene: Molecular indicators for photic zone anoxia. Geochim. Cosmochim. Acta **60:**4467-4496
25. **Krügel H., Krubasik P., Weber K., Saluz H.P. and Sandmann G.** (1999) Functional analysis of genes from *Streptomyces griseus* involved in the synthesis of isorenieratene,

a carotenoid with aromatic end groups, revealed a novel type of carotenoid desaturase. Biochim. Biophys. Acta **1439:**57-64

26. **Langworthy T.A.** (1985) Lipids of Archaebacteria, pp. 459-497. *In*: Woese C.R., Wolfe R.S. (eds.), The Bacteria, Vol. 8. Academic Press, New York, USA
27. **Lauterborn R.** (1906) Zur Kenntnis der sapropelischen Flora. Allg. Bot. **2:** 196 - 197
28. **Ludwig W., Strunk O., Klugbauer S., Klugbauer N., Weizenegger M., Neumaier J., Bachleitner M. and Schleifer K.H.** (1998) Bacterial phylogeny based on comparative sequence analysis. Electrophoresis. **19:**554-568
29. **Madigan M.T., Takigiku R., Lee R.G., Gest H. and Hayes K.M.** (1989.)Carbon isotope fractionation by thermophilic phototrophic sulfur bacteria: evidence for autotrophic growth in natural populations. Appl. Environ. Microbiol. **55:** 639-644
30. **Montesinos E., Guerrero R., Abella C. and 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
31. **Muyzer G., de Waal E.C. and Uitterlinden A.G.** (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
32. **Overmann J., Coolen M.J.L. and 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
33. **Overmann J., Cypionka H. and Pfennig N.** (1992) An extremly low-light-adapted phototrophic sulfur bacterium from the Black Sea. Limnol. Oceanogr. **32:**150-155
34. **Overmann J. and Garcia-Pichel F.** (2001) The phototrophic way of life. *In*: Dworkin M. et al. (eds.), The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community, 3rd edition (latest update release 3.11, September 2002). Springer, New York, USA, 2000 (http://ep.springer-ny.com:6336/contents/)
35. **Overmann J., Beatty J.T., Hall K.J., Pfennig N. and Northcote T.G.** (1991) Characterization of a dense, purple sulfur bacterial layer in a meromictic salt lake. Limnol. Oceanogr. **36:**846-859
36. **Overmann J. and Pfennig N.** (1989) *Pelodictyon phaeoclathratiforme* sp. nov., a new brown-colored member of the Chlorobiaceae forming net-like colonies. Arch. Microbiol. **152:**401-406

37. **Overmann J. and Schubert K.** (2002) Phototrophic consortia: model systems for symbiotic interrelations between prokaryotes. Arch. Microbiol. **177:**201-208
38. **Overmann J. and Tilzer M.M.** (1998) Control of primary productivity and the significance of photosynthetic bacteria in a meromictic kettle lake. Mittlerer Buchensee, West-Germany. Aquatic. Sci. **51:**261-278
39. **Overmann J. and Tuschak C.** (1997) Phylogeny and molecular fingerprinting of green sulfur bacteria. Arch. Microbiol. **167:**302-309
40. **Overmann J., Tuschak C., Fröstl J.M. and Sass H.** (1998) The ecological niche of the consortium "*Pelochromatium roseum*". Arch. Microbiol. **169:**120-128
41. **Overmann J. and van Gemerden H.** (2000) Microbial interactions involving sulfur bacteria: implications for the ecology and evolution of bacterial communities. FEMS Microbiol. Rev. **24:**591-599
42. **Pancost R.D, Freeman K.H., Patzkowsky M.E., Wavrek D.A. and Collister J.W.** (1998) Molecular indicators of redox and marine photoautotroph composition in the late Middle Ordovician of Iowa, USA Org. Geochem. **29:**1649-1662
43. **Passier H.F., Bosch J.-H., Nijenhuis L.J.L., Böttcher M.E., Leenders A., Sinninghe Damsté J.S., de Lange G.J. and de Leeuw J.W.** (1999) Sulphidic mediterranean surface waters during pliocene sapropel formation. Nature. **397:**146-149
44. **Pfennig N.** (1980) Syntrophic mixed cultures and symbiotic consortia with phototrophic bacteria: A Review. pp. 127-131 *In*: Gottschalk et al. (eds.), Anaerobes and Anaerobic Infections. Gustav Fischer Verlag, Stuttgart, New York
45. **Porter K.G. and Feig Y.S.** (1980) Use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25:**943-948
46. **Putschew A., Scholz-Böttcher B.M. and Rullkötter J.** (1996) Early diagenesis of organic matter and related sulfur incorporation in surface sediments of meromictic Lake Cadagno in the Swiss Alps. Org. Geochem. **25:**379-390
47. **Quandt L., Gottschalk G., Zielger H. and Stichler W.** (1977) Isotope discrimination by photosynthetic bacteria. FEMS Microbiol. Lett. **1:**125-128
48. **Rau G.** (1978) Carbon-13 depletion in a subalpine lake: carbon flow implications. Science **201:**901-902
49. **Rost R., Zondervan I. and Riebesell U.** (2002) Light-dependent carbon isotope fractionation in the coccolithophorid *Emiliania huxleyi.* Limnol. Oceanogr. **47:**120-128

50. **Schaeffer P., Adam P., Wehrung P. and Albrecht P.** (1997) Novel aromatic carotenoid derivatives from sulfur photosynthetic bacteria in sediments. Tetrahedron Lett. **48:**8413-8416
51. **Schidlowski M.** (1988) A 3,800-million-year isotopic record of life from carbon in sedimentary rocks. Nature. **333:**313-318
52. **Schink B.** (1991) Syntrophism among prokaryotes. In: Balows, A., H. G. Trüper, M. Dworkin, W. Harder and K.-H. Schleifer (Eds.): The Prokaryotes. Springer Verlag, New York, Berlin, Heidelberg
53. **Schouten S., Rupstra W.I.C., Kok M., Hopmans E.C., Summons R.E., Volkman J.K. and Sinninghe Damsté J.S.** (2001) Molecular organic tracers of biogeochemical processes in a saline meromictic lake (Ace Lake). Geochim. Cosmochim. Acta **65:**1629-1640.
54. **Schouten S., van Kaam-Peters H.M.E., Schoell M. and Sinninghe Damsté J.S.** (2000) Effects of an oceanic anoxic event on Early Toarcian carbon. Am. J. Sci. **300:**1-22.
55. **Sinninghe Damsté J.S. and Köster J.** (1998) A euxinic southern North Atlantic Ocean during the Cenomanian/Turonian oceanic anoxic event. Earth Planet. Sci. Lett. **158:**165-173.
56. **Sinninghe Damsté J.S., Schouten S. and van Duin A.C.T.** (2001) Isorenieratene derivatives in sediments: Possible controls on their distribution. Geochim. Cosmochim. Acta **65:**1557-1571.
57. **Sinninghe Damsté J.S., Wakeham S.G., Kohnen M.E.L., Hayes J.M. and de Leeuw J.W.** (1993) A 6,000-year sedimentary molecular record of chemocline excursion in the Black Sea. Nature. **362:**827-829
58. **Sirevåg R.** (1995) Carbon metabolism in green bacteria, pp. 871-883. *In*: Blankenship R.E., Madigan M.T. and Bauer C.E. (eds.), Anoxygenic photosynthetic bacteria, Advances in Photosynthesis, Vol. II. Kluwer Academic Publishers, Dordrecht, The Netherlands
59. **Sirevåg R., Buchanan B.B., Berry J.A. and Troughton J.H.** (1977) Mechanisms of CO2 fixation in bacterial photosynthesis studied by the carbon isotope fractionation technique. Arch. Microbiol. **112:**35-38
60. **Steenbergen C.L.M. and Korthals H.J.** (1982) Distribution of phototrophic microorganisms in the anaerobic and microaerophilic strata of Lake Vechten (The

Netherlands). Pigment analysis and the role in primary production. Limnol. Oceanogr. **27:**883-895

61. **Tornabene T.G., Langworthy T.A., Holzer G. and Oró J.** (1979) Squalenes, phytanes and other isoprenoids as major neutral lipids of methanogenic and thermoacidophilic "Archaebacteria". J. Mol. Evol. **13:**73-83
62. **Trüper H.-G. and Genovese S.** (1968) Characterization of photosynthetic sulfur bacteria causing red water in Lake Faro (Messina, Sizily). Limnol. Oceanogr. **13:**225-232
63. **Tuschak C., Glaeser J. and Overmann J.** (1999) Specific detection of green sulfur bacteria by *in situ*-hybridization with a fluorescently labeled oligonucleotide probe. Arch. Microbiol. **171:**265-272
64. **van der Meer M.T.J., Schouten S. and Sinninghe Damsté J.S.** (1998). The effect on the reversed tricarboxylic acid cycle on the 13C contents of bacterial lipids. Org. Geochem. **28:**527-533
65. **van Gemerden H. and Mas J.** (1995) Ecology of phototrophic sulfur bacteria, pp. 49-85. *In*: Madigan M.T., Bauer C.E. (eds.), Anoxygenic Photosynthetic Bacteria. Kluwer Academic Publishers, Dotrecht, the Netherlands
66. **Wachniew P. and Rózanski K.** (1997) Carbon budget of a mid-latitude, groundwater-controlled lake: Isotopic evidence for the importance of dissolved inorganic carbon recycling. Geochim. Cosmochim. Acta **61:**2453-2465

Chapter 4

The significance of organic carbon compounds for *in situ* metabolism and chemotaxis of phototrophic consortia

INTRODUCTION

Phototrophic consortia are regularly structured aggregates containing a colorless, rod-shaped, central bacterium, which is covered by numerous epibiotic green or brown bacteria (24, 25, 29, 31, 33, 36). Phototrophic consortia have been found in numerous stratified lakes and ponds worldwide (30), where they may contribute up to two-thirds of total prokaryotic biomass (15, 16). This high abundance indicates that phototrophic consortia may be significant participants in the biogeochemical transformations of carbon and sulfur compounds. Cell division in phototrophic consortia proceeds in a highly coordinated fashion (30) and evidence exists for a rapid signal transfer between the epibionts and the central bacterium (13).

A majority of the phototrophic consortia known to date are barrel-shaped and motile (compare Fig. 2D). Although light microscopical investigations indicate that the central rod-shaped bacterium is the motile component of the consortia (30), the precise insertion point and the number of flagella still need to be determined. Even less is known with respect to the physiology of the bacterial cells comprising the phototrophic consortia. Motile phototrophic consortia were described in the early 20th century (4, 21), but became amenable to molecular studies only recently after laboratory cultures had been established. As expected (8, 33, 36), the epibionts of phototrophic consortia were subsequently identified as green sulfur bacteria (43), but represent novel and unique phylotypes within the radiation of green sulfur bacteria (14). Unexpectedly, the central bacterium is a member of the β-Proteobacteria (14). Since the

typical sulfur- and sulfate-reducing bacteria belong either to the δ-subgroup of the Proteobacteria or to the low GC Gram-positive bacteria, the phylogenetic affiliation of the central bacterium is not consistent with the internal sulfur cycle that had previously been postulated for intact phototrophic consortia (33).

The carbon metabolism of the epibionts in a natural population of the brown-colored phototrophic consortium "*Pelochromatium roseum*" has recently been studied by analysing the stable carbon isotope discrimination values of their specific biomarker bacteriochlorophyll (BChl) *e* (16). These data strongly indicate that epibionts grow photoautotrophically under *in situ* conditions and incorporate CO_2 via the reverse tricarboxylic acid cycle. Furthermore, electron transport in cells of the epibionts of the green-colored consortium "*Chlorochromatium aggregatum*" was stimulated by the addition of sulfide to the culture medium as demonstrated by the reduction of the artificial electron acceptor 5-cyano-2,3-di-4-tolyl-tetrazolium chloride (CTC) (13). Physiologically, the green sulfur bacterial epibionts in phototrophic consortia appear to be very similar to their free-living counterparts. Nevertheless, the epibionts represent phylogenetic lineages distinct from free-living green sulfur bacteria, indicating that they have adapted to the life in association. To date, the selective advantage for the green sulfur bacteria in the associated state has remained obscure.

In cultures of "*C. aggregatum*" growth of intact consortia was only observed in the presence of both light and 2-oxoglutarate, and the consortia exhibited a chemotactic behavior towards 2-oxoglutarate (13). Given the autotrophic growth of epibionts in natural populations, it is unknown whether organic carbon compounds are also significant for the chemotaxis and growth of phototrophic consortia in the environment. In order to gain more insight into the physiology of the bacterial partners in the associated state and under natural conditions, we investigated the chemotactic behavior and the uptake of organic carbon compounds in a natural population of the phototrophic consortium "*P. roseum*".

MATERIAL AND METHODS

Study site

Eutrophic Lake Dagow (surface area, 0.24 km^2; maximum depth, 9.5 m) is located approximately 100 km north of Berlin, Germany (7). Each year, an anoxic, sulfide-containing hypolimnion develops after the onset of summer stratification and a dense accumulation of phototrophic consortia forms at a depth between 6.0 and 8.0 m (16, 30). During the present study, the lake was visited between July 2 and 7, 1998.

Water sampling

Water samples were obtained from the deepest part of the lake employing a bilge pump connected to gas-tight isoversinic tubing. A sampling depth of 6.3 m was chosen based on the vertical distribution of anoxygenic photosynthetic activity as determined in a parallel study (16). The inlet of the tubing consisted of two polyvinyl chloride (PVC) cones, spaced 1 cm apart from each other (19). This device allowed the reproducible sampling of different water layers in 5 cm intervals. The samples were filled into autoclaved 1 liter glass bottles, which were kept in the dark to avoid the damage of the phototrophic bacteria caused by the high light intensities at the lake surface (30). The bottles were gas tight sealed to prevent abiotic oxidation of sulfide and brought back to the laboratory in an insulated container which maintained *in situ* temperatures.

Environmental parameters and bacterial cell numbers

A LI-1000 spherical quantum sensor (wavelength range 400-700 nm) connected to a data logger (Li Cor, Lincoln, Nebr., USA), was used to determine underwater irradiance. Conductivity and temperature were measured with a LF 196 conductivity meter (WTW, Weilheim, Germany), pH with an pH/T electrode connected to a microprocessor pH meter 196T (WTW), and oxygen concentrations with an Oximeter Oxi 196 (WTW). Sulfide concentrations were determined by the methylene blue method (9). To avoid sulfide oxidation, 5 to 20 ml aliquots were collected directly from the pump outlet into glass tubes containing basic zinc acetate solution (final concentrations, 10 mM zinc acetate, 1 mM NaOH).

Total cell numbers of bacteria and numbers of phototrophic consortia were determined by epifluorescence microscopy (Zeiss Axiolab; Zeiss, Oberkochen, Germany) on black polycarbonate membrane filters (0.2 μm pore size, Isopore GTBP membranes; Millipore, Eschborn, Germany) after staining with 4′, 6-diamidino-2-phenylindole (DAPI) (34), employing the Zeiss filter set no. 1. In samples with high numbers of phototrophic consortia, numbers were determined directly by counting individual consortia in each microscopic field at a magnification of 1000x. For samples with low numbers of phototrophic consortia, a total of 100 microscopic fields were examined, the presence or absence of consortia was recorded, and numbers were calculated by applying the most-probable-number formula of Button et al. (6) as described previously (30).

Electron microscopy

Flagella of phototrophic consortia were visualized by transmission electron microscopy after negative staining of bacterial cells from the chemocline water. Single drops of chemocline water were transferred to Formvar-coated copper grids (150 µm mesh size) using an inoculation loop. Bacterial cells were allowed to settle on the copper grid for 5 min and the water was subsequently removed with a tissue paper. Uranyl acetate solution (5%, w/v in water) was then applied to the grid for 1 min at room temperature. After draining off the uranyl acetate, the copper grids were washed with double distilled water, and air-dried. Samples were analyzed in a Zeiss EM109 electron microscope. The phototrophic consortia were identified according to their distinct morphology and the presence, number and insertion points of bacterial flagella recorded.

In situ *chemotaxis assay*

The chemotactic response of phototrophic consortia was tested *in situ* by incubation of capillaries filled with different test compounds in the chemocline of Lake Dagow. Sterile 100 mM stock solutions of sulfide, thiosulfate, glycerol, lactate, propionate, citrate, succinate, 2-oxoglutarate and glycine were prepared anoxically in Hungate tubes sealed with butyl rubber septa, and flushed with N_2. For preparation of organic acids, the corresponding sodium salts were used and the pH of the stock solutions was adjusted to 7.3 with NaOH. This pH value was chosen based on the pH values of 7.3 to 7.4 as determined previously for chemocline water in Lake Dagow (16; 30). All compounds were diluted to a final concentration of 500 µM with filter sterilized (cellulose nitrate membrane filters, pore size 0.2 µm; Sartorius, Göttingen, Germany), anoxic chemocline water. In the water sample used for dilution, original sulfide concentrations were ≤ 5 µM and oxygen was below the detection limit (≤ 5µM) (see Fig. 1A).

Each incubation rack consisted of a solid PVC-block (length, 480 mm; height, 50 mm; width, 28 mm), which had 15 x 3 mm diameter holes drilled in its upper side. The holes were spaced 3 cm apart from each other in order to avoid interference of individual chemotaxis assays and were filled with plasticine. Rectangular capillaries (length, 50 mm; inside diameter, 0.1 x 1.0 mm; capacity 5 µl, Vitro Dynamics, Rockaway, N.J., USA) were filled with the diluted test substances. Controls were filled with sterile, anoxic chemocline water only. The capillaries were immediately sealed with plasticine (Idena, Berlin, Germany) at one end and then fixed with the closed end in the PVC incubation rack. For incubation, two 1 m-steel wires were fixed to the ends of the incubation rack and their other end attached to a calibrated steel wire which in turn was connected to a float at the lake surface. With this

setup, the chemotaxis assays could be positioned in the Lake Dagow chemocline with a vertical precision of better than 5 cm. Directly after filling the capillaries, the incubation rack was lowered into the water column. Incubation lasted for 3 to 5 hours.

After incubation, the rack was retrieved from the chemocline; each capillary was recovered from the rack and immediately closed with plasticine. The capillaries were then stored in a cool box and transported back to the laboratory where they were analyzed within 1 hour following completion of the incubation. Phototrophic consortia were counted directly in each capillary by phase-contrast and dark-field microscopy at a magnification of 100x and 400x.

Heterotrophic potential of 2-oxoglutarate uptake

The significance of 2-oxoglutarate for microbial metabolism in the chemocline was assessed by incubation of water samples with different concentrations of radioactively labeled 2-oxoglutarate. A stock solution of 2-[^{14}C(U)]-oxoglutarate (specific activity 10.4 GBq·mmol^{-1}, concentration 356 µM; DuPont, Bad Homburg, Germany) was diluted 1:100 using sterile filtered water from a depth of 6.3 m. The working solution was added to 18 ml-samples from 6.3 m depth to yield final concentrations of 2, 5, 10, 20 and 50 nM. Three replicates were prepared for each concentration. One replicate received formaldehyde at a final concentration of 4% (v/v) to stop microbial activity. All samples were incubated *in situ* (6.3 m) employing glass tubes sealed with teflon-lined screw caps. After an incubation period of 1.2 hours, the tubes were retrieved and the active samples also preserved by addition of formaldehyde.

Subsequently, the samples were transferred to serum bottles and acidified with 0.5 ml HCl to a final pH of 1.8. $^{14}CO_2$ was extracted with N_2 and trapped in Vigreux columns filled with 10 ml Carbosorb E (Canberra-Packard, Dreieich, Germany). Radioactivity of these samples was counted by liquid scintillation after adding one volume of PermaFluorE$^+$ (Packard) to the trapping solution. The extracted samples were filtered through 0.2 µm pore size membrane filters (cellulose nitrate, Ø 25 mm; Schleicher and Schuell, Dassel, Germany), the filters placed in glass vials containing 10 ml of scintillation solution (Aquasafe 300, Packard) and counted in a Packard 1600TR scintillation counter. For calculation of 2-oxoglutarate uptake, values of formaldehyde-treated controls were subtracted. Since 2-oxoacids are unstable and are decarboxylated or decarbonylated under strongly acidic conditions, we also determined the chemical degradation of 2-oxoglutarate in a separate experiment. Radioactively labeled 2-oxoglutarate was added to 18 ml aliquots of double distilled water at the same concentration as in the chemocline experiments, and the liberation of $^{14}CO_2$ monitored after acidification with HCl in the presence and absence of 4% v/v

glutaraldehyde. In the absence of formaldehyde, the amount of CO_2 liberated by abiotic processes reached 2% of the values determined for physiologically active chemocline bacteria and therefore proved to be insignificant. In the presence of formaldehyde, however, 48% of the CO_2 liberated was due to chemical degradation of 2-oxoglutarate. In this case, the respiration data were corrected for abiotic degradation of the radiolabeled substrate.

Since the natural concentration of 2-oxoglutarate was unknown, its turnover time and the affinity of bacterial uptake systems were estimated according to the method of Wright and Hobbie (46):

$$\frac{t_{inc}}{F} = \frac{K_t + S_n}{V_{\max}} + \frac{1}{V_{\max}} \cdot A \qquad (1)$$

where t_{inc} is the incubation time, F the ratio of 2-oxoglutarate utilized (i.e., degraded plus assimilated) to 2-oxoglutarate added, K_t the half saturation constant for the substrate uptake, S_n the (unknown) concentration of 2-oxoglutarate *in situ*, V_{max} the maximum uptake rate for the substrate, and A the concentration of the radioactively labeled substrate added. The turnover time of the substrate at its natural concentration can be estimated as the y-axis intercept after linear regression of t_{inc}/F versus A. The x-axis intercept (K_t+S_n) provides a maximum estimate for the half saturation constant K_t of 2-oxoglutarate uptake (46).

Uptake of 2-oxoglutarate by phototrophic consortia

Radioactively labeled 2-[^{14}C(U)]-oxoglutarate (specific activity 10.4 GBq·mmol^{-1}, DuPont, Bad Homburg, Germany) was added to water samples to a final activity of 2.6 kBq·ml^{-1}, corresponding to a concentration of 250 nM 2-oxoglutarate. Samples were incubated anoxically in screw-cap glass tubes with gas tight rubber seals (22.5 ml; Fisher Scientific, Elancourt, France) at 10 °C (the temperature *in situ*) at a light intensity of 5 µmol quanta·m^{-2}·s^{-1} (18 W daylight fluorescent bulb L, Osram, München, Germany). This light intensity was chosen since photosynthetic activity of phototrophic consortia at *in situ* light intensity (1 µmol quanta·m^{-2}·s^{-1}, see Results section) is light limited (16) and since previous studies had demonstrated that intensities ≥ 10 µmol quanta·m^{-2}·s^{-1} inhibit phototrophic consortia (30). A second set of samples was incubated in the dark. In a third experiment, incubation tubes received sulfide at a final concentration of 200 µM prior to the addition of radioactively labeled 2-oxoglutarate. A neutralized stock solution of sulfide (pH 7.4; 37) was used in order not to change the pH of the water samples. After 2, 10, 30, 60 and 180 min, the uptake of the radioactive label was stopped by the addition of formaldehyde to a final concentration of 4%. Controls for the unspecific adsorption of radiolabeled substrate were

directly treated with formaldehyde and then incubated in the light for 2 or 180 min. All experiments were run in duplicate.

Excess radioactivity was removed from all samples by three washes of the cell material by repeated centrifugation (13000 x g, 4°C, 5 min) and resuspension of the pellets in 500 µl artificial freshwater (3). Afterwards, the bacterial cells were immobilized on acetone cleaned microscopic slides for two minutes at 70°C. The samples were stored in boxes with blue silica gel at room temperature until further processing.

For microautoradiography, the microscopic slides were coated with autoradiographic coating solution EM-1 (Amersham International, Buckinghamshire, UK). The solution was heated for 15 min at 43°C in a darkroom and the microscopic slides were dipped into the coating solution using a Coplin jar. Excess solution was drained off from the backside of the slides using a tissue paper. Slides were placed horizontally on a cooled (-20°C) metal plate for 10 min to solidify the coating solution, and were subsequently dried at ambient temperature for 2 hours. Exposure of the autoradiographic emulsion was performed at 4 °C in the dark in closed plastic containers over blue silica gel (1, 2).

Initial microautoradiography experiments demonstrated that sufficiently strong signals could only be obtained after an exposure time of 21 days. This time was used in all subsequent experiments. After samples had warmed up to room temperature the emulsion was developed for 4 min with Kodak 19 developer (40 $g \cdot l^{-1}$; Kodak, Stuttgart, Germany), followed by incubation for 1 min in acetic acid (0.5 % w/v) and by fixation in sodium thiosulfate solution (30% w/v). Finally, the slides were washed for 20 min in tap water, then for 2 min in double distilled water and dried for 2 h at ambient temperature in the dark.

For post-staining, an aqueous solution of DAPI (final concentration, 0.2 $\mu g \cdot ml^{-1}$) was directly applied to the dried slides (2). After drying of the staining solution, one drop of paraffin oil was pipetted on top of the emulsion and the sample sealed with a cover slip. The slides were inspected with epifluorescence, phase-contrast and bright field microscopy. Phototrophic consortia and purple sulfur bacteria were investigated with regard to the formation of silver grains on top of and around the cells by phase contrast microscopy. Uptake of radioactive label was recorded for each "*P. roseum*" consortium detected. For comparison, the uptake of label by *Chromatium* cells was assessed. On each microscopic slide, a total of 50 "*P. roseum*" consortia and 50 *Chromatium* cells were investigated and the percentage of consortia and *Chromatium* cells which were associated with silver grains was determined.

RESULTS

Environmental parameters and vertical distribution of bacteria in the chemocline

In July 1998, pronounced vertical gradients of temperature and conductivity indicated a persistent stratification of the water column below a depth of 4 m in Lake Dagow (data not shown). Molecular oxygen was depleted at a depth of 6.0 m (Fig. 1A). Sulfide was detected in water layers below this depth and reached concentrations of 240 µM above the lake bottom.

Epifluorescence microscopy revealed a dense accumulation of "*P. roseum*" between depths of 6.3 and 6.6 m. "*P. roseum*" was most abundant at 6.4 m where a total of $5.5 \cdot 10^5$ consortia per milliliter were counted (Fig. 1B). The total cell count at this depth was $5.7 \cdot 10^7$ per milliliter (Fig. 1B). Based on a ratio of 20 epibionts per central bacterium (30), epibionts accounted for 16 % and central bacteria for 0.8% of the total bacterial numbers at the depth of the highest abundance of "*P. roseum*". At a depth of 6.5 m, epibionts contributed an even larger part to the bacterial community and accounted for 42% of all bacteria. The water temperature at the depth of maximum "*P. roseum*" numbers was between 9 and 10°C and the

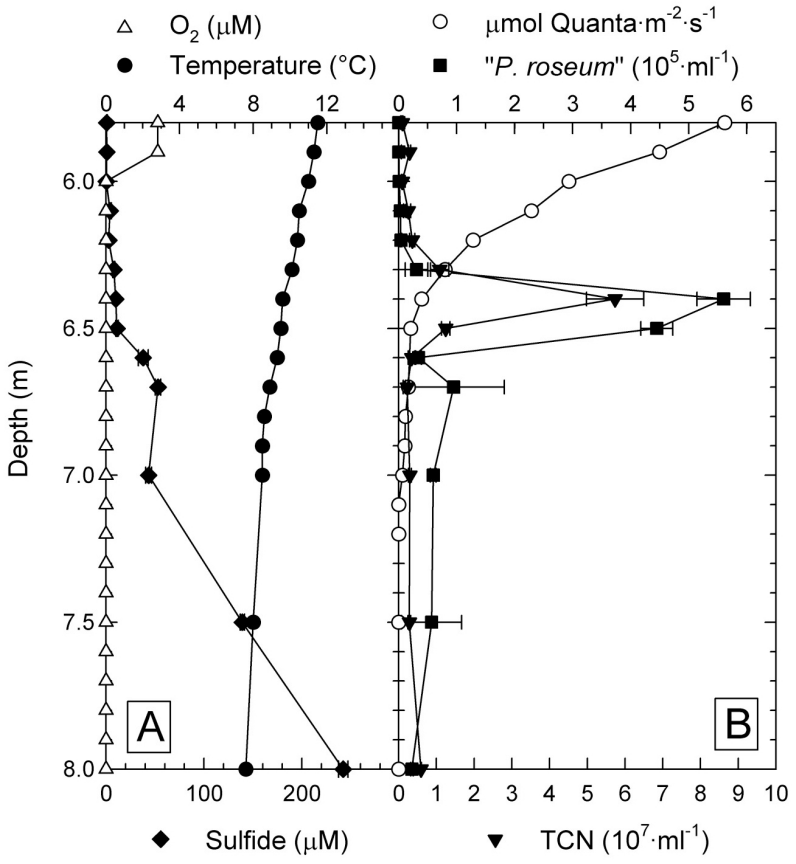


Figure 1. Physical, chemical and biological parameters in the hypolimnion of Lake Dagow on July 4, 1998. **A.** Vertical distribution of temperature, and of oxygen and sulfide concentrations. **B.** Vertical distribution of light intensities, numbers of "*P. roseum*" consortia and total cell numbers (TCN). Error bars represent standard deviations of two measurements.

pH was between 7.3 and 7.4. Sulfide concentrations in the chemocline accumulation of "*P. roseum*" consortia were very low and ranged between 0.1 and 5 µM (Fig. 1A).

Similar to other chemocline communities of anoxygenic phototrophic bacteria (45), the cell-specific physiological activity of phototrophic consortia was found to be highest at 6.3 m, which is above their biomass maximum (16). Consequently, samples for physiological experiments were taken from this depth. At 6.3 m depth, epibionts accounted for 5.4 % and central bacteria for 0.3% of the total cell count. The light intensity at this depth was 0.8 µmol quanta·m^{-2}·s^{-1} (Fig. 1B).

Flagellation of phototrophic consortia

Flagella were visualized by transmission electron microscopy of negatively stained "*P. roseum*" consortia. Based on their larger size and their spindle-shaped morphology, the central bacteria could be distinguished from epibionts. This was especially evident in partially disaggregated consortia where few epibiont cells had remained attached to the central bacterium (Fig. 2A). Analyses revealed a monopolar monotrichous flagellation of the central bacterium (Fig. 2B and C). On average, the flagellum measured 2.5 times the length of the "*P. roseum*" consortium.

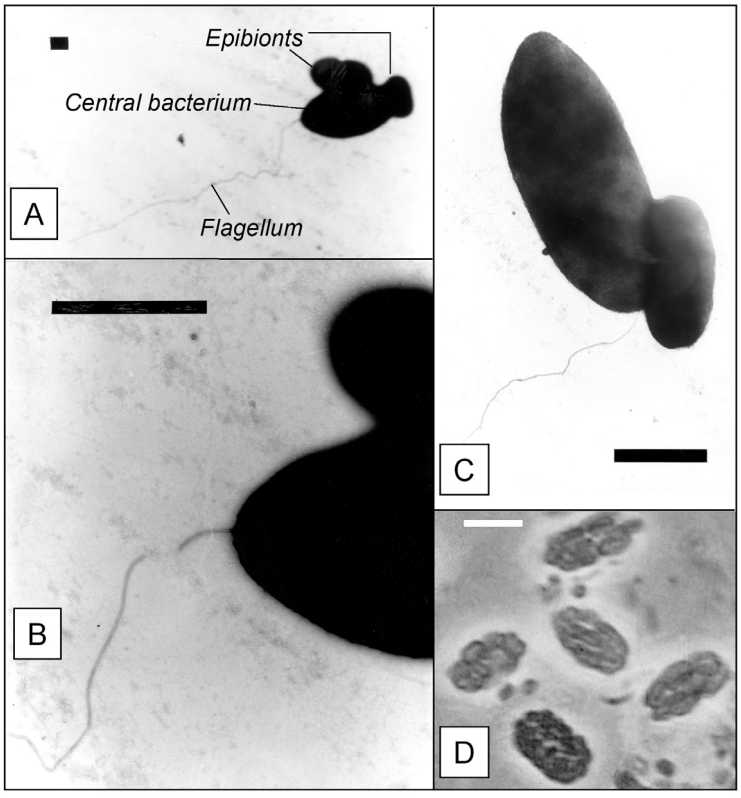


Figure 2. A - C. Electron micrograph of negatively stained and partially disaggregated phototrophic consortia obtained from chemocline water samples, showing the monopolar monotrichous flagellation of the central bacterium. Bars represent 1 µm. **D.** Phase contrast photomicrograph of "*P. roseum*" from Lake Dagow. Bar represents 5 µm.

Chemotaxis of "P. roseum"

Intact "*P. roseum*" consortia exhibited pronounced positive chemotaxis towards sulfide and 2-oxoglutarate (Fig. 3). Numbers of "*P. roseum*" consortia in capillaries containing 500 µM sulfide or 500 µM 2-oxoglutarate exceeded those in the control by a factor of 4 and 2.5, respectively. In contrast, capillaries filled with succinate harbored significantly less consortia then the control. Succinate thus acted as a repellent *in situ*.

Heterotrophic potential of 2-oxoglutarate uptake

Incubation with 2-[^{14}C(U)]-oxoglutarate revealed that the chemocline microbial community was capable of utilizing this substrate even at nanomolar concentrations (Fig. 4). When plotting the ratio *tinc*/*F* for incorporated 2-[^{14}C(U)]-oxoglutarate against the concentration *A* of the radioactive substrate added, the regression yielded

$$t_{inc}/F = 8.71\text{h} + 0.50(\text{h}\cdot\mu\text{M}^{-1})\cdot\text{A}(\mu\text{M}) \qquad (r^2=0.987,\ p<0.01),$$

with a turnover time of T = 8.7 h, and a maximum uptake rate of V_{max} = 2 nM·h^{-1}. The calculated maximum for the half saturation constant (estimated as the x-axis intercept, K_t+S_n, Fig. 4) was 17.4 nM. Of the 2-oxoglutarate used, 44.4 ± 8.6 % was trapped as $^{14}CO_2$ and hence had been respired by the microbial community. The respiration measurements yielded

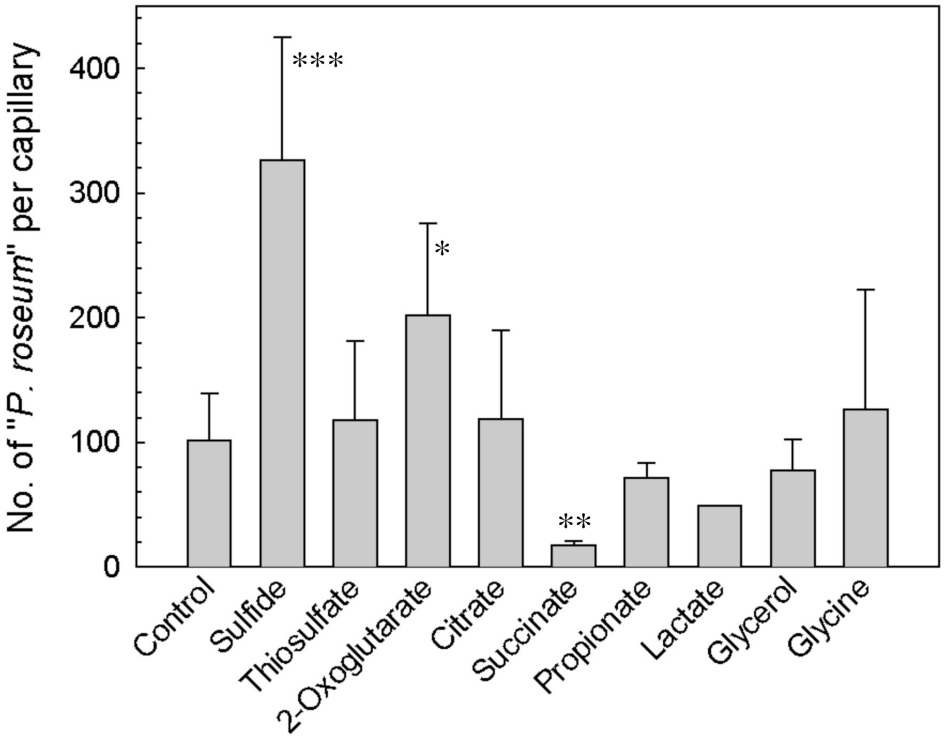


Figure 3. *In situ* chemotaxis of "*P. roseum*". The bars represent numbers of "*P. roseum*" consortia counted in capillaries after incubation for 5 h in the chemocline of Lake Dagow. Error bars represent standard deviations of the counts. Values significantly different from the control are indicated by asterisks (*, $p < 0.1$; **, $p < 0.05$, ***, $p < 0.01$).

data with a higher experimental variability. When uptake rates of 2-oxoglutarate were corrected for the amount of substrate respired, the resulting regression was

$$t_{inc}/F = 6.12\text{h} + 0.18(\text{h}\cdot\mu\text{M}^{-1})\cdot\text{A}(\mu\text{M}) \qquad (r^2=0.785,\ p<0.05).$$

Based on this second dataset, the turnover time of 2-oxoglutarate in the chemocline of Lake Dagow was 6.1 hours, the maximum uptake rate was 5.6 $nM\cdot h^{-1}$, and the maximum for the half saturation constant 34 nM (Fig. 4). Theoretically, the latter value should be the same as the K_t+S_n value estimated from net incorporation data without respiration (i.e. 17.4 nM); however, the two estimates are rarely similar (18).

Uptake of exogenous 2-oxoglutarate by intact consortia

Uptake of 2-oxoglutarate by individual phototrophic consortia could be detected after water samples from the chemocline of Lake Dagow were incubated with 2-[^{14}C(U)]-oxoglutarate for 180 min, and after the exposure time for microautoradiography was extended to 21 days. Even after this prolonged incubation, the background in silver grain formation remained very low and hence did not interfere with our microautoradiography analysis.

After microautoradiography, "*P. roseum*" consortia were easily identified by their peculiar cell arrangement (e.g. Fig. 5A, arrows) and by the color of the epibiotic green sulfur bacteria in transmitted light (not visible in the epifluorescence photomicrographs of Fig. 5).

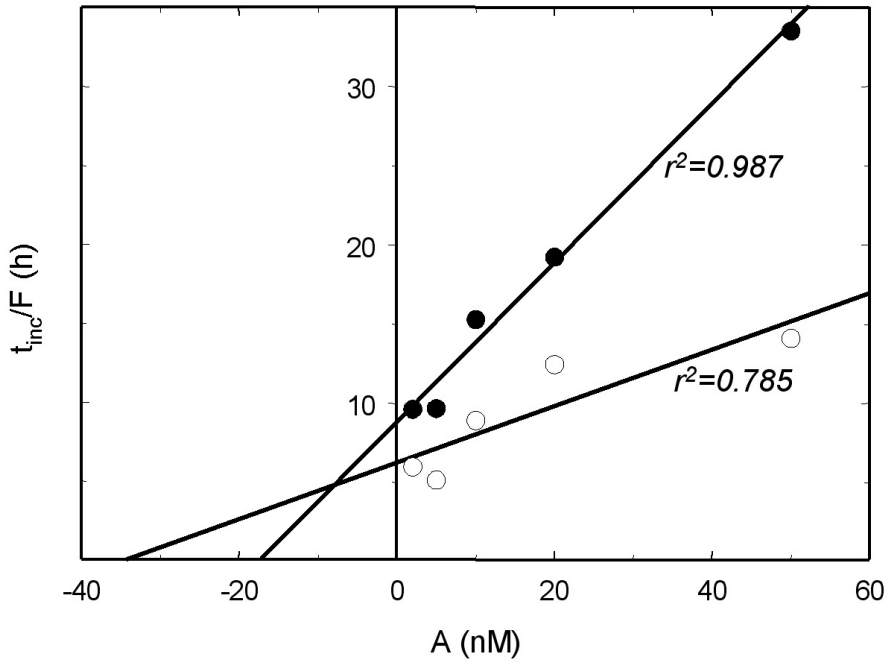


Figure 4. Heterotrophic potential for 2-oxoglutarate utilization of the bacterial community in the chemocline of Lake Dagow. According to the method of Wright and Hobbie (46), the ratio of incubation time to fraction of radiolabel utilized (t_{inc}/F) is plotted against the concentration of radiolabeled 2-oxoglutarate added (A) (see Materials and Methods). Filled circles show values for incorporation only, hollow circles the data for incorporation plus respiration of 2-oxoglutarate. For each data set, the linear regression is plotted.

Uptake of 2-[^{14}C(U)]-oxoglutarate was clearly detectable through silver grain formation on top and around "*P. roseum"* consortia (Fig. 5D). On average, 87.5% of all "*P. roseum*" consortia incorporated radioactive label when sulfide and light were present (Fig. 5D and 6). In contrast, ≤ 1.4% of "*P. roseum*" consortia resulted in the formation of silver grains when either (1) incubated in the light but in the absence of sulfide (Fig. 5A and B), or (2) kept in the dark in the presence of sulfide, or (3) in the dark without sulfide, or (4) when treated with formaldehyde prior to the addition of 2-[^{14}C(U)]-oxoglutarate (Fig. 6).

For comparison, the uptake of radioactive label by purple sulfur bacteria was investigated. In the chemocline of Lake Dagow, purple sulfur bacteria were present at smaller numbers than "*P. roseum*" consortia (16, 30). The rod-shaped cells were closely related to *Chromatium okenii* as shown by culture-independent molecular methods (27). In contrast to "*P. roseum*" consortia, 62 to 84% of *Chromatium* cells incorporated radioactive label under the different conditions tested, irrespective of the presence of light or sulfide (Fig. 6). A very weak uptake of radioactive label by 0.5 % of the *Chromatium* cells was observed when cells

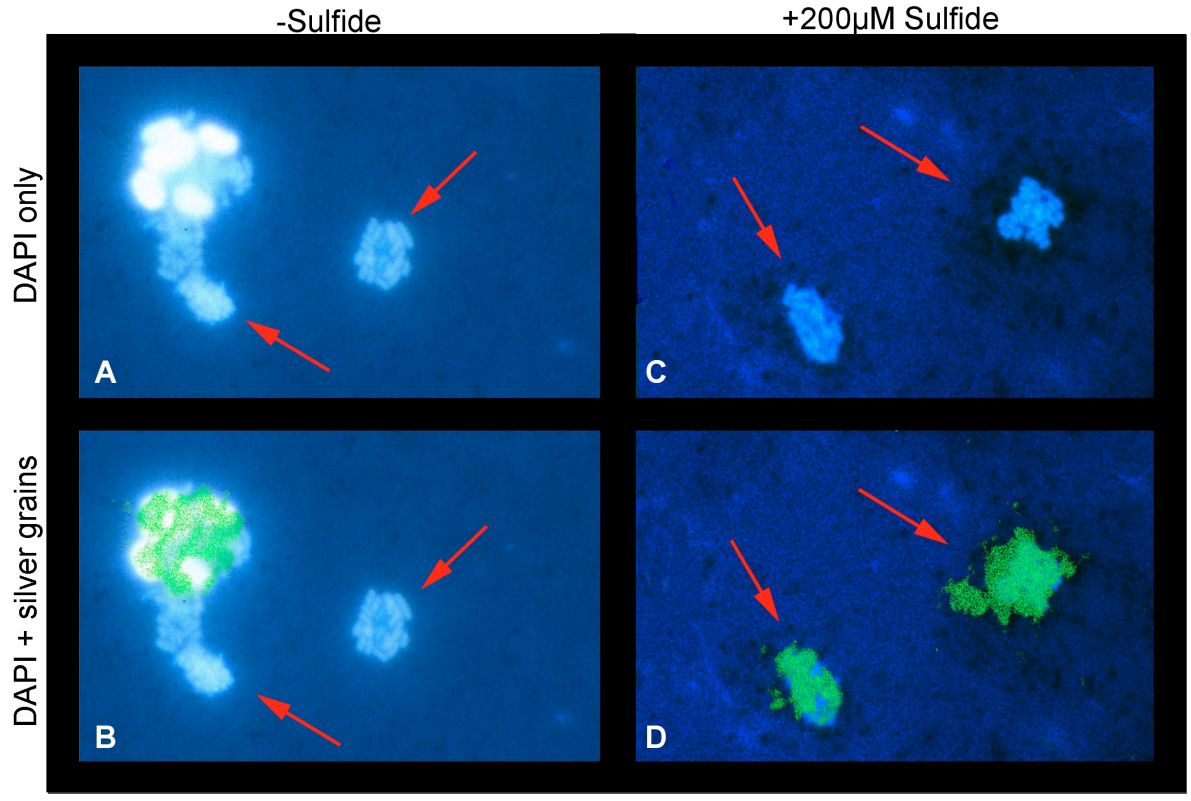


Figure 5. Uptake of 2-[^{14}C(U)]-oxoglutarate by "*P. roseum*" consortia as detected by microautoradiography. Upper microphotographs in **A** and **C** show bacterial cells as observed by epifluorescence microscopy after DAPI staining. Lower photomicrographs (**B, D**) indicate the position of silver grains (depicted in the false color green) and were created by overlaying bright-field pictures of the silver-grain layer with images of the bacterial cells. Red arrows point towards individual phototrophic consortia. The very bright cells in **A** are *Chromatium* cells.

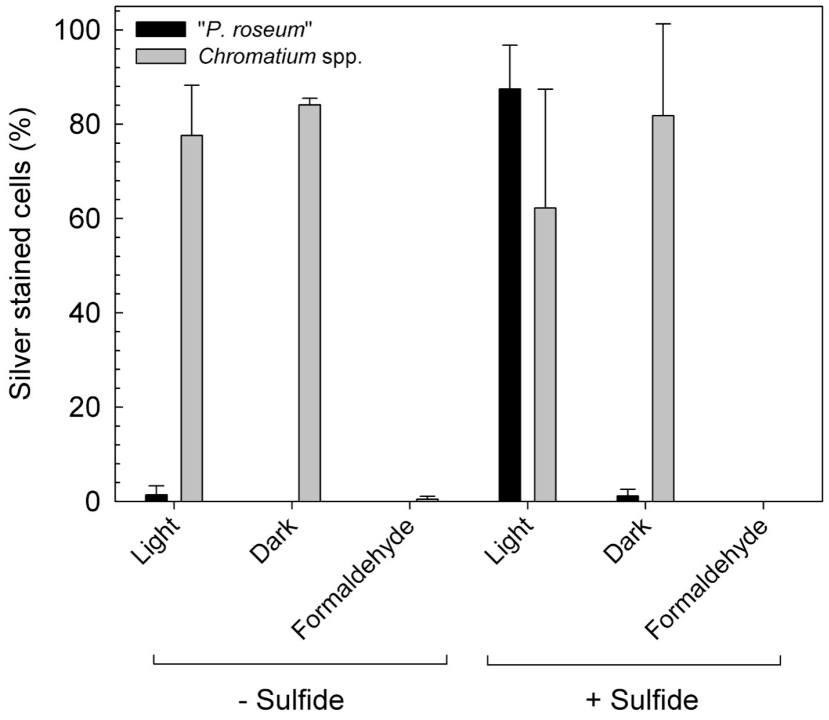


Figure 6. Percentage of silver grain formation by "*P. roseum*" consortia and *Chromatium* cells in the absence and presence of sulfide. The fraction of cells, which developed silver grains in the photographic emulsion, was determined by microscopic counting.

were killed by formaldehyde prior to the incubation with 2-[^{14}C(U)]-oxoglutarate (Fig. 6). Hence, non-specific adsorption of 2-oxoglutarate to bacterial cells was negligible in our microautoradiography experiments.

DISCUSSION

In a parallel study, stable carbon isotope analyses revealed that the epibionts of "*P. roseum*" consortia in Lake Dagow grow photolithoautotrophically (16). In laboratory cultures of the green-colored "*C. aggregatum*", however, growth of intact consortia was only observed in the presence of both, light and 2-oxoglutarate. In addition, "*C. aggregatum*" exhibits a chemotactic behavior towards 2-oxoglutarate (13). The aim of the present study was therefore to obtain new insights into the significance of organic carbon compounds for the metabolism and chemotaxis of "*P. roseum*" consortia under natural conditions. Previous investigations of the Lake Dagow chemocline demonstrated a low diversity of green sulfur bacteria (27) and a numerical dominance of "*P. roseum*" epibionts among the phototrophic bacteria community (16). We chose this natural accumulation of "*P. roseum*" as a well-suited experimental system for culture-independent studies of the physiology of the consortium "*P. roseum*" and the interaction of the two symbiotic partners.

Motility and chemotaxis of phototrophic consortia

Although previous studies had provided preliminary evidence for a monopolar flagellation of phototrophic consortia (4, 30), the precise insertion and number of flagella could not be shown unambiguously to date. The present study for the first time demonstrates a monopolar monotrichous flagellation of the central bacterium. In contrast, the epibionts of phototrophic consortia were never observed to carry flagella, similar to all described species of green sulfur bacteria (25).

In combination with the chemotaxis towards sulfide, the motility of the phototrophic consortia provides a selective advantage for the otherwise immotile green sulfur bacterial epibionts. Some free-living species of green sulfur bacteria form gas vesicles, thereby decreasing the buoyant density of the cells. Cells of *Pelodictyon phaeoclathratiforme* increase their gas vesicle content upon transfer to low light intensities, but these changes in buoyancy occur over a time period of several days (28) and thus are too slow compared to the diurnal fluctuations in light intensities and sulfide concentrations. In contrast, vertical migration of phototrophic consortia by flagellar motility is orders of magnitude faster and hence will confer a selective advantage on the green sulfur bacterial epibionts over their free-living relatives. Motility will also counteract the sedimentation of the rather dense consortia (30) out of the chemocline. Together with the scotophobic response, the chemotaxis towards sulfide are likely explanations for the dominance of "*P. roseum*" consortia in Lake Dagow where sulfide is strongly depleted during daytime and reaches concentrations of only ≤ 5 µM in the layer of anoxygenic phototrophic bacteria.

Our *in situ* experiments revealed clear chemotaxis of "*P. roseum*" towards 2-oxoglutarate. However, lower numbers of consortia accumulated in the capillaries containing 2-oxoglutarate as compared to those containing sulfide. It is feasible that this diminished response towards 2-oxoglutarate is caused by the specific carbon metabolism of the epibionts. Free-living green sulfur bacteria have been shown to excrete up to 30% of the photosynthetically fixed carbon (11), mainly as 2-oxo-3-methylvalerate and 2-oxoglutarate (40). The latter compound is a direct intermediate of the reverse tricarboxylic acid cycle which is employed by green sulfur bacteria for CO_2 fixation (12, 40). Since the epibionts in the natural population of "*P. roseum*" consortia of Lake Dagow also grow autotrophically (16), they may excrete 2-oxo acids similar to their free-living relatives. The 2-oxoglutarate excreted would then interfere with sensing of exogenous 2-oxoglutarate thereby leading to a reduced chemotactic response.

So far, chemotaxis towards 2-oxoglutarate is only known from one type of phototrophic consortia, namely laboratory enrichments of "*C. aggregatum*" (13). Although the epibionts of "*C. aggregatum*" and "*P. roseum*" are phylogenetically rather distantly related (16), the respective consortia both exhibit the same chemotactic behavior. Possibly, chemotaxis towards 2-oxoglutarate represents a common property of phototrophic consortia. In the aquatic environment, particulate matter represents a major source of dissolved carbon substrates (42). Organic particles accumulate in the chemocline of stagnant water bodies (10), where they can create a horizontally heterogeneous environment (26). Chemotrophic aquatic bacteria have been shown to occur in patches extending over tens to hundred µm (20). In a similar manner, chemotaxis of motile phototrophic consortia would enable the cells to rapidly accumulate in those parts of heterogeneous environments where organic carbon compounds are released by other microorganisms.

Relevance of 2-oxoglutarate in the natural habitat

Growth of intact phototrophic consortia in laboratory enrichments of "*C. aggregatum*" depends on the addition of exogenous 2-oxoglutarate (13). It is unknown however whether 2-oxoglutarate is directly incorporated by the phototrophic consortia and whether this organic compound is of significance under *in situ* conditions. Therefore, we investigated whether the cells of "*P. roseum*" consortia are not only capable of sensing, but also of incorporating this chemoattractant under *in situ* conditions.

In order to investigate whether 2-oxoglutarate is of relevance in the chemocline carbon cycle of Lake Dagow, the kinetics of 2-oxoglutarate utilization by the microbial community was determined. A pre-requisite for a reliable estimate of kinetic parameters by this method is that the turnover time T of the substrate at its natural concentration (A=0) is significantly longer than the time of incubation. In our case, T was >6 hours and hence clearly exceeded the incubation time used for the incubation experiments. Based on our analysis of the heterotrophic potential, chemocline bacteria express high affinity uptake systems for 2-oxoglutarate (average half saturation constant ≤ 10 - 40 nM) and utilize this carbon compound at an average rate of ~6 nM·h^{-1}. By comparison, aerobic freshwater and marine bacterioplankton has been shown to utilize glucose at maximum rates of 0.13 - 7.4 nM·h^{-1}, acetate at a V_{max} of 0.32 - 88.3 nM·h^{-1} (17, 46), and aspartate at a V_{max} of 0.54 - 17 nM·h^{-1} (5, 18). The maximum estimates for half saturation constants in these cases were 8.6-38 nM glucose, 117-250 nM acetate, and 2 - 526 nM aspartate, respectively. Turnover times of the three substrates ranged between 2 and 430 hours. Similar V_{max} values of 0.02 - 3.1 nM·h^{-1}

have recently been reported for the uptake of amino acids by freshwater bacterioplankton (38).

In conclusion, our data indicate that free dissolved 2-oxoglutarate is present at nanomolar concentrations, is rapidly turned over under anoxic conditions and thus is an important intermediate of the carbon cycle in the chemocline of Lake Dagow. Microautoradiography revealed that phototrophic consortia were capable of incorporating 2-[^{14}C(U)]-oxoglutarate at extremely low concentrations. Thus, high affinity uptake systems for 2-oxoglutarate must be present in either the central bacterium or the epibionts, or in both. Since incorporation of 2-oxoglutarate was also observed for the accompanying *Chromatium* cells, chemocline bacteria compete for this substrate in their natural environment.

Because of its limited spatial resolution (22, 23) the microautoradiography technique cannot be used to monitor the incorporation of radioactively labeled substrates by individual bacterial cells when they are in close proximity. Therefore, it is presently unclear whether 2-oxoglutarate is incorporated by the epibionts or the central bacterium. Several observations suggest, however, that 2-oxoglutarate most likely is incorporated by the latter. First, the stable carbon isotope ratios of photosynthetic pigments of the epibionts of "*P. roseum*" provide strong evidence for photoautotrophic growth *in situ* (16). Second, none of the described species of green sulfur bacteria utilize 2-oxoglutarate (25, 35, 39, 41, 44). The epibiont of "*P. roseum*" is presently not available in pure culture. However, the epibiont of "*C. aggregatum*" has recently been isolated and shown to exclusively assimilate acetate and peptone, but not 2-oxoglutarate (K. Vogl, J. Glaeser, J. Overmann, unpublished data). Third, the reduction of the artificial electron acceptor CTC by the epibionts of "*C. aggregatum*" was dependent on the addition of sulfide but not 2-oxoglutarate (13). This indicates that the electron flow in the epibionts was not stimulated by 2-oxoglutarate.

Implications for the signal exchange between the cells in "*P. roseum*"

In phototrophic consortia, light is sensed and absorbed by the photosynthetic antenna bacteriochlorophyll of the epibiont (13). It appears most likely that 2-oxoglutarate is incorporated by the central bacterium (see above). However, the incorporation of 2-oxoglutarate by intact "*P. roseum*" consortia was strictly dependent on the simultaneous presence of light and sulfide. If indeed the central bacterium utilizes 2-oxoglutarate, our data would suggest a signal transfer between the epibionts and the central bacterium. By this mechanism, the physiological state of the epibiont would control the incorporation of 2-oxoglutarate of the central bacterium. Previously, a rapid signal exchange between epibionts and the central bacterium has been demonstrated to occur during the scotophobic

accumulation of phototrophic consortia in the light (13). The present data indicate that communication between the partner bacteria in phototrophic consortia is not limited to the scotophobic response, but also occurs during metabolism of organic compounds. An exchange of organic carbon compounds, vitamins and chelators has been suggested for other consortia, like the associations of heterotrophic bacteria with filamentous cyanobacteria (32).

REFERENCES

1. **Amersham International plc.** (1989) Nuclear emulsions for high resolution microautoradiography. Product information and guidelines for the LM-1 and EM-1 emulsions. Amersham International plc. Buckinghamshire, England.
2. **Andreasen K. and Nielsen P.H.** (1997) Application of microautoradiography to the study of substrate uptake by filamentous microorganisms in activated sludge. Appl. Environ. Microbiol. **63:**3662-3668
3. **Bartscht K., Cypionka H. and Overmann J.** (1999) Evaluation of cell activity and methods for the cultivation of bacteria from a natural lake community. FEMS Microbiol. Ecol. **28:**249-259
4. **Buder J.** (1914) Chloronium mirabile. Ber. Deut. Bot. Ges. **31:**80-97
5. **Burnison B.K and Morita R.Y.** (1974) Heterotrophic potential for amino acid uptake in a naturally eutrophic lake. Appl. Environ. Microbiol. **27:**488-495
6. **Button D.K., Schut F., Quang P., Martin R., and Robertson B.R. (**1993) Viability and isolation of marine bacteria by dilute culture: theory, procedures and initial results. Appl. Environ. Microbiol. **59:**881-891
7. **Caspar S.J.** (ed.) (1985) Lake Stechlin - a temperate oligotrophic lake. Dr. W. Junk Publishers, Dordrecht, The Netherlands
8. **Caldwell D.E. and Tiedje J.M.** (1974) A morphological study of anaerobic bacteria from the hypolimnia of two Michigan lakes. Can. J. Microbiol. **21:**362-376
9. **Cline J.D.** (1969) Spectrophotometric determination of hydrogen sulfide in natural water. Limnol. Oceanogr. **14:**454-458
10. **Culver D.A. and Brunskill G.J.** (1969) Fayetteville Green Lake, New York. 5. Studies of primary production and zooplankton in a meromictic marl lake. Limnol. Oceanogr. **14**:862-873
11. **Czeczuga B. and Gradski F.** (1972) Relationship between extracellular and cellular production in the sulphuric green bacterium *Chlorobium limicola* Nads. as compared to primary production of phytoplankton. Hydrobiologia **42:**85-95
12. **Evans M.C.W., Buchanan B.B. and Arnon D.I. (**1966) A new ferredoxin dependent carbon reduction cycle in a photosynthetic bacterium. Proc. Natl. Acad. Sci. USA **55:**928-934
13. **Fröstl J. and Overmann J.** (1998) Physiology and tactic response of "*Chlorochromatium aggregatum*". Arch. Microbiol. **169:**129-135

14. **Fröstl J. and Overmann J.** (2000) Phylogenetic affiliation of the bacteria that constitute phototrophic consortia. Arch. Microbiol. **174:**50-58
15. **Gasol J.M., Jürgens K., Massana R., Calderón-Paz J.I. and Pedrós-Alió C.** (1995) Mass development of *Daphnia pulex* in a sulfide-rich pond (Lake Cisó). Arch. Microbiol. **132:**279-296
16. **Glaeser J. and Overmann J.** (2003) Characterization and *in situ* carbon metabolism of phototrophic consortia. Appl. Environ. Microbiol. **69:**3739-3750
17. **Gocke K., Dawson R. and Liebezeit G.** (1981) Availability of dissolved free glucose to heterotrophic microorganisms. Mar. Biol. **62:**209-216
18. **Hobbie J.E. and Crawford C.C.** (1969) Respiration corrections for bacterial uptake of dissolved organic compounds in natural waters. Limnol. Oceanogr. **14:**528-532
19. **Jørgensen B.B., Kuenen J.G. and Cohen Y.** (1979). Microbial transformation of sulfur compounds in a stratified lake (Solar Lake, Sinai). Limnol. Oceanogr. **24:**799-822
20. **Krembs C., Juhl A.R., Long R.A. and Azam F.** (1998) Nanoscale patchiness of bacteria in lake water studied with the spatial infromation preservation method. Limnol. Oceanogr. **43:**307-314
21. **Lauterborn R.** (1906) Zur Kenntnis der sapropelischen Flora. Allg. Bot. **2:**196-197
22. **Lee N., Nielsen P. H., Andreasen K.H., Juretschko S., Nielsen J.L., Schleifer K.-H. and Wagner M.** (1999) Combination of fluorescent *in situ* hybridization and microautoradiography - a new tool for structure-function analyses in microbial ecology. Appl. Environ. Microbiol. **65:**1289-1297
23. **Nielsen P.H., Aquino de Muro M. and Nielsen J.L.** (2000) Studies on the *in situ* physiology of *Thiothrix* spp. present in activated sludge. Environ. Microbiol. **2:**389-398
24. **Overmann J.** (2001a) Phototrophic consortia: a tight cooperation between non-related eubacteria, pp. 239-255. *In*: Seckbach J. (ed.), Symbiosis. Mechanisms and model systems. Kluwer, Dordrecht, The Netherlands
25. **Overmann J.** (2001b) Green sulfur bacteria, pp. 601-623. *In*: Garrity G.M., et al. (Eds.) Bergey´s manual of systematic bacteriology, vol 1. Williams and Wilkins, Baltimore, USA
26. **Overmann J., Beatty J.T. and Hall K.J.** (1994) Photosynthetic activity and population dynamics of *Amoebobacter purpureus* in a meromictic saline lake. FEMS Microbiol. Ecol. **15:**309-320

27. **Overmann J., Coolen M.J.L. and 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
28. **Overmann J., Lehmann S. and Pfennig N.** (1991) Gas vesicle formation and buoyancy regulation in *Pelodictyon phaeoclathratiforme* (Green sulfur bacteria). Arch. Microbiol. **157:**29-37
29. **Overmann J. and Schubert K.** (2002) Phototrophic consortia: model systems for symbiotic interrelations between prokaryotes. Arch. Microbiol. **177:**201-208
30. **Overmann J., Tuschak C., Fröstl J.M. and Sass H.** (1998) The ecological niche of the consortium "*Pelochromatium roseum*". Arch. Microbiol. **169:**120-128
31. **Overmann J. and van Gemerden H.** (2000) Microbial interactions involving sulfur bacteria: implications for the ecology and evolution of bacterial communities. FEMS Microbiol. Rev. **24:**591-599
32. **Paerl H.W. and Pinckney J.L.** (1996) A mini-review of microbial consortia: their roles in aquatic production and biogeochemical cycling. Microb. Ecol. **31:**225-247
33. **Pfennig N.** (1980) Syntrophic mixed cultures and symbiotic consortia with phototrophic bacteria: A Review. pp. 127-131. *In*: Gottschalk G., Pfennig N. and Werner H. (eds.), Anaerobes and Anaerobic Infections. Gustav Fischer Verlag, Stuttgart, New York, USA
34. **Porter K.G. and Feig Y.S.** (1980) Use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25:**943-948
35. **Quandt L., Gottschalk G., Zielger H. and Stichler W.** (1977) Isotope discrimination by photosynthetic bacteria. FEMS Microbiol. Lett. **1:**125-128
36. **Schink B.** (1991) Syntrophism among prokaryotes, pp. 276-299. *In*: Balows A., Trüper H.G., Dworkin M., Harder W. and Schleifer K.-H. (eds.), The Prokaryotes. Springer Verlag, New York, Berlin, Heidelberg
37. **Siefert E. and Pfennig N.** (1984) Convenient method to prepare neutral sulfide solution for cultivation of phototrophic sulfur bacteria. Arch. Microbiol. **139:**100-101
38. **Simon M.** (1998) Bacterioplankton dynamics in a large mesotrophic lake: II. Concentrations and turnover of dissolved amino acids. Arch. Hydrobiol. **144**:1-23
39. **Sirevåg, R.** (1995) Carbon metabolism in green bacteria. pp. 871-883. *In*: Blankenship R.E., Madigan M.T. and Bauer C.E. (eds.), Anoxygenic photosynthetic bacteria, Advances in Photosynthesis, Vol. II. Kluwer Academic Publishers, Dordrecht, The Netherlands

40. **Sirevåg R. and Ormerod J.G.** (1970) Carbon dioxide-fixation in photosynthetic green sulfur bacteria. Science. **169:**186-188

41. **Sirevåg R., Buchanan B.B., Berry J.A. and Troughton J.H.** (1977) Mechanisms of CO2 fixation in bacterial photosynthesis studied by the carbon isotope fractionation technique. Arch. Microbiol. **112:**35-38

42. **Smith D.C., Simon M., Alldredge A.L. and Azam F.** (1992) Intense hydrolytic enzyme activity on marine aggregates and implications for rapid particle dissolution. Nature. **359:**139-142

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

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

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

46. **Wright R.T. and Hobbie J.E.** (1966) Use of glucose and actetate by bacteria and algae in aquatic ecosystems. Ecology. **47:**447-464

Chapter 5

Diversity and biogeography of phototrophic consortia

INTRODUCTION

Motile phototrophic consortia are associations of bacterial cells in which a flagellated colorless β-proteobacterium is surrounded by numerous cells of green sulfur bacteria, the so-called epibionts (41, 43). The barrel-shaped, motile phototrophic consortia were first described in the early 20th century (7, 34). Since their discovery, seven different morphotypes, namely "*Chlorochromatium aggregatum*", "*C. glebulum*", "*C. magnum*", "*C. lunatum*", "*Pelochromatium roseum*", "*P. roseo-viride*" and "*P. selenoides*" were described according to the morphology and cellular arrangement of their epibionts (41). In "*C. aggregatum*", the colorless bacterium is surrounded by green-colored rod-shaped bacteria, while brown epibionts are found in "*P. roseum*". Consortia of the type "*C. glebulum*" are bent and contain gas-vacuolated green epibionts. "*C. magnum*" contains a larger number (i.e. ~ 36) of green epibionts than "*C. aggregatum*" and has a globular morphology. "*P. roseo-viride*" carries an inner layer of brown-colored cells and an outer one consisting of green bacteria. In "*C. lunatum*" and "*P. selenoides*", epibionts are half-moon-shaped, and either green or brown, respectively. The seven types of phototrophic consortia have been reported to occur in numerous freshwater lakes and ponds worldwide (8, 39, 42). In addition, two immotile associations ("*Chloroplana vacuolata*" and "*Cylindrogloea bacterifera*") have been described, but these morphotypes appear to occur more rarely.

The cellular arrangement in phototrophic consortia cells is very regular and the cell division of the partner bacteria proceeds in a highly coordinated fashion (42). Furthermore, a rapid signal transfer between the epibionts and the central bacterium has been demonstrated (19). These observations indicate that the two bacterial partners are tightly and specifically associated. Phototrophic consortia occupy a narrow and well-defined ecological niche: they were found to thrive exclusively in the chemocline of freshwater lakes and only if concentrations of dissolved sulfide and light intensities are low (42).

One of the fundamental paradigms of microbiology has been that bacteria, in contrast to higher organisms, are ubiquitous (2, 4). Under these conditions, species with an identical ecological niche would be expected to outcompete each other rapidly, resulting in a low overall diversity. On the opposite, endemism of microorganisms would result in a significantly higher global diversity (16, 17), since geographic isolation would be a major driving force maintaining this biodiversity (47). In the case of phototrophic consortia, it is unknown whether a particular morphotype always harbors the same types of bacteria, or whether the diversity is actually higher than suggested by the microscopic appearance of the consortia.

From the high specificity of the bacterial association in phototrophic consortia, it may also be concluded that this type of interaction arose only rarely, if not once, during evolution. Indeed, initial analyses of the phylogenetic affiliation of 5 types of phototrophic consortia from two lakes indicated that the epibionts form a single cluster within the green sulfur bacterial radiation, whereas most other free-living members of this phylum are more distantly related (20). Since only three morphotypes of phototrophic consortia in two lakes could be analyzed, however, the available data are not sufficient to permit conclusions regarding the overall diversity of epibionts, their alleged monophyletic origin, and their biogeographical distribution.

The aims of the present investigation were therefore (1) to obtain a first quantitative estimate of the global diversity of epibionts, (2) investigate the extent of competitive exclusion between the different morphological types of phototrophic consortia, (3) to analyze whether this particular type of symbiosis developed only once during evolution, or arose convergently in different branches of the green sulfur bacterial radiation, and (4) to elucidate whether geographic isolation has played a significant role during the evolution of epibionts of phototrophic consortia.

We chose a culture-independent approach (20), by which different types of phototrophic consortia are mechanically isolated by micromanipulation and 16S rRNA gene sequences of

epibionts are obtained after a highly sensitive group-specific amplification step, followed by a separation by denaturing gradient gel electrophoresis (DGGE). This study revealed an unexpected diversity and a distinct biogeographic distribution of the epibionts of phototrophic consortia.

MATERIAL AND METHODS

Study sites

Fourteen lakes and ponds were selected based on previous studies and data in the literature. The study sites are located in six different geographic areas (Fig. 1) and comprise three lakes in Germany, two in Spain, one pond in Massachusetts, seven lakes in Michigan and one lake in the State of Washington (Tab. 1). In each of the lakes, an anoxic, sulfide-containing hypolimnion builds up after the onset of summer stratification. Concomitantly, dense accumulations of anoxygenic phototrophic bacteria and phototrophic consortia develop in the chemocline (5, 13, 23, 24, 25, 26, 42, 50). The physicochemical conditions in the chemocline during summer stratification were comparable among the lakes (Tab. 1). In Oyster Pond (Woods Hole, Mass., USA), the surface sediment usually becomes anoxic during summer and is subsequently colonized by anoxygenic phototrophic bacteria. Although not detectable directly by microscopy in sediment samples, phototrophic consortia can be enriched from this sediment if the samples are incubated under appropriate conditions (J. Overmann, unpublished observations; see Tab. 1). During the present study, the sampling locations were visited in the years 1998 to 2001 and between the months of July and October (Tab. 1).

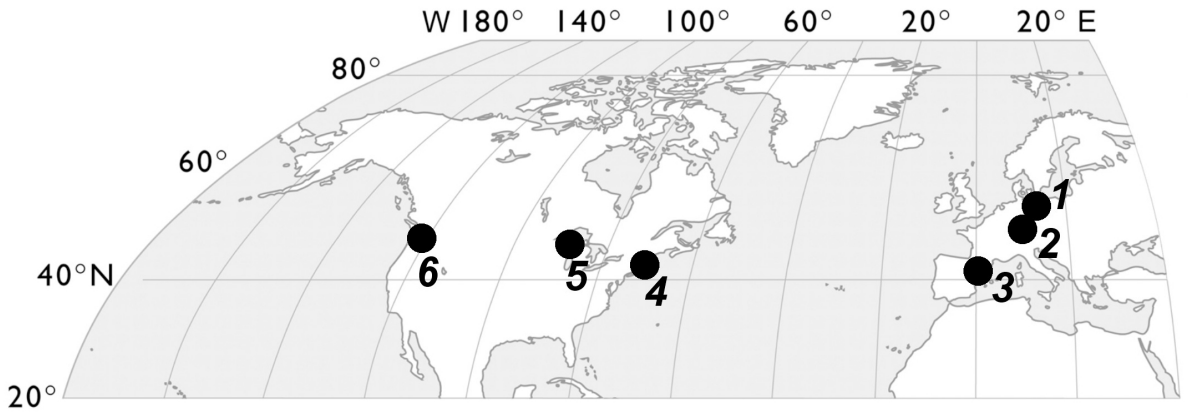


Figure 1. Geographic areas sampled during the present study. Numbers refer to Table 1 which lists the lakes studied and provides details on environmental parameters.

Table 1. Selected physicochemical parameters and phototrophic consortia detected in the different lakes

Lake	Characteristic environmental parameters[1] Chemocline (m)	Temperature (°C)	pH	Light intensity (µmol·m^{-2}·s^{-1})	Sulfide (µM)	Reference	Sampling Date	Depth (m)	Morphotype	Epibiont phylotype[2]
Geographical region 1[3]										
Dagow	6.0	10	7.35	0.7	10	(26)	07.07.1998	6.4	"*C. aggregatum*"	M
									"*C. magnum*" CmD	E
									"*P. roseum*" PRDBD7	K
									"*P. latum*" PRDBD8	N
Haussee	6.0	13.7	7.5	2.4	-	H.-D. Babenzien, pers.comm.	20.07.1999	6.5	"*C. aggregatum*" CaH	C
									"*P. roseum*" PrH	G
									"*P. selenoides*" PSH	D
Geographical region 2										
Schleinsee	6	8 - 10	7.8	0.5 - 5	30	(13)	09.09.2001	7	"*P. roseum*" 25	K
Geographical region 3										
Cisó	2.75	15	7.2	-	200	unpubl.data	23.08.1999	2.75	"*C. aggregatum*" CaS	P
Coromina	1.5	20	7.4	0.01	10	(24)	22.08.1999	2.5	"*C. aggregatum*" CaC	P
Geographical region 4										
Oyster Pond[4]	n.a.	23	7	7	100	unpubl.	30.6.2000	0.01	"*C. aggregatum*" CaSp	L
Geographical region 5										
Wintergreen	3.5	17	8.1	0.95	192	(25)	04.09.2001	4.2	"*C. aggregatum*" 7b-2	M
									"*C. aggregatum*" 8a-2	N
									"*C. magnum*" 13-2	H
									"*P. latum*" 29-2	N
Baker	7	6	7.5	2.94	102	(25)	04.09.2001	6	"*C. aggregatum*" 11b	D
									"*C. magnum*" 14-2	H
									"*P. roseum*" 1a-2	O
									"*P. latum*" 1b-2	N

Table 1. continued

Sheffer	-	-	-	-	-	-	04.09.2001	5.8	"*C. aggregatum*" 9b-2 D "*C. magnum*" 9a H "*P. roseum*" 18a O "*P. roseum*" 18c-2 B "*P. latum*" 18b N "*Clp. vacuolata*" D
Mud	4	15	7	0.4	8	(25)	04.09.2001	3.5	"*C. magnum*" 52 A
Leach	3.5	21	7.2	1.52	87	(25)	04.09.2001	7	"*C. aggregatum*" 10b-2 N "*C. magnum*" 10a H "*P. roseum*" 20 O
Round	4.5	12	7.5	3.27	133	(25)	04.09.2001	4.7	"*C. aggregatum*" 4b-2 N "*C. magnum*" 14-2 H "*P. latum*" 19-2 N
Cassidy	8	5	7.4	0.05	62	(25)	04.09.2001	6.7	"*C. aggregatum*" 5-2 D "*C. magnum*" 10 H "*C. lunatum*" 12-2 D "*P. roseum*" 16a-2 O "*P. latum*" 2b N
Geographical region 6									
Echo	-	-	-	-	-	-	10.10.1998	10	"*C. aggregatum*" CaEL J "*C. glebulum*" ELGSB5 F "*C. magnum*" ELGSB2 I "*P. roseum*" PrEL B

[1] data from the literature, average values for summer months are given. See also literature cited for geographical coordinates of individual lakes.
[2] compare phylogenetic tree in Fig.5
[3] compare Fig. 1
[4] phototrophic consortia not detectable in sediment samples, but enriched under the conditions provided
n.a., not applicable
-, no information available

Water sampling

Water samples were obtained from the deepest part of the lakes employing a bilge pump connected to gas-tight isoversinic tubing. The inlet of the tubing consisted of two polyvinyl chloride (PVC) cones, spaced 1 cm apart from each other (33). This device allowed the reproducible sampling of different water layers at 5 cm intervals. Sampling depths were chosen based on the vertical distribution of anoxygenic photosynthetic bacteria as determined by light and phase-contrast microscopy.

Samples from European lakes were filled into autoclaved 1 liter glass bottles and kept in the dark to avoid damage of the phototrophic bacteria which occurs at the high light intensities at the lake surface (42). The bottles were sealed gas tight to prevent abiotic oxidation of sulfide, transported back to the laboratory at *in situ* temperatures and processed within 8 hours after sampling. Samples obtained from North American lakes were transferred to 10 ml gas-tight screw cap glass tubes and anoxic conditions were maintained by the addition of 200 µM neutralized (pH 7.3) sulfide solution (44). The glass tubes were shipped on artificial ice and by courier to the home laboratory, and processed within 48 h.

Isolation of intact phototrophic consortia and molecular fingerprinting of epibionts

Phototrophic consortia were mechanically separated from the chemocline microbial community using a micromanipulator connected to an inverted microscope (18, 19). At first, water samples containing phototrophic consortia were evenly spread on coverslips by squeezing 100 µl of water sample between two acetone-cleaned coverslips (60 x 20 mm). Excess water was removed with a paper tissue and the coverslips separated again and air-dried aseptically. After drying, different morphotypes of phototrophic consortia could be distinguished by bright-field and phase-contrast microscopy according to their size and according to the color, number and shape of the epibionts.

Batches containing between 5 and 45 consortia with identical morphology were collected by micromanipulation and directly subjected to PCR amplification. 540 bp-long 16S rRNA gene fragments were amplified employing oligonucleotide primers GC 357f and GSB 840r and the PCR conditions published previously (40). Our method permits the highly sensitive and specific amplification of 16S rRNA gene fragments of the epibionts (20). The particular fragment was chosen since it includes the region V3, which is the largest of the highly variable regions within the 16S rRNA gene (11). In order to check for the presence of different epibionts in the same morphotype of phototrophic consortia, the resulting amplification products were separated by denaturing gradient gel electrophoresis (DGGE)

according to their melting behavior (38). The 6% (wt/vol) polyacrylamide DGGE-gels contained a linear gradient of 35 % to 70 % denaturing agents.

Phylogenetic analyses of 16S rRNA gene sequences of the epibionts

After staining the gels with ethidium bromide, individual DNA bands were excised from the DGGE gel, recovered by electroelution, reamplified and sequenced as described (40).

Phylogenetic analysis of 16S rRNA gene sequences of epibionts was performed using the ARB phylogeny package (36). The program Fast Aligner V1.03 was used for the alignment of all complete 16S rRNA gene sequences of green sulfur bacteria as available through the National Center for Biotechnology Information website (1). The sequence of *Chloroherpeton thalassium* ATCC 35110T was chosen as the outgroup. The alignment was manually corrected based on secondary structure information of *Chlorobium vibrioforme* ATCC 6030 and a phylogenetic tree was constructed from all sequences longer than 1300 base pairs using the maximum likelihood program DNA_ML. Afterwards, the partial 16S rRNA gene sequences of epibionts were inserted into the phylogenetic tree employing the Parsimony Interactive tool without changing the tree topology. The resulting alignments were corrected again manually and the overall tree calculated.

Comparison of epibionts in different geographical regions

The presence or absence of distinct types of epibionts was used to compare the communities of phototrophic consortia in lakes within the same biogeographical region or in lakes of different regions.

Binary similarity of communities in different lakes was computed employing the coincidence index of Dice (12)

$$D = \frac{2a}{2a + b + c} \qquad (1)$$

where a denotes the number of epibiont types present in two environments compared, b the number of types found exclusively in the first, and c the number of epibiont types present in the second environment.

Chemotaxis assays

The chemotactic response of phototrophic consortia was tested by incubation of capillaries filled with different test compounds. Sterile 100 mM stock solutions of sulfide, thiosulfate,

glycerol, acetate, pyruvate, lactate, propionate, citrate, succinate, 2-oxoglutarate and glycine were prepared anoxically in Hungate tubes sealed with butyl rubber septa, and flushed with N_2. For the preparation of organic acids, the corresponding sodium salts were used and the pH of the stock solutions was adjusted to 7.3 with NaOH. This pH value was chosen based on the pH values of 7.2 to 7.4 as determined previously for the chemocline water Lake Dagow and Lake Sisó (Tab. 1) (26, 42). All compounds were diluted to a final concentration of 500 µM with filter sterilized (cellulose nitrate membrane filters, pore size 0.2 µm; Sartorius, Göttingen, Germany), anoxic chemocline water.

Rectangular capillaries (length, 50 mm; inside diameter, 0.1 x 1.0 mm; capacity 5 µl, Vitro Dynamics, Rockaway, N.J., USA) were then filled with the diluted test substances. Controls were filled with sterile, anoxic chemocline water only. The capillaries were immediately sealed with plasticine (Idena, Berlin, Germany) at one end and then inserted with their open end into the sample containing the consortia (19). Incubation lasted for 3 to 5 hours.

After incubation, each capillary was recovered and immediately closed with plasticine. Phototrophic consortia were counted directly in each capillary by phase-contrast and dark-field microscopy at a magnification of 100x and 400x.

RESULTS AND DISCUSSION

Individual populations of phototrophic consortia as detected by molecular fingerprinting

Although they represent highly ordered structures, phototrophic consortia could theoretically form randomly from bacterial cells which encounter each other accidentally. Phototrophic consortia formed in this random fashion would harbor different types of green sulfur bacteria as epibionts. An earlier investigation of phototrophic consortia obtained from a single lake (Echo Lake) had suggested that all epibiont cells present in a given type of phototrophic consortium actually belong to one single phylotype (20). In the present study, phototrophic consortia from different lakes and two different continents were analyzed by 16S rRNA gene fingerprinting and sequencing in order to assess whether (1) all morphologically identical consortia from one lake contain one single type of epibiont, and (2) whether the same type of phototrophic consortium always harbors the same epibiont in different lakes.

Different numbers of "*Chlorochromatium magnum*" were picked from Lake Cassidy, the 16S rRNA gene fragments of the epibionts were amplified and subsequently separated by DGGE. Independent of the sample size, all samples analyzed yielded fingerprints with the same melting behavior (Fig. 2A). Sequencing confirmed that each band contained the same

16S rRNA gene sequence. A similar result was obtained for "*Pelochromatium roseum*" isolated from Lake Dagow. Identical 16S rRNA gene fingerprints were obtained for batches of 10 and 45 individual consortia (data not shown). Evidently, phototrophic consortia with the same morphology which share the same habitat contain only one single epibiont phylotype.

When phototrophic consortia were collected from different lakes, however, phylogenetic fingerprinting revealed that morphologically similar consortia actually contained different epibionts (Fig. 2B). For example, seven different phylotypes were recorded for "*Chlorochromatium aggregatum*". These consortia were usually indistinguishable by bright field and phase contrast light microscopy with respect to their overall shape, and the arrangement and color of the epibionts (Fig. 2C). Similarly, four different fingerprint patterns were detected for "*Chlorochromatium magnum*" from eight North American lakes and Lake

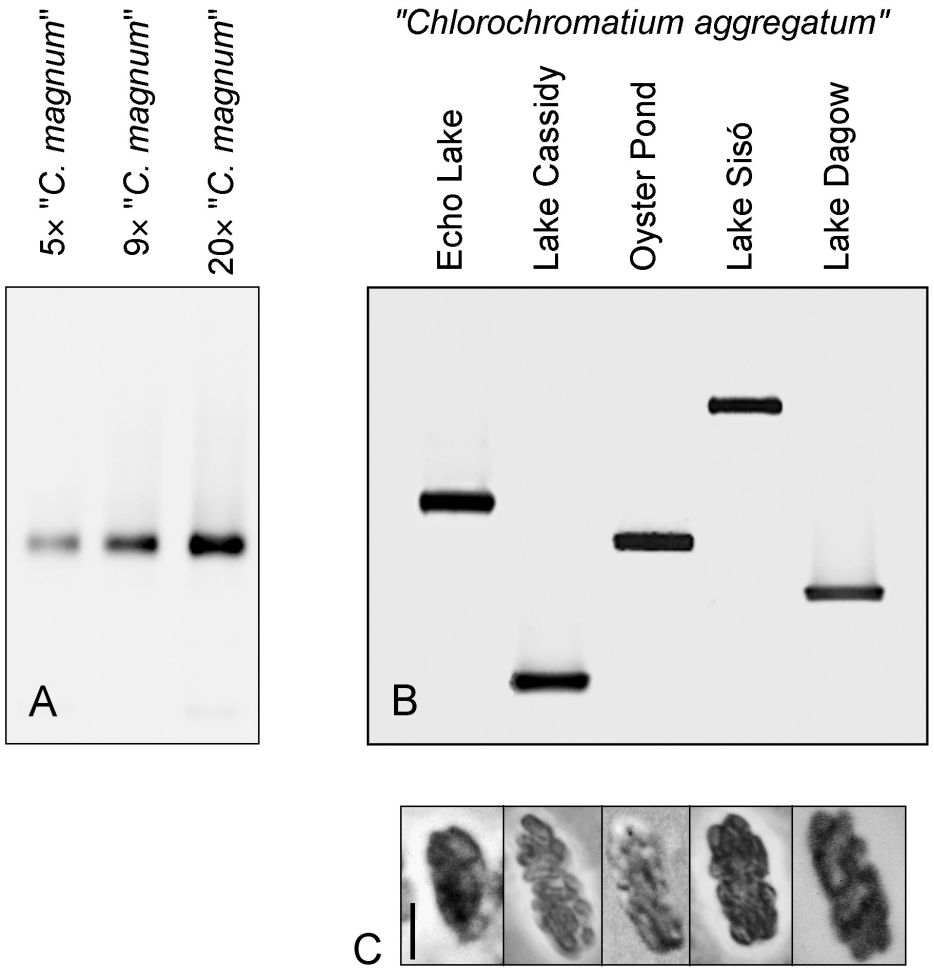


Figure 2. A. Epibiont 16S rRNA gene fragments amplified from increasing numbers of "*Chlorochromatium magnum*" sampled in Lake Cassidy and separated by DGGE. **B.** Epibiont 16S rRNA gene fragments amplified from the same morphotype "*Chlorochromatium aggregatum*" as found in four different lakes in North America and one lake in Eastern Germany. Fragments were separated by DGGE. The gradient of denaturant in A. and B. extends from 40% (top) to 60% (bottom of gel). Negative images of ethidium bromide-stained gels are shown. **C.** Phase-contrast photomicrographs of zje different "*C. aggregatum*" corresponding to the DGGE fingerprints from the five lakes in **B**. Bar, 5 µm.

Dagow, and for the brown-colored "*Pelochromatium roseum*" from three German and five North American lakes (Tab. 1). To date, only seven types of motile phototrophic consortia are distinguished based on the morphology and number of their epibionts (20, 39, 41). Our results demonstrate that the phylogenetic diversity of the epibionts in phototrophic consortia significantly surpasses the diversity as deduced solely from their morphology.

In nine of the 14 lakes sampled, different morphological types of phototrophic consortia were found to co-occur (Tab. 1, 2). Molecular fingerprinting revealed that each morphological type of consortium also contained its specific epibiont (Fig. 3). Notable exceptions were the epibionts of some "*Chlorochromatium aggregatum*" consortia, of "*C. lunatum*", "*Pelochromatium selenoides*" and of "*Chloroplana vacuolata*" which had all identical 16S rRNA fingerprints (phylotype D in Tab. 2, Fig. 5). Also, epibionts from another "*C. aggregatum*" and from "*P. latum*" were phylogenetically identical (phylotype N in Tab. 2). In these cases, sequencing of the corresponding DGGE bands confirmed the

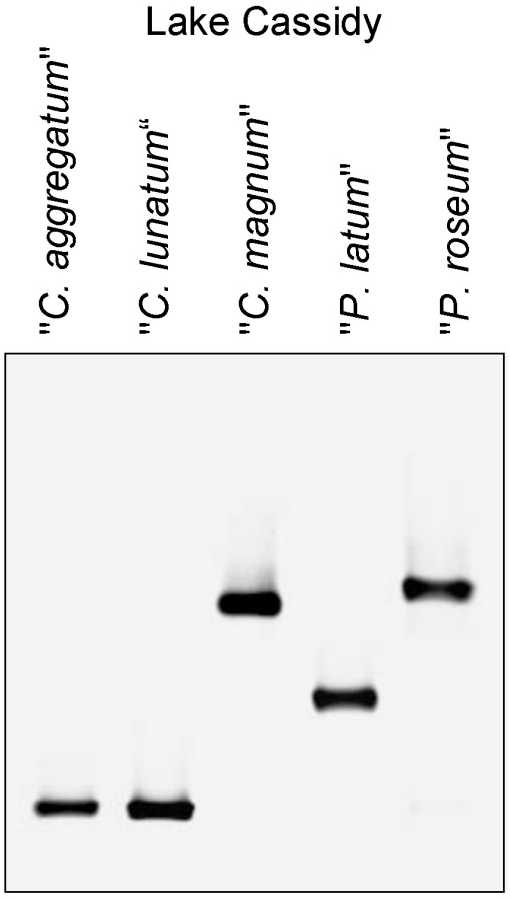


Figure 3. Diversity of epibionts of phototrophic consortia in Lake Cassidy. 16S rRNA gene fragments were amplified from all different morphotypes of phototrophic consortia present and subsequently separated by DGGE. A negative image of an ethidium bromide-stained gel is shown.

presence of identical partial 16S rRNA gene fragments of the epibionts in the different morphological types of phototrophic consortia (Fig. 5).

Our detailed analysis of the phototrophic consortia in five Michigan lakes and Lake Dagow (Eastern Germany) also revealed the presence of a previously undescribed morphological type of phototrophic consortium. This type consisted of 44 to 58 cells of a brown-colored epibiont which were associated with a single central colorless motile bacterium (Fig. 4A, B). Epibionts were arranged in several layers around the central rod, resulting in a more stumpy shape of the intact consortium (Fig. 4A) as compared to "*Pelochromatium roseum*" (Fig. 4C, D). 16S rRNA gene fingerprinting demonstrated that the epibionts present in the novel type of consortium differed from all other consortia containing brown-colored epibionts. However, the novel type of consortium contained always the identical 16S rRNA gene sequences in Michigan as well as in German lakes (Tab. 1; Fig. 5, phylotype N). Based on these data, the name "*Pelochromatium latum*" is proposed for the novel phototrophic consortium.

Diversity of epibionts of phototrophic consortia

Our systematic survey of phototrophic consortia from the 14 lakes yielded a total of 41 partial 16S rRNA gene sequences (Fig. 5, Tab. 1). Among these, 16 distinct sequence types (phylotypes, capital letters in Fig. 5, Tab. 1 and 2) were detected. A cumulative plot of the phylotypes against the sequence numbers reached saturation (Fig. 6, filled circles). This

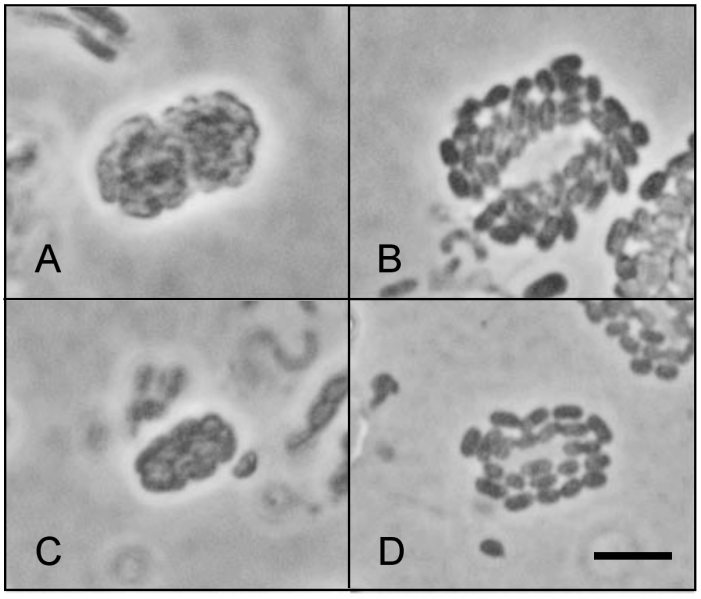


Figure 4. Comparison of the two morphotypes "*Pelochromatium latum*" (**A**, **B**) and "*Pelochromatium roseum*" (**C**, **D**). Phase contrast photomicrographs of consortia in the intact (**A**, **C**) and the disaggregated state (**B**, **D**) are shown. Bar, 5 µm.

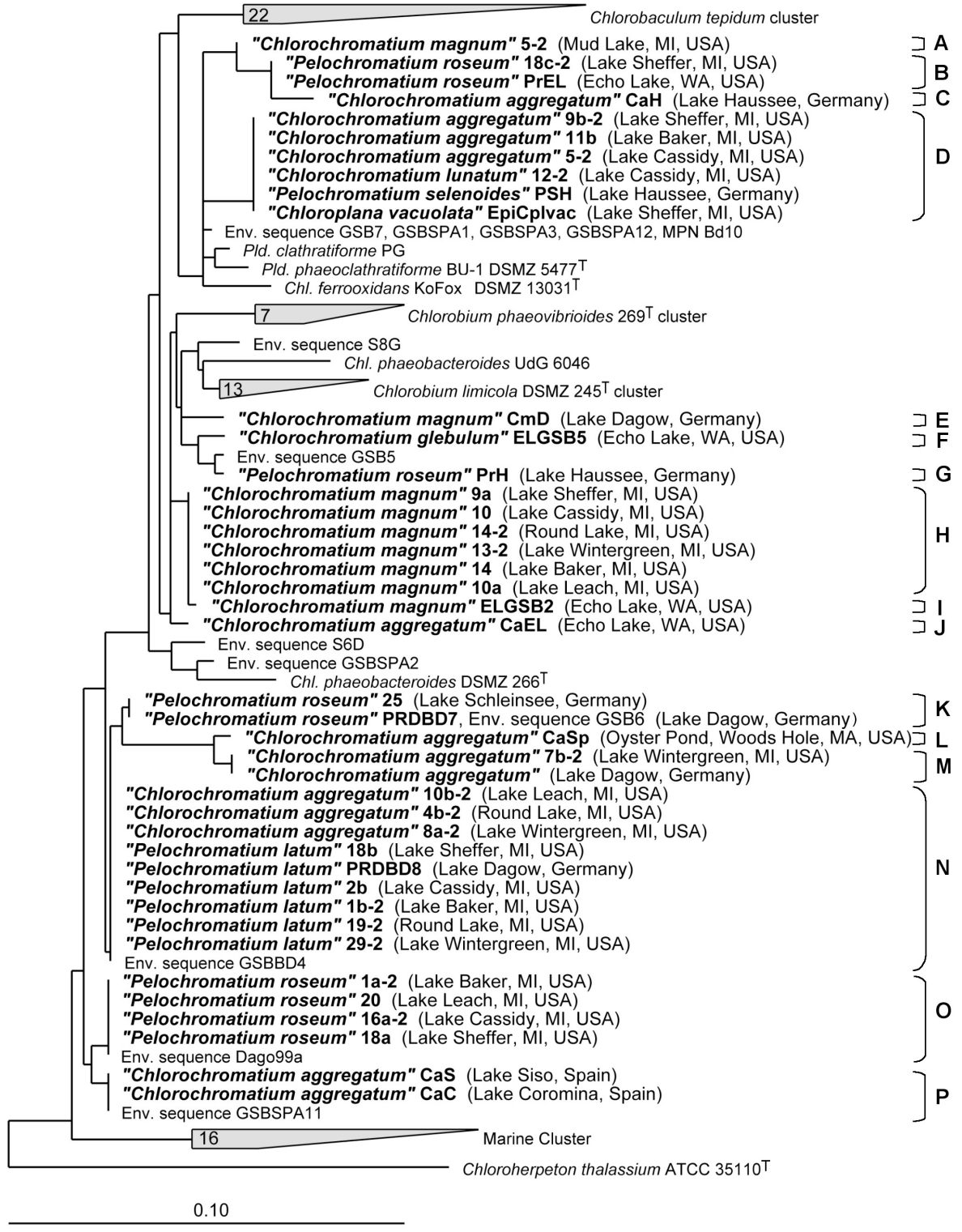


Figure 5. Phylogenetic tree of 16S rRNA gene sequences of green sulfur bacterial epibionts and relatives. Distinct phylotypes are indicated by capital letters A through P. Bar indicates 0.1 fixed point mutations per nucleotide position. Kajetan Vogl provided the sequence of the epibiont of "*Chloroplana vacuolata*".

Table 2. 16S rRNA signature sequences and biogeographical distribution of the different epibiont types

Phylotype		16S rRNA signature nucleotide positions[1]											Morphotype	Phylo	Biogeographical region					
	Acc.No.	*409*	*453-454*	*476*	*479*	*613*	*624*	*627*	*638*	*658*	*746*	*748*		-Morphotype	1	2	3	4	5	6
A	AJ578401												"*C. magnum*"	A					×	
B	AJ578402												"*P. roseum*"	B					×	×
C	AJ578404												"*C. aggregatum*"	C	×					
D	AJ578405												"*P. selenoides*"	D1	×					
													"*C. lunatum*"	D2					×	
													"*C. aggregatum*"	D3					×	
													"*Clp. vacuolata*"	D4					×	
E	AJ272094												"*C. magnum*"	E	×					
F	AJ272092						***G***						"*C. glebulum*"	F						×
G	AJ578410												"*P. roseum*"	G	×					
H	AJ578411								***U***				"*C. magnum*"	H					×	
I	AJ272093								***U***				"*C. magnum*"	I						×
J	AJ272091												"*C. aggregatum*"	J						×
K	AJ578417												"*P. roseum*"	K	×	×				
L	AJ578418		***AC***		***A***		***G***	***C***[3]		***A***		***U***	"*C. aggregatum*"	L				×		
M	AJ272090		***AC***		***A***		***G***	***C***		***A***	***G***	***U***	"*C. aggregatum*"	M	×				×	
N	AJ578423												"*C. aggregatum*"	N1					×	
													"*P. latum*"	N2	×				×	
O	AJ578428	***C***			***A***								"*P. roseum*"	O					×	
P	AJ578432				***A***								"*C. aggregatum*"	P			×			
													Phylo-Morphotypes per continent			*8*			*14*	
													Phylo-Morphotypes per region		*7*	*1*	*1*	*1*	*10*	*4*
													Lakes sampled		*2*	*1*	*2*	*1*	*7*	*1*
													Phylo-Morphotypes per lake[2]		*3.5*	*1*	*1*	*1*	*3.7*	*4*

[1] *E. coli* numbering

[2] as calculated from Table 1

[3] changes are complementary according to secondary structure (compare ref. 20)

indicates that the majority of epibiont phylotypes which were present in the biogeographical regions had been recovered by our approach.

Some epibionts had identical partial 16S rRNA gene sequences but exhibited clearly different morphology with respect to cell shape (rod-shaped versus half-moon-shaped, in "*Chlorochromatium aggregatum*" and "*Chlorochromatium lunatum*", respectively) or color (brown- versus green-colored, "*Pelochromatium latum*" and "*Chlorochromatium aggregatum*, respectively) (Fig. 5, phylotypes D and N). It is well established that green-colored strains of the green sulfur bacteria harbor bacteriochlorophyll (BChl) *c* and *d* and the carotenoids chlorobactene, OH-chlorobactene and γ-carotene as light-harvesting pigments. In contrast, brown-colored strains contain BChl *e* and the carotenoids isorenieratene and β-isorenieratene. Both sets of pigments are mutually exclusive (39) suggesting that green sulfur bacteria employ either the chlorobactene or the isorenieratene branch of the carotenoid biosynthetic pathway (49). The different morphology of epibionts with identical partial 16S rRNA gene sequences thus indicates that these epibionts are also genetically different and warrants a further distinction. In order to arrive at a more appropriate estimate of total diversity, those epibionts with identical phylotypes but different morphology were considered as different types (D1-D4 and N1-N2, Tab. 2). As a result, a total of 20 different types can be recognized in our dataset (Fig. 6, triangles). Each of these types represents a distinct epibiont with a unique combination of 16S rRNA gene sequence and morphology ("phylo-morphotype"). The cumulative plot of the different phylo-morphotypes also reached saturation, indicating that

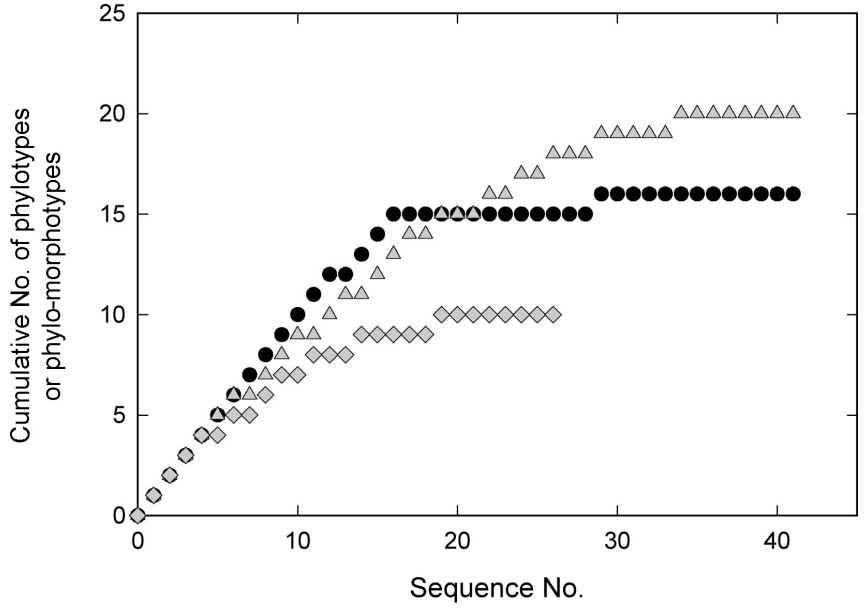


Figure 6. Comparison of the total diversity of phylotypes of epibionts (●), and of phylotypes with distinct morphology (phylo-morphotype, ▲) present in the data set. Also included is the cumulative plot for epibiont types found in Michigan lakes (◆). Random numbers were generated and assigned to the strains (Sequence No.), and the sequential detection of different phylotypes and phylo-morphotypes plotted.

further sampling in the six geographical regions at best would yield only few and very rare additional types of epibionts.

Biogeography of phototrophic consortia

It is mostly assumed that physical or geographical barriers do not exist for prokaryotes (35) and it has been suggested that the high population densities of microorganisms drives a rapid, large scale dispersal across the physical and geographical barriers (16, 17). Species like *Escherichia coli*, *Salmonella enterica* or *Haemophilus influenzae* consist of a limited number of clones, each, however, with a worldwide distribution (37). For *Neisseria meningitidis*, particular genotypes were detected on different continents (9). Evidently, dispersal of such human pathogens and commensals is rapid enough to permit a widespread geographic distribution not only of bacteria with identical 16S rRNA gene sequences (phylotypes) but even of cells with the same genotype.

While studies of marine and freshwater microorganisms indicate that most of the species are ubiquitous, too (3, 14, 15, 16, 17, 21, 22, 45, 51), some soil bacteria may have a more limited distribution (10, 46). A limited migration between North and Central America has been shown for *Rhizobium leguminosarum* biovar *phaseoli* (46). For most free-living or non-human pathogens and symbionts investigated, endemicity seems to be limited to the level of genotypes (strains or subspecies) rather than bacterial species (phylotypes). This is true for physiologically and phylogenetically very different bacteria like fluorescent pseudomonads, the Gram-positive *Renibacterium salmoninarum*, or strains of the symbiotic cyanobacterium *Nostoc* sp (10, 29, 31). However, cosmopolitan species could not be detected among psychrophilic sea ice bacteria from Arctic and Antarctic samples (48). Similarly, 16S rRNA gene sequencing of different populations of *Achromatium oxaliferum* suggested a high degree of endemism since identical sequences were never recovered from geographically separated freshwater sediments (28). Hence, endemic species of bacteria may exist in some rare cases.

Endemism of a particular group of bacteria can be inferred if the corresponding DNA sequences form a separate phylogenetic cluster, indicating that strains sampled at a certain location evolved from a common ancestor in geographically independent clades. Although the partial 16S rRNA gene sequences of some epibionts of phototrophic consortia were closely related, a geographic clustering of the sequences was only observed for the two European clusters K and P which, however, may not be complete (see below). Two types in the larger clusters, namely the epibiont type M of "*Chlorochromatium aggregatum*", and type N2 of "*Pelochromatium latum*" were present on both continents (Fig. 5, Tab. 2). Consequently,

geographic isolation is not an appropriate explanation for the high diversity and for the phylogenetic relationships observed for the epibionts of phototrophic consortia.

Among the individual biogeographic regions, the highest number of morphologically different phototrophic consortia was encountered in Michigan lakes (Tab. 2). In this region, the 26 phototrophic consortia analyzed yielded a cumulative number of 10 different types of epibionts. The repeated detection of identical epibiont types (Fig. 6, diamonds) indicates that essentially the entire diversity of epibionts had been recovered from this biogeographical area. In contrast, eight different types of epibionts were detected in a total of 10 analyses of consortia in the European lakes. Consequently, the cumulative plot of epibiont types did not reach saturation (not depicted in Fig. 6) and even more types of epibionts are to be expected to occur in other European lakes.

From the above data, several conclusions can be drawn with respect to the biogeography of phototrophic consortia. A pairwise comparison of the types of epibionts between the Michigan lakes (excluding Mud Lake which contained only a single type of epibiont) yielded a mean value for the Dice coefficient of 0.582 ± 0.162 (n=15). This high value shows that communities of phototrophic consortia in lakes within this particular biogeographical region are highly similar. In contrast, only two types of epibionts (M and N2) were found to occur on both continents investigated. The corresponding Dice coefficient for the pairwise comparison between North American and European epibionts was only 0.182. Theoretical considerations indicate that a higher similarity between North American and European lakes is unlikely. A high similarity, i.e. a Dice coefficient of 0.58, between North American and Europena lakes would only be possible if at least 20 different types of epibionts would occur in Europe, and at least 50% of these were also present in North American lakes. In reality, much less (25%) of the epibiont types detected in Europe were also present in North America. While a higher diversity of ≥ 20 epibiont types cannot be excluded for European lakes (since the dataset for this continent is incomplete, see above), it appears highly unlikely that our random sampling procedure selectively excluded just those epibionts which are common to both, North American and European lakes.

Implications for the ecology and evolution of phototrophic consortia

Our study revealed that a single lake harbors distinct populations of phototrophic consortia, whereby each population contains only one particular type of epibiont. It is unlikely that additional types of epibionts exist in the same consortium but were missed by our approach because of the following considerations. DGGE fingerprinting has been shown to detect those 16S rRNA gene sequences which represent more than 1 of all sequences present in a DNA

sample (38). The mean number of epibionts per consortium was determined to be 19.9 ± 4.4 in "*Pelochromatium roseum*" and 36 ± 4.4 in "*Chlorochromatium magnum*" (42). Based on its sensitivity, the DGGE fingerprinting method would therefore have been suitable to detect even a single cell of a second phylotype per phototrophic consortium.

Epibionts of those consortia investigated to date represent unique 16S rRNA sequence types and do not occur in a free-living state. Together with the rapid signal transfer demonstrated earlier (19), our findings support the view that the interaction between green sulfur bacteria and chemotrophic motile bacteria in phototrophic consortia is an obligate one. Based on our data, the green sulfur bacterial epibionts must have specifically adapted to the life in association. Nevertheless, epibionts of phototrophic consortia form several distinct phylogenetic clusters (Fig. 5). According to our phylogenetic analysis, green sulfur bacteria arose from different ancestors which independently developed the ability to form obligate associations with motile chemotrophic bacteria.

If the phototrophic consortia thus formed would occupy the same ecological niche, competitive exclusion would be expected to lead to a rapid extinction of some types and would ultimately result in a low overall diversity of phototrophic consortia. Our data on the diversity and distribution of phototrophic consortia imply that competitive exclusion exists only in a few cases, "*Chlorochromatium magnum*" being the most obvious example. For this consortium, different types of epibionts were never detected in the same lake (Tab. 1, 2). Although epibiont type H of "*Chlorochromatium magnum*" was one of the two most frequent types in Michigan lakes, it was never detected in other lakes. Possibly, the different epibionts of "*Chlorochromatium magnum*" therefore represent vicarious types.

As described in the preceding section, the results of our phylogenetic analysis of epibiont 16S rRNA gene sequences are inconsistent with a geographical isolation of individual epibiont types over geological time scales. Nevertheless, a nonrandom biogeographical pattern and a low similarity of epibionts between the two continents does exist. It has been shown that common soil bacteria, like *Curtobacterium citreum*, *Bacillus megaterium*, *Arthrobacter globiformis*, *Microbacterium* spp., *Sphingomonas* spp., *Sinorhizobium* spp. and *Paracoccus* spp. and fungal pathogens of plants are transported on dust particles and are capable of surviving the long-range transport through the atmosphere (6, 30). Transport between continents occurs on a time scale of ~6 days (32). The high similarity between assemblages of phototrophic consortia in neighboring lakes and the pronounced intercontinental differences of phylotypes suggests that the dispersal of phototrophic consortia

is comparatively slow and cannot compensate for phylotypes lost by competitive exclusion in a distantly located environment.

However, the simultaneous presence of up to four different types of consortia in the same lake indicates that competitive exclusion of phototrophic consortia is not the rule and that cells in morphologically distinct types of consortia also differ with respect to their physiology. Previous analyses suggested that consortia with brown-colored epibionts are adapted to lower light intensities than their green counterparts (42). More direct evidence for

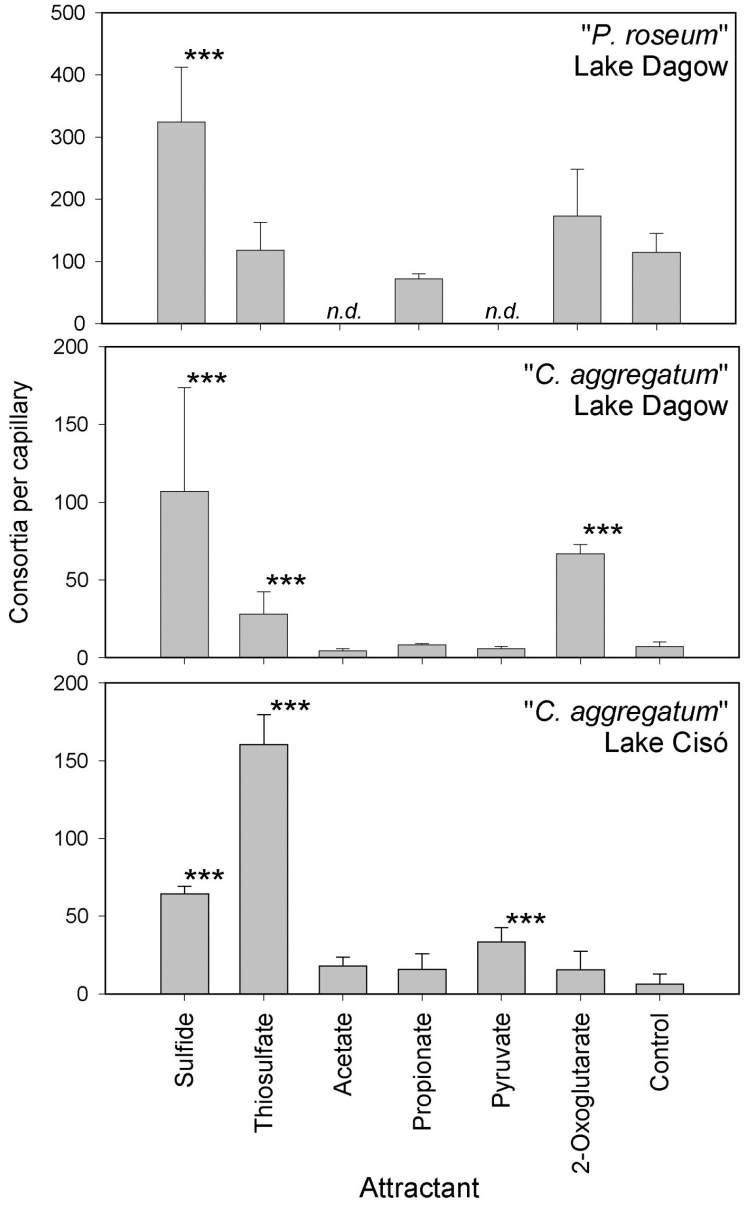


Fig. 7. Chemotactic accumulation of three different types of motile consortia from two different lakes. Bars represent numbers of phototrophic consortia counted in capillaries after incubation for 3-5 h. Experiments were run in three to four parallels. Values for "*C. aggregatum*" from Lake Dagow were recalculated from (19), those for "*P. roseum*" were taken from (27). Error bars represent standard deviations of the counts. Values differing from the control at a high level of significance are indicated by asterisks (***, $p < 0.001$). *n.d.*, not determined.

physiological differences between different consortia comes from our chemotaxis experiments with three phototrophic consortia (Fig. 7). Intact "*Pelochromatium roseum*" consortia exhibited a pronounced chemotaxis towards sulfide, and weakly responded to 2-oxoglutarate. A strong chemotactic response towards sulfide, thiosulfate and 2-oxoglutarate was detected for "*Chlorochromatium aggregatum*" from Lake Dagow, whereas the morphologically similar " *Chlorochromatium aggregatum*" from Lake Sisó was attracted by pyruvate instead of 2-oxoglutarate (Fig. 7). Since the chemotaxis towards organic carbon compounds is likely to be mediated by the central motile bacterium (27), differences in ecological niches at least in part are caused by differences in the physiology of the chemotrophic partner bacterium.

The presence of one single type of epibiont in each type of phototrophic consortium appears to be a general feature of all types of phototrophic consortia investigated (Figs. 2, 3). Each type of consortium hence represents a highly specific association of a particular green sulfur bacterium with a colorless motile bacterium. The colorless central bacteria of different morphological types of consortia have been shown to belong to the β-*Proteobacteria* (20). It remains to be elucidated, however, whether the same colorless motile bacterium occurs in different phototrophic consortia, or whether each of the different types of epibionts is associated with another, particular chemotroph as suggested by the results of our chemotaxis experiments.

REFERENCES

1. **Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Miller W. and Lipman D.J.** (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic. Acids. Res. **25:**3389-3402
2. **Baas-Becking L.G.M.** (1934) Geobiologie of Inleiding Tot de Milieukunde. W.P. van Stockum & Zoon N.V., The Hague, The Netherlands
3. **Beeder J., Nilsen R.K., Rosnes J.T., Torsvik T. and Lien T.** (1994) *Archaeoglobus fulgidus* isolated from hot North Sea oil field waters. Appl. Environ. Microbiol. **60:**1227-1231
4. **Beijerinck M.W.** (1913) De infusies en de ontdekking der backteriën, Jaarboek van de Koninklijke Akademie v. Wetenschappen. Müller, Amsterdam, The Netherlands
5. **Borrego C.M., Garcia-Gil J., Cristina X.P., Vila X. and Abella C.A.** (1998) Occurrence of new bacteriochlorophyll *d* forms in natural populations of green photosynthetic sulfur bacteria. FEMS Microbiol. Ecol. **26:**257-267
6. **Brown J.K.M. and Hovmøller M.S.** (2002) Aerial dispersal of pathogens on the global and continental scales and its impact on plant disease. Science. **297:**537-541
7. **Buder J.** (1914) Chloronium mirabile. Berichte Dtsch. Bot. Ges. **31:**80-97
8. **Caldwell D.E. and Tiedje J.M.** (1975) A morphological study of anaerobic bacteria from the hypolimnia of two Michigan lakes. Can. J. Microbiol. **21:**362-376
9. **Caugant D.A., Mocca L.F., Frasch C.E., Frøholm L.O., Zollinger W.E. and Selander R.K.** (1987) Genetic structure of *Neisseria meningitidis* populations in relation to serogroup, serotype, and outer membrane protein pattern. J. Bacteriol. **169:**2781-2792
10. **Cho J.-C. and Tiedje J.M.** (2000) Biogeography and degree of endemicity of fluorescent *Pseudomonas* strains in soil. Appl. Environ. Microbiol. **66:**5448-5456
11. **Dams E., Hendriks L., Van de Peer Y., Neefs J.-M., Smits G., Vandenbempt I. and De Wachter R.** (1988) Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Res. **16:**87-173
12. **Dice L.R.** (1945) Measures of the amount of ecologic association between species. Ecology. **26:**297-302
13. **Eichler B. and Pfennig N.** (1990) Seasonal development of anoxygenic phototrophic bacteria in a holomictic drumlin lake (Schleinsee, F.R.G.) Arch. Hydrobiol. **119:**369-392

14. **Eguchi M., Ostrowski M., Fegatella F., Bowman J., Nichols D., Nishino T. and Cavicchioli R.** (2001) *Sphingomonas alaskensis* strain AF01, an abundant oligotrophic ultramicrobacterium from the North Pacific. Appl. Environ. Microbiol. **67:**4945-4954
15. **Field K.G., Gordon D., Wright T., Rappé M., Urbach E., Vergin K. and Giovannonni S.J.** (1997) Diversity and depth-specific distribution of SAR11 cluster rRNA genes from marine planktonic bacteria. Appl. Environ. Microbiol. **63:**63-70
16. **Finlay B.J.** (2002) Global dispersal of free-living microbial eukaryote species. Science **296:**1061-1063
17. **Finlay B.J. and Clarke K.J.** (1999) Ubiquitous dispersal of microbial species. Nature. **400:**828
18. **Fröhlich J. and König H.** (1999) Rapid isolation of single microbial cells from mixed natural and laboratory populations with the aid of a micromanipulator. System. Appl. Microbiol. **22:**249-257
19. **Fröstl J.M. and Overmann J.** (1998) Physiology and tactic response of the phototrophic consortium "*Chlorochromatium aggregatum*". Arch Microbiol **169:**129-135
20. **Fröstl J.M. and Overmann J.** (2000) Phylogenetic affiliation of the bacteria that constitute phototrophic consortia. Arch. Microbiol. **174:**50-58
21. **Fuhrman J.A., McCallum K. and Davis A.A.** (1993) Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific Oceans. Appl. Environ. Microbiol. **59:**1294-1302
22. **Garcia-Pichel F., Prufert-Bebout L. and Muyzer G.** (1996) Phenotypic and phylogenetic analyses show *Microcoleus chthonoplastes* to be a cosmopolitan cyanobacterium. Appl. Environ. Microbiol. **62:**3284-3291
23. **Gasol J.M., Jürgens K., Massana R., Calderón-Paz J.I. and Pedrós-Alió C.** (1995) Mass development of *Daphnia pulex* in a sulfide-rich pond (Lake Cisó). Arch. Hydrobiol. **132:**279-296
24. **Gich F.** (2001) Ecologia i caracterització pigmentària de bacteris fotosintètics amb nou tipus de bcl *d*. Ph.D. Thesis, Institut d´Ecologia Aquatica, Universitat de Girona, Girona. 283 pp.
25. **Gich F., Garcia-Gil J. and Overmann J.** (2001) Previously unknown and phylogenetically diverse members of the green nonsulfur bacteria are indigenous to freshwater lakes. Arch. Microbiol. **177:**1-10

26. **Glaeser J. and Overmann J.** (2003) Characterization and *in situ* carbon metabolism of phototrophic consortia. Appl. Environ. Microbiol. **69:**3739-3750
27. **Glaeser J. and Overmann J.** (2003b) The significance of organic carbon compounds for *in situ* metabolism and chemotaxis of phototrophic consortia. Environ. Microbiol. (in press)
28. **Gray N.D., Howarth R., Rowan A., Pickup R.W., Jones J.G. and Head I.M.** (1999) Natural communities of *Achromatium oxaliferum* comprise genetically, morphologically, and ecologically distinct subpopulations. Appl. Environ. Microbiol. **65:**5089-5099
29. **Grayson T.H., Cooper L.F., Atienzar F.A., Knowles M.R. and Gilpin M.L.** (1999) Molecular differentiation of *Renibacterium salmoninarum* isolates from worldwide locations. Appl. Environ. Microbiol. **65:**961-968
30. **Griffin D.W., Garrison V.H., Herman J.R. and Shinn E.A.** (2001) African desert dust in the Carribbean atmosphere: Microbiology and public health. Aerobiologia **17:**203-213
31. **Guevara R., Armesto J.J. and Caru M.** (2002) Genetic diversity of *Nostoc* microsymbionts from *Gunnera tinctoria* revealed by PCR-STRR fingerprinting. Microb. Ecol. **44:**127-136
32. **Jaffe D., Anderson T., Covert D., Kotchenruther R., Trost B., Danielson J., Simpson W., Berntsen T., Karlsdottir S., Blake D., Harris J., Carmichael G. and Uno I.** (1999) Transport of Asian air pollution to North America. Geophys. Res. Lett. **26:**711-714
33. **Jørgensen B.B., Kuenen J.G. and Cohen Y.** (1979) Microbial transformations of sulfur compounds in a stratified lake (Solar Lake, Sinai). Limnol. Oceanogr. **24:**799-822
34. **Lauterborn R.** (1906) Zur Kenntnis der sapropelischen Flora. Allg. Bot. **2:**196 - 197
35. **Lawrence J.G.** (2001) Catalyzing bacterial speciation: correlating lateral transfer with genetic headroom. Syst. Biol. **50:**479-496
36. **Ludwig W., Strunk O., Klugbauer S., Klugbauer N., Weizenegger M., Neumaier J., Bachleitner M. and Schleifer K. H.** (1998) Bacterial phylogeny based on comparative sequence analysis. Electrophoresis **19:**554-568
37. **Maynard Smith J., Smith N.H., O´Rourke M. and Spratt B.G.** (1993) How clonal are bacteria? Proc. Natl. Acad. Sci. **90:**4384-4388

38. **Muyzer G., de Waal E.C. and Uitterlinden A.G.** (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

39. **Overmann J.** (2001) Green sulfur bacteria. *In*: Garrity, G.M. (ed.) Bergey´s manual of systematic bacteriology, vol 1. Williams and Wilkins, Baltimore, pp. 601-623

40. **Overmann J., Coolen M.J.L. and 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

41. **Overmann J. and Schubert K.** (2002) Phototrophic consortia: model systems for symbiotic interrelations between prokaryotes. Arch. Microbiol. **177:**201-208

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

43. **Pfennig N.** (1980) Syntrophic mixed cultures and symbiotic consortia with phototrophic bacteria: a review, pp. 127-131. *In*: Gottschalk G., Pfennig N., Werner H. (eds.), Anaerobes and anaerobic infections. Gustav Fischer Verlag, Stuttgart, New York

44. **Siefert E. and Pfennig N.** (1984) Convenient method to prepare neutral sulfide solution for cultivation of phototrophic sulfur bacteria. Arch. Microbiol. **139:**100-101

45. **Snyder A.R., Williams H.N., Baer M.L., Walker K.E. and Stine O.C.** (2002) 16S rDNA sequence analysis of environmental *Bdellovibrio*-and-like organisms (BALO) reveals extensive diversity. Int. J. Syst. Evol. Microbiol. **52:**2089-2094

46. **Souza V., Nguyen T.T., Hudson R.R., Piñero D. and Lenski R.E.** (1992) Hierarchical analysis of linkage disequilibrium in *Rhizobium* populations: Evidence for sex? Proc. Natl. Acad. Sci. **89:**8389-8393

47. **Staley J.T.** (1999) Bacterial biodiversity: a time for place. ASM News **65:** 681-687

48. **Staley J.T. and Gosink J.J.** (1999) Poles apart: Biodiversity and biogeography of sea ice bacteria. Annu. Rev. Microbiol. **53:**189-215

49. **Takaichi S.** (1999) Carotenoids and carotenogenesis in anoxygenic photosynthetic bacteria. In: Frank, H.A., Young, A.J., Britton, G., Cogdell, R.J. (eds.) The photochemistry of carotenoids. Kluwer Academic Publishers, Dordrecht, boston, London. pp. 39-69

50. **Vila X., Abella C.A., Figueras J.B. and Hurley J.P.** (1998) Vertical models of phototrophic bacterial distribution in the metalimnetic microbial communities of several freshwater North-American kettle lakes. FEMS Microbiol. Ecol. **25:**287-299

51. **Zwart G., Hiorns W.D., Methé B.A., van Agterveld M.P., Huismans R., Nold S.C., Zehr J.P. and Laanbroek H.J.** (1998) Nearly identical 16S rRNA sequences recovered from lakes in North America and Europe indicate the existence of clades of globally distributed freshwater bacteria. System. Appl. Microbiol. **21:**546-556

Chapter 6

Summary

Novel bacteriochlorophyll* e *structures and species-specific variability of pigment composition in green sulfur bacteria

The relative composition of bacteriochlorophyll (BChl) homologs in five different strains of brown-colored green sulfur bacteria was investigated by HPLC-MS/MS and NMR analyses. In addition, the effect of incubation light intensities on homolog distribution was studied in one of the strains (strain Dagow III). A total of 23 different BChl *e* structures were detected and comprise four homologous porphyrin ring systems and eight different esterifying alcohols. Several BChl *e* structures are novel. These include a C-8 ethyl, C-12 methyl [E,M] BChl e_F homolog which was identified by ^{1}H-NMR analyses of the isolated, main farnesyl homologs (BChl e_F). In addition, five previously unknown homolog series with dodecanol, pentadecenol, tetradecanol, hexadecenol and phytol as the esterifying alcohols were detected. The composition of BChl *e* homologs from the five strains of green sulfur bacteria differed with respect to the relative abundance of the homologs (BChl e_F: 25.6 to 67.0% of total BChl *e* content in stationary cultures). In strain Dagow III, the abundance of BChl e_F homologs decreased upon entry into the stationary phase. In all free-living strains, the abundance of BChl e_F was increased when the relative carotenoid content was low. The present results provide a more detailed picture of pigment composition in chlorosomes and thus will help to elucidate their structure and function. Furthermore, the newly discovered BChl *e* molecules are valuable biomarkers for the study of the occurrence and the metabolism of green sulfur bacteria in past and present ecosystems.

Characterization and* in situ *carbon metabolism of phototrophic consortia

A dense population of the phototrophic consortium "*P. roseum*" was investigated in the chemocline of a temperate holomictic lake (Lake Dagow, Brandenburg, Germany). Fluorescent *in situ* hybridization revealed that the brown-colored epibionts of "*P. roseum*" constituted up to 37 % of total bacterial cell numbers and up to 88 % of all green sulfur bacteria present in the chemocline. Specific amplification of 16S rRNA gene fragments of green sulfur bacteria and DGGE fingerprinting yielded a maximum of 4 different DNA bands depending on the year of study, indicating a low diversity of green sulfur bacteria. The 465 bp-long 16S rRNA gene sequence of the epibiont of "*P. roseum*" was obtained after sorting individual consortia by micromanipulation, followed by a highly sensitive PCR. The sequence obtained represents a new phylotype within the radiation of green sulfur bacteria. A maximum of light-dependent $H^{14}CO_3^-$ fixation in the chemocline in the presence of DCMU suggested anaerobic autotrophic growth of the green sulfur bacteria. The metabolism of the epibionts was further studied by determination of stable isotope ratios ($\delta^{13}C$) of their specific biomarkers. Analysis of photosynthetic pigments by HPLC revealed the presence of high concentrations of BChl *e* and smaller amounts of BChl *a*, *d* and Chl *a* in the chemocline. Unexpectedly, isorenieratene and β-isorenieratene, carotenoids typical for other brown-colored members of the green sulfur bacteria, were absent. Instead, four different esterifying alcohols of BChl *e* were isolated as biomarkers of green sulfur bacterial epibionts, and their $\delta^{13}C$ measured. Farnesol, tetradecanol, hexadecanol and hexadecenol all were significantly enriched in ^{13}C compared to bulk dissolved and particulate organic carbon, and compared to the biomarkers of purple sulfur bacteria. The difference ($\Delta\delta^{13}C$) between $\delta^{13}C$ values of farnesol, the major esterifying alcohol of BChl *e*, and CO_2 was -7.1‰, which provides clear evidence for a photoautotrophic mode of growth of the green sulfur bacterial epibionts of "*P. roseum*" *in situ*.

The significance of organic carbon compounds for* in situ *metabolism and chemotaxis of phototrophic consortia

The significance of organic carbon substrates for the chemotaxis and physiology of phototrophic consortia was investigated in a dense chemocline community of "*Pelochromatium roseum"*. For the first time, the monopolar monotrichous flagellation of the central bacterium could be visualized. *In situ*, intact motile "*P. roseum*" consortia were strongly attracted by sulfide and 2-oxoglutarate, which indicated a potential role of these compounds in the metabolism of "*P. roseum*". In chemocline water samples, 2-[$^{14}C(U)$]-oxoglutarate was utilized at nanomolar concentrations (half saturation constant of uptake

Kt ≤ 10 - 40 nM), and at a maximum uptake rate of *Vmax* ≈ 6 nM·h-1. The calculated turnover of 2-oxoglutarate at *in situ* concentrations was ~6 hours. Microautoradiography of chemocline water samples revealed that 87.5 % of the "*P. roseum*" consortia incorporated 2-oxoglutarate when both light and sulfide were present, whereas uptake was detected in less than 1.4% of the consortia if either light or sulfide were absent. Since the green sulfur bacterial epibionts in "*P. roseum*" have been shown to grow autotrophically, 2-oxoglutarate most likely is taken up and utilized by the central bacterium. Thus, our results indicate that incorporation of 2-oxoglutarate by the central bacterium is regulated by the metabolic state of the green sulfur bacterial epibionts.

Diversity and biogeography of phototrophic consortia

Motile phototrophic consortia are highly regular associations in which numerous cells of green sulfur bacteria surround a flagellated colorless β-proteobacterium in the center. To date, seven different morphological types have been described. In addition, two immotile associations involving green sulfur bacteria are known. Employing a culture-independent approach, different types of phototrophic consortia were mechanically isolated by micromanipulation from 14 freshwater lakes and ponds and partial 16S rRNA gene sequences of the green sulfur bacterial epibionts were determined. In all cases, phototrophic consortia with the same morphology and from the same habitat contained only one single epibiont phylotype. None of the epibiont 16S rRNA gene sequences have so far been detected in free-living green sulfur bacteria, indicating that the interaction between epibionts and chemotrophic bacteria in the phototrophic consortia is an obligate one. Morphologically indistinguishable phototrophic consortia collected from different lakes contained different epibionts. Overall, 20 different types of epibionts were detected in the present study. Based on our phylogenetic analysis, the epibiont sequences are not monophyletic and arose from different ancestors which independently developed the ability to form symbiotic associations. In the majority of lakes investigated, different types of phototrophic consortia were found to co-occur. Based on this finding, morphologically distinct types of consortia occupy different ecological niches. This is supported by the results of chemotaxis assays which revealed that different populations of the consortia "*Chlorochromatium aggregatum*" and "*Pelochromatium roseum*" were attracted by different organic carbon substrates and different reduced sulfur compounds. The present study thus demonstrates a high diversity, the presence of multiple niches, and a nonstatistical biogeographical distribution of phototrophic consortia in the natural environment.

ACKNOWLEDGEMENTS

I am indebted to Prof. Dr. Jörg Overmann for his confidence, for giving me the opportunity to work on a fascinating project and for getting me interested in the ecology of phototrophic bacteria. In the same manner I am indebted to Prof. Dr. Heribert Cypionka (Oldenburg) for the stimulating time in his group during the first half of my thesis.

I am thanking Prof. Dr. Jürgen Rullkötter (Oldenburg) for providing MS facilities, Dr. Kai Mangelsdorff for GC-MS measurements, Dr. Heike Rütters and Thomas Möhring for the help with the HPLC-MS measurements and Dr. Ute Güntner and Dr. Monika Segl (Bremen) for measuring $\delta^{13}C$ values of CO_2.

Without the generous help of Dr. Hans-Dieter Babenzien (Neuglobsow) who provided laboratory facilities during field studies at Lake Dagow most of this work would not have been possible.

I am thanking Prof. Dr. Jesus Garcia-Gil and his group (Girona, Spain) for fruitful discussions and for organizing the sampling lakes nearby Girona to obtain phototrophic consortia. I would also like to thank Prof. Dr. Carles Abella (Girona) for kindly providing chemocline samples from Michigan lakes and Prof. J. Staley (Seattle, USA) for the water sample from Echo lake. Especially I would like to thank Dr. Lluis Bañeras (Girona) for his help with the HPLC of photosynthetic pigments.

For fruitful and valuable discussions I would like to thank Dr. Elke Jaspers, Dr. Henrik Sass, and Dr. Karin Schubert. Also, I would like to thank Ann-Katrin Manske for assistence with the construction of the phylogenetic tree of epbibiotic green sulfur bacteria.

The colleagues in Oldenburg in Munich have contributed to this work with discussions, technical advice and their friendship. Your company made science fun and the laboratory a great place!

My special thanks are devoted to Christian, Judit, Willm, Frederic, Anna and Ann for their friendship and the good time in Munich. I am thanking Elke for her help and the time we spent together.

My family was a continuous source of strength during the last years and made my life much happier.

At this point I would like to thank my friends and all the people that have supported me during the last years.

Martina! Thank you for always being there when I need you, thank for your patience, your help, support and confidence throughout the last 18 month. Life without you would be very empty!

CURRICULUM VITAE

Personal data

Name	Jens Glaeser
Date of birth	05 June 1972
Place of birth	Haselünne
Parents	Peter Glaeser and Ilse Glaeser (maiden name: Oldenhage)

Education

1978 - 1982	Grundschule Bippen
1982 - 1991	Gymnasium Leoninum in Handrup: Abitur

Social service

1991 - 1992	Emergency rescue assistant

Academic education

1992 - 1999	Study of Biology at the Carl von Ossietzky University in Oldenburg Vordiplom: 28/08/1994
08/1996 - 07/1997	Studies in microbial ecology and evolution at the University of Oregon, Eugene, USA
29/01/1999	Graduation (Diplom) from the Carl von Ossietzky University in Oldenburg. Major subject: microbiology Minor subjects: genetics, ecology and biochemistry Title of Diploma work: "Isolation and characterization of new phototrophic sulfur bacteria and and their ecological significance"
02/1999 - 12/2000	Ph.D. student at the Carl von Ossietzky University in Oldenburg
01/2001 - 06/2002	Ph.D. studies continued at the Ludwig-Maximilians-Universität München (inscribed from 03/04/2001until 30/09/2003)
since 15/10/2002	Research collaborator at the Justus-Liebig-Universität in Gießen