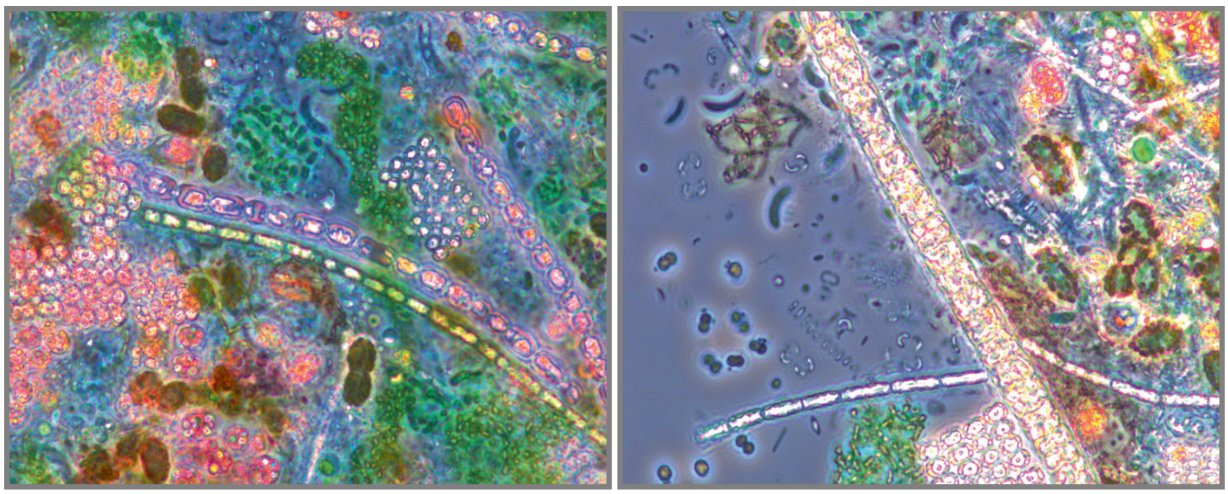
Ludwig-Maximilians-Universität München

Fakultät für Biologie

CHARACTERIZATION OF THE SYMBIOTIC BACTERIAL PARTNERS IN PHOTOTROPHIC CONSORTIA



Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades

vorgelegt von

Kristina Renate Pfannes

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im Juli 2007

„WENN DU EIN SCHIFF BAUEN WILLST, DANN TROMMLE NICHT MÄNNER ZUSAMMEN, UM HOLZ ZU BESCHAFFEN, AUFGABEN ZU VERGEBEN UND DIE ARBEIT EINZUTEILEN, SONDERN LEHRE SIE DIE SEHNSUCHT NACH DEM WEITEN, ENDLOSEN MEER"

ANTOINE DE SAINT-EXUPERY
FRZ. FLIEGER & SCHRIFTSTELLER (1900-1944)

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PUBLICATIONS ORIGINATING FROM THIS THESIS

CHAPTER 3

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CHAPTER 4

Pfannes KR, Vogl K, Overmann J (2007) The heterotrophic symbionts of phototrophic consortia belong to a highly diverse subgroup of *Betaproteobacteria* characterized by a tandem *rrn* operon structure. *Environ Microbiol* 9:2782-2794

CHAPTER 5

Vogl K, Glaeser J, **Pfannes KR**, Wanner G, Overmann J (2006) *Chlorobium chlorochromatii* sp. nov., a symbiotic green sulfur bacterium isolated from the phototrophic consortium "*Chlorochromatium aggregatum*". *Arch Microbiol* 185:363-372

CHAPTER 6

Pfannes KR, Müller JF, Zikeli K, Bayer AI, Vogl K, Wenter R, Hütz KA, Grossart H-P, Sandmann G, Takaichi S, Overmann J (2007) Oxygen tolerance and physiological properties of symbiotic and free-living green sulfur bacteria. (manuscript)

CONTRIBUTIONS

CHAPTER 3

The chemotaxis experiments were performed by Birgit Kanzler. She also did the *in situ* hybridization and the phylogenetic analyses under supervision of Kajetan Vogl. Kristina Pfannes established the enrichment and isolation of genomic DNA of the central bacterium via CsCl equilibrium density gradient centrifugation, determined the mol% G+C content and performed the real-time PCR.

CHAPTER 4

Kristina Pfannes performed the amplification and cloning of the rRNA operon. Moreover, she did the screening and analysis of the natural bacterial communities and conducted the *in situ* hybridization as well as the phylogenetical and statistical analyses. Kajetan Vogl developed the new cultivation approach of consortia cultures.

CHAPTER 5

The epibiont was isolated and phylogenetically analysed by Kajetan Vogl in cooperation with Jens Glaeser. Kajetan Vogl also did the substrate test and the growth experiments (temperature optimum, light intensity optimum, pH optimum) and the pigment analyses. Prof. Dr. Gerhard Wanner took the electron microscopy pictures. The contributions of Kristina Pfannes were the determination of the cell surface hydrophobicity and the salinity optimum, the pigment extraction, the KOH-string test and the Gram-staining.

CHAPTER 6

Kristina Pfannes, Kerstin Zikeli and Johannes Müller conducted the oxygen tolerance test. The enzyme assays were established and performed by Kristina Pfannes and Johannes Müller. Roland Wenter did the RT-PCR. Kristina Pfannes performed the genome analyses and the phylogenetic studies. Also, she prepared the samples for the analysis of the carotenoids and the determination of the amino acid and carbohydrate excretion. Prof. Dr. Gerhard Sandmann and Prof. Dr. Shinichi Takaichi analysed the carotenoids. Kerstin Zikeli and Katharina Hütz were responsible for the chlorosome analysis. The carbohydrate excretion and the amino acid excretion was measured by Dr. Hans-Peter Grossart and Prof. Dr. Meinhard Simon, respectively. Anne Bayer did the amino acid utilization experiment.

I hereby confirm the above statements

Kristina Pfannes Prof. Dr. Jörg Overmann

TABLE OF CONTENTS

I. ABBREVIATIONS

°C	degree Celsius
µ	micro
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
B. subtilis	*Bacillus subtilis*
BChl	Bacteriochlorophyll
bp	base pairs
C.	*Chlorochromatium, Clostridium*
Cba.	*Chlorobaculum*
Chl.	*Chlorobium*
CR	central rod
CTAB	Cetyl trimethyl ammonium bromide
D	Dagow
DAPI	4',6-diamidino-2-phenylindol
DNA	Desoxyribonucleic acid
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen
EDTA	Ethylendiaminotetraacetic acid
e.g.	*exempli gratia* (for example)
et al.	*et alia* (and others)
F	forward
FISH	fluorescence *in situ* hybridization
FMO	Fenna-Matthews-Olson
g	gram
GSB	Green Sulfur Bacteria
H	Helper oligonucleotide
HPLC	High performance liquid chromatography
IOS	interoperon spacer
iPCR	inverse polymerase chain reaction
ITS	internal transcribed spacer

kb	thousand base pairs
l	liter (analogously ml and µl)
M	mol $\cdot l^{-1}$ (analogously mM, µm, nm)
Mb	million base pairs
MPN	most probable number
n.d.	not determined
P.	*Pelochromatium*
PCR	Polymerase chain reaction
r	reverse
Rfx.	*Rhodoferax*
rrf	5S ribosomal ribonucleic acid
rrl	23S ribosomal ribonucleic acid
rrn	ribosomal ribonucleic acid
rRNA	ribosomal ribonucleic acid
rrs	16S ribosomal ribonucleic acid
SD	standard deviation
v/v	volume / volume
w/v	weight / volume

1 SUMMARY

Bacterial interactions play a major role in nature, but are poorly understood, because of the lack of adequate model systems. Phototrophic consortia represent the most highly developed type of interspecific bacterial association due to the precise spatial arrangement of phototrophic green sulfur bacteria (GSB) around a heterotrophic central bacterium. Therefore, they are valuable model systems for the study of symbiosis, signal transduction, and coevolution between different bacteria. This thesis summarizes a series of laboratory experiments with the objective of elucidating the molecular, physiological and phylogenetical properties of the two bacterial partners in the symbiotic phototrophic consortium "*Chlorochromatium aggregatum*".

The central bacterium of "*C. aggregatum*" had been identified as a *Betaproteobacterium*, however, it could not be characterized further due to the low amount of consortia in enrichment cultures. In this work a suitable method for enrichment and isolation of DNA of the central bacterium of "*C. aggregatum*" has been established using cesium chloride-bisbenzimidazole equilibrium density gradient centrifugation (Chapter 3). In density gradients, genomic DNA of the central bacterium of "*C. aggregatum*" formed a distinct band, which could be detected by real-time PCR. Using this method, the GC-content of the central bacterium was estimated to be 55.6%. Furthermore, its precise phylogenetic position was determined and it was shown to represent a novel and phylogenetically isolated lineage of the *Comamonadaceae* within the β-subgroup of the *Proteobacteria.*

Chapter 4 describes the detection of a new, highly diverse subcluster of *Betaproteobacteria*, which contains several central bacteria of phototrophic consortia. Genomic DNA of the central bacterium of "*C. aggregatum*" was enriched several hundred fold by employing a selective method for growth of consortia in a monolayer biofilm followed by a purification of the central bacterial genome by density gradient centrifugation. A combination of molecular methods revealed that two *rrn* operons of the central bacterium are arranged in a tandem fashion. This rare gene order was exploited to screen various natural microbial communities. A diverse and previously unknown subgroup of *Betaproteobacteria* was discovered in the chemocline of Lake Dagow, Eastern Germany. All 16S rRNA gene

sequences recovered are related to that of the central bacterium of "*C. aggregatum*". Phylogenetic analyses showed that the central, chemotrophic symbionts of phototrophic consortia have a polyphyletic origin, just like their phototrophic counterparts. This indictates that not only different GSB but also different *Betaproteobacteria* have adapted to life in this type of symbiosis.

Chapter 5 focuses on the isolation of the epibiont of "*C. aggregatum*" from a consortia enrichment culture and its description as *Chlorobium chlorochromatii* strain CaD. It represents a novel species within the genus *Chlorobium* and is characterized by physiological properties typical for GSB. However, the symbiotic strain differs from free-living GSB in the distribution of its chlorosomes and the presence of a conspicuous additional structure at the attachment-site to the central bacterium. Its capability to grow in pure culture indicates that it is not obligately symbiotic.

The natural habitat of GSB and phototrophic consortia is the chemocline of stratified lakes. Therefore, the physiological response to oxygen exposure of the epibiont and the free-living GSB *Chlorobium limicola* has been investigated (Chapter 6). It was shown that GSB are able to survive oxygen exposure and have developed several strategies for oxygen detoxification. Genome annotation revealed the presence of several enzymes involved in oxygen detoxification in all currently sequenced GSB genomes. Phylogenetic analyses showed that most of these enzymes likely were present in the common ancestor of this group. The activity of some of those enzymes could be confirmed. Since carotenoids also act as antioxidants, the carotenoid composition of the epibiont was investigated. In contrast to all other GSB it lacks chlorobactene, the major carotenoid in green-coloured GSB. In addition, 7,8-dihydro-γ-carotene has been identified in the epibiont as a novel carotenoid in nature.

Substantial progress has been made in the course of this study not only with the establishment of a method facilitating genome sequencing of the central bacterium of "*C. aggregatum*", but also with the developement of a molecular screening tool for central bacteria of phototrophic consortia. The resulting sequences will enable the direct comparison of the phylogeny of both bacterial partners in different phototrophic consortia and hence will provide the unique opportunity to assess for the first time the process of the coevolution of a bacteria-bacteria-symbiosis.

2 INTRODUCTION

2.1 DIVERSITY OF SYMBIOSES

In nature, organisms live in close association with their surrounding physical environment as well as with other organisms. Therefore, organisms in an ecosystem interact with each other, resulting in a great variety of biological interactions. Biological interactions can be classified on the basis of either their effects or the mechanisms of the interaction (Abrams 1987). Today, both of these classifications are used and frequently combined to categorize interactions. Common interaction categories include competition, predation, parasitism, disease, herbivory and symbiosis (Krebs 2001).

Symbiosis represents an important form of biological interaction. The concept is derived from the Greek words *syn* (with/plus) and *bio* (life) and designates an interaction between two dissimilar organisms. While the original meaning is simply "living together" of two or more organisms of different species (DeBary 1879, Pierantoni 1910), today the term "symbiosis" is mostly used in the sense of mutualism – that is living together for the benefit of both partners (Krebs 2001, Moran 2006). The difference from the frequently used term synergism is, that symbiotic relationships are obligatory under certain conditions and highly specific, and one member of the association ordinarily cannot be replaced by another related species. Therefore, symbiotic relationships can be considered extended synergism (Atlas and Bartha 2005). Sometimes, the interactions between organisms cannot exactly be defined or it is not possible to clearly differentiate between the single categories, since the boundaries are fluid (Hacker and Heeseman 2000, Krebs 2001, Pfannes 2003). Therefore, terms for the classifications of interactions are in general loosely used. In this context it was suggested to consider an interaction as symbiotic if it is difficult to determine whether the relationship is obligatory for all participants (Atlas and Bartha 2005).

The age of symbiotic associations has been traced back to as much as 250 million years (Baumann *et al.* 1998). Over this span of time, coevolution occurred (Bandi *et al.* 1996, Moran 2006, Sauer *et al.* 2000). Coevolution is a driving force for the emergence of specific mechanisms of organismic interactions and novel physiological capabilities of the symbiotic

system as compared to the individual partners (Overmann 2006). In some cases, diversification of host and symbiont has occurred in parallel, through the history of constant association, which is referred to as codiversification. This demonstrates, that symbiosis has been crucial in adaptive radiation, evolution of lineages and ecological diversification (Margulis and Fester 1991). Hence, the diversity of symbioses is striking.

Symbioses occur in all domains of living organisms. They are ubiquitous in terrestrial and aquatic communities and have played a key role in the emergence of major life forms on earth and the generation of biological diversity (Moran, 2006). While the tropics count for their extraordinary richness not only in species diversity but also in the diversity of symbiotic associations - only to mention the incredible richness of myrmecophtyes (Bequaert 1922, Chapman and Margulis 1998, Davidson and McKey 1993, Pfannes and Baier 2002) and termite-protozoa symbioses (Dolan 2001, Ohkuma 2003), - some temperate aquatic environments are likewise outstanding e.g. with essentially all cells of green sulfur bacteria, that usually are free-living bacteria, occurring in the symbiotic state (Glaeser and Overmann 2003a).

2.2 SIGNIFICANCE OF PROKARYOTIC SYMBIOSES

In contrast to microbial pathogenicity, which was the first-studied field of microbiology and therefore has attracted most attention, the investigation of symbiotic interactions lags behind. Considering that only 200 bacterial species are regarded as human pathogens (Lengler *et al.* 1999) whereas the number of commensal and symbiotic bacteria species associated just with the human intestinal tract is more than three times higher (Suau *et al.* 1999) this seems unwarranted. One exception is the well studied complex of *Rhizobium*-legume symbioses. This is mainly due to their importance in agriculture, since these associations are responsible for the reduction of 120 million tonnes of atmospheric nitrogen to ammonia each year (Freiberg *et al.* 1997). Recently, it has been recognized that even if the outcome in symbiotic and pathogenic systems is completely different, common molecular mechanisms are involved to mediate the communication between the interacting partners. Specifically, nitrogen-fixing bacterial symbionts of legume plants, collectively termed rhizobia, and phytopathogenic bacteria have adopted similar strategies and genetic traits to colonize, invade and establish a chronic infection in the plant host (Soto *et al.* 2006).

The disregard of prokaryotic symbioses is largely due to technical limitations, because it is very difficult to maintain symbiotic associations in the laboratory (Overmann 2006). Free-living organisms, free of complex interdependencies, are relatively easy to grow and hence, commonly studied in the laboratory. However, the awareness, that bacterial symbioses have an enormous impact for many ecosystems and are important driving forces in evolution (Gross *et al.* 2003), has emerged since the endosymbiotic origin of eukaryotic organelles was proposed (Margulis and Bermudes 1985). Today, there is growing consensus that the mitochondria and chloroplasts of eukaryotes arose from bacteria that became established intracellularly in primitive single celled organisms 1-2 billion years ago (Margulis and Bermudes 1985, Margulis 1993, Margulis and Sagan 2002). As early as 1905 it was postulated by Mereschkowsky that plastids evolved from cyanobacterial ancestors and later, that mitochondria are descended from ancient aerobic free-living bacteria (since specified as α-Proteobacteria) (Wallin 1927). The "serial endosymbiosis hypothesis" is derived from this hypothesis, which collectively expounds on the origin of eukaryotic cell organelles resulting in novel organisms that were capable of invading novel metabolic and ecological niches (Kutschera and Niklas 2005).

There exist an immense variety of microbial symbioses, ranging from symbioses with animals, over symbioses with plants to symbioses involving only microbes. An outstanding example for intermicrobial symbioses is for instance the relationship between certain algae or cyanobacteria and fungi that result in the formation of lichens (Ahmadjian 1993, Lamb 1959). Another symbiotic association involving algae is that of *Paramecium*, which can host numerous cells of the alga *Chlorella* within its cytoplasm (Ball 1969). There, the algae provides the protozoan with organic carbon and oxygen, and the protozoan provides protection, motility and carbon dioxide. Furthermore, it is reported that *Parachlamydia* and *Neochlamydia*, close relatives of pathogenic *Chlamydia*, can live in obligate intracellular symbiotic association with environmental and clinical amoebae such as *Acanthamoeba* spp. (Fritsche *et al.* 2000, Pfannes 2003). By far less common as eubacterial symbionts are symbiotic associations with archaebacteria. However, symbiotic consortia of archaea and sulphate–reducing bacteria, that are most likely the biological agents of anaerobic methane oxidation, are a contemporary example (Hinrichs *et al.* 1999, Boetius *et al.* 2000).

Likewise, the omnipresence of symbiotic bacteria is reflected by the fact that they affect a variety of diverse fields; for one to mention development. The aphid symbiont *Buchnera aphidicola* governs bacteriocyte development and *Vibrio fischeri* was similarly shown to mediate the development resulting in either inflammation or in a mutualistic animal-microbe association by releasing tracheal cytotoxin (Braendle *et al.* 2003, Koropatnick *et al.* 2004). Aside, a diverse population of symbiotic bacteria provides genetic and metabolic attributes including the ability to harvest otherwise inaccessible nutrients (Backhed *et al.* 2005). Furthermore symbiotic bacteria like *Wolbachia* and *Rickettsia* affect reproduction and speciation in arthropods by male-killing, parthenogenesis induction, and feminization (Bandi *et al.* 2001, Hurst and Jiggins 2000, Stouthamer *et al.* 1999). Some bacterial symbionts mediate defence against natural enemies as for instance aphid resistance to fungal pathogens mediated by the endosymbiont *Regiella insecticola* or antitumor polyketides of the pederin familiy, isolated from beetles (Kellner 2001, Piel 2002, Scarborough *et al.* 2005). Moreover, symbiotic bacteria are known to affect immunity, as exemplifies the gut commensal flora, by shaping the intestinal immune system (Cash and Hooper 2005, Macdonald and Monteleone 2005).

Most microbial symbioses have a physiological basis in that compounds produced by one partner are useful for the other. However, symbioses can also entail the transfer of genes from one partner to the other, which in some cases cements two cells to a bipartite, co-evolving unit (Hoffmeister and Martin 2003). Fortunately, by now the complete genome sequences for some of the prokaryotic symbionts are available, which enables the investigation of symbiosis on a genome level. As of June 8, 2007, out of 524 completed microbial genome sequences (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi), 251 belonged to pathogens and 57 to symbionts. Nowadays, horizontal gene transfer of so-called "symbiosis islands", mobile genetic entities that were initially identified in *Mesorhizobium loti* (Sullivan and Ronson 1998) recieves increased attention. Symbiosis islands seem to act like pathogenicity islands, which have been shown to play an important role in promoting the adaptive evolution of commensal, symbiotic and environmental bacteria (Dobrindt *et al.* 2004). Advances in molecular symbiosis research do also permit a comparison across different symbiotic systems as well as pathogenic systems (Overmann 2006). Amino acid metabolism appears to be a key feature of some bacteria-insect symbioses, which is for

instance revealed by the genome sequence of the obligate aphid endosymbiont *Buchnera* sp. (Lai *et al.* 1994) and the obligate endosymbiont of carpenter ants *Blochmannia* sp., which are closely related (Sauer *et al.* 2002, Shigenobu *et al.* 2000). The genome of the endosymbiont *Wigglesworthia glossinidia*, which is symbiotically associated with the tsetse fly, indicates that the basis of the symbiosis lies on vitamin biosynthesis rather than amino acid biosynthesis (Akman *et al.* 2002). The latter microbes are all characterized by highly reduced genomes. So is the genome of *Buchnera aphidicola* with 618 kb significantly smaller than that of free-living enteric bacteria such as *Escherichia coli* (Akman *et al.* 2001, van Ham *et al.* 2003). Considerable genome reduction of obligate intracellular pathogens or symbionts has been reported frequently (Gross *et al.* 2003, Moran 2002, van Ham *et al.* 2002). In contrast to the symbionts *Buchnera* and *Blochmannia*, obligate parasites such as *Rickettsia* and *Mycoplasma* have lost many biosynthetic capabilities, making them dependent on compounds supplied by the parasitized host (Ochman and Moran 2001). The smallest microbial genome sequenced to date is that of the obligate symbiont *Nanoarchaeum equitans* (490 kb), which encodes the machinery for information processing and repair, but lacks genes for lipid, cofactor, amino acid, or nucleotide biosynthesis (Das *et al.* 2006, Waters *et al.* 2003). Another molecular principle of organismic interaction seems to be the presence of RTX–like components in symbiotic associations. They have been detected in phototrophic consortia (Overmann 2006, Vogl *et al.* in prep.) and the association of *Riftia pachyptila* with chemoautotrophic bacteria (Cavanaugh 2004), two very different symbiotic systems. This implies that genome structure and properties frequently reflect the lifestyle of the organism.

Recapitulating, these examples make clear, that symbiosis represents a typical way of prokaryotic life (Overmann 2006). They also show that most microbial interactions involve other organisms than bacteria. There are only few purely bacterial symbioses known. One is the symbiosis of mealybugs, which are associated with betaproteobaterial endosymbionts that in turn harbour G*ammaproteobacteria* inside (Dohlen *et al.* 2001, Hoffmeister and Martin 2003). The second is the symbiosis of phototrophic consortia, object of this study, which involves two unrelated bacteria. In contrast to most symbiotic systems, the latter can be kept in laboratory culture, what makes it an adequate model system to generally study the basis of symbiosis.

2.3 PHOTOSYNTHESIS AND PHOTOTROPHY IN BACTERIA

Photosynthesis is the most important biochemical pathway on Earth since nearly all life depends on it. It is the reduction of carbon dioxide into biomass using energy derived from light. Photosynthesis can be divided into the light reaction and ATP-synthesis and the dark reaction involving carbon dioxide fixation (van Niel 1931) and occurs in higher plants, algae, protists and bacteria, organisms collectively referred to as photoautotrophs. Phototrophy is defined as a metabolic mode in which organisms convert electromagnetic energy into chemical energy for growth (Bryant and Frigaard 2006, Eiler 2006, Madigan *et al.* 2000). There exist two distinct mechanisms to accomplish phototrophy. The simplest employs retinal-binding proteins, like bacteriorhodopsin or proteorhodopsin, that respond to light stimuli, so-called rhodopsins (Lanyi 2004). Because rhodopsins do not mediate electron transfer, phototrophic organisms employing this mechanism are not considered to be photosynthetic (Bryant and Frigaard 2006). A more complex process to empower phototrophy implicates the (bacterio)-chlorophyll based-energy conversion, which depends on photochemical reaction centres and is found in five bacterial phyla encompassing the *Chlorobi*, *Chloroflexi*, *Proteobacteria, Firmicutes* and *Cyanobacteria*.

Phototrophic bacteria do not form a phylogenetically homogenous and closely related group, but belong to distantly related bacterial lineages (Fig. 1). Whereas all currently described strains of the *Chlorobi* and *Cyanobacteria* have a photoautotrophic lifestyle, only some strains of the *Chloroflexi* (the filamentous anoxygenic phototrophs), *Proteobacteria* (purple sulfur and purple non-sulfur bacteria) and *Firmicutes* (Heliobacteria) are phototrophic (Bryant and Frigaard 2006, Overmann and Garcia-Pichel 2001, Yurkov and Beatty 1998). Because of its complexity it is assumed that bacterial photosynthesis has not been evolved more than once (Woese 1987). The origin of photosynthesis has been traced back to the *α-Proteobacteria* by comparison of multiple photosynthesis genes (Xiong and Bauer 2002) and it is likely that lateral gene transfer has spread the capacity for photosynthetic growth across the domain bacteria (Blankenship 1992, Nagashima *et al.* 1997, Raymond *et al.* 2002).

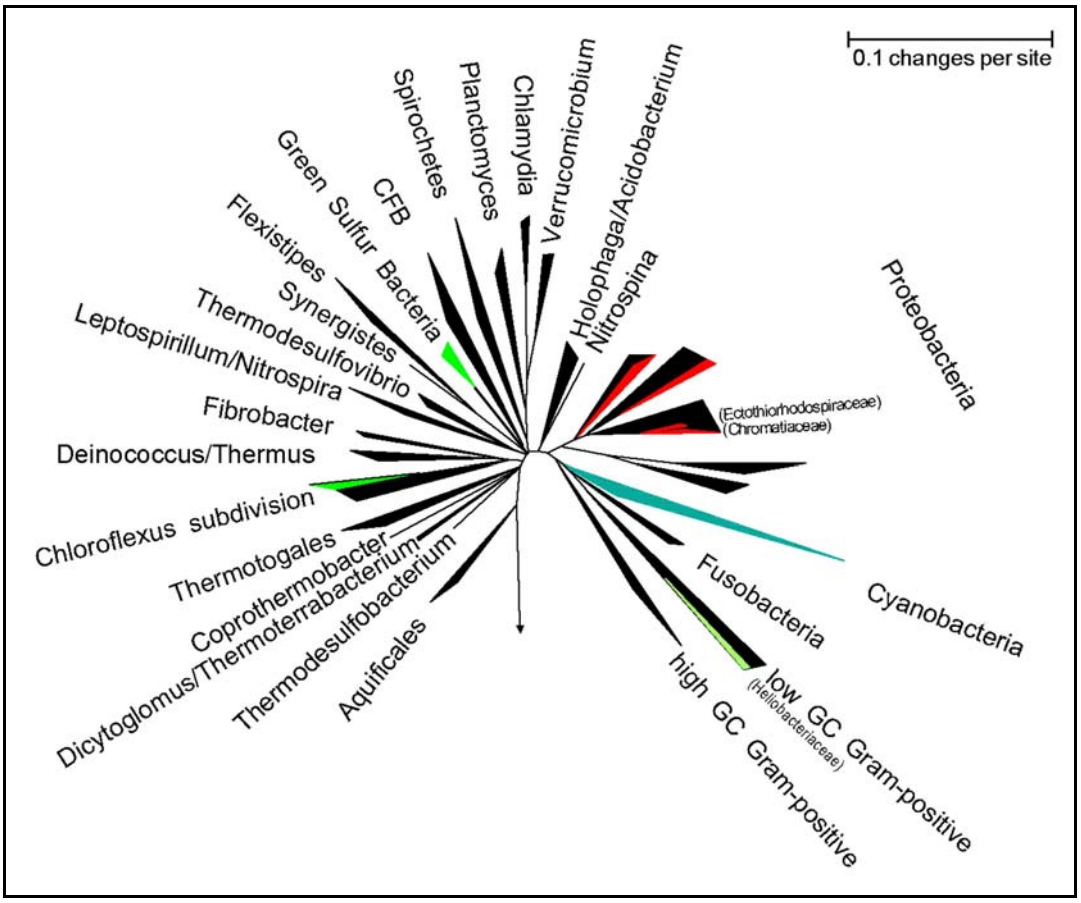


Figure 1. Phylogenetic tree of the domain Bacteria based on 16S rRNA sequences (adopted from Overmann and Garcia-Pichel 2001). Phyla containing phototrophic bacteria are coloured. *Light green*: Bacteria containing chlorosomes as light-harvesting antenna. *Red*: Bacteria containing antenna complexes within the cytoplasmic membrane and quinone/pheophytin-type reaction centres. *Medium green*: Gram-positive bacteria with FeS-type reaction centres. *Petrol*: Bacteria containing the two types of reaction centres. Width of coloured wedges indicates the phylogenetic diversity.

Photosynthetic organisms produce a variety of light harvesting structures like chlorosomes, phycobilisomes and bacteriochlorophylls (BChls) to enhance the rate of light-driven energy transport (Blankenship 2002, Green and Parson 2003). Chlorosomes are only found in the green sulfur bacteria and the green non-sulfur bacteria and phycobilisomes from the *Cyanobacteria*, red algae and glaucophytes. In contrast, different bacteriochlorophylls (BChl *a* – BChl *e*) are broadly distributed over the phototrophic bacteria. Additionally, various carotenoids function in photosynthetic organisms as light-harvesting molecules and have protective funtion to prevent harmful photooxidation related to the presence of singlet oxygen (Fraser *et al.* 2002, Lengeler *et al.* 1999).

The major difference between the groups of photosynthetically active organisms is the formation of oxygen in oxygenic photosynthesis or the absence of oxygen production in anoxygenic photosynthesis (Lengeler *et al.* 1999). Eukaryotic photosynthesizers and prokaryotic *Cyanobacteria* use water as an electron donor and produce oxygen as a waste product during hydrolysis. Whereas plants contribute the major part of the photosynthetic carbon dioxide fixation in terrestrial environments *Cyanobacteria* accomplish most of the marine primary production (Campbell *et al.* 1994, Goericke and Welschmeyer 1993, Liu *et al.* 1997). By doing so, *Cyanobacteria*, green plants and algae are the source of nearly all the oxygen in the atmosphere. In contrast, oxygen is not formed in anoxygenic photosynthesis, which is believed to be the precursor of oxygenic photosynthesis (Blankenship 1992, Xiong *et al.* 1998). While anoxygenic photosynthesizers today play a minor role in primary production, they were probably more predominant in ancient times (Overmann and Garcia-Pichel 2001).

In contrast to oxygenic photosynthesis, which involves two coupled chlorophyll-based photosystems, anoxygenic photosynthesizers contain only one type of bacteriochlorophyll-based photosystem, which requires electron donors for carbon dioxid fixation with lower standard reduction potential than water (Overmann 2001a, Overmann and Garcia-Pichel 2001). Therefore, reduced sulfur compounds (e.g. H_2S), and several simple organic compounds serve as electron donors in anoxygenic photosynthesis (Brune 1989, Pfennig 1978). Anoxygenic phototrophs are distributed in all lineages of phototrophic bacteria except *Cyanobacteria* (Fig. 1). With the exception of the green sulfur bacteria forming a coherent group, the four lineages of anoxygenic bacteria are only distantly related (Stackebrandt *et al.* 1996, Woese 1987).

2.4 GREEN SULFUR BACTERIA

Green sulfur bacteria (GSB) represent a metabolically limited, physiologically well-defined and genetically closely related bacterial group. The GSB are all members of the phylum *Chlorobi*, which shares a common root with the *Bacteroidetes* (Garrity and Holt 2001). The group of the GSB is represented by the family *Chlorobiaceae* and forms a phylogenetic branch distinct from other main phylogenetic lines. The existing species have been described as obligately anaerobic photoautotrophs that oxidize sulfur compounds and fix carbon by the

reverse tricarboxylic acid cycle. They are gram-negative bacteria and except for representatives of the genus *Chloroherpeton*, which moves by gliding, all genera are nonmotile. Some species, however, possess gas vesicles, which enable them to float at a desired depth in the water (Overmann 2001b).

Although widely distributed, GSB are restricted to anoxic habitats exposed to light. Thus, they are found in brackish waters, marine lagoons, intertidal sandflats, hypersaline habitats and freshwater lakes (Martinez-Alonso *et al.* 2004, Matheron and Baulaigue 1972, Overmann 2001b, Suckow 1966, Trüper 1970). Typically, populations of GSB are found below layers of purple sulfur bacteria, reflecting their adaptation to low light intensities (Montesinos *et al.* 1983). Due to this adaptation and their affinity to high sulfide concentrations, these bacteria prevail in the deeper anoxic layers of lagoons and lakes, in the Black Sea, as well within the top millimetres of sediments with an oxic/anoxic boundary (Trüper 1970, Vila *et al.* 2002). GSB are either green or brown-coloured. The major component of the green strains is BChl *c* or *d*, whereas brown species mainly contain BChl *e*. Small amounts of BChl *a* are present in both, green-coloured and brown-coloured strains. (Gloe *et al.* 1975, Overmann 2001b). One of the properties responsible for the specific low-light adaptation of GSB is the presence of characteristic light harvesting structures, the chlorosomes, which are attached to the cytoplasmic membrane and contain BChl *c*, *d* or *e* molecules (Frigaard 2003, Frigaard and Bryant 2006, Cohen-Bazire *et al.* 1964, Staehelin *et al.* 1978). These light harvesting features are the reason for the dominance of GSB in natural communities under low light conditions, since GSB need only one fourth of the light intensity to reach similar growth rates like e.g. *Chromatiaceae* in the laboratory (Biebl and Pfennig 1978). Whereas a chlorosome contains about 10000 BChl molecules and is linked to 5–10 reaction centres (Abella *et al.* 1998), the light harvesting antenna of *Chromatiacae* contains 10 times less BChls per reaction center (Abella *et al.* 1998). Although chlorosomes are found in both the GSB and the green non sulfur bacteria, the FMO protein (Fenna-Matthews-Olson) is unique to the GSB. It is an unusual water-soluble bacteriochlorophyll-binding protein that transfers excitation energy from the chlorosomes to the reaction centre. Due to its uniqueness it has been used as genetic marker to study the phylogeny and the environmental diversity of GSB communities (Imhoff 2003, Alexander and Imhoff 2006). Moreover, phylogenetic analyses based on the FMO protein have been the motive to propose a new taxonomy for

GSB, since the traditional classification system based on cell morphology, pigments and substrate utilization did not concur with their molecular phylogeny. Besides pigments directly involved in light harvesting, GSB also contain different types of carotenoids, which participate in the energy-transfer process and are efficient free-radical scavengers. Wheareas the major and characteristic carotenoid of green-coloured GSB is chlorobactene, the main carotenoid of brown-coloured species is isorenieratene (Liaaen-Jensen *et al.* 1964, Liaaen-Jensen 1965, Wahlund *et al.* 1991, Overmann et al. 1992, Frigaard *et al.* 2004, Imhoff 1995). These carotenoids are unusual by having aromatic end-groups and are, besides in green sulfur bacteria, found only in some actinomycetes and a few sponges (Armstrong 1999, Britton 1998).

The significance of the GSB can be illustrated on the basis of different examples. *Chlorobium tepidum* has become the model organism for the GSB due to the availability of an efficient natural transformation system. In comparison to other GSB strains, it grows rapidly and easily in the laboratory. Moreover, it was the first representative of the GSB of which the genome was sequenced (Eisen *et al.* 2002). The bioluminescent deep-sea fish *Malocosteus* is reported to make extraordinary use of the BChls of GSB, concentrating and modifying it to use as a pigment for visualizing red light (Dyer 2003). It is also known, that GSB form a variety of syntrophic associations. In the laboratory, *C. limicola* f. sp. *thiosulfatophilum* can only grow in co-culture with the purple sulfur bacterium *Chromatium vinosum*. This reflects the situation in the environment where coexistence of purple and GSB is widespread (Overmann 2001b). The cause of the interaction is assumed to lie in the higher sulfide affinity of *Chlorobium*, which cannot exploit polysulfides in the presence of hydrogen sulfide. This is compensated by the rapid polysulfide utilization of *Chromatium* (van Gemerden and Mas 1995). Moreover, stable syntrophic associations of sulfur- and sulfate reducing bacteria are reported, which are based on a closed sulfur cycle (Overmann 2001b, Pfennig 1980). Finally, GSB occur in associations with a single colourless bacterium as phototrophic consortia, which are the subject of this thesis.

2.5 PHOTOTROPHIC CONSORTIA

By definition, consortia are close associations of microbial cells in which two or more different microorganisms form an organized structure by maintaining a permanent cell-to-cell contact (Schink 1991, Trüper and Pfennig 1971). Besides these phototrophic anoxygenic consortia there exist several other types of bacterial consortia (Overmann 2006). The most prevalent ones are consortia containing Cyanobacteria (Paerl and Kellar 1978), consortia containing colourless sulfur bacteria (Jørgensen and Gallardo 1999), consortia containing nitrifying bacteria (Mobarry *et al.* 1996) and consortia containing methanogenic bacteria (Leadbetter and Breznak 1996). Phototrophic consortia, which were described for the first time over one century ago (Lauterborn 1906), represent a special type of microbial consortia since they are considered to be the most highly developed symbiotic association between two different prokaryotic species (Overmann and Schubert 2000, Schink 1991). Among all consortia, phototrophic consortia stand out by the highest degree of mutual interdependence between non-related bacteria (Overmann 2006). They consist of a fixed number of GSB epibionts, which are arranged in a highly ordered fashion around a central colourless chemotrophic rod-shaped bacterium.

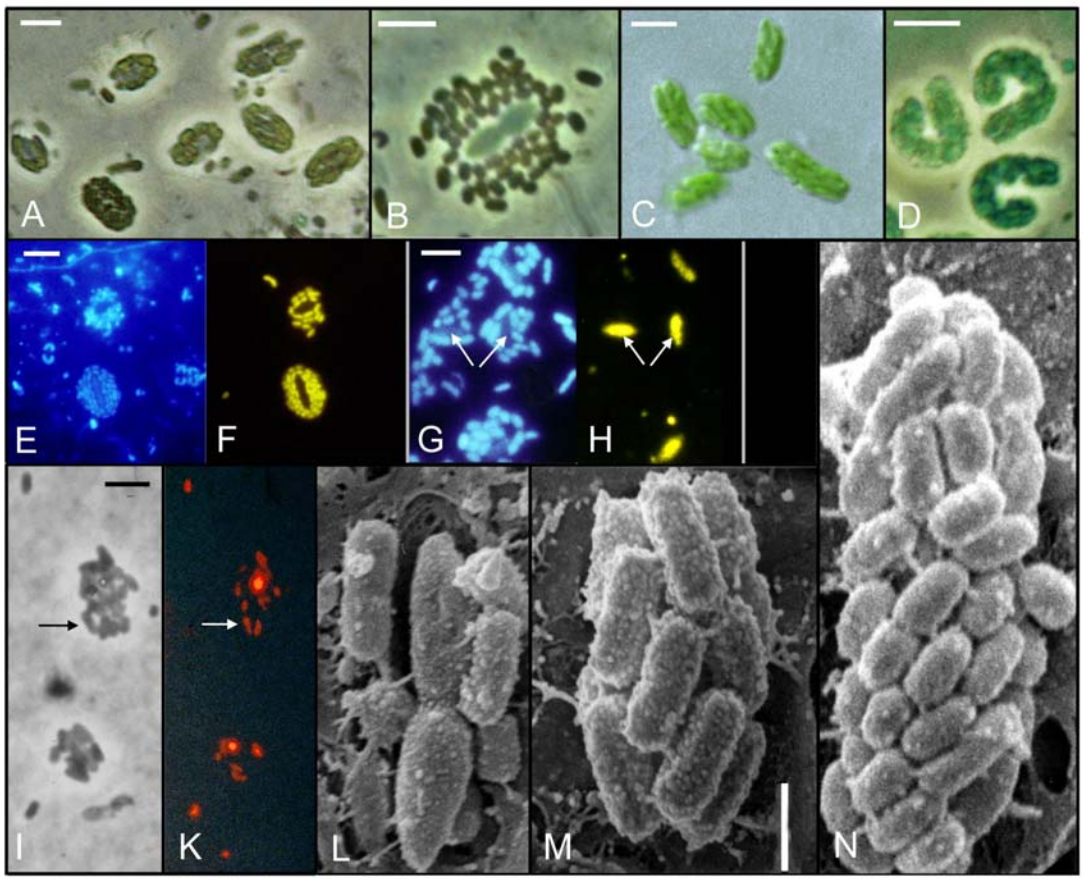
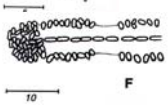


Figure 2. A. Phase contrast photomicrograph of intact "*P. roseum*". **B.** "*P. roseum*" after disaggregation on an agar coated microscope slide. **C.** Differential interference contrast image of "*C. aggregatum*" consortia. **D.** Phase contrast photomicrograph of "*C. glebulum*". **E, F.** Bacterial cells from the chemocline from Lake Dagow hybridisized with a Cy3-labeled probe specific for GSB (GSB-532) and counterstained with DAPI. **E.** DAPI fluorescence. **F.** Cy3 fluorescence. **G, H.** Same as in E, F but with a *Betaproteobacteria* specific probe (BET42a). Arrows indicate the positions of the central bacteria. **I, K.** Reduction of the fluorochrome CTC by epibionts (arrows) in the presence of light and sulfide. **I**: Phase contrast photomicrograph of "*C. aggregatum*". **K.** Fluorescence of CTC. Bars 5 μm. **L-N.** Scanning electron micrographs of consortia from Lake Dagow. **L.** Consortium with exposed central bacterium. **M.** Intact consortium. **N.** Consortium after division of epibiont cells (Overmann and Schubert 2002). Bar 1 = μm

To date, a total of 19 morphologically different types of bacterial consortia are recognized based on the taxonomy and the arrangement of the participating bacteria (Glaeser and Overmann 2004, Huber *et al.* 2002, Overmann 2001a). Of these, 10 are phototrophic consortia (Table 1) (Overmann 2006). One possibility to differentiate between the consortia are their epibionts. Usually all epibionts in one consortium are equally coloured and the first constituent of the naming indicates the color of the epibionts with "*Chlorochromatium*" for green cells, e.g. "*Chlorochromatium aggregatum*", and "*Pelochromatium*"

for brown cells, e.g. "*Pelochromatium roseum*". However, an outer green layer and an inner brown layer of epibionts distinguish "*Pelochromatium roseo-viridae*" from all other phototrophic consortia (Gorlenko and Kusnetzow 1972). Contrary to all other know types of phototrophic consortia, those contain only one layer of epibionts (Fröstl and Overmann 2000, Overmann and van Gemerden 2000). While the smallest phototrophic consortia in average consist of 13 epibionts *("C. aggregatum")* the largest aggregates contain up to 400 ("*Chloroplana vacuolata*") (Overmann *et al.* 1998) (Table 1). Despite these differences among the consortia, the number of epibionts within one type of consortium is fairly stable (Overmann *et al.* 1998). Another obvious variation in phototrophic consortia is the general shape of the aggregates, the shape of the single epibionts, the shape of the central bacteria and its ability to move. Whereas the majority of the phototrophic consortia are motile due to their flagellated central bacterium, some consortia can change their location due to gas vacuoles (e.g. "*Chloroplana vacuolata*") (Table 1) (Dubinia and Kuznetzow 1976). "*C. aggregatum*" and "*P. roseum*" are both motile, barrel-shaped and represent the most abundant phototrophic consortia in the chemocline of freshwater lakes (Fig. 2) (Overmann *et al.* 1998). "*Chlorochromatium glebulum*" contains green epibionts, is comparatively larger and is easily distinguished from other consortia by its bent shape (Skuja 1957, Fröstl and Overmann 2000). Similar in size, but rather globular in shape are "*Chlorochromatium magnum*" (Fröstl and Overmann 2000), "*Pelochromatium roseo-viridae*" (Gorlenko and Kusnetztow 1972) and "*Pelochromatium latum*", (Glaeser and Overmann 2004). "*Chlorochromatium lunatum*" and "*Pelochromatium selenoides*" resemble a pine-cone and contain half-moon shaped epibionts (Abella *et al.* 1998) (Table 1). Moreover, two non-flagellated morphotypes of phototrophic consortia have been characterized that differ in the arrangement of their associated bacteria. "*Chloroplana vacuolata*", with up to 400 gas-vacuolated cells the largest of the phototrophic consortia, is sheet-like structured. "*Cylindrogloea bactifera*" consists of green-coloured cells surrounding a central chain of colourless capsulated bacteria (Perfiliev 1914, Skuja 1957). Phototrophic consortia have been designated with species names although these names have no scientific value since they consist of two types of bacteria (Tüpfer and Pfennig 1971). Hence, scientific names of consortia are without standing in nomenclature and given in quotation marks.

Table 1. Overview of phototrophic consortia (modified from Fröstl and Overmann 2000, Overmann and van Gemerden 2000, Overmann 2006). n.d. = not determined

Consortium	Morphology	Colour of epibionts	Number of epibionts	Gas vesicles	References
"Chlorochromatium aggregatum"		green	12.9 ± 4.5	-	Lauterborn 1906 Fröstl & Overmann 2000
"Chlorochromatium glebulum"		green	7 - 40	+	Skuja 1957 Fröstl & Overmann 2000
"Chlorochromatium magnum"		green	36 ± 4.4	+	Froestl & Overmann 2000
"Chlorochromatium lunatum"		green	68.6 ± 2.5	+	Abella *et al.* 1998
"Pelochromatium roseum"		brown	19.4 ± 4.4	-	Lauterborn 1913 Tuschak *et al.* 1999
"Pelochromatium selenoides"		brown	44.5 ± 3.5	+	Abella *et al.* 1998
"Pelochromatium roseo-viride"		green/brown	n.d.	+	Gorlenko & Kusnetzov 1972
"Pelochromatium latum"		brown	brown	?	Glaeser & Overmann 2004
"Chloroplana vacuolata"		green	= 400	+	Dubinia & Kusnetzov 1976
"Cylindrogloea bacterifera"		reen	n.d.	-	Perfiliev 1914 Skuja 1957

Depending on seasonal development, phototrophic consortia thrive in the chemocline of stratified freshwater lakes in about 5-8 meters of depth. It has been reported, that in these environments, their biomass can amount to as much as two-thirds of the total bacterial biomass (Gasol *et al.* 1995). The cell aggregates have been observed to occur in various locations worldwide (Overmann *et al.* 1998). While there have been a variety of investigations regarding the physiology, diversity, and phylogenetic relationships of the epibionts, the heterotrophic partner bacteria are poorly investigated. At the beginning of this study, 19 types of phototrophic consortia were distinguished based on the different 16S rRNA gene sequences of their epibionts. Biogeographic studies revealed that phototrophic consortia with the same morphology that share the same habitat contain only one single type of epibiont. Moreover, no epibiont has so far been shown to occur in the free-living state according to 16S rRNA analyses (Glaeser and Overmann 2004). This raises the question of which factors select for the dominance of epibionts of phototrophic consortia over free-living green sulfur bacteria. In this context it is most likely, that the motility gained by the nonmotile epibionts due to the association with the flagellated central bacterium results in a selective advantage in the chemocline. Evidence for this is the occurrence of a rapid exchange of multiple signals between both bacterial partners which includes the perception and reaction towards light and chemical stimuli (Fröstl and Overmann 1998). Phototrophic consortia are therefore regarded as the most highly developed symbiosis between different types of bacteria (Schink 1991). However, phototrophic consortia have not been investigated in detail before „*C. aggregatum*" was available in enrichment culture as the first representative of phototrophic consortia (Fröstl and Overmann 1998). With only one more exception (*Anabaena* sp., Pearl and Kellar 1978) this consortium provides the rare opportunity to study molecular principles of bacteria-bacteria interactions in comparison to other symbioses involving prokaryotes.

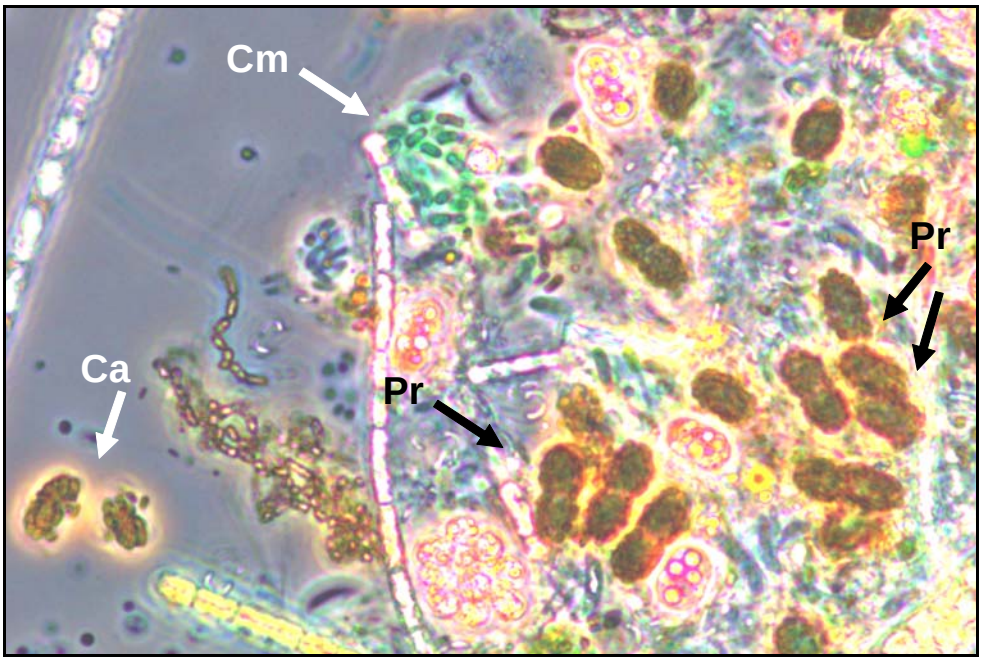


Figure 3. Phase contrast photomicrograph of the bacterial community from the chemocline of Lake Dagow. The dominant, brown-coloured cell aggregates represent "*Pelochromatium roseum*" (Pr). The two greenish consortia on the left edge are "*Chlorochromatium aggregatum*" (Ca). In the upper center there is a disaggregated single "*Chlorochromatium magnum*" (Cm) consortium. The green epibionts are clearly visible. Bar = 5 µm

2.6 "*CHLOROCHROMATIUM AGGREGATUM*" AS A MODEL ORGANISM

Although phototrophic consortia have already been known for one century, the first stable laboratory culture was established only recently when "*Chlorochromatium aggregatum*" was isolated from the mesophilic Lake Dagow, located approximately 100 km north of Berlin (Fröstl and Overmann 1998). Since then a great effort has been made to elucidate the functionality of this symbiosis starting with the examination of the phylogeny of both participants.

Based on 16S rRNA analyses, fluorescence *in situ* hybridization, the presence of chlorosomes and other specific bacteriochlorophyll pigments, the epibionts of phototrophic consortia belong to the GSB (Overmann 2001a, Tuschak *et al.* 1999). It has been shown they grow obligately photolithoautotrophically, as do all other known *Chlorobiaceae* under natural conditions (Glaeser and Overmann 2003a, Overmann 2001). Meanwhile a stable pure culture of the epibiont from „*C. aggregatum*" has been obtained (Vogl *et al.* 2006). In contrast to the

epibionts, the central bacteria of phototrophic consortia could neither be enriched nor isolated to date. Against former suggestions (Pfennig 1980) fluorescence *in situ* hybridization revealed that the central colourless bacterium is affiliated with the *Betaproteobacteria* (Fröstl and Overmann 2000). Despite these first steps into the characterization of the single partners in this heterogeneous association, the physiological and genetic interactions, especially on the part of the central bacterium, are still poorly understood.

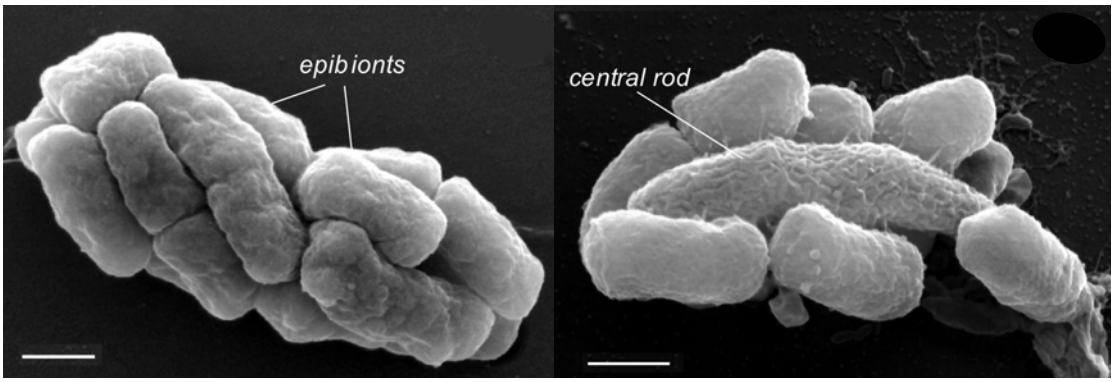


Figure 4. Scanning electron micrographs of "*C. aggregatum*". Left: Intact consortium. Right: Consortium with exposed central bacterium (courtesy of K. Vogl and Prof. Dr. G. Wanner). Bar = 1 μm

It has been shown, that there has to be an exchange of multiple signals between the bacterial partners within this close interaction. When an enrichment culture of „*C. aggregatum*" is illuminated the consortia exhibit a sudden change in the direction of movement, which results in a reversion of the movement when entering the dark (Buder 1914, Fröstl and Overmann 1998). Yet, only the central bacterium is motile (Overmann *et al.* 1998, Glaeser & Overmann 2003b) and only the epibiont is photoreactive (Overmann 2001a). In addition to the perception and reaction towards light and chemical stimuli, other properties imply that both, close metabolic and signal exchange, occur. Examples are synchronized multiplication (Overmann *et al.* 1998), chemotactic behavior towards sulfide (Fröstl and Overmann 1998) as well as the incorporation of 2-oxoglutarate (Glaeser and Overmann 2003b). Intact consortia incorporate the latter, as revealed by microautoradiography. As the green sulfur bacterial epibionts grow autotrophically and cannot utilize 2-oxoglutarate, it must be taken up by the central bacterium and since this

uptake occurs only in the presence of both light and sulfide, the incorporation of 2-oxoglutarate by the central bacterium is regulated by the metabolic state of the epibionts, in turn suggesting a specific signal exchange between the partner bacteria (Glaeser and Overmann 2003b). In conclusion, further studies are needed to gain further insight into the physiology and diversity of central bacteria of consortia and to elucitade the molecular basis of this outstanding symbiosis.

2.7 AIM OF THIS WORK

Intraspecific communication between bacteria has been studied intensively, whereas much less is known about interspecific interactions between different types of bacteria. This gap in knowledge is in sharp contrast to the important role phototrophic consortia play as model systems for the investigation of symbiosis. Despite previous research on this association, the molecular principles of the interaction between the epibiont and the central bacterium in phototrophic consortia are not understood in detail. The overall focus of this thesis therefore was a detailed molecular, physiological, and phylogenetical characterization of both partners in the symbiosis of the phototrophic consortium "*C. aggregatum*". For that purpose, cultures of the epibiont, enrichment cultures of "*C. aggregatum*", as well as natural populations of phototrophic consortia have been used to study the interaction by complementary experimental approaches.

This thesis is structured in two parts. First, the central rod is investigated with regard to its phylogenetic characterization as well as to the isolation and enrichment of its DNA for subsequent genome sequencing (Chapter 3 and Chapter 4). The second focus of this work is the characterization of the epibiont of "*C. aggregatum*", *Chl. chlorochromatii* strain CaD, and the investigation of the physiological factors that underlie the symbiotic properties of *C. chlorochromatii* (Chapter 5 and Chapter 6).

The first section (Chapter 3) of this work deals with the characterization and classification of the central bacterium. In contrast to the epibionts, the central bacteria of phototrophic consortia could neither be enriched nor isolated to date. As a consequence, a main subject of this thesis was to establish a method for the recovery of sufficient genomic DNA of the central bacterium for further genome sequencing and molecular analyses. After the previous failure of various different attempts, due to the low frequency of central

bacteria of "*C. aggregatum*", with only about 0.07% in the enrichment cultures, eventually, CsCl-bisbenzimidazole equilibrium density centrifugation turned out to be successful. Additionally, this method was exploited to determine genome properties of the central rod, such as its GC-content. In contrast to earlier suggestions (Pfennig 1980), fluorescence *in situ* hybridization (FISH) showed the central bacterium to be affiliated with the *Betaproteobacteria.* However, it could not be characterized further (Fröstl and Overmann 2000). Therefore, the phylogenetical position of the central bacterium of "*C. aggregatum*" should be determined more precisely in this study. Phylogenetic analyses of the 16S rRNA gene revealed that the central bacterium of "*C. aggregatum*" represents a so far isolated phylogenetic lineage within the family *Comamonadaceae*.

Nothing is known so far about the genome properties of the central bacteria of phototrophic consortia. Consequently, Chapter 4 is dedicated to genome analyses of the central bacterium of "*C. aggregatum*" starting with molecular analyses of the rRNA operon. The most significant obstacle to this study was the low concentration of central rod DNA in comparison to DNA of the contaminants. A method was developed to grow intact "*C. aggregatum*" in a biofilm, which can be harvested for subsequent experiments. After CsCl-bisbenzimidazole equilibrium density gradient centrifugation, genomic DNA of the central bacterium could be highly enriched and different complementary approaches were applied to elucidate the rRNA operon of the central bacterium. The operon structure turned out to be tandemly arranged in a manner only rarely occurring in other prokaryotes. This information was exploited to develop a molecular screening tool for the detection of rRNA sequences of central bacteria in other types of phototrophic consortia. By this means, several sequences were obtained from the environment and could be verified by FISH, indicating that not only different green sulfur bacteria, but also different *Betaproteobacteria* have adapted to life in this type of symbiosis. Subsequently, the microdiversity of these betaproteobacterial chemocline communities was investigated and phylogenetic analyses of the unexpectedly diverse set of bacterial clones were done. All clones were shown to be closely related to the central bacterium of „*C. aggregatum*". This was an important step to elucidate the origin, the diversity, and the phylogeny of central rods of phototrophic consortia, which so far never could be investigated due to inadequate methods for identification and recovery.

The following section (Chapter 5) of this work describes for the first time the isolation

and subsequent description of an epibiont in a phototrophic consortium and its physiological characterization. After its isolation from an enrichment culture of the phototrophic consortium "*C. aggregatum*", a broad spectrum of physiological experiments was conducted. 16S rRNA analyses showed, that the strain is distantly related to *Chlorobium* species within the phylum of the GSB. The epibiont was therefore described as a novel species within the genus *Chlorobium* and named *Chlorobium chlorochromatii* CaD.

The next chapter (Chapter 6) is dedicated to the investigation of the physiology of the strain *Chl. chlorochromatii* CaD in more detail. For that purpose, comparisons of the free-living epibiont, the epibiont in the associated state, and other free-living GSB have been conducted. A main focus laid on the physiological reponse to oxygen exposure, because GSB are inevitably exposed to oxygen thriving in their natural habitat, the chemocline of stratified lakes. Since carotenoids are antioxidants, playing a role in oxygen detoxification, the investigations were also focused on the determination of the carotenoid composition of the epibiont. Pigment analyses revealed the presence of 7,8-dihydro-γ-carotene, a carotenoid yet unknown in nature.

In the course of my investigations arose a variety of questions, which in part elucidate small aspects of this symbiosis, but cannot be embedded within a single chapter of this work. Therefore, in Chapter 7 some particular relevant data are presented. Their consideration for future studies will certainly be of great interest in further understanding of the interaction in *„C. aggregatum"*.

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3 MOLECULAR CHARACTERIZATION OF THE NON-PHOTOSYNTHETIC PARTNER BACTERIUM IN THE CONSORTIUM "*CHLOROCHROMATIUM AGGREGATUM*"

3.1 ABSTRACT

Phototrophic consortia represent valuable model systems for the study of signal transduction and coevolution between different bacteria. The phototrophic consortium "*Chlorochromatium aggregatum*" consists of a colorless central rod-shaped bacterium surrounded by about 20 green-colored epibionts. Whereas the epibiont was identified as a member of the green sulfur bacteria, and recently isolated and characterized in pure culture, the central colorless bacterium has been identified as a β-*Proteobacterium* but so far could not be characterized further. In the present study, "*C. aggregatum*" was enriched chemotactically and the 16S rRNA gene sequence of the central bacterium was elucidated. Based on the sequence information, fluorescence *in situ* hybridization (FISH) probes targeting four different regions of the 16S rRNA were designed and shown to hybridize exclusively to cells of the central bacterium. Phylogenetic analyses of the 1437 bp-long sequence revealed that the central bacterium of "*C. aggregatum*" represents a so far isolated phylogenetic lineage related to *Rhodoferax* spp., *Polaromonas vacuolata* and *Variovorax paradoxus* within the family *Comamonadaceae*. The majority of relatives are not-yet-cultured and were found in low-temperature aquatic environments, or aquatic environments containing xenobiotica or hydrocarbons. In CsCl-bisbenzimidazole equilibrium density gradients, genomic DNA of the central bacterium of "*Chlorochromatium aggregatum*" formed a distinct band, which could be detected by Quantitative PCR using specific primers. Using this method, the mol% G+C content of the central bacterium was determined to be 55.6%.

3.2 INTRODUCTION

During the course of evolution, prokaryotes have entered into numerous symbiotic relationships. So far, mostly the symbioses between bacteria and eukaryotes have been investigated (Overmann and Schubert 2002). On the contrary, interactions between different prokaryotes have received much less attention, such that only the syntrophic associations of anaerobic chemotrophic bacteria with archaea are understood in sufficient detail (Schink 1991). Microscopic studies have revealed, however, that morphologically highly structured associations of different prokaryotes exist in natural habitats (Overmann 2001a, Overmann and Schubert 2002). In these so-called consortia, prokaryotes maintain a permanent cell-to-cell contact; hence their mutual interdependence may be obligatory.

Of the consortia known, only two phototrophic consortia have been cultivated in the laboratory (Fröstl and Overmann 1998, Glaeser *et al.* 2002). Phototrophic consortia consist of a colorless central rod-shaped bacterium surrounded by 13-69 green- or brown-colored epibionts (Overmann 2001a), and typically occur in the chemocline of many stratified lakes (Caldwell and Tiedje 1974, Croome and Tyler 1984, Gasol *et al.* 1995, Glaeser and Overmann 2003a, Glaeser and Overmann 2004) where they may constitute up to 66% of the total bacterial biomass (Gasol *et al.* 1995).

Several lines of evidence indicate that a direct communication exists between the two different types of bacteria in phototrophic consortia (Fröstl and Overmann 1998, Glaeser and Overmann 2003b). Intact consortia accumulate scotophobically in the light, at wavelengths, which correspond to the absorption maxima of the bacteriochlorophylls present in the epibionts (Fröstl and Overmann 1998). Epibiont cells are nonflagellated, however, whereas the central bacterium is motile by means of a single polar flagellum (Glaeser and Overmann 2003a, Overmann *et al.* 1998). Consequently, the scotophobic response must involve signal exchange between the epibionts and the central bacterium. As a second observation, intact phototrophic consortia take up 2-oxoglutarate, most likely mediated by the central bacterium. This uptake is strictly dependent on the presence of sulfide and light, both utilized by the epibionts (Glaeser and Overmann 2003b). Accordingly, the physiological state of the epibiont cells appears to control the 2-oxoglutarate uptake by the central bacterium. Phototrophic consortia thus represent valuable model systems for the study of signal

transduction and coevolution between different bacteria.

Using 16S rRNA-based methods, the epibionts of phototrophic consortia have been identified as green sulfur bacteria (Fröstl and Overmann 2000, Tuschak *et al.* 1999). In the associated state, epibionts grow photoautotrophically like their free-living green sulfur bacterial relatives (Glaeser and Overmann 2003a). Recently, the epibiont of the phototrophic consortium "*Chlorochromatium aggregatum*" could be isolated in pure culture and its physiology characterized in detail (Vogl *et al.* 2006). Also, the genome sequence of the epibiont has just been completed (http://genome.jgi-psf.org/finished_microbes/chlag/chlag.download.html). In contrast, only very little is known of the central bacterium. By fluorescence *in situ* hybridization (FISH), it could be identified as a member of the β-subclass of the *Proteobacteria* (Fröstl and Overmann 2000). However, its precise phylogenetic position could not be determined because of the notoriously low cell numbers of central bacteria which are present in the available "*C. aggregatum*" cultures.

3.3 MATERIAL AND METHODS

3.3.1 Source of organisms

Enrichment cultures of "*Chlorochromatium aggregatum*" were established previously from a sediment sample of the eutrophic Lake Dagow (100 km north of Berlin) (Fröstl and Overmann 1998). "*C. aggregatum*" consists of a colorless central rod and approximately 20 green-pigmented epibionts. A recently isolated pure culture of the epibiont strain CaD of „*C. aggregatum*" (Vogl *et al.* 2006), and cultures of *Rhodocyclus tenuis* DSMZ 109^T, *Ralstonia eutropha* DSMZ 428, *Chlorobium phaeobacteroides* DSMZ 266^T and *Clostridium acetobutylicum* DSMZ 792 were used for reference.

3.3.2 Media and growth conditions

„*C. aggregatum*" was grown in K4-medium of the following composition (components in grams per liter): KH_2PO_4, 0.25; NH_4Cl, 0.05; $MgCl_2 \cdot 6\ H_2O$, 0.05; $CaCl_2 \cdot 2\ H_2O$, 0.05; HEPES, 2.38; $NaHCO_3$, 0.84. After autoclaving, the medium was cooled under an N_2/CO_2 atmosphere, and sterile sulfide solution ($Na_2S \cdot 9\ H_2O$, 0.12 g in 20 ml), 1 ml of a seven-vitamin solution (Pfennig 1978), 1 ml trace element solution SL10 (Widdel *et al.* 1983), and 0.25 ml lipoic acid

solution (100 mg·l^{-1}) were added. The pH was adjusted to 7.4 and the medium dispensed into air-tight, screw-capped bottles. Prior to inoculation with 5% (v/v) of an enrichment culture of „*C. aggregatum*", the medium was supplemented with 0.05% (v/v) of trace element solution SL12B (Overmann *et al.* 1992) and 0.5 mM 2-oxoglutarate (final concentrations).

Cultures were incubated at 15°C and at 20 µmol quanta m^{-2} s^{-1} of a daylight fluorescent tube (Lumilux de Lux, 18W, Osram, Munich, Germany). Light intensities were monitored using a LiCor LI-250 lightmeter equipped with the pyranometer sensor PY38153 (Walz, Effeltrich, Germany). During exponential growth, cultures received 0.5 mM neutral sulfide solution (Siefert and Pfennig 1984) and 0.5 mM 2-oxoglutarate every two days.

3.3.3 Enrichment by chemotaxis

For subsequent molecular analyses, „*C. aggregatum*" was enriched exploiting the chemotactic behaviour of the intact consortia. The original method (Fröstl and Overmann 1998) was modified. All manipulations were carried out in an anaerobic chamber under an atmosphere of 95% N_2 and 5% H_2. 100 ml Meplats bottles with twelve bore holes (Fröstl and Overmann 1998) were filled with 20 ml of a densely grown culture of „*C. aggregatum*" containing 2.6 · 10^5 consortia per ml. Sulfide solution (1 mM) was prepared in sterile filtered (0.1 µm pore Durapore membrane filters; Millipore, Eschborn, Germany) culture supernatant. Flat rectangular capillaries (length 50 mm; inside diameter 0.1 x 1.0 mm or 0.1 x 2.0 mm; Vitrocom, New Jersey, USA) or round capillaries (volume 5 µl, 10 µl, 20 µl and 100 µl; Servoprax, Wesel, Germany; Brand, Wertheim, Germany; or Assistent, Sondheim/Röhn, Germany) were filled by capillary action with the sulfide solution and sealed at one end with plasticine (Münchner Künstler Plastilin, Munich, Germany). Capillaries were then inserted through the holes in the Meplats bottle until their open end reached the culture liquid, and fixed with plasticine. Incubations proceeded overnight at 15°C and an ambient light intensity of 20 µmol quanta m^{-2} s^{-1}. Afterwards, the content of the capillaries was transferred into 100 µl of sterile double-distilled water and centrifuged for 15 min at 13,000 rpm. The cell pellet was resuspended in 10 µl of double-distilled water and stored at -20°C.

3.3.4 PCR

Chemotaxis enrichments were lysed by five consecutive freeze-thaw cycles (each cycle consisting of a 3 min-incubation each at 100°C and at -20°C). One µl of the cell lysate was used for amplification. Amplification reactions for pure cultures received 50 ng of genomic DNA. Standard PCR conditions (Overmann *et al.* 1999) were performed with a DNA thermal cycler (GeneAmp PCR-system 2400, Applied Biosystems, Forster City, USA) and PCR-products were analyzed by standard agarose gel electrophoresis.

For amplifications with primers GC341f or 341f and 907r (Muyzer *et al.* 1995), the cycling conditions described previously were employed (Overmann and Tuschak 1997). For the specific amplification of betaproteobacterial sequences, primers Beta680f (Overmann *et al.* 1999) and an improved version of primer 13R (Aakra *et al.* 1999) (5′-TCGCCAAGGCATCCA-CC-3′, *E. coli* position 23-39 of the 23S rRNA) were used. The step down PCR program comprised 10 cycles with denaturation at 94°C for 30 s, primer annealing at 61°C for 1 min, and elongation at 72°C for 3 min, followed by 25 cycles with the annealing temperature changed to 56°C. Two primers CRa641f (5′-ACTGCAGATGCTAGAGTA-3′) and CRa641r (5′-CGTACTCTAGCATC-TGCAGT-3) were designed in the present study and are specific for the 16S rRNA gene sequence of the central bacterium of "*C. aggregatum*". The specific primers were combined either with GC341f, or with universal primers 8f or 1492r (Lane 1991). For amplification with primer pair 8f / CRa641r, the optimized step down program comprised 10 cycles with denaturation at 94°C for 30 s, primer annealing at 70°C for 30 s, and elongation at 72°C for 2 min, followed by 20 cycles with the annealing temperature changed to 65°C. Cycling conditions for primer pair GC341f / CRa641r were: 10 cycles with denaturation at 94°C for 30 s, primer annealing at 58°C for 45 s, and elongation at 72°C for 1 min, followed by 30 cycles with the annealing temperature changed to 53°C. For amplification with the primer pair CRa641f / 1492r, the annealing temperature was set to 60°C for 30 s during the first 10 cycles, and to 55°C for the subsequent 25 cycles. In this case, elongation proceeded for 1 min at 72°C.

3.3.5 Quantitative PCR

The relative amount of genomic DNA of the central bacterium from „*C. aggregatum*" in CsCl-bisbenzimidazole density gradients was determined by quantitative PCR (iQ™ iCycler, BIO-RAD, München, Germany) employing the primer pair 341f / CRa641r and the SYBR Green Supermix (BIO-RAD) for the detection of double-stranded PCR products. Each reaction received 4 ng of template DNA. For standardization of the values, tenfold dilutions of genomic DNA from the „*C. aggregatum*" culture (concentration range 4 pg to 400 ng) were measured in parallel. Cycling conditions included 5 min of denaturation (95°C), 45 s of annealing (67°C, 40 cycles) and 1 min of elongation (72°C). All measurements were done in quadruplicate and negative controls were included throughout. The relative enrichment factor for genomic DNA of the central bacterium was derived from a comparison of the Ct values determined directly for the enrichment culture and the Ct values determined for DNA fractions from the CsCl-gradients.

3.3.6 Denaturing gradient gel electrophoresis (DGGE)

16S rRNA gene fragments amplified were separated by DGGE (Muyzer *et al.* 1993, Muyzer *et al.* 1995), conducted in the Ingeny phorU2 system (Ingeny International BV, Goes, The Netherlands) for 15 min at 200 V, then 12 h at 180 V at a constant temperature of 60°C. After staining with SYBRGold (MoBiTec, Göttingen, Germany) the DNA bands were visualized on a UV-transilluminator and DNA fragments of interest were excised with a sterile scalpel. Gel pieces were incubated for 1 h at 65°C in 20 µl of 2 mM Tris/HCl (pH 8.0), the eluted DNA reamplified, and the amplification products purified for sequencing using the QiaQuick PCR purification kit (Qiagen GmbH, Hilden, Germany). Gel images were captured with a digital camera (Spot RT color; Diagnostic Instruments Inc., USA) and processed with the Spot RT version 3.1 software.

3.3.7 Cloning

PCR-products were cloned through chemical transformation with the TOPO TA Cloning-Kit (version P; Invitrogen, Carlsbad, California, USA). Plasmids were extracted with the QIAprep Spin Miniprep-Kit (Invitrogen) and the presence of inserts verified by digestion with *EcoRI, PvuI, HaeIII* (MBI Fermentas, St. Leon-Rot, Germany).

3.3.8 Sequencing

Sequencing was performed by the dideoxynucleotide method (Sanger *et al.* 1977) using the AmpliTaq FS BigDyeTerminator Cycle Sequencing Kit according to the protocol of the manufacturer and the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany). Additionally to the primers described above, oligonucleotides 926f, 1055f and 1055r (Amann e*t al.* 1995, Lane 1991) were employed in the sequencing reactions. The computer program Lasergene (Seqman II, DNASTAR Inc., Madison, Wisconsin, USA) was used for sequence editing.

3.3.9 Phylogenetic analyses

16S rRNA sequences were analyzed using the software packages ARB (Ludwig *et al.* 2004) and PHYLIP (Phylogeny Inference Package, version 357c; Felsenstein 1989). Sequences of the 50 phylogenetically closest relatives of the central bacterium were retrieved from the GenBank database employing BLAST 2.0.4. (Altschul *et al.* 1997) and imported into the ARB database. The Fast Aligner V1.03 tool was used for automatic sequence alignment. The latter was checked and manually corrected based on secondary structure information, yielding an alignment of 1542 informative nucleotide positions.

Phylogenetic trees were constructed using the Maximum Likelihood and Maximum Parsimony algorithms within the ARB package and the Neighbour Joining and distance-based methods (DNADIST plus FITCH) as implemented in the PHYLIP software package. To identify variable branching points, the phylogenetic trees generated were compared pairwise employing the COMPARE TOPOLOGY function of the ARB program. In addition, reproducibility of the branching pattern of the Maximum Likelihood tree was tested by bootstrap analysis, generating a set of 100 resamplings. Those branches which were observed to vary between the four methods and which had low bootstrap support were collapsed with deeper branching points to yield multifurcations, using the ARB software (W. Ludwig, pers. communication).

3.3.10 Fluorescence *in situ* hybridization (FISH)

In order to verify the 16S rRNA gene sequence determined for the central rod of „C. aggregatum", specific FISH-probes were created with the DESIGN PROBES function of the ARB software package. The accessibility of the target sites was checked based on data available for *Escherichia coli* (Fuchs *et al.* 1998). For probes targeting sites with limited accessibility, corresponding helper oligonucleotides (Fuchs *et al*. 2000) were designed. Overall, four specific probes and 8 helper oligonucleotides were used (Table 1). Fluorescence *in situ* hybridization was carried out on black polycarbonate filters resulting in disintegration of the phototrophic consortia, thereby exposing the central rod (Tuschak *et al.* 1999). Twenty ng each of the Cy3-labeled probe and the corresponding helper oligonucleotides at the appropriate hybridization stringency were used (Table 1). Hybridization stringency was tested and optimized by varying the formamide concentrations between 5 and 35%. After counterstaining with 4′,6-diamidino-2-phenylindole (DAPI), hybridizations were analyzed by epifluorescence microscopy.

3.3.11 CsCl – bisbenzimidazole density gradient centrifugation

The mol% G+C content of the central bacterium was determined in cesium chloride-bisbenzimidazole equilibrium density gradients (Bostock-Smith and Searle. 1999, Holben and Harris 1995, Holben *et al.* 2004, Nüsslein and Tiedje 1998, Vinograd and Hearst 1962), using a modified protocol. Ten µl of bisbenzimidazole stock solution (Hoechst # 33258; 25 $\mu g \cdot \mu l^{-1}$) were added to 10 ml of CsCl-TE (1.3g CsCl · ml^{-1} in TrisHCl/EDTA 10 mM/1 mM). The mixture was transferred to a 16 x 76 mm ultracentrifuge Quick-Seal tube (# 342413; Beckman Coulter, USA), overlaid with 1 ml of DNA extract containing 150 µg DNA from a consortia culture, the headspace completely filled with paraffin oil, balanced and sealed. Centrifugation proceeded at 232.000 × g for 48 h at 15°C in a fixed-angle rotor (70.1 Ti, Beckman Coulter, USA). DNA bands were visualized under UV at 365 nm and 100 µl fractions were collected from below via a 21-G needle connected to silicon tubing, employing a peristaltic pump.

Subsequently, the refractive index of each fraction was determined. Bisbenzimidazole and CsCl were removed using three repeated extractions in CsCl-saturated isopropanol,

followed by two wash steps with Tris-HCl in Centricon-50 ultrafiltration units (Millipore, Eschborn, Germany). The DNA content of all purified fractions was determined fluorimetrically using PicoGreen (Molecular Probes, Eugene, Oregon, USA). For standardization, genomic DNA of bacterial strains with known GC content (*Clostridium acetobutylicum*, mol% G+C = 30.9; *Chlorobium phaeobacteroides*, mol% G+C = 49.0; *Ralstonia eutropha*, mol% G+C = 64.4) was separated in the same gradient and a standard curve established correlating the known GC contents to the refractive index.

3.3.12 Accession numbers

The almost full-length 16S rRNA gene sequence of the central bacterium of "*C. aggregatum*" has been deposited in the EMBL database under accession number DQ009030. Partial sequences of the accompanying bacteria are deposited under numbers DQ009027–DQ009029 and DQ009031-DQ009034.

Table 1. Fluorescently labeled probes, helper oligonucleotides and hybridization conditions employed for FISH

Probe/helper[a]	sequence	T (°C)	Formamide
CR-207	5′- CGC GCG AGG CCC TCT -3′	48	20%
CR-207-H1	5′- CAG GTC CCC CGC TTT CAT -3′		
CR-207-H2	5′- CTG ATA TCA GCC GCT CCA AT -3′		
CR-442	5′- AAG GCT GTT TCG CTC CGT -3′	45	20%
CR-641	5′- TAC TCT AGC ATC TGC AGT -3′	45	20%
CR-641-H1	5′- CAC AAA TGC AAT TCC CAG -3′		
CR-641-H2	5′- GTT GAG CCC GGG GAT -3′		
CR-641-H3	5′- TTC ACA TCC GTC TTA CAG -3′		
CR-641-H4	5′- CAT CCC CCT CTG CCG -3′		
CR-1282	5′- CGA CTG ACT TTA TGG -3′	45	5%
CR-1282-H1	5′- GGT TGG CTC CCT CTC -3′		
CR-1282-H2	5′- CTG CGA TCC GGA CTA -3′		
Cont-645	5′- TGC CAT ACT CTA GCC TTC -3′	45	20%
Cont-645-H1	5′- CAG TCA CAA GCG CAG TT -3′		
Cont-645-H2	5′- CCC AAG TTG AGC CCG -3′		
Cont-645-H3	5′- GGG ATT TCA CGC CTG -3′		
Cont-645-H4	5′- AAT TCC ACC CCC CTC -3′		
Cont-995	5′- CTT CAG GCT CCT GGA CAT -3′	45	20%
Cont-995-H1	5′- GTC AAG GGT AGG TAA GGT TTT-3′		
Cont-995-H2	5′- TCG GGC ACA CCC AAA TCT-3′		
Cont-995-H3	5′- CCT GTG TTC CAG TTC CCT T-3′		

[a]Numbers indicate 5′-end according to *E. coli* numbering. Suffix "CR" denotes probes specific for the central rod-shaped bacterium of "*C. aggregatum*", "Cont" for probes specific for free-living *Betaproteobacteria*. Helper oligonucleotides are denoted by the suffix "H".

3.4 RESULTS AND DISCUSSION

3.4.1 Improved chemotactic enrichment of intact phototrophic consortia

Based on DAPI counting of standard enrichment cultures of "*Chlorochromatium aggregatum*", cells of the central bacterium reach ≤ (0.071±0.003)% of total cell counts. At such low frequencies, 16S rRNA gene sequences cannot be detected by PCR/DGGE (Muyzer *et al.* 1993, Straub *et al.* 1998). Therefore, the chemotaxis of "*C. aggregatum*" towards sulfide (Fröstl and Overmann 1998) was exploited as a rapid means to selectively enrich intact consortia. In small flat rectangular capillaries (0.1 mm x 1.0 mm), "*C. aggregatum*" accumulated within the first 3 mm from the capillary opening. However, these enrichments also contained a high number of motile chemotrophic bacteria, which still amounted to 85% of all cells (similar to the accumulation depicted in Fig. 1B). Experiments with round capillaries of different sizes and volumes did not yield suitable enrichments. In flat capillaries with a larger width (0.1 mm x 2.0 mm), however, "*C. aggregatum*" accumulated in two distinct zones. In addition to the primary enrichment detected near the capillary opening, a second zone of accumulation formed in the center of the capillary, at a distance approximately 1 cm from the primary accumulation. Direct phase contrast microscopy of the capillaries revealed that much less accompanying bacteria were present in this secondary accumulation (Fig. 1A) than in the accumulation near the capillary end (Fig. 1B).

In order to gather sufficient material for subsequent molecular analyses, chemotactic enrichments from 48 flat rectangular capillaries were collected by breaking off the capillaries in the center (Fig. 1A), and blowing out their contents into an eppendorf vial using sterile filtered air. Then, the cells were concentrated by centrifugation to a final titer of 10^7 consortia ml^{-1}.

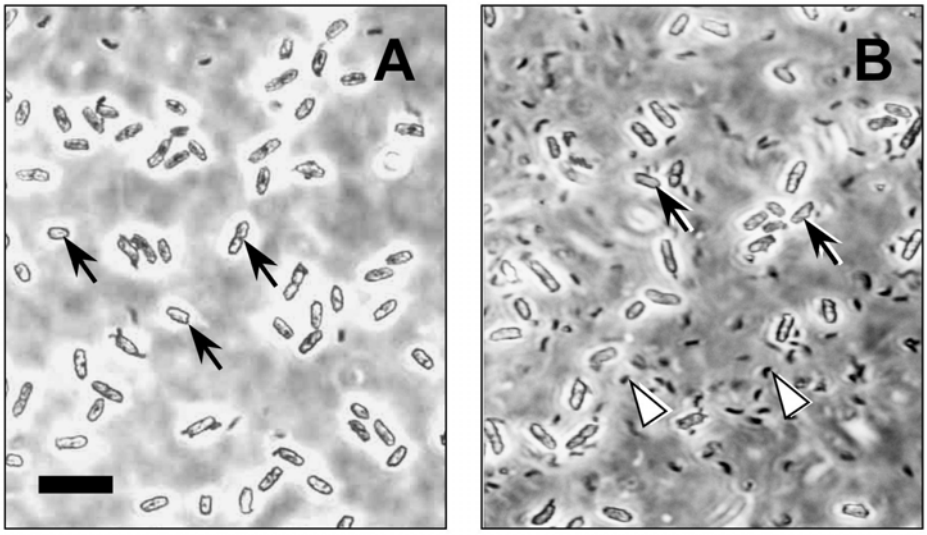

Fig. 1. **A.** Phase contrast photomicrograph of the accumulation of "*Chlorochromatium aggregatum*" (black arrows) formed in the center of the 0.1 x 2 mm rectangular capillary. **B.** Photomicrograph of the bacteria accumulated at the opening of the capillary. Besides phototrophic consortia (black arrows), numerous motile contaminants (white arrow heads) are present in this accumulation. Bar = 20 μm

3.4.2 Analysis of the 16S rRNA gene sequence of the central bacterium of "*C. aggregatum*"

In a first step, partial 16S rRNA genes were amplified from the chemotactic enrichment of "*Chlorochromatium aggregatum*" using primers GC341f and 907r. Subsequent separation by denaturing gradient gel electrophoresis revealed the presence of 7 different DNA fragments among the amplification products (Fig. 2, bands *a* through *g*). All bands were excised, reamplified and sequenced. In parallel, a 1400 bp-long DNA fragment was amplified with the betaproteobacterial primer pair Beta680f/13R, and was also sequenced.

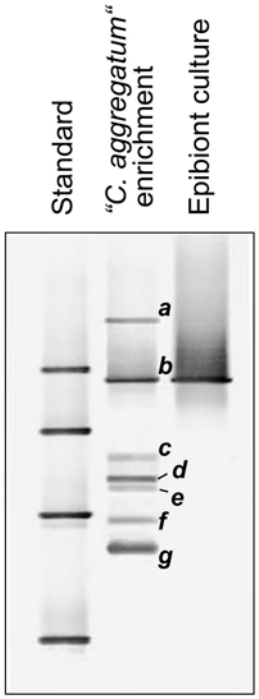

Fig. 2. Separation by DGGE of 16S rRNA gene fragments amplified with primers GC341f and 907r from the chemotaxis enrichment of "*Chlorochromatium aggregatum*" and from a pure culture of the epibiont of "*Chlorochromatium aggregatum*". Italic letters denote DNA bands, which were excised and sequenced. A negative image of an SYBRGold-stained gel is shown.

Band *b* could be identified as the 16S rRNA gene fragment of the epibiont (Fig. 2), which was confirmed by sequence comparison. Of the remaining sequences, three (*c*, *d* and *e*) were affiliated with the *Betaproteobacteria*, two (*f* and *g*) with the δ-*Proteobacteria*, and one (*a*) with the ε-*Proteobacteria*. In previous FISH-analyses of the "*C. aggregatum*" enrichment culture, the central bacterium could be identified as a member of the *Betaproteobacteria* (Fröstl and Overmann 2000). In order to investigate whether the 16S rRNA sequence of the central bacterium was present among sequences *c* – *e*, specific oligonucleotide probes were constructed for each of the sequences and used to analyze the central bacterium by FISH. The sequence of the long DNA fragment amplified with the betaproteobacterial primer pair matched sequence type *c* and was therefore used to construct a specific probe (Cont-995) for this sequence type.

Probes Cont-995 and Cont-645, targeting sequence type *c* and type *d*, respectively, hybridized only to free-living bacteria but not to the central bacterium of "*C. aggregatum*", and hence must originate from accompanying bacteria present in the enrichment. In contrast,

probes CR-442 and CR-641, targeting sequence type *e*, hybridized exclusively to the central bacterium (Fig. 3A-D).

Table 2. Phylogenetic affiliation of partial 16S rRNA gene sequences from DGGE fingerprints (compare Figs. 3 and 6)

Melting type	Closest relative	(Sub)Phylum	Accession number	Similarity (%)
a	*Sulfurospirillum arsenophilum MIT-13*	ε-*Proteobacteria*	U85964	97.8
b	*Chlorobium chlorochromatii* CaD*	*Chlorobi*	AJ578461	100.0
c	*Azonexus fungiphilus* MFC-EB24	*β-Proteobacteria*	AJ630292	99.0
d	*Quadricoccus australiensis* Ben177	*β-Proteobacteria*	AY007722	98.5
e	*Rhodoferax ferrireducens* DSMZ15236^T	*β-Proteobacteria*	AF435948	94.8
f	*Desulfovibrio aerotolerans* DvO5	*δ-Proteobacteria*	AY746987	98.9
g	*Desulfovibrio aerotolerans* DvO5	*δ-Proteobacteria*	AY746987	97.2
h	*Cellulomonas terrae* DB5	*Actinobacteria*	AY884570	97.1

* = epibiont of "*Chlorochromatium aggregatum*"

Subsequently, the missing sequence stretches at the beginning and the end of the 16S rRNA gene of the central bacterium were amplified. To this end, PCR primers identical (CRa641r) or complementary (CRa641f, see Materials and Methods section) to the specific probe CR-641 were combined with primers 8f and 1492r, respectively. Genomic DNA of the two *Betaproteobacteria Rhodocyclus tenuis* DSMZ 109^T and *Ralstonia eutropha* DSMZ 428 was employed to establish highly specific PCR conditions. Sequencing of the resulting amplification products yielded a 650 bp-long sequence from the 5′-end, and a 850 bp-long sequence from the 3′-end of the 16S rRNA gene. Both sequences showed 100% identity in the overlapping regions to the central fragment (sequence *e*) obtained by PCR-DGGE.

Finally, the origin of the two 16S rRNA gene sequences was verified by FISH. Two probes were designed for the terminal sequence regions (CR-207 and CR-1282; Table 1) and were found to hybridize exclusively to cells of the central bacterium of "*C. aggregatum*" (Fig. 3E,F). Assembling all three 16S rRNA gene sequence fragments of the central bacterium yielded an almost complete 16S rRNA gene sequence of a total length of 1437 bp.

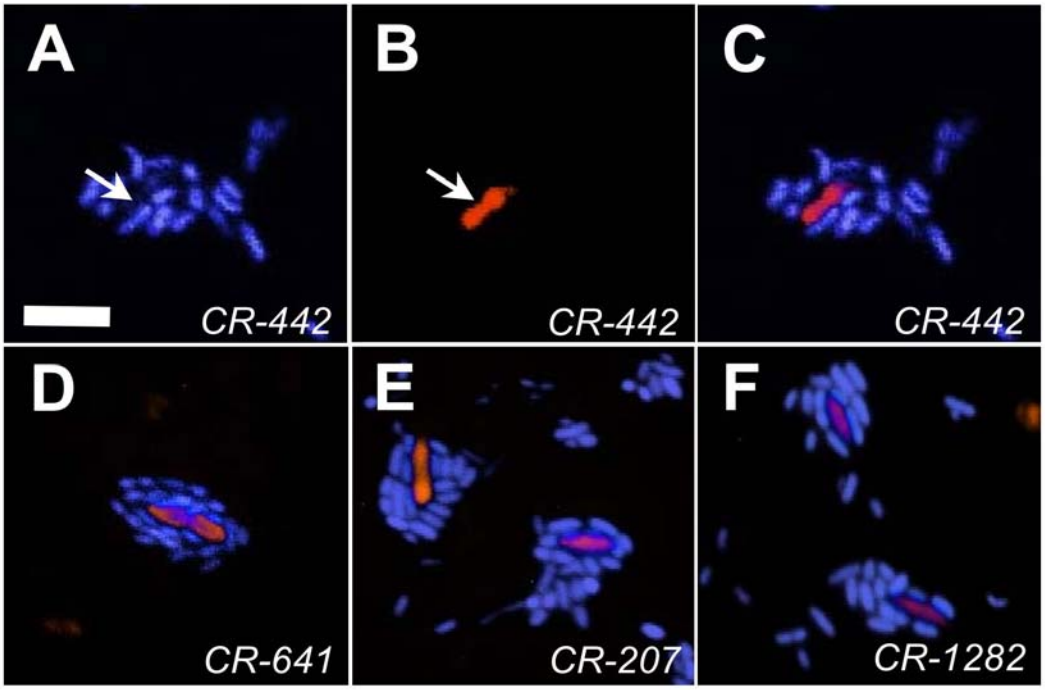

Fig. 3. Specific detection of the central bacterium of "*Chlorochromatium aggregatum*" by FISH with four different oligonucleotide probes (compare Table 1). All bacterial cells were stained with DAPI. **A.** FISH with probe CR-442, DAPI fluorescence. Arrow indicates location of the central bacterium. **B.** Same field of view as in A., but showing Cy3 fluorescence. **C.** Overlay of A. and B. **D.** Overlay of DAPI fluorescence and Cy3-fluorescence after FISH using probe CR-641. **E.** Overlay of DAPI fluorescence and Cy3-fluorescence after FISH using probe CR-207. **F.** Overlay of DAPI fluorescence and Cy3-fluorescence after FISH using probe CR-1282. Bar = 10 μm

3.4.3 Phylogenetic classification of the central rod

Based on 16S rRNA gene sequence comparisons, the phylogenetically closest relatives of the central bacterium are *Rhodoferax ferrireducens*, *Rfx. antarcticus* and a variety of not-yet-cultured bacteria. Sequence similarity was always lower than 95%, however (the closest cultured relative *Rfx. ferrireducens* DSMZ 15236^T being 94.77% similar). According to our phylogenetic analyses (Fig. 4), the central rod of "*C. aggregatum*" represents a so far isolated phylogenetic lineage, and clusters with the genera *Rhodoferax* and *Polaromonas* within the family *Comamonadaceae* (Beta I – subgroup; Glöckner *et al.* 2000). The majority of relatives are not-yet-cultured and were found in low-temperature aquatic environments, or aquatic environments containing pollutants like monochlorobenzene and tetrachloroethene, or hydrocarbons (Fig. 4) (Alfreider *et al.* 2002, Brinkmeyer *et al.* 2003, Finneran *et al.* 2003, Hiraishi *et al*. 2001, Jung *et al*. 2004, Watanabe *et al.* 2000).

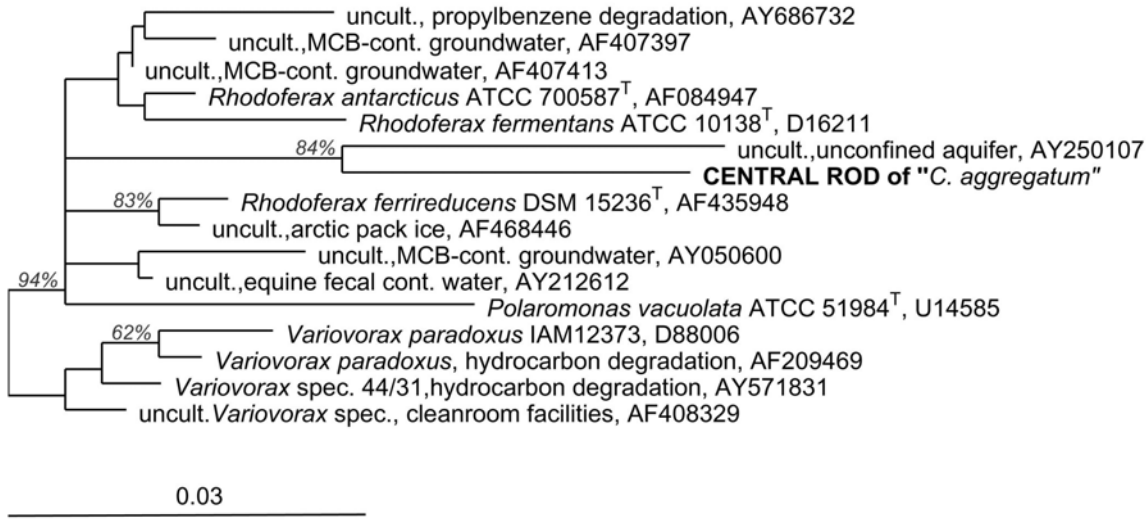

Fig. 4. Phylogenetic affiliation of the central bacterium of "*Chlorochromatium aggregatum*". The consensus tree was constructed in ARB, based on Maximum Likelihood, Maximum Parsimony, Neighbor Joining and distance-based phylogenetic analyses (see Materials and Methods section). Percentages at nodes indicate bootstrap values out of 100 bootstrap resamplings as determined for the Maximum Likelihood tree. Only values above 50% are shown. Uncult., uncultured bacteria detected as environmental clones. Bar indicates 3% fixed point mutations per nucleotide base.

3.4.4 GC-content and enrichment of genomic DNA of the central bacterium

CsCl-bisbenzimidazole equilibrium density centrifugation was used to determine the mol% G+C content of the central bacterium and to establish a large-scale purification method for its genomic DNA. Due to the inherently small volumes, which can be generated in the chemotaxis enrichments, the DNA had to be extracted directly from conventional laboratory cultures of "*C. aggregatum*" in this case.

Density gradient centrifugation separated the genomic DNA into four distinct bands as visualized by UV illumination (bands 1 through 4, Fig. 5A). Since the fluorescence of the DNA-bisbenzimidazole complex is proportional to the amount of bisbenzimidazole bound and thus decreases with mol% G+C content (Holben adn Harris 1995), genomic DNA with a high mol% G+C content is barely detectable. The gradient was separated into 54 fractions and the DNA content of each fraction was determined by PicoGreen dye binding (Fig. 5B, filled circles). This second method confirmed the presence of band 4 and demonstrated its high DNA content, whereas band 2 was barely detectable with PicoGreen. Each fraction was

PCR amplified with eubacterial primers, the products separated by DGGE and sequenced. Five major phylotypes could be distinguished (melting position *a*, *b*, *d*, *g*, *h*; Fig. 5C). With one exception (sequence type *h*), these sequences could be assigned to those identified in the chemotaxis enrichment. The genomic DNA of the epibiont of "*C. aggregatum*" (type *b*) accumulated in the center of the gradient, corresponding to the GC content of 46.7 mol% as recently determined (Vogl *et al.* 2006). Band 1 of the CsCl gradient was formed mainly by genomic DNA from the accompanying *Sulfospirillum arsenophilum* (sequence type *a*). According to our analysis, band 2 contained a mixture of genomic DNA from *Ssp. arsenophilum*, the epibiont and a relative of *Desulfovibrio aerotolerans* (sequence types *a*, *b*, and *g*). In band 3, predominantly *Desulfovibrio aerotolerans* was detected, whereas band 4 also contained DNA from an actinobacterium related to *Cellulomonas terrae* (sequence type *h*).

The genomic DNA of the central bacterium of "*C. aggregatum*" in the density gradients did not reach a concentration sufficient for the detection with eubacterial primers. However, amplification with the specific primer pair GC341f / CRa641r yielded amplification products for several fractions of the gradient. DGGE analysis revealed the presence of only one melting type (Fig. 5D) and sequencing confirmed that it was indeed identical to that of the central bacterium of "*C. aggregatum*". The specific amplification protocol was therefore used to quantify the enrichment factor for genomic DNA of the central bacterium in the CsCl-gradient by Quantitative PCR (Fig. 5B, diamonds). Compared to the original enrichment culture of "*C. aggregatum*", genomic DNA of the central bacterium was enriched 150-fold in fractions 27 – 29. At an enrichment factor of 150x and a frequency of 0.07% in the original culture, the genomic DNA of the central bacterium therefore amounted to approximately 10% of the total DNA in these fractions. Despite this relatively high frequency, the 16S rRNA gene sequence of the central bacterium could not be detected in the complex bacterial communities by PCR-DGGE with eubacterial primers. Our result is in line with data from another study, in which a detection limit of 9% was determined (Straub *et al.* 1998).

The distinct banding of the genomic DNA of the central bacterium of "*Chlorochromatium aggregatum*" in CsCl density gradients was used to estimate its GC content, using the median of the refractive indices of all ten fractions with enrichment factors ≥50 (Fig. 5B). This yielded a mol% G+C content of the central bacterium of "*C. aggregatum*" of

55.6%, which is commensurate with the values determined for other *Comamonadaceae*, spanning a range between 52 and 70% (Hiraishi *et al.* 1991, Irgens *et al.* 1996, Jung *et al*. 2004, Willems *et al*. 1991). Whereas the most closely related *Rhodoferax* species have a GC-content of 59.8-61.5%, values of *Polaromonas vacuolata* strains are between 52 and 57 mol% and those of *Variovorax paradoxus* strains range between 67 and 69 mol%.

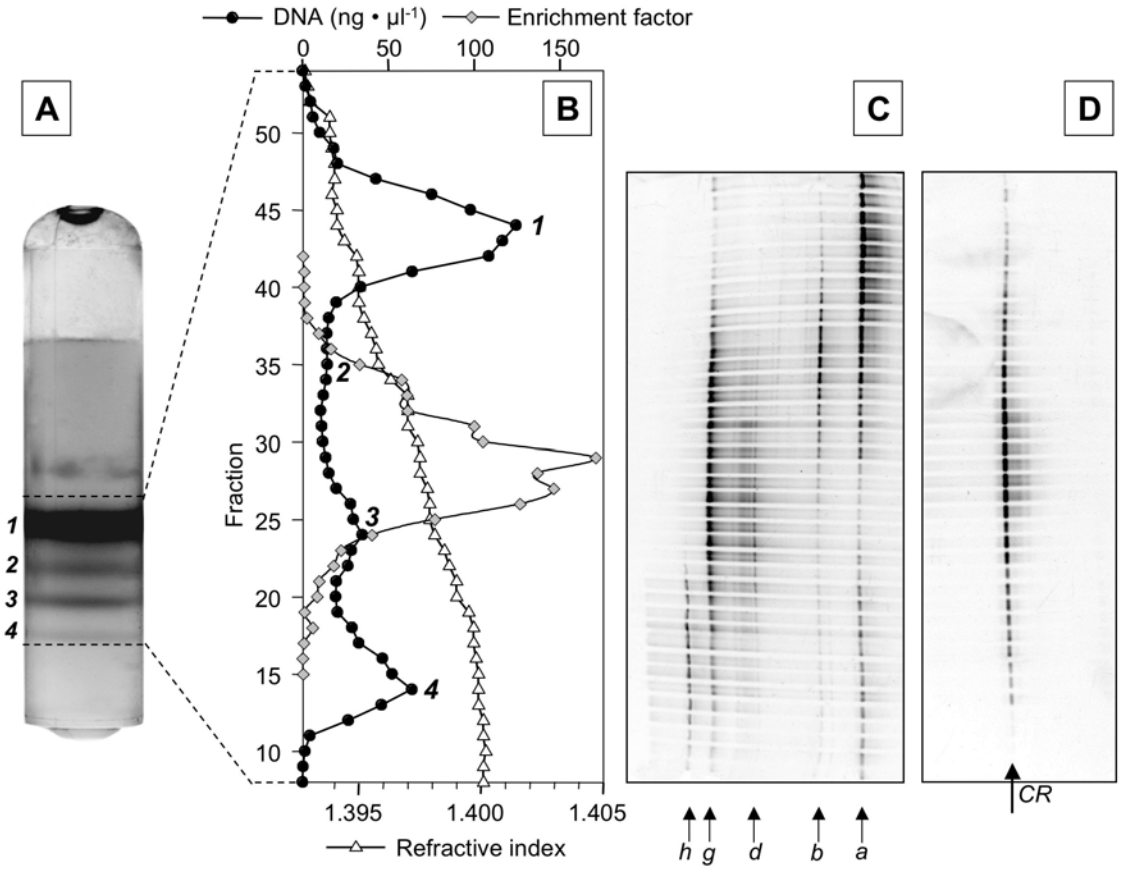

Fig. 5. **A.** Separation of genomic DNA of different bacteria present in the enrichment culture of "*Chlorochromatium aggregatum*" using CsCl-bisbenzimidazole gradient density centrifugation. A negative image of DNA bands visualized by fluorescence under UV illumination is shown. **B.** DNA concentration (●), refractive index (Δ), and enrichment factor of the genomic DNA from the central bacterium (♦) along the CsCl-gradient. DNA concentrations are presented as moving averages (n = 3). Numbering 1 to 4 refers to the respective bands in **A. C.** DGGE fingerprinting of 16S rRNA gene fragments amplified from the different fractions using eubacterial primers GC341f and 907r. Melting types a to h represent DNA fragments from accompanying bacteria. **D.** DGGE fingerprinting of 16S rRNA gene fragments of the central bacterium amplified with primers GC341f and the specific primer CR641r. CR, melting position of the DNA fragment of the central bacterium.

3.4.5 Relevance for future studies

For future enrichment and isolation attempts of the central bacterium of "*Chlorochromatium aggregatum*", it is important to resolve stimulating effects of accompanying bacteria in the enrichment culture. Interestingly, one bacterium which reacted chemotactically towards sulfide was identified as *Sulfurospirillum arsenophilum*. A similar bacterium has been detected as an essential partner in coculture with the green sulfur bacterium *Chlorobium ferrooxidans*, where it is thought to provide the green sulfur bacterium with an essential growth factor (Heising *et al.* 1999). It therefore appears possible that the accompanying bacteria fulfil similar functions in the "*Chlorochromatium aggregatum*" enrichment culture. Also, sulfur cycling between the *Sulfurospirillum* and consortia may occur in the enrichment cultures, since the phylogenetically related sulfur-reducing *Sulfurospirillum deleyianum* DSMZ 6946^T is known to grow syntrophically by sulfur cycling with green sulfur bacteria (Wolfe and Pfennig 1977). Another interesting finding is the presence of the β-*Proteobacterium Azonexus fungiphilus*, which is known to require 2-oxoglutarate for growth (Reinhold-Hurek and Hurek 2000), which may explain the failure to eliminate this bacterium from the enrichment cultures.

So far, only very little is known of the physiology of the central rod-shaped bacterium of phototrophic consortia. Due to the large phylogenetic distance to other known bacteria, however, physiological properties cannot be inferred from its phylogenetic position. Phototrophic consortia exhibit a chemotactic response towards 2-oxoglutarate (Fröstl and Overmann 1998, Glaeser and Overmann 2004), which is taken up by the cells (Glaeser and Overmann 2003b). Recently, the epibiont of "*C. aggregatum*" could be isolated in pure culture and was found to be incapable of using 2-oxoglutarate (Vogl *et al.* 2006). Taken together, these findings suggest that 2-oxoglutarate is utilized by the central bacterium. The specific oligonucleotide probes developed in the present work now allow to perform enrichment experiments with different substrates and to selectively and sensitively screen for the growth of the central bacterium alone. One question central to the understanding of the bacterial association in phototrophic consortia is whether the association is an obligatory one. The specific oligonucleotide probes now available permit to track the central bacterium in its natural habitat in order to determine whether it occurs in the free-living state. Finally, the CsCl-bisbenzimidazole density gradient centrifugation is suitable for the separation of

genomic DNA of the central bacterium from DNA of some of the accompanying bacteria and therefore is relevant for subsequent genome sequencing efforts.

3.5 ACKNOWLEDGEMENTS

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4 THE HETEROTROPHIC SYMBIONTS OF PHOTOTROPHIC CONSORTIA BELONG TO A HIGHLY DIVERSE SUBGROUP OF *BETAPROTEOBACTERIA* CHARACTERIZED BY A TANDEM *RRN* OPERON STRUCTURE

4.1 ABSTRACT

Phototrophic consortia represent the most highly developed type of interspecific association of bacteria and consist of green sulfur bacterial epibionts attached around a central colourless rod-shaped bacterium. Based on 16S rRNA gene sequencing, the central bacterium of the consortium "*Chlorochromatium aggregatum*" was recently shown to represent a novel and phylogenetically isolated lineage of the *Comamonadaceae* within the β-subgroup of the *Proteobacteria*. To date, 19 types of phototrophic consortia are distinguished based on the different 16S rRNA gene sequences of their epibionts, but the diversity and phylogenetic relationships of the heterotrophic partner bacteria are still unknown. We developed an approach based on the specific *rrn* (ribosomal RNA) operon structure of the central bacterium of "*C. aggregatum*" to recover 16S rRNA gene sequences of other central bacteria and their close relatives from natural consortia populations. Genomic DNA of the central bacterium of "*C. aggregatum*" was first enriched several hundred fold by employing a selective method for growth of consortia in a monolayer biofilm followed by a purification of the genome of the central bacterium by cesium chloride-bisbenzimidazole equilibrium density gradient centrifugation. A combination of inverse PCR, cloning and sequencing revealed that two *rrn* operons of the central bacterium are arranged in a tandem fashion and are separated by an unusually short intergenic region of 195 base pairs. This rare gene order was exploited to screen various natural microbial communities by PCR. We discovered a diverse and previously unknown subgroup of *Betaproteobacteria* in the chemoclines of freshwater lakes. This group was absent in other freshwater and soil samples. All the 16S rRNA gene sequences recovered are related to that of the central bacterium of "*C. aggregatum*". Fluorescence *in situ* hybridization indicated that two of these sequences

originated from central bacteria of different phototrophic consortia, which, however, were only distantly related to the central bacterium of "*C. aggregatum*". Based on a detailed phylogenetic analysis, these central bacterial symbionts of phototrophic consortia have a polyphyletic origin.

4.2 INTRODUCTION

Phototrophic consortia were first described one century ago (Lauterborn 1906) and subsequently have been detected in many freshwater lakes worldwide (Caldwell and Tiedje 1974, Croome and Tyler 1984, Gorlenko 1988, Overmann and Tilzer 1989, Gasol *et al.* 1995, Overmann *et al.* 1998, Glaeser and Overmann 2004). Phototrophic consortia represent symbiotic associations between a colourless chemotrophic central bacterium surrounded by a fixed number of green sulfur bacteria, so-called epibionts (Pfennig 1980). The epibionts represent unique phylotypes among green sulfur bacteria and so far have only been observed in the associated state (Fröstl and Overmann 2000, Glaeser and Overmann 2004).

Recently, the epibiont of the phototrophic consortium "*Chlorochromatium aggregatum*" could be isolated in pure culture, which allowed the detailed investigation of its physiological and molecular properties (Vogl *et al.* 2006) and the sequencing of its genome (http:// genome.jgi-psf.org/draft_microbes/chlag/chlag.home.html). A combination of micromanipulation and 16S rRNA gene analyses of natural populations of phototrophic consortia uncovered an unexpected large phylogenetic diversity among the green sulfur bacterial epibionts (Glaeser and Overmann 2004). As a result, 19 different types of phototrophic consortia are recognized to date. A detailed phylogenetic analysis revealed that epibionts are not monophyletic, indicating that the ability to form symbiotic associations either arose independently from different ancestors, or was present in a common ancestor prior to the radiation of the green sulfur bacteria and the transition to the free-living state in the independent lineages (Glaeser and Overmann 2004).

In contrast to the epibionts, very little is known of the phylogeny and molecular biology of the central bacterium. This has to be attributed to the fact that this bacterium could not be cultured separately from its epibionts and that it usually represents less than 0.1% of all cells in typical enrichment cultures (Kanzler *et al.* 2005). The central bacterium is colourless and is assumed to grow chemoheterotrophically. Based on indirect evidence obtained by

microautoradiography, it is capable of assimilating 2-oxoglutarate (Glaeser and Overmann 2003). Fluorescence *in situ* hybridization (FISH) with group-specific oligonucleotides demonstrated that the central bacteria of different consortia belong to the β-subgroup of the *Proteobacteria* (Fröstl and Overmann 2000). Recently, the 16S rRNA gene of the central bacterium of "*C. aggregatum*" was sequenced (Kanzler *et al.* 2005). Sequence analysis confirmed its affiliation with the *Betaproteobacteria* and revealed that it represents an isolated phylogenetic lineage within the family *Comamonadaceae.* So far, nothing is known of the diversity of central bacteria in the different consortia. It is therefore still unknown whether different types of heterotrophic partner bacteria exist in phototrophic consortia and, if the latter is the case, whether the different heterotrophic partners are phylogenetically closely related.

Since central bacteria of phototrophic consortia constitute only a minor fraction of the cells in natural bacterial communities, highly specific molecular methods are required to selectively retrieve their 16S rRNA gene sequences. In search for flanking DNA sequences which would allow the specific amplification of the 16S rRNA genes of the central bacteria, a method for large-scale enrichment and purification of genomic DNA of the central bacterium of "*C. aggregatum*" was established and applied to analyse the structure of its rRNA operons and the genome organisation in its vicinity. Our study of the genome of the central bacterium of "*C. aggregatum*" revealed a tandem operon organisation, which so far has been observed only rarely among prokaryotes. The sequence obtained from the central bacterium of "*C. aggregatum*" is unique due to the extremely small size of the intergenic region between both *rrn* operons. Exploiting this unusual operon structure in a culture-independent PCR approach enabled us to detect a highly diverse and novel subgroup of aquatic *Betaproteobacteria*, and to recover and analyse two novel phylotypes of central bacteria of phototrophic consortia.

4.3 MATERIALS AND METHODS

4.3.1 Source of organisms and genomic DNA extraction and enrichment

The enrichment culture of "*Chlorochromatium aggregatum*" was originally obtained from the eutrophic Lake Dagow (Brandenburg, eastern Germany, Fröstl and Overmann 1998). Cultures were grown in K4 medium (Kanzler *et al.* 2005) in 10 l glass bottles at 15°C and under continuous illumination at 20 μmol quanta · m^{-2} · s^{-1}. In order to create a homogenous light field, the glass bottles were wrapped in one layer of white paper and illuminated by four 25 W tungsten light bulbs, which were placed at regular distances around the bottles. In contrast to the standard enrichment cultures, which frequently contain only a small number of intact "*C. aggregatum*" consortia, a dense monolayer biofilm of "*C. aggregatum*" formed on the walls of the vessels during four weeks of cultivation. This wall coating was harvested for DNA extraction and the supernatant discharged.

DNA was extracted by a standard protocol using CTAB (Ausubel 1995). Briefly, cell pellets were resuspended in Tris/EDTA buffer containing 0.5% SDS and 100 μg proteinase K·ml^{-1}. After addition of NaCl and CTAB, the crude DNA was extracted with chloroform/isoamylalcohol, precipitated with isopropanol and finally washed with 70% (v/v) ethanol. Genomic DNA of the central bacterium was subsequently separated from that of accompanying bacteria using the CsCl-bisbenzimidazole equilibrium density centrifugation method established previously (Kanzler *et al.* 2005). The efficiency of purification was checked against the chemotaxis enrichment method used in earlier investigations (Kanzler *et al.* 2005) by amplifying the 16S rRNA gene fragments and separating them by denaturing gradient gel electrophoresis (Muyzer *et al.* 1995). Whereas the chemotaxis enrichment method yields only a faint band of the 16S rRNA gene fragment of the central bacterium of "*C. aggregatum*", this fragment clearly dominated in DNA extracts from the monolayer biofilms after separation by density gradient centrifugation (Fig. 2, arrow).

Environmental genomic DNA was extracted from freshwater and sediment samples collected from Lake Dagow (in July 1996 and September 2006), Jones Lake (September 1998; Michigan, USA), Silver Lake (September 1998; Wisconsin, USA), Lake Cisó (March 2005; near

Girona, NE-Spain), mesotrophic prealpine Lake Starnberger See (March 2004; near Starnberg, S-Germany), oligotrophic alpine Walchensee (March 2004; near Kochel am See, S-Germany), from an alpine soil from Jochberg (February 2002; near Kochel am See, S-Germany) and a forest soil from Staudach (October 2005; near Starnberg, S-Germany). Genomic DNA from *Ralstonia eutropha* DSMZ 428 was used for reference.

4.3.2 PCR amplification, cloning of the rRNA operon

The rRNA operon gene sequence was recovered by a combination of inverse PCR, the construction of specific PCR primers, and their subsequent utilization in PCR. Ten μg of genomic DNA were digested with KpnI (MBI Fermentas, St. Leon-Rot, Germany) overnight and the digest was purified with the QIAquick PCR-purification kit (Qiagen, Hilden, Germany). Within the 16S rRNA gene of the central bacterium, the KpnI restriction site is located at position 482-487 (*E. coli* numbering). Three μg of the resulting DNA fragments were diluted in 1.5 ml of Tris-buffer and circularised with 30 units T4 DNA ligase (MBI Fermentas) at 15°C for 16 h. The DNA was then purified in a Centricon 100 ultrafiltration unit (Millipore, Eschborn, Germany) and concentrated to a final volume of 100 μl. Of the resulting DNA, 200 ng were applied in an iPCR amplification, which was performed in a GeneAmp 9700DNA thermal cycler (Applied Biosystems, Weiterstadt, Germany) and employing primers CR-442f and CR-207r (Kanzler *et al.* 2005, Supplementary table 1) and Ampli*Taq* Gold polymerase (Applied Biosystems). Cycling conditions comprised 30 cycles with a denaturation step at 95°C for 30 s, annealing at 51°C for 1 min, elongation at 72°C for 4 min, and a final extension step at 72°C for 10 min. In order to achieve a higher stringency of the iPCR, acetamide was added to a final concentration of 5% (Tuschak *et al.* 2005).

Supplementary table 1. Oligonucleotide primers used for amplification and sequencing of different portions of the *rrn*-operons of *Betaproteobacteria*

Primer	Sequence (5′-3′)	Target	Reference
CR-207r	CGC GCG AGG CCC TCT	16S rDNA	Kanzler *et al.* 2005
CR-442f	ACG GAG CGA AAC AGC CTT	16S rDNA	Kanzler *et al.* 2005
β-680r	TCA CTG CTA CAC GYG	16S rDNA	Overmann *et al.* 1999
907r	CCG TCA ATT CCT TTG AGT TT	16S rDNA	Muyzer *et al.* 1995
1055r	AGC TGA CGA CAG CCA T	16S rDNA	Amann *et al.* 1995
CR-1282f	CCA TAA AGT CAG TCG	16S rDNA	Kanzler *et al.* 2005
CR-ITS1-42f	ATC GGA AGG TGC GGC TGG	ITS1	this study
CR-ITS1-90f	GAC ACC CAC ACT TAT CGG	ITS1	this study
CR-23S-575r	AAT GTA AGT CGC TGA CCC	23S rDNA	this study
CR-23S-632r	ACG CCC TAT TCG GAC TCG	23S rDNA	this study
β-23S-1027r	GCC TCC CCA CTT CGT TT	23S rDNA	Manz *et al.* 1992
CR-23S-1288f	AAG GTT TTC TAC GCA ACG	23S rDNA	this study
CR-23S-1388f	ACG TGT AGT GCG ATG TGG	23S rDNA	this study
CR-23S-1694f	TAG GTG AAG TCC CTA GCG	23S rDNA	this study
CR-23S-2201r	TCA AAC TGC CTA CCA TGC	23S rDNA	this study
CR-23S-2241r	TCG AAC TCC TCC GTT ACG	23S rDNA	this study
CR-ITS2-52r	AAC CAA TTT ACG GAG ATG	ITS2	this study
β-5S-30f	CCA CYC CTT CCC WTC CCG	5S rDNA	this study
β-5S-44f	TCC CGA ACA GGA CMG TGA AAC	5S rDNA	this study
CR-5S-55f	GGA CCG TGA AAC ACC TTC	5S rDNA	this study

"CR" denotes primers specific for the central rod-shaped bacterium of "*C. aggregatum*"; "β" indicates primers are specific for *Betaproteobacteria*. Within genes numbers at the end of each primer designation indicate binding position according to *E. coli* numbering. For primers targeting internal described spacers, numbers denote position on the actual sequence of the central bacterium.

Our analysis revealed a previously unknown tandem *rrn* operon structure for the central bacterium of "*Chlorochromatium aggregatum*". Three different approaches were used to confirm this result (compare Fig. 3). Firstly, inverse PCR was repeated with the isoschizomeric enzyme Acc65I (MBI Fermentas) and the sequence of the resulting DNA fragments was compared to that of the first iPCR. Secondly, primers specifically targeting the novel 5S rRNA gene sequence (CR-5S-55f) were constructed based on the determined sequence (Supplementary table 1). This primer was combined with primer CR-1282r which targets the 16S rRNA gene sequence of the central bacterium (Kanzler *et al*, 2005) and direct amplification trials were performed with genomic DNA from the central bacterium, using a step down PCR protocol with 40 cycles, which comprised a denaturation step at 95°C for 30 s, annealing at 60°C (first 10 cycles), then at 55°C (following 30 cycles) for 1 min, elongation at 72°C for 1 min, and a final extension step at 72°C for 10 min. Thirdly, a primer pair consisting of the *Betaproteobacteria*-specific primer β-23S-1027f (binding within the 23S rRNA gene; Manz *et al.*, 1992) and of CR-207r (Supplementary table 1) were used in a step down PCR protocol under the above conditions.

For the purpose of recovering the portion downstream of the 16S rRNA gene of the second *rrn* operon which in most bacteria includes the ITS1 and the 23S rRNA gene regions, genomic DNA from the central bacterium of "*C. aggregatum*" was amplified with primer β-23S-1027r and CR-1282f (Kanzler *et al.* 2005). In this amplification, the step down PCR program included 10 cycles with denaturation at 94°C for 30 s, primer annealing at 58°C for 1 min and elongation at 72°C for 2 min, followed by 30 cycles with the annealing temperature changed to 53°C.

4.3.3 Analysis of natural bacterial communities

In order to screen natural bacterial communities for the occurrence of *Betaproteobacteria* exhibiting a similar tandem *rrn* operon structure, we designed two primers, β-5S-30f and β-5S-44f (Supplementary table 1), which specifically target the 5S rRNA gene sequence of all known *Betaproteobacteria*. Each of these primers was combined with either the *Betaproteobacteria*-specific primer β-680r (Overmann *et al.* 1999), universal primer 907r (Muyzer *et al.* 1995) or eubacterial primer 1055r (Amann *et al.* 1995) (Supplementary table 1). The step down PCR program for amplification included 10 cycles with denaturation at 94°C

for 30 s, primer annealing at 58°C for 1 min and elongation at 72°C for 2 min, followed by 30 cycles with the annealing temperature set to 53°C.

All PCR products generated were examined by standard 1.4% (wt/vol) agarose gel electrophoresis and were cloned using the TOPO TA cloning kit (version R; Invitrogen, Carlsbad, CA). Plasmids were extracted with a QIAprep spin miniprep kit (Qiagen), and the presence of inserts was verified by digestion with EcoRI (MBI Fermentas). Two separate clone libraries were established for the Lake Dagow samples obtained in 1996 and 2006.

4.3.4 Sequencing and phylogenetic analyses

PCR products were sequenced by the dideoxynucleotide method (Sanger *et al.* 1977) using an ABI Prism 3730 genetic analyzer (Applied Biosystems) and the Ampli*Taq* FS Big Dye Terminator cycle sequencing kit according to the protocol of the manufacturer. The Vector NTI computer package was used for sequence assembly and editing (Invitrogen). Sequencing of the ITS1 region was accomplished by primer walking using four custom designed primers (CR-ITS1-42f, CR-ITS1-90f, CR-23S-575r, and CR-23S-632r; Supplementary table 1). For covering the complete *rrn* tandem operon, primer walking was conducted with the following primers: CR-23S-2201r, CR-23S-2241r, CR-23S-1288f, CR-23S-1388f, CR-23S-1694f, CR-ITS2-52r (Supplementary table 1). All 16S rRNA gene sequences obtained in the present study were checked for possible chimeras by using the CHIMERA-CHECK online analysis program of the RDP-II database (Maidak *et al.* 2001). No chimeras were identified.

tRNAs were identified using the program tRNAscan-SE, version 2.21 (Lowe and Eddy 1997). Phylogenetic relationships were analysed using the software packages ARB (Ludwig *et al.* 2004) and PHYLIP (Phylogeny Inference Package, version 3.57c; Felsenstein 1989). Sequences of the phylogenetically closest relatives of the central bacterium and the obtained clones were retrieved from the GenBank database using BLAST version 2.0.4 (Altschul *et al.* 1997) and imported into the ARB database. Phylogenetic trees were inferred using the maximum likelihood and maximum parsimony algorithms within the ARB package. Additionally, the distance-based methods DNADIST (Kimura) and FITCH were used.

To identify variable branching points, the phylogenetic trees generated were compared in a pairwise fashion employing the COMPARE TOPOLOGY function of the ARB program. Finally, the CONSENSE program implemented in the PHYLIP software package was applied

to generate a consensus tree resulting from the three phylogenetic trees. For this comparison, the majority rule was employed. In addition, reproducibility of the branching pattern obtained with the three methods was tested by bootstrap analysis, generating a set of 100 resamplings. In a final step, the consensus tree as well as the bootstrap values were used to correct the maximum likelihood tree. Those branches which were observed to differ between the three tree-building methods and which had only low bootstrap support, were collapsed with deeper branching points to yield a polytomic consensus tree.

4.3.5 Coverage and diversity estimates

We attempted to estimate the diversity of *Betaproteobacteria* with tandem *rrn* operons cooccurring in a single bacterial community. A sample was obtained from the chemocline of Lake Dagow in the year 2006 and a clone library of partial *rrn* operons with a tandem structure was generated as described above. Clonal richness was calculated in EstimateS (Version8.0.0) (Colwell 2005), using the Chao1 estimator (Chao 1984). Additionally, we used the abundance based coverage estimator (ACE, Chao and Lee 1992) to estimate minimum numbers of tandem *rrn* containing *Betaproteobacteria* in the chemocline of Lake Dagow. Sample order randomisation was set at 50 randomisations, and the coverage estimator was set at 12 (a setting of 2 did not change the results). Chao analysis was done without bias correction, using the classic formula. All 16S rRNA gene sequences of this clone library were analysed with the Analytic Rarefaction 1.3 program (Holland 2003).

4.3.6 Fluorescence *in situ* hybridisation (FISH)

Fluorescence *in situ* hybridisation was used to assign environmental 16S rRNA gene sequences of *Betaproteobacteria* to the different bacterial morphotypes present in the chemocline of Lake Dagow with the specific aim to identify sequences of the central heterotrophic symbionts of phototrophic consortia among our sequence collection.

The sequences generated from the first clone library of Lake Dagow were used to design specific FISH probes with the DESIGN PROBES function of the ARB software package. The accessibility of the target sites were checked based on data available for *Escherichia coli* (Fuchs *et al.* 1998). For probes targeting sites with limited accessibility, corresponding helper oligonucleotides (Fuchs *et al.* 2000) were designed. Overall, 8 specific probes and 24 helper

oligonucleotides were then used (Supplementary table 2) to analyse the chemocline bacterial community present in the chemocline of Lake Dagow in the year 2006. FISH was carried out on white polycarbonate filters (0.2 μm). Of each of the Cy3-labeled probe and the corresponding helper oligonucleotides, twenty nanograms were employed at the appropriate hybridisation stringency (Supplementary table 2). Hybridisation was conducted for 2h at 45°C. Stringency was tested and optimised by varying the formamide concentrations between 5 and 35%. After counterstaining with 4′,6-diamidino-2-phenylindole (DAPI), hybridisations were analysed by epifluorescence microscopy.

4.3.7 Nucleotide sequence accession numbers

The tandem rRNA operon sequence of the central bacterium of "*C. aggregatum*" has been assigned the GenBank accession number EF203797. Partial sequences consisting of identically organized tandem *rrn* operons recovered from the investigated freshwater lakes have been deposited under accession numbers EF203798-EF203865 and EF219476.

Supplementary table 2. Fluorescently labelled probes, helper oligonucleotides and hybridisation conditions

Probe or helper[a]	Sequence (5′-3′)	T_m (°C)	Formamide (%)
D1-444	**GGC AGA CCT TTT CGC TCC**	**58.4**	**35**
D1-444-H1	GTA CAA AAG CAG TTT ACA ACC	54.1	
D1-444-H2	GTC ATG AGC CCA CCG TAT TAG	60.0	
D1-444-H3	GGT GCT TAT TCT TAC GGT ACC	58.0	
D2a-454	**ATC GTA TTA GGA CAG ACC**	**51.5**	**25**
D2a-454-H1	GTT TCG TTC CGT ACA AAA G	52.4	
D2a-454-H2	CGG TAC CGT CAT TAG CCC	58.4	
D2a-454-H3	AGC CGG TGC TTA TTC TTA	51.5	
D2b-447	**TAA AGA GAG CCT TTT CGC**	**51.5**	**5**
D2b-447-H1	TCC GTA CAA AAG CAG TTT AC	53.3	
D2b-447-H2	GTC ATG AGC CCC CTG TAT	56.1	
D2b-447-H3	GCT TAT TCT TAC GGT ACC	51.5	
D3-210	**CCA TTC GCG CGA GGC CCT**	**62.9**	**20**
D3-210-H1	GCG AGT CCC CCG CTT TCA TC	63.6	
D3-210-H2	GCT AAT CTG ATA TCG GCC GCT	60.0	
D3-210-H3	GAG CTT TTA CCC CAC CAA ACT	58.0	
D4a-443	**TAA AGC CTT TTC GCT CCG**	**53.8**	**25**
D4a-443-H1	TAC AAA AGC AGT TTA CAA CCC	54.0	
D4a-443-H2	TCA TTA GCC TTA AGT ATT AGT	50.1	
D4a-443-H3	GTG CTT ATT CTT ACG GTA CCG	58.0	
D4b-453	**CTT TAT TAG AGC CCA CCG**	**53.8**	
D4b-453-H1	TTT CGT TCC GTA CAA AAG CAG	56.0	
D4b-453-H2	GGT ACC GTC ATT AGC AGA	53.8	
D4b-453-H3	GCC GGT GCT TAT TCT TAC	53.8	
27GS4-208	**CAA TCG CGC AAG GCT TTT**	**53.8**	**10**
27GS4-208-H1	GCA AGT CCC CTG CTT TTA	53.8	
27GS4-208-H2	CTA ATC TGA TAT CGG CCA CTC	56.0	
27GS4-208-H3	AGC TTT TAC CTC ACC AAC TAG	56.0	
27GS4-454	**CTT TCC GTA TTA GTT AAA GCT**	**52.1**	**10**
27GS4-454-H1	TTT TCG CTC CGT ACA AAA G	52.5	
27GS4-454-H2	CTT ACG GTA CCG TCA TTA G	52.5	
27GS4-454-H3	TAG TTA GCG GGT GCT TAT T	52.5	

Numbers indicate 5′- end according to *E. coli* numbering. Fluorescently labelled probes are denoted in bold. Helper oligonucleotides are denoted by the suffix "H".

4.4 RESULTS AND DISCUSSION

4.4.1 Isolation and purification of genomic DNA of the central bacterium of "*C. aggregatum*"

In the enrichment cultures of "*C. aggregatum*" grown under standard laboratory conditions, the cells of the central bacterium are accompanied by a larger number of free epibionts and additional chemotrophic bacteria and thus typically amount to only ≤ 0.1% of all cells present (Kanzler *et al.* 2005) (Fig. 1A). Previously, the chemotactic behaviour of intact consortia towards sulfide has been used to enrich them for subsequent PCR amplifications (Kanzler *et al.* 2005). However, these chemotaxis enrichments yielded numbers of consortia far too small for the preparation of genomic DNA of the central bacterium.

In the present study, we established a method to selectively grow large numbers of intact consortia in a biofilm on the walls of large (10 l) culture flasks. At homogenous illumination, consortia first grew in the suspended state until reaching an optical density (OD_{650}) of 1.0, after which they accumulated at the wall of the culture bottles due to the severe self-shading in the culture liquid (Fig. 1B). Within four weeks of cultivation, a dense monolayer biofilm had formed which consisted mostly of "*C. aggregatum*" (Fig. 1C). After extraction, the genomic DNA of the central bacterium was separated from that of the epibiont by the previously established CsCl-bisbenzimidazole equilibrium density centrifugation (Kanzler *et al.* 2005). The overall efficiency of enrichment of the combined method was compared to that of the chemotaxis method or the density gradient centrifugation method alone, by employing denaturing gradient gel electrophoresis (DGGE) fingerprinting of 16S rRNA gene fragments (Fig. 2). Our new combined approach is clearly superior to all previously used techniques and allows an enrichment of genomic DNA from the central bacterium by several hundred fold as estimated from the corresponding band intensities in Fig. 2.

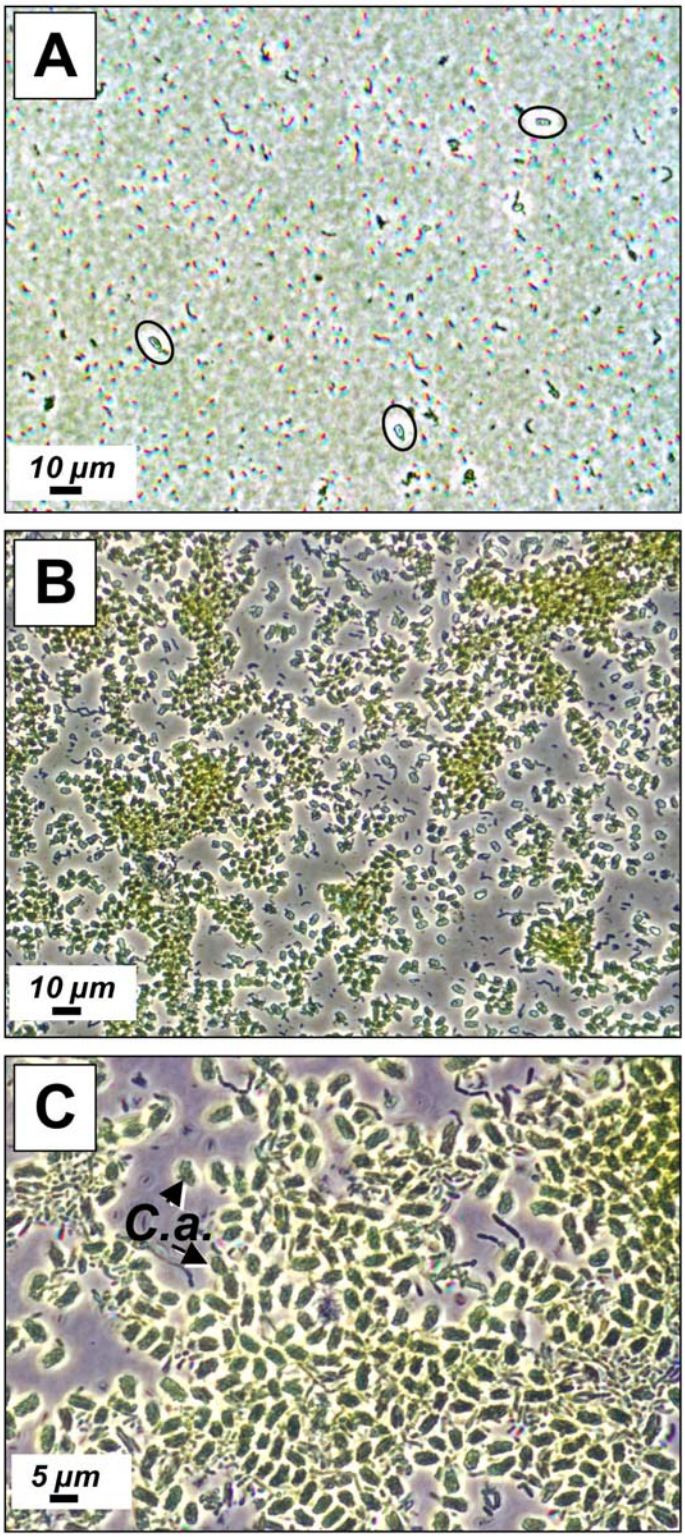

Fig. 1. **A.** Phase contrast micrograph of a typical enrichment culture of "*Chlorochromatium aggregatum*". Intact consortia are denoted by circles. **B.** Accumulation of "*C. aggregatum*" forming a monolayer biofilm at the wall of 10 l bottles after 4 weeks of incubation under light-limited conditions. **C.** Biofilm at higher magnification. The number of consortia is greatly increased relative to the number of accompanying free-living bacteria. *C.a.,* intact "*C. aggregatum*" consortia.

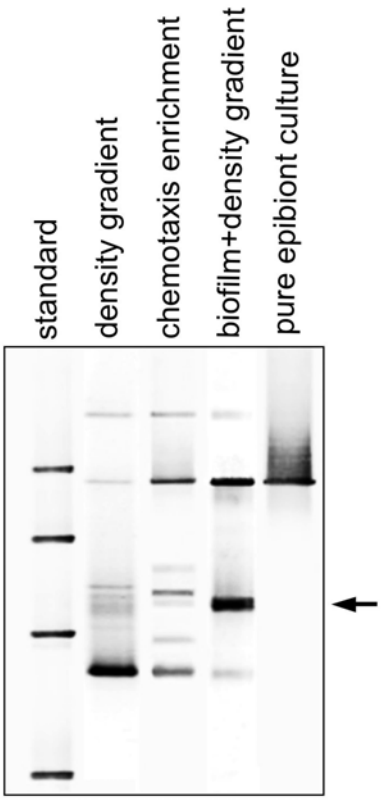

Fig. 2. Different methods for the enrichment and purification of genomic DNA of the central bacterium of "*C. aggregatum*", as analysed by DGGE-fingerprinting of 16S rDNA gene fragments after amplification with primers GC341f and 907r. Enrichments were conducted by CsCl-bisbenzimidazole density gradient centrifugation alone, by chemotaxis, and by growth of intact consortia in biofilms followed by CsCl-bisbenzimide density gradient centrifugation (see text). The arrow indicates the specific melting position of the fragment from the central bacterium of "*C. aggregatum*". The fingerprint of a pure culture of the epibiont *Chlorobium chlorochromatii* CaD is shown for comparison (rightmost lane).

4.4.2 Organisation of *rrn* operons in the central bacterium of "*C. aggregatum*"

The purified genomic DNA of the central bacterium of "*C. aggregatum*" was used to analyse the regions flanking its known 16S rRNA gene sequence. Inverse PCR amplification of the region located upstream of the 5′-end of the 16S rRNA gene yielded a 489 bp-long DNA fragment. As expected, the 3′-end of the amplified fragment represented the first 204 bp of the previously determined 16S rRNA (*rrs*) gene of the central bacterium (Kanzler *et al.* 2005). However, sequence analysis of the upstream portion of the fragment revealed that the first 90 bp were part of a 5S rRNA (*rrf*) gene, which was separated from the downstream *rrs* gene by a short 195 bp spacer sequence (Fig. 3). The spacer sequence did not contain start or stop codons and did not show homology to any open reading frame in the GenBank database. In

order to assess whether the *rrs* gene of the central bacterium is followed by a 23S rRNA gene (*rrl*) as in the vast majority of known bacterial *rrn* operons, the corresponding region was amplified with primers CR-1282f and β-23S-1027r (Supplementary table 1) and sequenced. Based on this analysis, the *rrs* gene of the central bacterium is 1533 bp long and is followed by a 662 bp-long internal transcribed spacer (ITS)1 region and the *rrl* gene (Fig. 3). The ITS1 region was found to contain two tRNA genes, $tRNA^{Ile}$ and $tRNA^{Ala}$. The presence of tRNA genes within the ITS1 spacer regions is a common, yet not universal, feature of rRNA operons (Krawiec and Riley 1990).

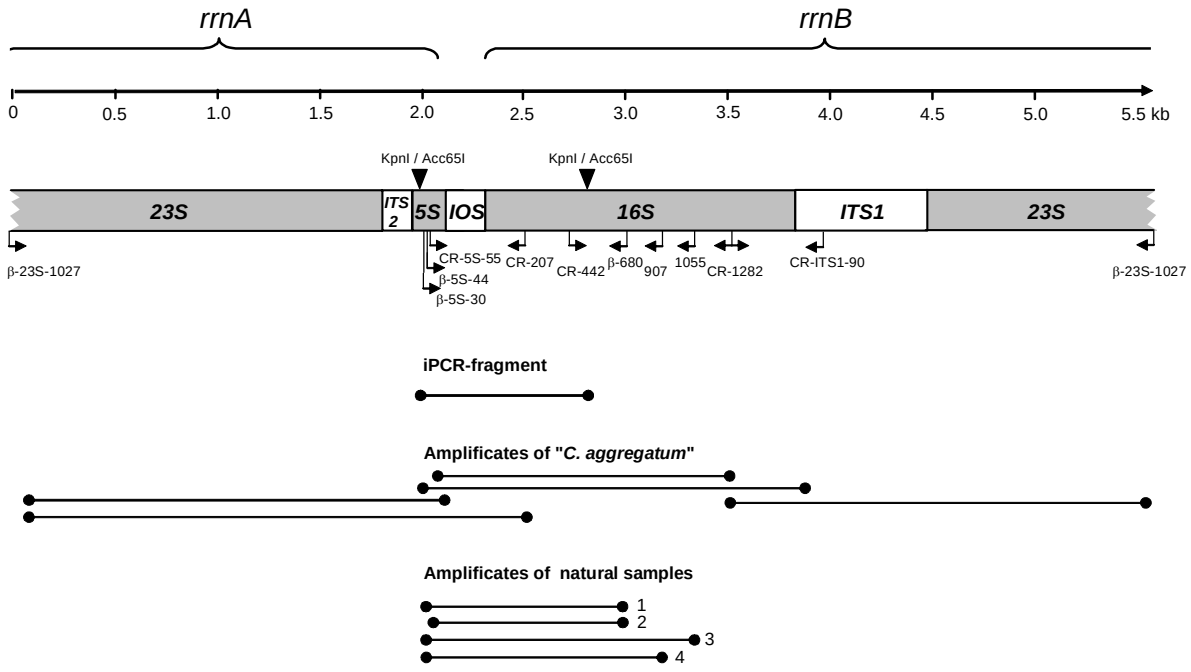

Fig. 3. Gene order and lengths of ribosomal RNA genes in the central bacterium of "*C. aggregatum*". The KpnI / Acc65I restriction sites for the generation of the iPCR fragment are indicated. Binding sites of the primers (compare Supplementary table 1) used for overlapping amplification and sequencing of the central bacterium of "*C. aggregatum*" and for the retrieval of *rrn* sequences from natural communities are indicated.

The canonical *rrn*-operon of *Bacteria* consists of genes coding structural 16S rRNA (*rrs*), 23S rRNA (*rrl*) and 5S rRNA (*rrf*) in the order *rrs* - ITS1 - *rrl* - ITS2 - *rrf* (Krawiec and Riley 1990; Srivastsava and Schlessinger 1990). Within the operon, the rRNA genes are separated by internal transcribed spacers which usually contain the genes for one or two tRNAs.

Our results suggested a tandem organisation of two *rrn* operons (*rrnA* and *rrnB*; Fig. 3) separated by only 195 bp. This unusually close tandem arrangement of two *rrn* operons was confirmed by several independent tests. First, the inverse PCR was repeated with an isoschizomeric enzyme (Acc65I). After cloning and sequencing of the respective PCR product, the sequence completely matched the previous amplicon. Second, a fragment spanning *rrfA*, the interoperon sequence and the beginning of the *rrsB* gene could be amplified using a primer targeting the novel 5S rRNA gene sequence of the central bacterium (CR-5S-55f) and a reverse primer specific for its 16S rRNA gene (CR-1282r) (amplification products indicated in Fig. 3). Third, the region between the *rrlA* and the *rrsB* genes could be amplified with the primer pair β-23S-1027f and CR-207r (Fig. 3). Sequencing of all amplificates obtained yielded an identical sequence of the 195 bp-long intergenic region.

All of the above results are in line with the gene order *rrlA* - *rrfA* - *rrsB*. The overall sequence information obtained in the present study spans a total of 5460 bp of the tandem *rrn* operon of the central bacterium of "*C. aggregatum*" (Fig. 3). The *rrlA* gene is separated by a 93 bp ITS2 from the 116 bp long *rrfA* gene. So far, there are no reports of the presence of any tRNA gene in the ITS2 of any bacterium (Chen *et al.* 2000). Correspondingly, no tRNA-like structures were identified within the ITS2. The 195 bp-long interoperon spacer (IOS) is followed by a 1533 bp long *rrs*. An ITS1 of 662 bp and a *rrl* gene follow. The available information indicates a canonical order *rrs* - ITS1 - *rrl* - ITS2 - *rrf* in the central bacterium of "*C. aggregatum*" as in most other bacteria investigated (Srivastsava and Schlessinger 1990). Comparison of the sequences of the more than 30 clones obtained from the central bacterium of "*C. aggregatum*" during this part of our study showed no sequence ambiguity. We therefore conclude that the sequence divergence of the *rrn* operons in this bacterium must be very small. Similarly to the central bacterium, the genome of the phylogenetically closely related *Rhodoferax ferrireducens* DSMZ15236^T habours *rrnA* and *rrnB* operons with identical sequences (National Center for Biotechnology Information (NCBI) Microbial Genome

database; *http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html*) which are, however, separated by a 350 bp-long intergenic region (Table 1).

Because of the unusual organisation of the *rrn* operon in the central bacterium of "*C. aggregatum*", all available rRNA operon sequences of the NCBI Microbial Genome database for eubacterial genomes (404 entries; as of January, 2007) were systematically examined for a tandem organisation of their *rrn* operons. This revealed that only 24 (5.9%) of the sequenced genomes harbour complete *rrn* operons in a tandem arrangement and with an interoperon spacer of ≤ 1000 bp (Table 1). Tandem *rrn* operons were found in the two Gram-positive phyla *Actinobacteria* and *Firmicutes* and in 4 classes of the phylum *Proteobacteria*, but occurred most frequently among the *Firmicutes* (10.5% of all genomes) and the *Gammaproteobacteria* (10% of all genomes) (Table 1). Most notably, of all 31 available genomes of *Betaproteobacteria*, only the closest relative of the central bacterium, *Rhodoferax ferrireducens* DSMZ15236^T, exhibits a tandem arrangement of *rrn* operons. None of the genomes of all other 16 phyla, which are currently represented in the NCBI Microbial Genome database contained closely spaced tandem *rrn* operons.

In conclusion, only few of the known bacterial genome sequences match that of the central bacterium of "*C. aggregatum*" with respect to its *rrn* operon organisation. Within this group, the central bacterium of "*C. aggregatum*" exhibits the shortest interoperon spacer, together with *Bacillus subtilis* subsp. *subtilis* strain 190.

Table 1. Overview about all sequenced bacteria with tandem *rrn* operons separated by an interoperon spacer (IOS) < 1000 bp

Phylum/Class and Strain [a]	IOS (bp) [b]	Total number of *rrn* operons
Actinobacteria (1 of 36 genomes)		
Bifidobacterium longum NCC2705	868	4
Firmicutes (10 of 95 genomes)		
Bacillus subtilis subsp. *subtilis* str. 190	190	10
Clostridium acetobutylicum ATCC 824^T	354, 354, 354, 354	11
Clostridium tetani E88	350	6
Lactobacillus johnsonii NCC 533	381	6
Lactobacillus gasseri ATCC 33323^T	409	6
Lactobacillus sakei subsp. *sakei* 23K	497	7
Staphylococcus aureus subsp. *aureus* COL	212	6
Staphylococcus aureus subsp. *aureus* MW2	212	6
Staphylococcus epidermidis RP62A	229	6
Staphylococcus saprophyticus subsp. *saprophyticus* ATCC 15305^T	296	6
α-Proteobacteria (1 of 54 genomes)		
Novosphingobium aromaticivorans DSMZ 12444^T	533, 533	3
β-Proteobacteria (1 of 31 genomes)		
Rhodoferax ferrireducens DSMZ 15236^T	350	2
γ-Proteobacteria (10 of 100 genomes)		
Colwellia psychrerythraea 34H	593, 546, 539	9
Photobacterium profundum SS9	419, 419, 409, 418, 419	14
Pseudomonas putida KT2440	463	7
Psychrobacter arcticus 273-4^T	773	4
Psychrobacter cryohalolentis K5^T	758	4
Vibrio cholerae O1 *biovar eltor* str. N16961	367	8
Vibrio fischeri ES114	404, 404, 405, 404, 404	11
Vibrio parahaemolyticus RIMD 2210633	319	11
Vibrio vulnificus CMCP6	330	8
Vibrio vulnificus YJ016	336	9
δ-Proteobacteria (1 of 14 genomes)		
Pelobacter carbinolicus DSMZ 2380^T	390	2

[a] All completely sequenced eubacterial genomes within the NCBI database were considered (404 entries; as of January 01, 2007). Numbers in parenthesis give number of genomes with tandem operons of total number of genome sequences of each phylum or class.

[a] Multiple numbers indicate the presence of different pairs of tandem operons with the exception of *Novosphingobium aromaticivorans* DSMZ 12444^T. In the latter, the three existing *rrn* operons all occur in close vicinity to each other.

4.4.3 Occurrence and phylogenetic diversity of tandem operons in chemocline microbial communities

The fact that the tandem *rrn* operon structure was found only once among the *Betaproteobacteria* and particularly in the closest relative of the central bacterium of "*C. aggregatum*" suggested that tandem *rrn* operons may represent a specific feature of the chemotrophic symbiont of phototrophic consortia and its relatives. These relatives potentially also include central bacteria of other phototrophic consortia. We therefore developed a PCR-based method to specifically recover fragments of tandem *rrn* operons from complex natural communities known to harbour different types of phototrophic consortia. To this end, two primers (β-5S-30f and β-5S-44f; Supplementary table 1) which target the 5S rDNA gene of all known *Betaproteobacteria* were designed based on an alignment of all available betaproteobacterial *rrf* sequences in the databases. These primers were then combined with two eubacterial 16S primers (907r or 1055r) or the betaproteobacterial primer β-680r. Initially, four different primer combinations (β-5S-30f/β-680r, β-5S-44f/β-680r, β-5S-30f/907r, and β-5S-30f/1055r) were tested, using DNA extracts from the chemocline of one lake (Lake Dagow). This revealed that betaproteobacterial sequences encompassing the *rrnA-rrnB* interoperon region could be recovered reproducibly if the two primer pairs β-5S-30f/907r and β-5S-30f/1055r were employed.

The two primer pairs were subsequently employed to screen eight different aquatic and soil bacterial communities for the presence of *Betaproteobacteria* with tandem rRNA operons. The sampling sites comprised six freshwater lakes (Dagowsee, Lake Cisó, Jones Lake, Silver Lake, Starnberger See, Walchensee), two different soils (Jochberg and Staudach) and one lake sediment (Walchensee). Four of these, Lake Dagow, Lake Cisó, Jones and Silver Lake represent typical oxic-anoxically stratified environments, and are known to harbour different types of phototrophic consortia (Glaeser and Overmann 2004). Indeed, amplicons were exclusively obtained from chemocline environments whereas all other samples did not yield any amplification product independently of the primer combination used. In the case of Lake Cisó, amplification products of different lengths (between 1300 and 1500 bp) were obtained.

All amplification products were cloned, the clones subjected to a restriction digest analysis and representative clones of each restriction type were sequenced. This first limited

sequence analyses yielded different 16S rRNA gene sequences for several of the individual restriction types, thereby indicating the presence of an unexpected diversity of bacteria with a tandem *rrn* operon structure in the chemocline microbial communities of the four stratified lakes. Clones from Lake Cisó exhibited the largest variation in size due to lengths of interoperon spacers of 333 bp and 466 bp, which is commensurate with the larger length variation observed for the amplification products from this lake. Our first screen yielded the highest number of different sequence types for the chemocline microbial community from Lake Dagow. In order to evaluate the diversity of bacteria with a tandem *rrn* operon structure in more detail, the chemocline of Lake Dagow was sampled again in the year 2006 and a larger clone library of *rrn* sequences after amplification with primer pairs β-5S-30f/907r and β-5S-30f/1055r was analysed. From this sample, 62 different clones yielded correct inserts and nonchimeric sequences. All 62 sequences were found to contain *rrf* and *rrs* genes separated by a short spacer.

The entire set of 16S rRNA gene sequences generated in the present study was subjected to detailed phylogenetic analyses, by employing maximum likelihood, maximum parsimony and distance-based algorithms. A comparison of the topology of the three different phylogenetic trees demonstrated that the branching patterns of many sequences, or groups of sequences, varied considerably between the trees. In addition, these variable branches had a very low bootstrap support independent of the phylogenetic method employed. Because of this uncertainty, a consensus phylogenetic tree (Fig. 4) was calculated employing the majority rule algorithm as implemented in the PHYLIP program package (Felsenstein 1989). Our analyses unequivocally demonstrated that all 74 individual 16S rRNA gene sequences which had been recovered from the different chemocline bacterial communities in the different years are closely related to each other and form a novel, distinct subcluster within the phylum *Betaproteobacteria* (Fig. 4). This novel subcluster also encompassed the sequence of the central bacterium of "*C. aggregatum*" (Fig. 4). *Rhodoferax ferrireducens* DSMZ 15236^T, one of the closest cultivated relatives of the novel subcluster is characterized by a tandem *rrn* operon, whereas the genome of the more distantly related *Polaromonas vacuolata* DSMZ 15660^T contains only a single *rrn* operon (*ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html*). Although information on the genome sequences of other members of this group is so far missing, it appears likely that other cultivated relatives of the novel betaproteobacterial

subcluster similarly bear tandem *rrn* operons in their genome. *Rhodoferax ferrireducens* DSMZ 15236^T possesses two *rrn* operons with identical nucleotide sequences. Furthermore, a close arrangement of three *rrn* operons was only observed in one single case out of the 404 genomes available in the database (*Novosphingobium aromaticivorans* DSMZ 12444^T; Table 1).

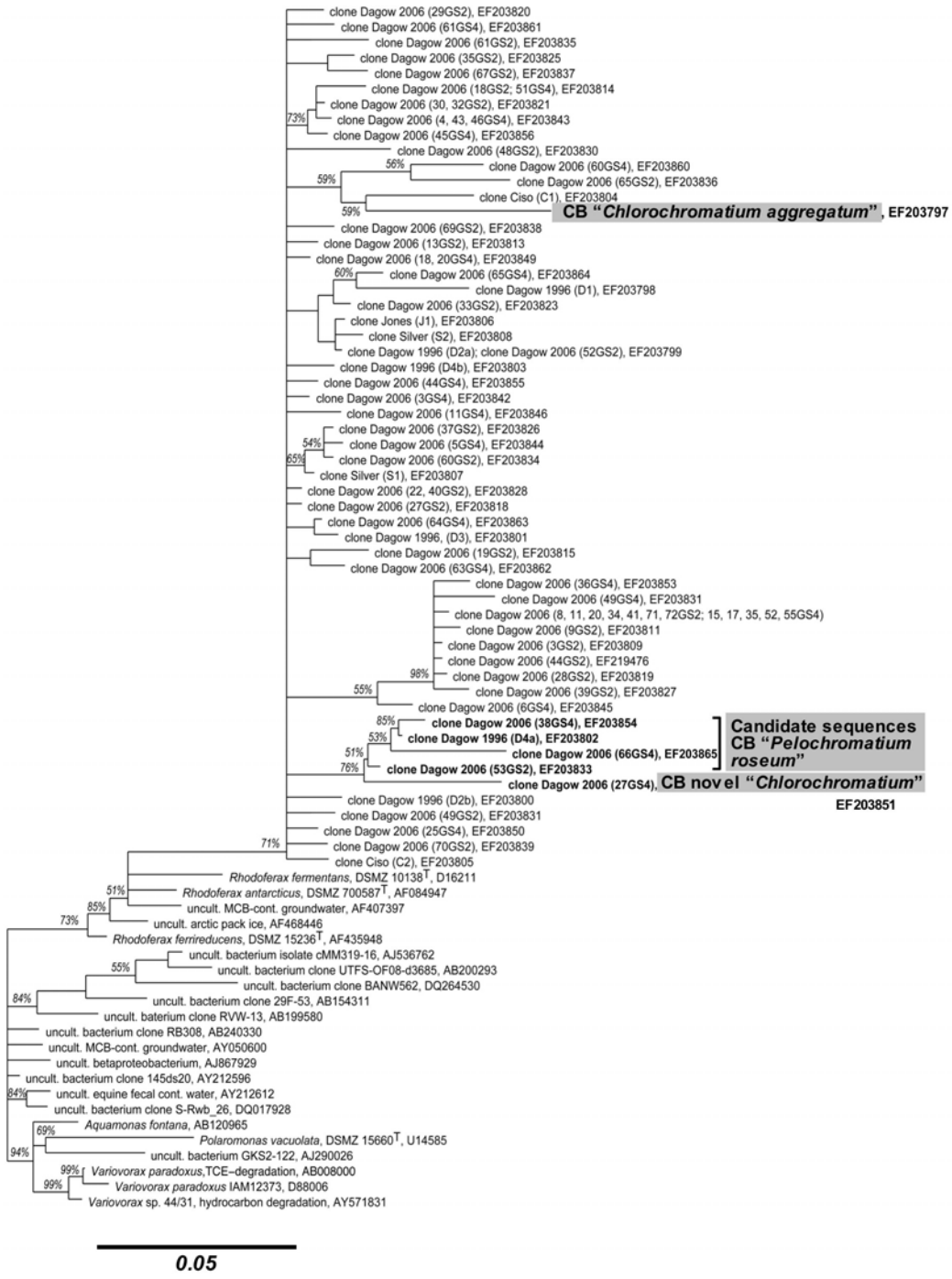

Fig. 4. Phylogenetic analysis of all 16S rRNA gene sequences obtained in the present study for *Betaproteobacteria* exhibiting a tandem *rrn* operon structure, and for their closest relatives. The consensus tree depicted was constructed based on individual maximum likelihood, maximum parsimony and distance-based phylogenetic trees employing the majority rule as implemented in the CONSENSE program of the PHYLIP package (see Material adn Methods). Percentages at nodes indicate bootstrap values out of 100 resamplings, as determined for all trees. Bootstrap values for maximum likelihood, maximum parsimony and distance-based trees differed by no more than 1%, therefore means of the three methods are given. Only values above 50% are shown. The phylogenetic position of the central bacteria (CB) of three different phototrophic consortia (including an novel type of "*Chlorochromatium*", see text) are highlighted. Bar indicates 5% fixed point mutations per nucleotide base.

Among the 62 clones retrieved from the chemocline of Lake Dagow in 2006, 39 clones were found to be unique, whereas one phylotype occurred 12 times, another one 3 times and four phylotypes were found twice among the clone library (Fig. 4). We used rarefaction (Holland 2003) to elucidate how well our clone library represented the total diversity of chemocline *Betaproteobacteria* harbouring tandem *rrn* operons. This analysis revealed that only a small fraction of the total sequence diversity of this group had been recovered as the rarefaction curve was far from saturation (Fig. 5). Clone library depth was analysed further employing the EstimateS software package (Version 8.0.0) (Colwell 2005). Employing the nonparametric Chao-1 estimator, we calculated the total diversity of *Betaproteobacteria* with tandem *rrn* operon organisation in the chemocline of Lake Dagow to amount to 235 species. Since the Chao-1 richness estimator tends to overestimate species richness in small samples, we also used the ACE estimator, which is based on the frequency of species with 10 or fewer individuals in the sample (Chao *et al.* 1993). Applying the ACE estimator, we arrived at a diversity value of 193 individual sequence types. Due to the particular design of our PCR, which relies on the presence of a *rrf* gene preceding the *rrs* gene, our PCR would retrieve only one out of the two *rrn* sequences from genomes with a tandem arrangement of canonical *rrn* operons. By inference, a major fraction of the sequence diversity discovered in the present work is likely to reflect the diversity of different bacteria rather than microheterogeneity of different *rrn* operons within the same genome. All of the above diversity analyses thus point towards an unexpectedly high diversity of cooccurring phylotypes of the newly discovered betaproteobacterial subcluster.

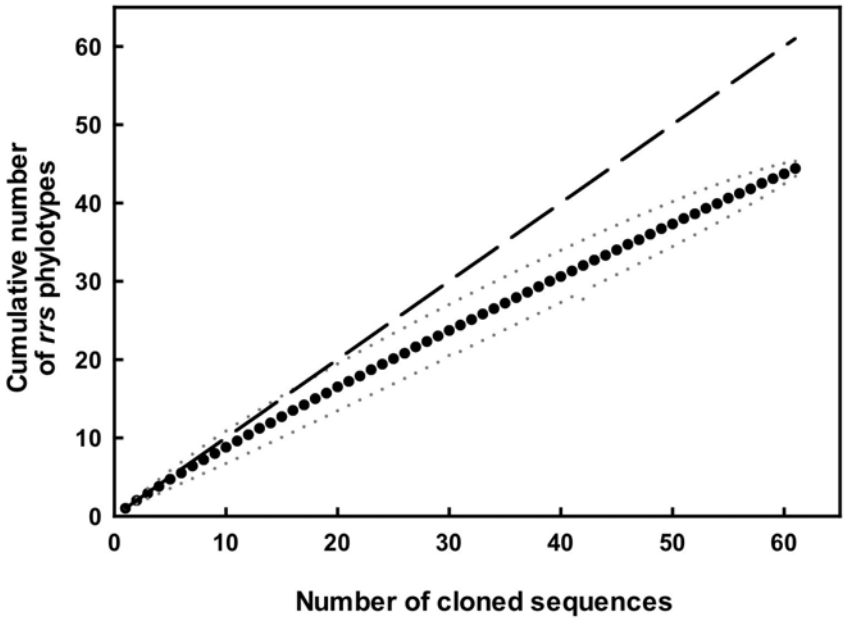

Fig. 5. Rarefaction curve of 63 *rrs*-sequence types originating from tandem *rrn* operon fragments as cloned from a chemocline water sample of Lake Dagow in the year 2006. Dotted lines represent the upper and the lower 95% confidence intervals. For comparison, the dashed line indicates a rarefaction curve for a set of unique phylotypes.

Whereas considerable microdiversity has been described for ribotype clusters of sulphate–reducing bacteria from salt marshes (Klepac-Ceraj *et al.* 2004), for coastal bacterioplankton communities (Acinas *et al.* 2004) and some open-ocean microbial groups (Rappé and Giovannoni 2003), virtually nothing is known so far of the microdiversity patterns of chemocline microbial communities in lacustrine (Overmann *et al.* 1999, Casamayor *et al.* 2000) and marine (Madrid *et al.* 2001, Sass *et al.* 2001, Vetriani *et al.* 2003) habitats. As revealed by our analysis, 45 different and novel phylotypes of *Betaproteobacteria* cooccurred in the same 100 ml water sample and hence in the same chemocline microbial community sampled in the same year. Of the combined dataset, 53 of the phylotypes were closely related and exhibited a sequence divergence of < 3% (Fig. 4), indicating that chemoclines of freshwater lakes may provide a multitude of ecological niches not only for phylogenetically distant and physiologically different groups like sulfate-reducing bacteria, methanogenic archaea, purple and green sulfur bacteria or sulfur oxidizing *Gamma-* or *Epsilonproteobacteria* (Overmann *et al.* 1999, Vetriani *et al.* 2003) but also for phylogenetically much closely related groups of aquatic bacteria. It remains to be elucidated whether the considerable microdiversity is limited to the newly discovered subcluster of *Betaproteobacteria* or also occurs among the other phylogenetic groups present in the chemocline.

Comparison of the interoperon spacer sequences of all our clones revealed a much higher sequence variability as compared to that of the 16S rRNA gene sequences. Moreover, the length of IOS regions varied considerably, ranging between 195 bp as in the case of the central bacterium of "*C. aggregatum*" to 466 bp in clone C1 from Lake Cisó (Fig. 6). The vast majority of interoperon spacers exhibited lengths between 340 and 349 bp.

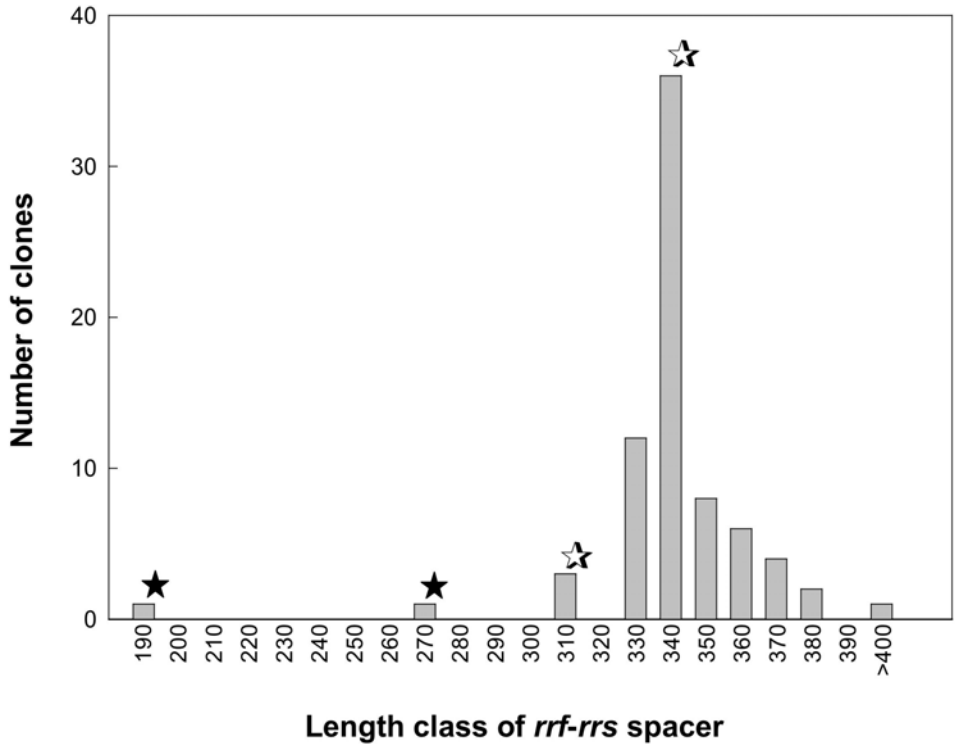

Fig. 6. Frequency distribution of lengths of all *rrf-rrs* spacer sequences cloned in the present study. The lower boundary of each length class is indicated (i.e., 190 for the length class 190-199). Filled asterisks (★) mark the 195 bp long *rrf-rrs* spacer sequence of the central bacterium of "*C. aggregatum*" and the 276 bp long *rrf-rrs* spacer sequence of the central bacterium of the newly discovered green "*Chlorochromatium*". Hollow asterisks (☆), indicate the length classes of the four clones which potentially originate from the central bacterium of "*Pelochromatium roseum*".

4.4.4 Identification of novel types of central symbionts of phototrophic consortia by FISH

One goal of the present investigation was to establish a method which permits a more selective retrieval of 16S rRNA gene sequences of the chemotrophic partner bacterium in phototrophic consortia and their phylogenetic relatives, and to subsequently analyse the phylogenetic relatedness of central bacteria originating from different types of phototrophic consortia.

We hypothesised that, if different phylotypes of central bacteria occur in different phototrophic consortia, they may exhibit a tandem arrangement of their *rrn* operons similar to the central bacterium of "*C. aggregatum*". Previous molecular analysis of the phototrophic consortia indigenous to Lake Dagow identified four different types, namely "*Chlorochromatium aggregatum*", "*Chlorochromatium magnum*", "*Pelochromatium roseum*" as well as "*Pelochromatium latum*" (Overmann *et al.* 1998, Glaeser and Overmann 2004). By comparison, the number of sequence types with tandem *rrn* arrangement in this lake was estimated to be two orders of magnitude larger (193 - 235; see preceding section). Of this large diversity, 45 different phylotypes, corresponding to only about 19 - 23% were covered by our clone library, implying that the probability to recover sequences of central bacteria of consortia was rather small.

Most notably, however, the interoperon spacer sequence of the central bacterium of "*C. aggregatum*" was the shortest among all clones of our libraries (195 bp; Fig. 6). We therefore employed fluorescence *in situ* hybridization (FISH) to check whether other clones with unusually short IOS sequences (marked by asterisks in Fig. 6) possibly originated from the central bacteria of other phototrophic consortia. Accordingly, FISH oligonucleotide probes were designed targeting a 16S rRNA sequence of a clone with a 276 bp long IOS sequence (clone 27GS4, Fig. 4; probes 27GS4-208 and 27GS4-454, Supplementary table 2) and two sequences with a 318 bp long IOS sequence (clones D4a and 53GS2, Fig. 4; probe D4a-443, Supplementary table 2). However, the latter probe also detected two closely related sequences (38GS4, 66GS4) which were present in clones with a 347 bp-long IOS sequence. In order to also cover some additional 16S rRNA sequence types representing different phylogenetic affiliations and different lengths (343-375 bp) of the IOS sequence, the five oligonucleotide probes (D1-444, D2a-454, D2b-447, D3-210, D4b-453; Supplementary table 2) were constructed to target the five 16S rRNA sequence types D1, D2a, D2b, D3, D4b (Fig. 4). All probes were then applied to a sample from the chemocline of Lake Dagow obtained in the year 2006.

Six out of the seven FISH probes or probe combinations yielded a positive FISH result (Fig. 7A-F). Only sequence type D4b, which had been detected in 1996, could not been found again in the chemocline community of the year 2006. Probes D1-444, D2a-454, D2b-447, D3-210 were observed to bind to long rod-shaped, short rod-shaped or coccal free-living

planktonic bacteria (Fig. 7A-D). In contrast, the oligonucleotide probes targeting phylotypes with the shortest IOS sequences (clone 27GS4, clones D4a/53GS2) specifically hybridized to the central rod-shaped bacteria of phototrophic consortia (Fig. 7E,F). The two probes targeting clone 27GS4 hybridised to the central bacterium of a novel type of phototrophic consortium with green epibionts (Fig. 7F). Second, the probe targeting the sequence types D4a/53GS2/38GS4/66GS4 hybridised to the majority of the phototrophic consortia present in the chemocline of Lake Dagow. In Lake Dagow, "*Pelochromatium roseum*" represents the by far dominating type of consortium (Overmann *et al.* 1998, Glaeser and Overmann 2004). Therefore, it is concluded that D4a/53GS2/38GS4/66GS4 represent candidate sequences for this brown-coloured phototrophic consortium.

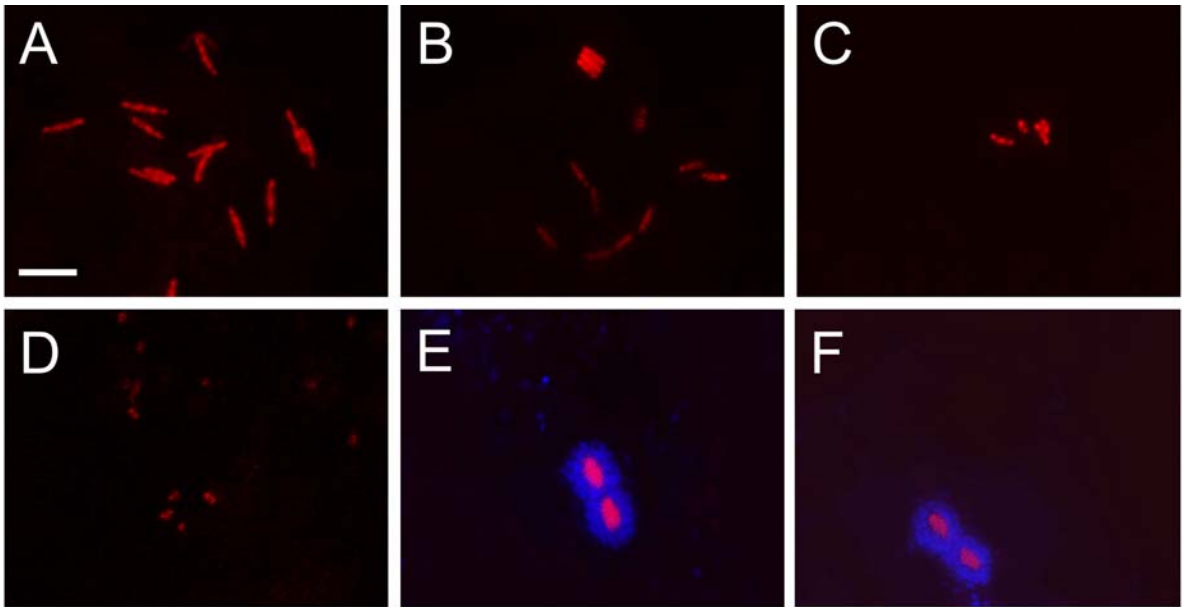

Fig. 7. **A–E**. Specific detection of different phylotypes obtained in the DNA sample from Lake Dagow (1996). All probes were labelled with Cy3. **A-D**. Free-living *Betaproteobacteria* detected with probe DagowD1-444 (**A**) Dagow2a-454 (**B**) Dagow2b-447 (**C**) and Dagow3-210 (**D**). **E.** Overlay of DAPI and Cy3 fluorescence image after FISH employing probe Dagow4a-443. **F.** Overlay of DAPI and Cy3 fluorescence image after FISH employing the two different oligonucleotide probes Dagow27GS4-208 and Dagow27GS4-454. Bar = 10 μm

Targeting specifically those environmental clones with a short intergenic spacer sequence of the tandem *rrn* operons thus resulted in the identification of two novel phylotypes of central bacteria in addition to the symbiont of "*C. aggregatum*". Interestingly, our comprehensive phylogenetic analysis demonstrated that the three known phylotypes of central bacteria (highlighted in Fig. 4) do not form a distinct group with the new subcluster of the *Betaproteobacteria*, indicating that these central bacteria of phototrophic consortia have a polyphyletic origin.

4.5 CONCLUSIONS

All chemotrophic symbionts of phototrophic consortia identified so far are members of a newly discovered subcluster *Betaproteobacteria* with a tandem *rrn* operon organisation. Our combined phylogenetic analyses as well as the different bootstrap analyses imply that the chemotrophic symbiont of the novel green-coloured "*Chlorochromatium*" is most closely related to that of "*Pelochromatium roseum*". However, both these phylotypes are more closely related to free-living *Betaproteobacteria* like phylotypes D1, D2a, D2b or D3, rather than the central bacterium of "*Chlorochromatium aggregatum*" (Fig. 5). The current phylogeny of the chemotrophic partner bacteria therefore reveals a polyphyletic origin of these bacteria, similar to the phylogeny of their phototrophic counterparts (Glaeser and Overmann 2004). This suggests that the ability to form a symbiotic association with green sulfur bacteria either arose independently from different ancestors or, alternatively, may have been present in a common ancestor prior to the radiation of the newly discovered subcluster of *Betaproteobacteria* and a transition to the free-living state in independent lineages.

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5 *CHLOROBIUM CHLOROCHROMATII* SP. NOV., A SYMBIOTIC GREEN SULFUR BACTERIUM ISOLATED FROM THE PHOTOTROPHIC CONSORTIUM "*CHLOROCHROMATIUM AGGREGATUM*"

5.1 ABSTRACT

A symbiotic green sulfur bacterium, strain CaD, was isolated from an enrichment culture of the phototrophic consortium "*Chlorochromatium aggregatum*". The capability of the epibiont to grow in pure culture indicates that it is not obligately symbiotic. Cells are Gram-negative, nonmotile, rod-shaped and contain chlorosomes. Strain CaD is obligately anaerobic and photolithoautotrophic, using sulfide as electron donor. Acetate and peptone are photoassimilated in the presence of sulfide and hydrogencarbonate. Photosynthetic pigments are bacteriochlorophylls *a* and *c*, and γ-carotene and OH-γ-carotene glucoside laurate as dominant carotenoids. In cells from pure cultures, chlorosomes are equally distributed along the inner face of the cytoplasmic membrane. In contrast, the distribution of the chlorosomes in symbiotic epibiont cells is uneven, with chlorosomes being entirely absent at the site of attachment to the central bacterium. Symbiotic epibiont cells display a conspicuous additional layered structure at the attachment site. The G+C content of genomic DNA of strain CaD is 46.7 mol%. On the basis of 16S rRNA sequence comparison, the strain is distantly related to *Chlorobium* species within the green sulfur bacteria phylum (≤ 94.6% sequence homology). The novel isolate is therefore described as a novel species within the genus *Chlorobium*, *Chlorobium chlorochromatii*.

5.2 INTRODUCTION

Green sulfur bacteria (*Chlorobiaceae*) are anoxygenic and obligately photoautotrophic bacteria. They occur in the chemocline of stratified lakes where light reaches sulfide-containing water layers (van Gemerden and Mas 1995). Within the bacterial radiation, all known green sulfur bacteria form a distinct phylum, which comprises also several environmental 16S rRNA gene clones (Overmann 2001a). Green and brown pigmented *Chlorobiaceae* species are known. The brown species contain bacteriochlorophyll (BChl) *e* and, with one known exception (Glaeser *et al*. 2002), the carotenoids isorenieratene and β-isorenieratene as the major light-harvesting pigments. Green-colored species contain bacteriochlorophyll *c* or *d* and chlorobactene and OH-chlorobactene as the dominant carotenoids (Imhoff 1995). In addition, BChl *a* is present in minor amounts in all green sulfur bacteria. The light-harvesting pigments are located in antenna structures called chlorosomes (Cohen-Bazire *et al*. 1964, Staehelin *et al.* 1978), which are 70-180 nm long and 30-60 nm wide ovoid bodies and are attached to the cytoplasmic face of the cellular membrane.

Particular green sulfur bacteria exist in a highly structured association with chemotrophic *Betaproteobacteria*. Most of these so-called phototrophic consortia consist of a motile, colorless, rod-shaped bacterium surrounded by 13-69 green sulfur bacteria (the epibionts) (Fröstl and Overmann 2000, Overmann 2001b, Overmann and Schubert 2002). Phototrophic consortia are meanwhile known for one century (Buder 1914, Lauterborn 1906). They occur in numerous stratified lakes worldwide (Glaeser and Overmann 2004, Overmann *et al*. 1998) where they can amount to two-thirds of the total bacterial biomass in the chemocline (Gasol *et al*. 1995). Phototrophic consortia are therefore likely to play a significant role in the biochemical cycles of stratified aquatic ecosystems. Fluorescence *in situ* hybridisation has revealed that up to 88% of all green sulfur bacteria in stratified lakes can be associated with phototrophic consortia (Glaeser and Overmann 2003a), indicating a considerable selective advantage of the symbiosis for the green sulfur bacterial partner.

None of the epibiont 16S rRNA gene sequences has been detected in free-living green sulfur bacteria so far (Glaeser and Overmann 2004), suggesting that the green sulfur bacterial epibionts are specifically adapted to life in the associated state. Nevertheless, based on stable carbon discrimination ($\delta^{13}C$) values of specific lipid biomarkers, epibionts of phototrophic

consortia grow photoautotrophically *in situ* like their free-living counterparts and do not seem to utilize organic carbon compounds supplied by the central bacterium (Glaeser and Overmann 2003a). However, several lines of evidence indicate that a rapid signal transfer occurs between the epibionts and the central bacterium (Fröstl and Overmann 1998, Glaeser and Overmann 2003b) and that cell division occurs in a tightly coordinated fashion (Overmann *et al*. 1998). In order to resolve the molecular basis of the interaction in phototrophic consortia, the biochemistry and physiology of both types of bacteria need to be elucidated in more detail. It is therefore necessary to isolate the partner bacteria in pure culture.

Almost 50 years ago, the isolation of a green sulfur bacterium from a natural population of the phototrophic consortium "*Chlorochromatium aggregatum*" was claimed (Mechsner 1957). Unfortunately the strain was lost before detailed physiological studies could be made. Here, we report the isolation in pure culture of the epibiont from "*C. aggregatum*" using improved cultivation techniques. We demonstrate that the strain reveals distinct differences to all validly decribed species of green sulfur bacteria. Consequently, the strain is described as a novel species.

5.3 MATERIALS AND METHODS

5.3.1 Source of organisms and cultivation

For isolation of the epibiont, a laboratory enrichment culture of "*Chlorochromatium aggregatum"* (Fröstl and Overmann 1998) was employed. The enrichment originated from lake Dagow (Brandenburg, Germany), a small eutrophic freshwater lake 100 km north of Berlin (Overmann *et al.* 1998). The defined mineral medium K3 was used for the maintenance of "*C. aggregatum*" enrichments and for the isolation of the epibiont. The medium contained per liter of double distilled water: KH_2PO_4, 0.25g; NH_4Cl, 0.05g; $MgCl_2 \cdot 6H_2O$, 0.05g; $CaCl_2 \cdot 2H_2O$, 0.05 g; and $NaHCO_3$, 1.5 g. After autoclaving, the medium was cooled to room temperature under an atmosphere of CO_2 (10 kPa, 30 min) and then kept under N_2/CO_2 (7 kPa/7 kPa). $NaHCO_3$ was autoclaved separately and added to the medium after cooling. Na_2S was used as reducing agent and as electron donor for anoxygenic photosynthesis at a final concentration of 0.5 mM. The pH was adjusted to 7.5 by addition of KOH solution.

Finally, sterile lipoic acid solution (0.25 ml of a 100 mM stock solution, Bast 2001), 1 ml seven vitamin solution (Pfennig 1978), and 1 ml trace element solution SL10 (Widdel *et al*. 1983) were added per liter of medium. Before inoculation with "*C. aggregatum*", 2-oxoglutarate was added to a final concentration of 0.5 mM from a freshly prepared and sterile filtered stock solution. In order to maintain a growing enrichment, cultures were supplemented with 0.5 mM of 2-oxoglutarate every second day.

Chlorobium limicola DSMZ 245^T was grown under standard conditions in sulfide-containing media supplemented with 3 mM acetate (Overmann and Pfennig 1989). *Chlorobaculum tepidum* ATCC 49652^T was grown at 46°C and a light intensity of 1000 μmol·m^{-2}·s^{-1} of tungsten lamp bulbs in modified CP-medium (Frigaard and Bryant 2001).

5.3.2 Isolation of the epibiont

In order to generate a highly enriched inoculum for the subsequent isolation of the epibiont, phototrophic consortia were first concentrated exploiting their chemotactic behavior. Chemotaxis experiments were set up essentially as described earlier (Fröstl and Overmann 2000, Glaeser and Overmann 2003b). Flat microscopic glass chambers (area 20 x 20 mm; height 3 mm) were custom-made from a microscopic slide, using coverslips as spacers and as the top lid. Three sides were sealed with paraffin and the chambers filled with the "*C. aggregatum*" enrichment culture. Rectangular capillaries (length, 50 mm; inner diameter, 0.1 x 1.0 mm; capacity 5 μl; Vitro Dynamics, Rockaway, NJ, USA) were filled with K3 medium supplemented with 1 mM sulfide. The capillaries were sealed with plasticine at one end and then inserted with the open end into the chemotaxis chamber. Afterwards, the chamber was completely sealed with paraffin and incubated for 2 hours at 15°C in dim light. The chemotactic enrichment of consortia was monitored microscopically in dark field. Capillaries containing accumulated consortia were withdrawn, their surface cleaned with 70% (v/v) ethanol and the first 5 mm from their open end were clipped off in order to separate contaminating bacteria. The remaining volume of the capillaries was used to inoculate fresh K3 medium.

Pure cultures were obtained by repeated deep agar dilution series (Pfennig 1978). To maintain highly reducing conditions throughout, 200 μM dithionite was added to the K3 medium immediately before preparation of the dilution series. A variety of carbon substrates

were tested for stimulation of the growth of the epibiont. 2-oxoglutarate, pyruvate, propionate or acetate were added in separate trials to a final concentration of 1 mM. Fermented rumen extract and fermented yeast extract were tested as growth factors. Rumen fluid is a well-established source of growth factors (volatile fatty acids, vitamins and hemin) and known to stimulate growth of various anaerobes like *Bacteroides*, *Eubacterium*, *Ruminococcus*, *Selenomonas* and *Treponema* species (Caldwell and Bryant 1966). For the preparation of fermented rumen extract, fresh rumen fluid was incubated for 4 days in the dark at 32°C, the liquid was clarified by centrifugation (20000 x g for 30 min at 4°C), the supernatant filtered in sterilized glass bottles (Millipore polycarbonate filters; pore size, 0.1 μm) and stored at -20°C until usage. For production of fermented yeast extract, 20 ml of swamp sediment was mixed with 8 g yeast extract, 80 ml tap water were added and the mixture incubated for 3 days at 25°C. The supernatant was clarified and sterile filtered as described above. Both growth factor solutions were used at a final concentration of 0.002% (v/v).

After incubation for 6 weeks in the light, macroscopically visible green-colored colonies had appeared in some of the agar tubes. Individual colonies were withdrawn from the agar with sterile Pasteur pipettes and resuspended in 5 ml of K3 medium containing glass beads. The colonies were broken up by shaking and 0.5 ml aliquots of the resulting cell suspension were used for immediate inoculation of a second series of deep agar tubes.

5.3.3 Confirmation of the isolation and phylogenetic characterization of the epibiont

In order to confirm the origin of the epibiont, the 16S rRNA gene sequence of the isolate was compared to that of the epibiont present in intact consortia. Individual "*Chlorochromatium aggregatum*" consortia were mechanically separated from the laboratory enrichment using a micromanipulator connected to an inverted microscope (Fröstl and Overmann 2000). Ten consortia were collected in a PCR tube, and directly subjected to PCR amplification. In parallel, the 16S rRNA gene sequence of the isolate was amplified from a cell pellet of the pure culture. Bacterial sequences of *Chlorobiaceae* were selectively amplified by employing oligonucleotide primers GC 357f and GSB 840r and specific PCR conditions (Overmann *et al*. 1999) in a GeneAmp PCR system 9700 (Applied Biosystems, Weiterstadt, Germany). The

generated DNA fragments were then separated and compared by PCR-DGGE fingerprinting using a 6% (w/v) polyacrylamide DGGE gel containing a linear gradient of 35% to 70% denaturing agents which was run at 200 V for 12 hours (Overmann *et al*. 1999). After staining with ethidium bromide, DNA bands were cut from the DGGE gel, recovered by electroelution, reamplified, and fragments with identical melting behaviour were sequenced to confirm sequence identity (Overmann et al. 1999).

5.3.4 Growth experiments

Pure cultures of the epibiont were grown in standard SL10 medium for green sulfur bacteria (Overmann and Pfennig 1989) supplemented with 3 mM Na-acetate and with the pH adjusted to 7.2. Cultures of the epibiont were maintained at 25°C in continuous light (50 μmol quanta·m^{-2}·s^{-1}; Osram tungsten lamp, 60 W). Light intensities were determined with a Li Cor LI-189 quantum meter equipped with a LI-200 SA pyranometer sensor (wavelength range, 380 – 1100 nm; Li Cor, Lincoln, Neb., USA). Optimum conditions for growth of the epibiont were determined with pure cultures incubated in 22 ml screw cap tubes. Growth was followed by measuring optical density at 750 nm (Bausch & Lomb Spectronic 20 photometer). At this wavelength and in media containing acetate, the formation of extracellular sulfur globules did not interfere with OD measurements.

Photoassimilation of organic carbon substrates was tested for 110 different compounds (the complete list is available upon request). In addition, the utilization of Fe^{2+} chelated by different agents was tested in growth experiments. For the preparation of Fe^{2+}-complexes, chelating agents (0.1 mM) were dissolved under anoxic conditions in an anaerobic chamber and an equimolar amount of $FeCl_2$ was added. Afterwards, the solutions were filtered (Millipore polycarbonate filters; pore size, 0.1 μm).

5.3.5 Pigment analyses

Absorption spectra were monitored in a Lambda 25 UV/VIS spectrophotometer (Perkin Elmer, Rodgau-Jügesheim, Germany). Spectra of whole cells were determined during exponential growth using cells resuspended in 60% (w/v) sucrose solution. For HPLC analyses, bacterial cells were harvested by centrifugation (15 min at 9770 × g and 4°C) using Pyrex glass centrifuge tubes and the pellets extracted in the dark in HPLC grade

acetone/methanol (7/2, v/v) for 30 min at 4°C in a sonification bath and then at 4°C over night. The extract was clarified by centrifugation and the supernatant transferred to a brown glass tube and dried in a stream of nitrogen gas. The pigments were redissolved in methanol/acetonitrile (80/15, v/v) and 0.1 volume of 1 M aqueous ammonium acetate solution was added as an ion pairing agent 10 min before injection into an HPLC system (Dionex, Munich, Germany). The composition of pigment extracts was analyzed by the method B of Airs *et al*. (2001). Pigments were separated using a multospher 120 RP18 (4.6 × 250 mm, 5 µm mesh) HPLC column (CS- Chromatography, Langerwehe, Germany), starting with 0.01 M ammonium acetate / methanol / acetonitrile (5/80/15, v/v/v) as the mobile phase for 5 min. Subsequently, the composition of the solvent was changed linearly to 0.01 M ammonium acetate / methanol / acetonitrile / ethyl acetate (1/32/15/52, v/v/v/v) over 76 min. Analyses were performed at a flux rate of 0.7 ml·min^{-1} and at 20°C. Pigment absorption spectra were recorded between 270 and 800 nm using a diode array spectrophotometer (PDA-1000, Dionex). For identification of individual carotenoids, extracts *Chlorobium tepidum* ATCC 49652^{T} with a known composition (Frigaard *et al*. 2004) were analyzed in parallel. For quantification of the carotenoids, the following absorption coefficients were used (in l·g^{-1}·cm^{-1}): for chlorobactene and γ-carotene, 265 at a wavelength of 490 nm; for OH-γ-carotene glucoside laurate, 158 at 490 nm (Frigaard *et al.* 2004); for unidentified carotenoids, 250 at 490 nm (Züllig 1985).

5.3.6 Cell surface hydrophobicity

The cell-surface hydrophobicity of the epibiont was determined in a quantitative assay with *n*-hexadecane (Rosenberg *et al*. 1980). The OD_{650} of an epibiont culture was measured and 3 ml subsamples were transferred into screw cap test tubes containing 0.5 ml of *n*-hexadecane. The tubes were flushed with nitrogen for 3 minutes, incubated for 10 minutes, and then homogenized on a vortex mixer for 2 minutes. After phase separation the lower aqueous phase was carefully removed with a Pasteur pipette and its OD_{650} determined again. The hydrophobicity index was calculated from the OD of the original cell suspension (OD_{650}) and the OD after the treatment (OD_{650}') according to: $H\,[\%] = 100 \cdot (OD_{650} - OD_{650}')/OD_{650}$

5.3.7 Electron microscopy

Cells were fixed immediately with 2.5% glutardialdehyde in 75 mM sodium cacodylate, 2 mM MgCl2, pH 7.0, for 1 h at room temperature. After several rinses in fixative buffer, a post-fixation step followed for 1 h with 1% osmium tetroxide in fixative buffer at room temperature. After two washing steps in distilled water, the cells were stained *en bloc* with 1% uranyl acetate in 20% acetone for 30 min. Dehydration was performed with a graded acetone series. Samples were then infiltrated and embedded in Spurr's low-viscosity resin (Spurr 1969). After polymerisation, ultrathin sections (50 and 70 nm) were cut with a diamond knife and mounted on uncoated copper grids. The sections were post-stained with aqueous lead citrate (100 mM, pH 13.0). All micrographs were taken with an EM 912 electron microscope (Zeiss, Oberkochen, Germany).

5.3.8 Sequencing and phylogenetic analysis

Sequences were obtained employing the Big Dye™Terminator Cycle Sequencing Kit (Applied Biosystems) and the ABIPrism™ 310 genetic Analyzer (Applied Biosystems GmbH). The almost complete 16S rRNA gene sequence of the pure culture of the epibiont was obtained as outlined before for other pure cultures (Gich *et al*. 2005).

Phylogenetic analysis of 16S rRNA gene sequences was performed using the ARB phylogeny package (Ludwig *et al*. 1998). The program Fast Aligner V1.03 was employed for alignment of all 16S rRNA gene sequences of green sulfur bacteria as available through the National Center for Biotechnology Information website (Altschul *et al*. 1997). The sequence of *Chloroherpeton thalassium* ATCC 35110^T was chosen as the outgroup, since it has been shown to branch at the root of all known *Chlorobiaceae* (Overmann 2001a). The alignment was manually corrected based on secondary structure information for *Chlorobium vibrioforme* ATCC 6030, and a phylogenetic tree was constructed from all sequences longer than 1300 bp, using the maximum likelihood program Fast DNAML. Distance matrices were calculated employing the algorithm of Kimura (Kimura 1980) by using the Phylip Distance Matrix program, version 3.6a3 within the ARB phylogeny package.

The accession number of the almost full-length 16S rRNA gene sequence of the epibiont is AJ578461.

5.3.9 Determination of the mol% G + C content

The mol% guanidine plus cytosine of genomic DNA was determined according to Mesbah *et al*. (1989). DNA was hydrolyzed with P1 nuclease, the nucleotides dephosphorylized with bovine alkaline phosphatase, and the resulting deoxyribonucleosides were analyzed by HPLC. These analyses were performed by the DSMZ (Braunschweig, Germany).

5.4 RESULTS AND DISCUSSION

5.4.1 Isolation of the epibiont of "*Chlorochromatium aggregatum*"

The enrichment of "*C. aggregatum*" originating from Lake Dagow (Fröstl and Overmann 1998) was used for the isolation of the epibiont. In these enrichments, 10^5 to 10^6 consortia were present per ml. Similar to all other consortia investigated to date (Glaeser and Overmann 2004), "*C. aggregatum*" harbors only one single phylotype of green sulfur bacteria. This phylotype could be detected after separation of consortia by micromanipulation, amplification of 16S rRNA gene fragments of green sulfur bacteria by group-specific PCR and separation of the amplificates by DGGE (Fig. 1). Since a cultivation of consortia separated by micromanipulation never yielded growing cultures, the enrichments had to be used in subsequent cultivation attempts.

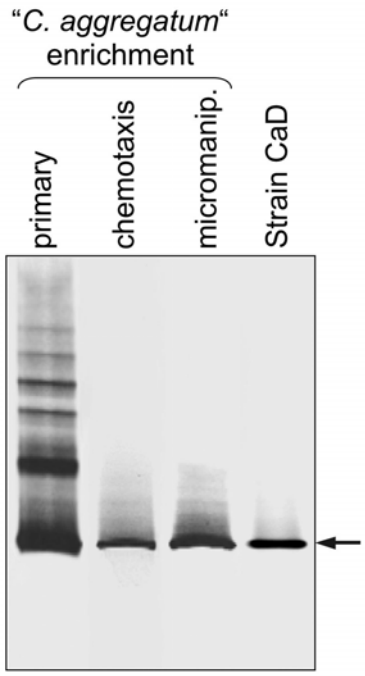

Figure 1. DGGE fingerprints of green sulfur bacteria from the primary enrichment (*primary*), from chemotactically enriched "*Chlorochromatium aggregatum*" (*chemotaxis*), from "*C. aggregatum*" mechanically separated by micromanipulation (*micromanip.*) as compared to the isolated epibiont CaD. *Arrow* indicates melting position of the 16S rRNA gene fragment of the epibiont as confirmed by sequence comparison.

However, analysis of the enrichment by PCR-DGGE revealed the presence of several different phylotypes of green sulfur bacteria (Fig. 1). Since all known free-living green sulfur bacteria are immotile, the chemotaxis of "*C. aggregatum*" consortia towards hydrogen sulfide (Kanzler *et al*. 2005) was exploited in the present work to separate live consortia from accompanying green sulfur bacteria. Individual capillaries containing 10^2-10^3 "*C. aggregatum*" consortia were used for subsequent inoculation of liquid K3 medium. After growth of these secondary enrichments, group-specific DGGE fingerprinting confirmed that only one single 16S rRNA gene sequence type of green sulfur bacteria was left. The melting behavior of this fragment was identical to that amplified from mechanically separated "*C. aggregatum*" (Fig. 1, arrow).

In the secondary enrichments, the green sulfur bacteria were also observed as single cells. This observation for the first time indicated that the epibiont was capable of growing in a non-associated state. Therefore, deep agar dilution series were employed to isolate the

epibiont in pure culture. Because the numbers of contaminating colorless bacteria exceeded those of free epibionts, differential centrifugation (650 x g, 5 min) was used to enrich the epibiont cells prior to dilution in deep agar media. The pellets thus generated contained mainly epibionts and phototrophic consortia and were directly transferred to liquefied agar media supplemented with various organic carbon sources and growth factors. After incubation for at least 4 weeks, small green colonies appeared in deep agar series, which had been supplemented with 200 μM dithionite and fermented rumen extract. None of the other additives promoted growth of the epibiont in the agar media. The dependence on highly reducing conditions for cultivation in deep agar media is not an unique property of strain CaD, but was also described to be essential for the isolation of two free living green sulfur bacteria, *Chl.* (formerly *Pelodictyon*) *phaeoclathratiforme* DSMZ 5477^T (Overmann and Pfennig 1989) and a low-light-adapted *Chlorobium* (strain MN1) from the Black Sea (Overmann *et al*. 1992).

Colonies from the highest dilutions were picked, resuspended in K3 medium, and used immediately for a subsequent passage through deep agar media. After growing in the second deep agar series, colonies were isolated from the highest dilutions and inoculated into liquid media. Similar to the agar media, green sulfur bacteria in the small picked colonies could divide exclusively in the presence of dithionite and fermented rumen extract. One of the resulting strains (CaD) was chosen for subsequent characterization. Its 16S rRNA gene fingerprint was identical to those of epibionts from consortia, which had been isolated by micromanipulation (Fig. 1, arrow). Sequencing of these fingerprints yielded identical nucleotide sequences, confirming that the generated culture indeed contained the epibiont of "*C. aggregatum*" (compare Fig. 4). Purity of the culture was checked by microscopic analysis. PCR/DGGE-fingerprinting of eubacterial 16S rRNA genes present in the culture of strain CaD yielded only one band (data not shown).

5.4.2 Morphology and cell surface hydrophobicity

Individual cells of the isolated epibiont were 2.7 (± 0.6) μm long and 0.5 (± 0.1) μm wide nonmotile rods. Some cells occurred in short chains (Fig. 2A). Cells were Gram variable upon staining (Bartholomew 1962). Similarly, the KOH test (Gregersen 1978) resulted in ambiguous results. Electron micrographs of thin sections revealed that epibiont cells exhibit

a typical Gram negative cell wall structure in which the outer and cytoplasmic membranes were clearly distinguishable (Fig. 2C). Electron microscopy also demonstrated the presence of chlorosomes (Fig. 2B), which represent the typical light harvesting structures of green sulfur bacteria (Cohen-Bazire and Sistrom 1966, Olson 1980). In pure cultures, chlorosomes were equally distributed over the inner face of the cytoplasmic membrane. In contrast, gaps devoid of chlorosomes were observed in epibiont cells associated with intact consortia (Fig. 2D). These gaps existed exclusively at the sites of cell-cell-contact to the central bacterium and featured an additional layered structure, which was never observed in epibiont cells of pure cultures (Fig. 2D, arrows). Intracellular sorting of chlorosomes and specific structures at the cell-cell-contact thus are specific for the symbiotic state. Based on our previous work, several lines of evidence indicate that a specific signal exchange occurs between the epibionts and the central bacterium in intact phototrophic consortia. Firstly, cell division of epibionts and the central bacterium proceed in a highly coordinated fashion (Overmann *et al*. 1998). Secondly, phototrophic consortia exhibit a scotophobic response in which epibionts function as light sensors whereas the central bacterium confers motility to the entire cell aggregate (Fröstl and Overmann 1998). Thirdly, incorporation of 2-oxoglutarate of intact consortia most likely is mediated by the central bacterium, but occurs only in the presence of light and sulfide, which are utilized by the epibionts (Glaeser and Overmann 2003b). Our present data now indicate that signal exchange between the bacterial partners in phototrophic consortia also causes an asymmetric distribution of chlorosomes and hence conspicuous changes in the cellular morphology of the epibiont.

The hydrophobicity index of free epibiont cells was 8.6%. Formation of aggregates due to cell-to-cell adhesion in the purple sulfur bacterium *Amoebobacter purpureus* (Overmann and Pfennig 1992), interactions between bacteria and phagocytes (Cunningham *et al*. 1975), and adhesion to substrates (Bryant *et al*. 1983) or surfaces (Marshall *et al*. 1971, Fletcher and Floodgate 1973) are partially mediated by hydrophobic interactions. Aggregating *Amb. purpureus* cells exhibit a hydrophobicity index of 96% (Overmann and Pfennig 1992). By comparison, the low hydrophobicity index of the epibiont cells indicates that they, at least under the conditions tested in pure culture, do not form a sufficiently hydrophobic cell surface, which would enable the formation of consortia based on hydrophobic interactions alone.

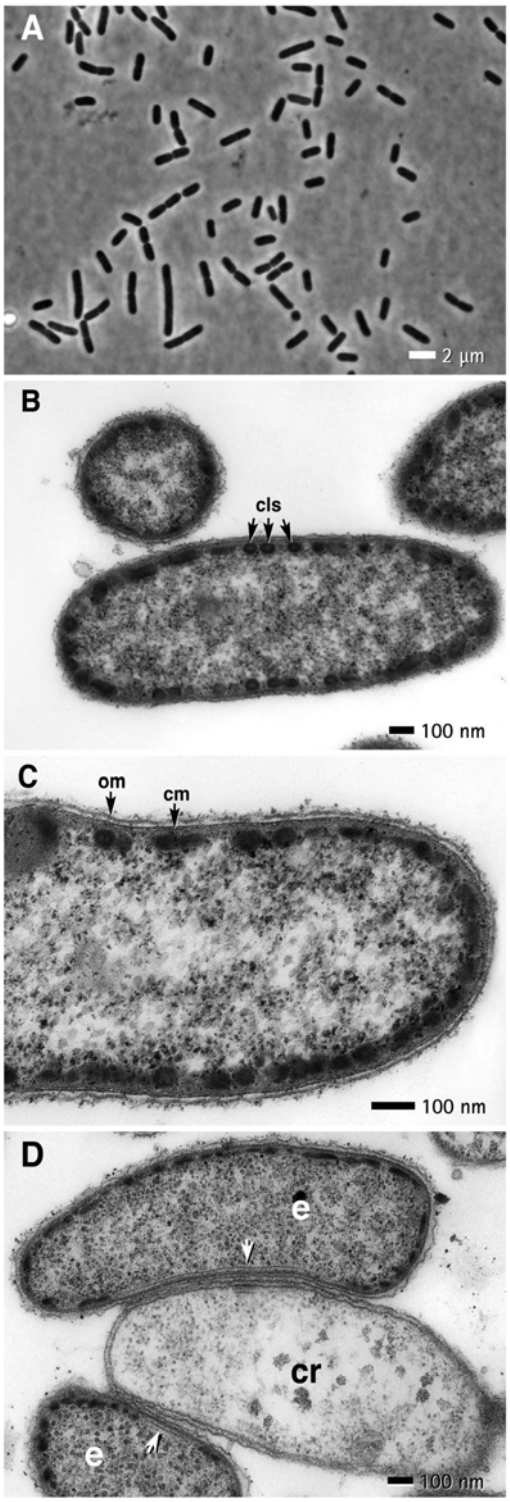

Figure 2. A. Phase-contrast micrograph of cells of a pure culture of strain CaD. **B.** Transmission electron micrograph of ultrathin sections of cell in a pure culture of strain CaD. Note the presences of chlorosomes (*arrows*). **C.** Detail of one epibiont cell in pure culture. The outer and cytoplasmic membranes are visible. **D.** Transmission electron micrograph of two epibiont cells associated to a central rod in an intact consortium. The attachment sites (*arrows*) are characterized by laminar layers and the absence of chlorosomes. *Cls*, chlorosomes, *cm*, cytoplasmic membrane, *cr*, central rod, *e*, epibiont, *om*, outer membrane.

5.4.3 Photosynthetic pigments

Densely grown cultures exhibited a green to olive green color. The absorption spectrum of whole cells showed maxima at 748 and 453 nm. Cell extracts in acetone revealed major peaks at 662 and 433 nm. Both results indicated the presence of bacteriochlorophyll *c* (Fig. 3).

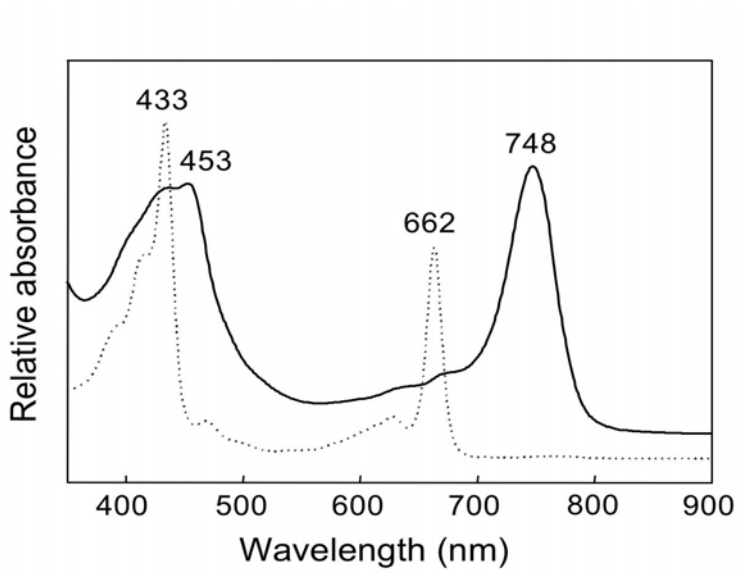

Figure 3. Absorption spectra of whole cells (—) and of acetone extract (••••) of strain CaD.

Four different bacteriochlorophyll *c* homologs esterified with farnesol, [E, M] BChlc_F, [E, E] BChlc_F, [Pr, E] BChlc_F, and [I, E] BChlc_F were detected by HPLC analyses. Similar to *Cba. tepidum* ATCC 49652^T and *Chl. limicola* DSMZ 245^T (Borrego *et al*. 1999), [E, E] BChlc_F and [Pr, E] BChlc_F represented the dominant bacteriochlorophyll *c* homologs in strain CaD. In contrast, the elution profile monitored at 470 nm revealed a distinct carotenoid composition of epibiont strain CaD. A total of 12 different carotenoids could be distinguished. As compared to data from the literature (Frigaard *et al*. 2004) and to the corresponding elution profile of *Cba. tepidum* determined in parallel, two carotenoids had retention times and absorption spectra similar to *trans*- and *cis*-chlorobactene. Two other compounds were identified as OH-γ-carotene glucoside laurate and γ-carotene. Based on this tentative assignment, OH-γ-carotene glucoside laurate represents the major carotenoid in strain CaD, amounting to 24% of the total carotenoid content. The tentatively identified chlorobactene isomers together represents 13% of the total carotenoid content, and γ-carotene amounted to

11%, respectively. In contrast, *Cba. tepidum* contained chlorobactene as the dominant carotenoid which amounted to 61% of all carotenoids. By comparison, OH-γ-carotene glucoside laurate constituted only 7% and γ-carotene only 4% of all carotenoids in *Cba. tepidum*. Our values are comparable to those reported by Takaishi *et al*. (1997) and Frigaard *et al*. (2004) for the same strain. *Chl. limicola* DSMZ 245^T resembled *Cba. tepidum* in that chlorobactene amounted to 54%, OH-γ-carotene glucoside laurate represented 10% and γ-carotene 6% of all carotenoids. By comparison to the strains previously investigated, strain CaD thus contains a significantly higher amount of highly hydrophobic carotenoids, which also comprise six unidentified compounds. Two of these unidentified compounds so far have only been detected in the epibiont. Particular features are the low concentrations of chlorobactene and the absence of its derivatives OH-chlorobactene glucoside, OH-chlorobactene, OH-chlorobactene glucoside laurate and 1′,2′-dihydrochlorobactene. The distinct carotinoid composition of strain CaD indicates that the biosynthetic pathways of carotenoid biosynthesis differ from those found in other green sulfur bacteria.

5.4.4 Physiological properties

Photolithoautotrophic growth of the epibiont occurred only under strictly anoxic conditions with hydrogen sulfide as electron donor. Thiosulfate and elemental sulfur were not utilized. Also Fe^{2+} added in different types of complexes (deferoxamine·Fe or diethylenetriaminepentaacetic acid·Fe) was not used as electron donor.

In the presence of hydrogen sulfide and hydrogencarbonate, only acetate and peptone were photoassimilated. 108 of the substrates tested were not used, including (concentrations in mM) L-(+)-alanine (5), L-(+)-arginine (5), benzoate (2), butyrate (2.5), caproate (0.5; 5), caprylate (0.5; 5), casaminoacids (0.05%), citrate (2), crotonate (0.5; 5), formate (2.5), fructose (5), fumarate (5), glucose (5), L-(+)-glycine (5), glyoxylate (5), L-(+)-lysine (5), malate (5), ornithine (5), 2-oxobutyrate (5), 2-oxoglutarate (= 2-oxoglutarate) (5), 2-oxohexanoic acid (5), 2-oxoisocaproate (5), 2-oxooctanoic acid (5), 2-oxovalerate (5), L-(+)-proline (5), propionate (5), pyruvate (5), L-(+)-serine (5), succinate (10), tartrate (2), oxaloacetate (5), L-(+)-threonine (5), valerate (0.5; 5), L-(+)-valine (5) (the full list is available upon request). In addition, no growth stimulation occured with molecular hydrogen provided as a H_2:N_2 mixture of 20:80 (v/v).

2-oxoglutarate is known to be indispensable for the cultivation of "*Chlorochromatium aggregatum*" in enrichment culture (Fröstl and Overmann 1998). Based on microautoradiography after incubation with radioactively labelled 2-oxoglutarate, this substrate is incorporated by intact consortia (Glaeser and Overmann 2003b). Based on our present results, 2-oxoglutarate is not utilized by the isolated epibiont, however. It is therefore concluded that the uptake of 2-oxoglutarate observed for "*C. aggregatum*" is mediated by the central bacterium.

The limited range (two) of organic substrates utilized for photomixotrophic growth is commensurate with the very limited physiological flexibility of other *Chlorobiaceae*. In contrast to the present results, the use of thiosulfate and atypical substrates like glycerol and malate was reported for a culture obtained from "*C. aggregatum*" five decades ago (Mechsner 1957). It therefore appears possible that the earlier cultivation attempts did not yield the epibiont, but another type of green sulfur bacteria.

The epibiont was capable of nitrogen fixation and required only vitamin B_{12} for growth. With sulfide as electron donor, growth became light saturated above light intensities of 10 μmol quanta·m^{-2}·s^{-1}. A maximum growth rate of 0.04 h^{-1} (doubling time, 17.3 h) was obtained under light-saturated conditions. The epibiont had an optimal growth temperature of 25°C and showed no growth at temperatures ≥40°C. Optimum pH was 7.0 to pH 7.3 and no growth occurred at pH values lower than pH 6.4. In comparison with free-living species of green sulfur bacteria, which mostly exhibit a pH optimum of 6.8 (Overmann 2001a), the pH optimum of growth of the epibiont thus is slightly shifted towards the alkaline range. This may reflect an adaptation to the close association of the epibiont with the central rod in phototrophic consortia. At salinities between 0.4% and 0.7%, the epibiont showed comparable growth rates. A pronounced lag phase of up to 114 hours was observed for cultures growing at salinities between 0.5% and 0.7%. Growth rates declined rapidly at higher salt concentrations. No growth occurred above a salinity of 0.8%.

5.4.5 Phylogeny and GC-content of the epibiont

The newly isolated bacterium is Gram negative, rod shaped and nonmotile. It grows only under strict anaerobic conditions and is obligately phototrophic. The photosynthetic pigment bacteriochlorophyll *c* and chlorosomes are present. Based on its morphological and

physiological properties, strain CaD represents a member of the green sulfur bacteria (Overmann 2001a). So far, only a 540 bp-fragment of the 16S rRNA gene had been sequenced and analyzed phylogenetically (Fröstl and Overmann 2000, Glaeser and Overmann 2004). The phylogenetic analysis of the full-length 16S rRNA gene obtained in this study confirmed this identification, placing the new isolate in the phylum green sulfur bacteria. Strain CaD is related to green sulfur bacteria of group 2 and 3 (group designations according to Imhoff 2003) (Fig. 4). Since all green sulfur bacteria of these groups belong to the genus *Chlorobium*, strain CaD is classified as a member of this genus. However, no close relationship was found to any strain cultured so far; the highest sequence similarity (94.6%) was found to the 16S rRNA gene sequence of *Chlorobium phaeobacteroides* III (Tab. 1).

The G+C content of the epibiont was 46.7 mol%. This DNA base composition is the lowest value known for the genus *Chlorobium* (*Chlorobium clathratiforme* DSMZ 5477, 47.9 mol%) and it is one of the lowest among green sulfur bacteria as a whole (*Chloroherpeton* spp.: 45.0 - 48.2 mol%) (Overmann 2001a).

Table 1. Evolutionary distances between strain CaD and its closest cultured relatives. Values represent percentage of dissimilarity of the 16S rRNA gene sequences corrected for multiple base changes by the method of Kimura (1980)

	1	2	3	4	5	6	7	8	9	10	11	12
1 Strain CaD	-											
2 *Chl. phaeobacteroides* III	5.4	-										
3 *Chl. phaeovibrioides* DSMZ 261	5.5	3.3	-									
4 *Chl. limicola* DSMZ 1855	5.5	0.0	3.3	-								
5 *Chl. limicola* 1630	5.5	0.1	3.4	0.1	-							
6 *Chl. ferrooxidans* DSMZ 13031^T	5.6	4.4	3.5	4.3	4.4	-						
7 *Chl. phaeobacteroides* Glu	5.6	0.0	3.3	0.0	0.1	4.4	-					
8 *Chl. phaeovibrioides* DSMZ 269^T	5.6	3.5	0.0	3.3	3.4	3.6	3.5	-				
9 *Chl. phaeovibrioides* DSMZ 265	5.6	3.5	0.3	3.5	3.6	3.8	3.5	0.3	-			
10 *Chl. limicola* 9330	5.7	0.2	3.6	0.2	0.3	4.5	0.2	3.6	3.8	-		
11 *Chl. limicola* DSMZ 245^T	5.7	0.2	3.4	0.1	0.2	4.6	0.3	3.6	3.6	0.2	-	
12 *Chl. phaeobacteroides* DSMZ 266^T	5.9	3.9	3.9	3.6	3.7	4.1	4.2	3.9	4.3	3.7	3.9	-

The alleged isolation of a green sulfur bacterium from consortia was described almost 50 years ago (Mechsner 1957). However, the strain was lost before being characterized in detail. Whereas the present study did not reveal conspicuous differences with respect to physiology, strain CaD is clearly unique based on the cellular distribution of chlorosomes, the architecture of the cell-cell-adhesion site and the carotenoid composition. Based on these as well as the phylogenetic differences to validly described species of green sulfur bacteria, we propose a new species of the genus *Chlorobium*, *Chlorobium chlorochromatii* with strain CaD as the type strain. *Chlorobium* strain CaD is the first epibiont of phototrophic consortia, which is available in pure culture.

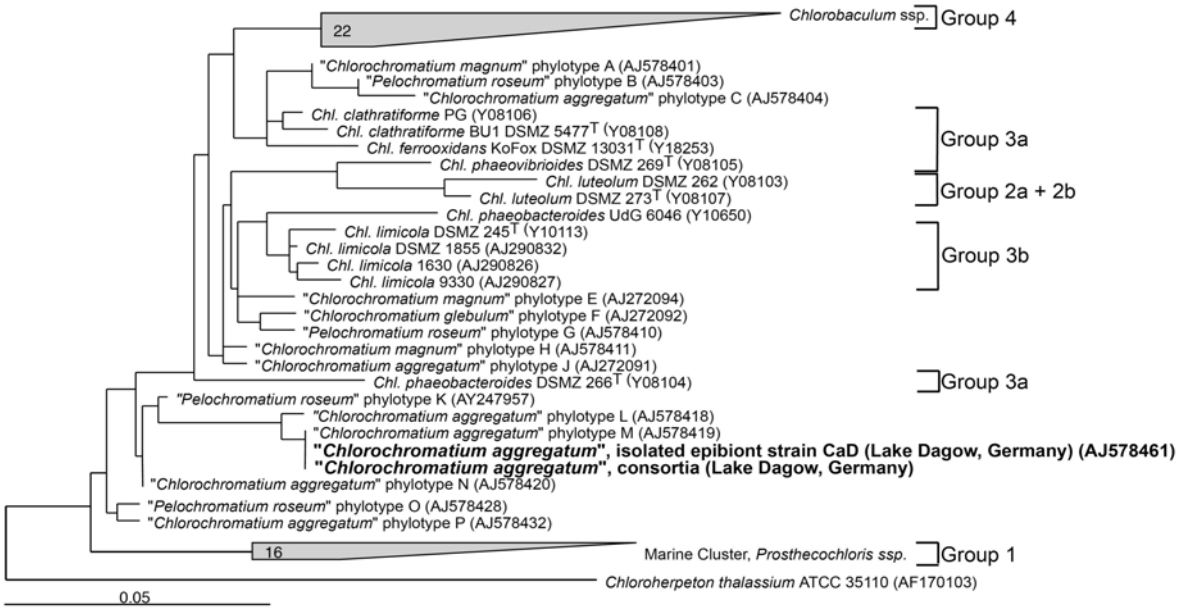

Figure 4. Phylogenetic affiliation of strain CaD. The 16S rRNA gene sequence of picked "*Chlorochromatium aggregatum*" consortia and of the isolate CaD were identical and are indicated in *bold type*. *Numbers* in polygons give number of phylotypes in the corresponding clusters. Groups of *Chlorobiaceae* are given according to Imhoff (2003). Bar = 0.05 fixed point mutation per nucleotide position.

5.4.6 Description of *Chlorobium chlorochromatii* sp. nov.

Chlorobium chlorochromatii sp. nov. chlo.ro′chro.ma′ti.i. Gr. adj. *chloros*, green, yellowish green; Gr. n. *chromatium* color, paint. M.L. gen. n. *chlorochromatii* of, originating from "*Chlorochromatium*".

In laboratory pure culture individual cells are rod–shaped. Cells are 2.7 (± 0.6) µm long and 0.5 (± 0.1) µm wide, nonmotile and Gram negative. In free-living cells, chlorosomes are equally distributed over the inner face of the entire cell. In contrast, cells associated with intact consortia "*Chlorochromatium aggregatum*" lack chlorosomes at the site of attachment to the central chemotrophic bacterium but instead possess additional layered structures at this site. Contains bacteriochlorophyll *c*. The major carotenoids are γ-carotene and its derivatives, mostly OH-γ-carotene glucoside laurate.

Strictly anaerobic and phototrophic. Growth occurs exclusively under anoxic and highly reducing conditions in the light. Electron donor sulfide. Thiosulfate, sulfur flower and molecular hydrogen not utilized. In the presence of sulfide and carbon dioxide, acetate and peptone are photoassimilated. Optimum pH for growth 7.0-7.3. Optimum temperature 25°C. Saturating light intensity 10 µmol quanta·m^{-2}·s^{-1}(daylight fluorescent tubes). NaCl is not required for growth. Growth is inhibited at a salinity of ≥ 0.8%. Vitamin B_{12} required for growth.

Habitat: Isolated from anoxic sulfide containing water of stratified lakes. In nature, the epibiont is so far only found in association with non-cultured *Betaproteobacterium* forming the phototrophic consortium "*Chlorochromatium aggregatum*".

DNA base ratio: 46.7 mol% G+C.

5.5 ACKNOWLEDGEMENTS

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6 OXYGEN TOLERANCE AND PHYSIOLOGICAL PROPERTIES OF SYMBIOTIC AND FREE-LIVING GREEN SULFUR BACTERIA

6.1 ABSTRACT

The physiology of the symbiotic green sulfur bacterium *Chlorobium chlorochromatii* CaD, which was recently isolated from the phototrophic consortium "*Chlorochromatium aggregatum*", has been investigated in order to identify specific adaptations to life in symbiosis. Green sulfur bacteria are inevitably exposed to oxygen thriving in their natural habitat, the chemocline of stratified lakes. Therefore, the effect of molecular oxygen on the obligately anaerobic bacteria was studied in particular. Survival experiments, genetic analyses, as well as enzyme assays revealed that the epibiont of "*C. aggregatum*" tolerates oxygen exposure up to 120 h. The carotenoid composition was determined by spectroscopic methods for *Chl. chlorochromatii* in the free-living as well as for the associated state. 7,8-dihydro-γ-carotene, a hitherto novel carotenoid in nature was detected independently of the growth state. Contrary to all other green sulfur bacteria, which contain chlorobactene as major carotenoid, this pigment is absent in *Chl. chlorochromatii*, indicating a new modified metabolic pathway. Moreover, several carbohydrate compounds could be identified, which are likely to be exchanged within the symbiosis of "*C. aggregatum*".

6.2 INTRODUCTION

Green sulfur bacteria (GSB, *Chlorobiaceae*) are strictly anaerobic organisms that occur in the chemocline, the interface between oxic and anoxic water layers of marine and freshwater ecosystems. The chemocline is characterized by steep, vertical gradients of physiochemical parameters, most importantly light, oxygen, sulfide, inorganic nitrogen compounds, and methane (Jørgensen *et al*. 1979, Overmann *et al*. 1991, van Gemerden and Mas 1995). GSB gain their energy by anoxygenic photosynthesis. They have a unique photosynthetic antenna structure, the chlorosome, which consists of a galactolipid monolayer membrane surrounding a large amount of antenna bacteriochlorophyll (BChl) and carotenoids

(Hirabayashi *et al.* 2004, Prokhorenko *et al.* 2000, Psencik *et al.* 2004). With respect to their pigment composition, GSB can be divided into green-coloured and brown-coloured species. While all GSB contain minor amounts of BChl *a* and Chl *a*, the green species contain BChl *c* or *d* and chlorobactene as major carotenoid, whereas Bchl *e* and isorenierantene is present in the latter group (Frigaard *et al.* 2004, Imhoff 1995). These carotenoids are special by having aromatic end groups and, besides in GSB, occur only in some actinomycetes and a few sponges (Armstrong 1999, Britton 1998).

Anaerobic organisms, like GSB, usually thrive in habitats deprived of oxygen and it was not observed until 1993 in sulfate reducing bacteria, that some anaerobic bacteria have a tolerance towards oxygen exposition (Marshall *et al.* 1993). It has been shown, that carotenoids can provide protection from activated oxygen species and play a role in oxygen detoxification by quenching singlet oxygen ($^1O_2$) (Schroeder and Johnson 1995). Oxidative stress functionally can be defined as an excess of activated oxygen. Oxygen radicals can cause damage to nucleic acids, proteins and lipids (Hill and Allen 1978). Singlet oxygen is generated in the dark by the decomposition of endoperoxides and other types of enzymatic and chemical reactions (Wasserman and Murray 1979). Moreover, it induces DNA damage and oxidizes proteins (Sies and Menck 1992). It has been recognized as a product of excited triplet state porphyrin type II photooxidation in photosynthetic systems and to react with carotenoids (Kautsky and de Bruijn 1931). Although the significance of light-catalyzed formation of $^1O_2$ has been widely discussed, less attention has been paid to $^1O_2$ generation in the dark (Schroeder and Johnson 1995).

Chl. chlorochromatii CaD is a mesophilic GSB that naturally forms a symbiotic association with a rod-shaped *Betaproteobacterium*, resulting in the phototrophic consortium "*Chlorochromatium aggregatum*". Phototrophic consortia consist of a motile, colourless, rod-shaped bacterium, surrounded by a distinct number of green sulfur bacterial epibionts (Fröstl and Overmann 2000). *Chl. chlorochromatii* has never been observed in the single state in nature and until its isolation from an enrichment culture of the phototrophic consortium "*C. aggregatum*", it has been considered as obligately symbiotic (Vogl *et al.* 2006). Nevertheless, no adaptations to symbiosis that could help to elucidate the basis of the symbioses on a physiological basis have been found so far, since its physiology did not show major differences from free-living GSB (Vogl *et al.* 2006). Therefore, in this study, the

physiology of the epibiont in pure culture has been investigated in more detail and compared to the epibiont in the associated state. To supplement our physiological experiments, we did comparative genome analyses of *Chl. chlorochromatii* and other GSB, whose sequences have been determined by the Joint Genome Institute (Walnut Creek, CA, USA; U.S. Department of Energy).

When bacteria are exposed to oxygen, radical oxygen species like superoxide and H_2O_2 are generated by the autooxidation of reduced FeS-proteins and flavoproteins (Storz and Imlay 1999). These reduced oxygen derivatives are strong oxidants, which can harm the cells if they are not immediately removed. Since the natural habitat of GSB is the chemocline, a boundary layer of oxic and anoxic water layers, we focused our study on oxygen tolerance. In this context we analysed the carotenoid composition of *Chl. chlorochromatii*, which is known to influence the detoxification process of active oxygen species. Moreover, the uptake and excretion of carbohydrates was investigated with respect to carbohydrate exchange within the symbiosis.

6.3 MATERIAL AND METHODS

6.3.1 Source of organisms and cultivation

In this study the epibiont of "*Chlorochromatium aggregatum*" was used in pure culture (*Chlorobium chlorochromatii* CaD) as well as in the symbiotic state. The experiments with the epibiont in the associated state were performed with enrichment cultures of "*C. aggregatum*", which were originally obtained from the eutrophic Lake Dagow (Brandenburg, eastern Germany; Fröstl and Overmann 1998). Cultures were grown as described previously in K4 medium at 20 µmol quanta · m^{-2} · s^{-1} (Pfannes *et al.* 2007). *Chl. chlorochromatii* was cultivated at 50 µmol quanta · m^{-2} · s^{-1} in SL10 medium (pH 7.2) for GSB supplemented with 3 mM Na-acetate (Overmann and Pfennig 1989). Light intensity was determined with a Li Cor LI-189 quantum meter equipped with a LI-200 SA pyranometer sensor (Li Cor, Lincoln, USA). *Chl. limicola* DSMZ 245^T was grown under the same conditions as depicted for *Chl. chlorochromatii* at a pH of 6.8.

Chlorobaculum tepidum ATCC 49652^T was cultivated at 46°C and a light intensity of 1000 µmol quanta · m^{-2} · s^{-1} of tungsten lamp bulbs in modified CP-medium (Frigaard and

Bryant 2001). *Bacillus subtilis* DSMZ 10, was grown in LB-medium on a shaker at 30°C. Growth conditions for *Clostridium acetobutylicum* DSMZ 792 were at 37°C under anoxic conditions in potato-medium.

6.3.2 Oxygen tolerance test

To determine the influence of oxygen exposition on the viability of associated versus free-living GSB cultures of *Chl. chlorochromatii* and *Chl. limicola* were harvested in the mid-exponential growth phase by centrifugation at 10000 x g for 20 min and resuspended in SL10 medium lacking the substrates Na_2S, $NaHCO_3$ and acetate. Subsequently, the cultures were split into four aliquots and incubated under different conditions. The first replicate of each organism was kept under oxygen saturation at regular illumination; the second replicate was cultivated in complete darkness. As a control both strains were incubated anoxically under the same conditions. Dilution series of samples taken at distinct time intervals were accomplished in SL10 medium. Three parallels, which were diluted tenfold in each dilution step, were carried out in gas tight 22.5 ml screw cap glass tubes. The test tubes were scanned for bacterial growth after two months of incubation and the results were analysed by the MPN (most probable number) method (Button *et al.* 1993).

6.3.3 Genome analyses and phylogeny

To identify mechanisms responsible for oxygen tolerance in *Chl chlorochromatii*, its genome was screened for relevant enzymes for oxygen detoxification and genes involved in carotenoid biosynthesis. Subsequently, comparisons with following GSB have been conducted: *Chlorobaculum tepidum* TLS ATCC 49652^T; *Chlorobium chlorochromatii* CaD; *Chlorobium limicola* DSMZ 245^T, *Chlorobium phaeobacteroides* DSMZ 266, ^{T} *Chlorobium phaeobacteroides* BS1, *Chlorobium luteolum* DSMZ 273^T, *Chlorobium clathratiforme* BU-1 DSMZ 5477^T, *Prosthecochloris aestuarii* DSMZ 271^T, *Chlorobium phaeovibrioides* DSMZ 265, and *Chlorobium ferrooxidans* DSMZ 13013. Prior to genome analyses, cultivation and DNA extraction for genome sequencing was done for all GSB but *Cba. tepidum* and *Chl. phaeobacteroides* BS1. The genome sequences have been determined or are in completion process by the Joint Genome Institute (Walnut Creek, CA, USA; U.S. Department of Energy; http://www.jgi.doe.gov). Manual genome annotation was conducted with the MANATEE

software available from TIGR (The Institute for Genomic Research, Rockville, USA).

All unique genes of *Chl. chlorochromatii* that are lacking in the genomes of the nine *Chlorobiaceae* mentioned above were identified employing *in silico* subtractive hybidization. The analysis was conducted with the "Compare Genomes" function on the IMG webpage (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi).

To identify the origin of relevant detoxifying enzymes in *Chl. chlorochromatii* and all other GSB, which have been sequenced so far (see above), protein sequences of the phylogenetically closest relatives were retrieved from the GenBank database using BLAST version 2.0.4 (Altschul *et al.* 1997). Phylogenetic trees were inferred using the maximum likelihood algorithm Protml within the software package PHYLIP (Phylogeny Inference Package, version 3.57c, Felsenstein 1989).

In order to detect a catalase gene in the genome of *Chl. limicola* amino acid sequences of catalase genes from *Chl. chlorochromatii* CaD, *Chl. phaeobacteroides* BS1, *Escherichia coli* 156 and *Bacillus subtilis* 168 were blasted against the genome of *Chl. limicola*. Additionally, random sequences of 10 amnino acids from these catalase genes were used for the BLAST search. The e-value used was $1e^0$.

6.3.4 Enzyme assays

For the determination of catalase and superoxide dismutase (SOD) activity of *Chl. chlorochromatii*, cultures were incubated at different conditions. Both, oxically and anoxically grown aliquots were kept at regular light intensity as well as in the dark. Samples were taken at 0 h, 48 h, and 96 h. The cells were harvested by centrifugation at 10000 x g for 20 min and suspended in phosphate buffer 50 mM pH 7.0. As a protease inhibitor, PMSF (polymethylsulfonylfluoride) was added to a final concentration of 1 mM. The suspension was treated with 1 min of sonification at 40% amplitude, a pulse of 0.5 sec and 1.0 sec pause between pulses. The suspension was then centrifuged at 10000 x g for 20 min and 4°C to remove cell debris and unbroken cells. The supernatant was used as cell extract. The protein content was determined by the method of Hartree (Hartree 1972), with bovine serum albumine as a standard. All enzyme activity measurements were referred to as units/mg protein.

Qualitative catalase activity was measured by adding one drop of 3% H_2O_2 to a pellet of cells spread on a glass slide. The immediate formation of bubbles, due to the release of oxygen, indicated the presence of catalase activity. Quantitative catalase activity was measured spectrophotometrically according to Beers und Sizer (1951) by following the decrease in absorbance at 240 nm caused by the reduction of H_2O_2 using a Perkin Elmer lambda 25 UV/VIS spectrometer. As a standard, catalase from bovine liver with 1340 U/mg solid was used. *B. subtilis* was used as positive control, *C. acetobutylicum* as negative control.

Superoxide dismutase (SOD) activity was determined by using the Ransod kit (RANDOX Laboratories LTD, Crumlin, United Kingdom). 12.5µl of the cell extract was mixed with 425 µl of the provided substrate and 62.5µl of the xanthin oxidase. The inhibition of the xanthin oxidase induced colour reaction was measured spectrophotometrically over a period of 8 min at 505 nm and 25°C. *Bacillus subtilis* DSMZ 10 was used as positive control, *C. acetobutylicum* as negative control.

6.3.5 RT-PCR

Superoxide dismutase, catalase and cytochrome-cbb_3 oxidase (subunit I) transcription in the isolated *Chl. chlorochromatii* and the associated epibiont within "*C. aggregatum*" was analyzed by reverse transcriptase-PCR (RT-PCR). RNA extraction of *Chl. chlorochromatii* was performed with the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad Laboratories, Hercules, USA) following the instruction manual, which includes a DNaseI digestion for removal of contaminating DNA. RT-PCR of 700 µg RNA was done with Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, USA) according to the manufacturers protocol. Subsequently, the cDNA was treated with 2 U RNase H (Roche Diagnostics GmbH, Mannheim, Germany) to remove RNA-cDNA-hybrids for improved sensitivity of the PCR amplification against the target cDNA. Specific primers for detection of cDNA of the investigated enzymes were designed (Table 2). The PCR mix contained 10 µl 10x buffer, 5 µl Q-Solution, 1 µl dNTPs-Mix (10 mM each), forward and reverse primers at 1 µM, 1.25 U Taq polymerase (Qiagen, Hilden, Germany), 1 µl 25 mM $MgCl_2$ and nuclease free water ad 50 µl (Ampuwa, FreseniusKabi GmbH, Bad Homburg, Deutschland).

Cycling conditions were initial denaturation at 94 °C for 3 min, followed by 80 °C for 2 min and 30 cycles of 94 °C (30 sec each), a 45 sec annealing step and elongation for 1 min at

72 °C (1 min). For the primer pairs EPI-*sod*-41f/EPI-*sod*-384r and EPI-*sod*-384f/EPI-*cbb*$_3$-582r annealing temperatures of 60 °C, for EPI-*kat*-1026f/EPI-*kat*-1544r of 65°C were used. PCR was performed with the GeneAmp-PCR-System 9700 (Applied Biosystems Co., Forster City, USA).

Table 1. Oligonucleotide primers used for amplification of relevant oxygen detoxifying enzymes in *Chl. chlorochromatii*

Primer	Sequence (5′-3′)	T_m (°C)
EPI-*sod*-41f	TTC CTT ATG CCG ACA CAG C	56.7
EPI-*sod*-384r	CCC TCA AGC ACT AAC CAA AGC	59.8
EPI-*cbb*$_3$-28f	GCG TTT ATG ACG ATA GGG	53.7
EPI-*cbb*$_3$-582r	CCA CCA CTC CAC ATT TGC	56.0
EPI-*kat*-1026f	ACC ACC GAC CTT GCT TTG C	58.8
EPI-*kat*-1544r	TAC GGT AAC CTT GTA GCC CGC	61.8

"EPI" denotes primers specific for *Chl. chlorochromatii*, the epibiont of "*C. aggregatum*". Numbers denote position on the actual sequence of *Chl. chlorochromatii*.

6.3.6 Respiration rate

Cells of *Chl. limicola* and *Chl. chlorochromatii* were harvested in the late logarithmic phase by centrifugation at 10000 x g for 20 min and suspended in Tris buffer 10 mM pH 7.5. The respiration rate was measured with a Clark-type oxygen electrode (Hansatech, King´s Lynn, England; Oxygraph plus version 1.01). As substrates, sulfide, acetate, L-glutamate, D-alanine, L-cysteine, L-isoleucin and L(+)-histidin were added to a final concentration of 10 mM. Additionally, either KCN 1 mM or 2,4,-dinitrophenol 0.05 mM were tested together with the substrates. As a positive control, cells of *B. subtilis* DSMZ 10, as negative control cells of *C. acetobutylicum* DSMZ 792 were treated in the same manner.

6.3.7 Pigment analysis

Freeze-dried cells were extracted with 6% KOH in methanol and heating for 20 min at 60 °C. This includes saponification of chlorophylls and fatty acid esters for easier determination and quantitation. After partitioning into 10% ether in petrol, carotenoids were separated and quantitated by HPLC on a Nucleosil C18 3-µm column (Macherey-Nagel, Düren, Germany)

with acetonitrile/methanol/2-propanol (85:10:5, by vol.) as eluent (Steiger *et al*. 2000). Absorbance spectra were recorded from the elution peaks using a Kontron diode-array detector model 440. Reference compounds were from *Cba. tepidum* (Takaichi *et al.* 1997) and neurosporene, γ-carotene and 1`-HO-γ-carotene was generated by complementation. For quantitation, γ-carotene and neurosporene were used.

6.3.8 Isolation of chlorosomes

Cultures of "*C. aggregatum*" and *Chl. chlorochromatii* were harvested in the exponential growth phase by centrifugation at 10000 rpm and 4°C. After resuspension in chlorosome isolation buffer (pH 7.4), comprising 10 mM Na-ascorbate, 2 mM Na-thiocyanate, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), and 2 mM dithiothreitol (DTT), the cells were disrupted by sonification for 5 min (60% pulse; 15 sec, pause, 30 sec) (Branson Sonic Power Company, Danbury, USA). Centrifugation at 10000 rpm for 10 min pelleted the cell debris. After adding 200 mM of Na-ethylenedinitrilotetraacetic acid (EDTA) and incubation for 15 min at room temperature, the suspension was centrifuged again and the supernatant was loaded onto a sucrose gradient (20-60 %). After centrifugation at 26000 rpm for 18 h at 4 °C, the resultant bands at 30 % sucrose were collected 1 mM PMSF and 2 mM DTT were added. Then the suspension was loaded on a flotation gradient (20-30 % sucrose) and centrifuged again at 26000 rpm for 18 h at 4 °C. Resultant bands were washed in phosphate buffered saline (pH 7.2) and chlorosomes were sedimented by ultracentrifugation at 59200 rpm and 4°C for 1.5 h. Chlorosomes were washed again in the same buffer and centrifuged at 55000 rpm.

Three different control approaches were conducted to assure successful isolation of the chlorosomes. Firstly, the *in vivo*-absorption spectrum of the epibiont and the absorption spectrum of the collected band was compared. For this purpose, cells were centrifuged for 5 min at 113200 rpm and resuspended in 100 µl supernatant. After the addition of 400 µl saturated sucrose, the *in vivo*-absorption was measured between 500 nm and 850 nm using a Lambda 25 UV/VIS Spectrometer (Perkin Elmer Instruments, Rodgau-Jügesheim, Germany).

Prior to the absorption-measurement of the collected band, 20 µl of the fraction were diluted in 230 µl of isolation buffer. Absorption was measured between 500 nm and 850 nm. The emission spectrum was measured at 450 nm (FluoroMax-3 Jobin, Yvon, Horiba). The

wavelength of the resulting emission-maximum for Bchl *c* served as excitation wavelength for the subsequent measurement of the excitation-spectrum.

As third approach to check if chlorosome isolation was successful, electron microscopy was performed (Fig. 8). For negative contrast, the chlorosome-containing fractions where diluted with Ampuva-water in a ratio of 1:25 and dropped on a collodium coated, carbon steamed grid. After one minute, the fraction was removed and the grid was dried at room temperature. Subsequently, one drop of contrast solution was applied and incubated for 1 min. Thereafter, the grid dried again at room temperature. Electron microscopy was conducted with a Zeiss EM 912 Electron microscope (Zeiss, Oberkochen, Germany).

6.3.9 Excretion of dissolved organic carbon (DOC)

For determination of total carbon (TOC), 125 ml lyophilized samples (of 0.2 μm filtrates) were analysed by high temperature combustion (Shimadzu TOC-5000). The standard deviation between three injections per sample was < 1%. The instrument blank (8–12 μM C) was measured using UV-irradiated Milli-Q water and was subtracted from each sample. For determination of dissolved organic carbon (DOC), the samples were acidified with 100 μl of 85% H_3PO_4 before analysis by high temperature combustion.

6.3.10 Excretion of neutral and acid carbohydrates

Neutral and acid carbohydrates of 125 ml lyophilized samples (of 0.2 μm filtrates) were analysed by HPLC using a Carbopac PA 10 column (Dionex, Sunnyvale, USA) and pulsed amperometric detection (Mopper *et al.* 1992). NaOH (250 mM) and Na-acetate (1 M) were used as eluents in the following manner: 0-20 min 6.3% NaOH, 20-21 min increase of NaOH to 41% and Na-acetate to 5%, 21-22 min 41% NaOH and 5% Na-acetate, 22-42 min 41% NaOH and increase of Na-acetate to 30%, 42-49 min 41% NaOH and increase of Na-acetate to 60%, 49-50 min increase of NaOH to 100% and decrease of Na-acetate to 0%, 50-52 min 100% NaOH, 52-53 min decrease to 6.3% NaOH, 53-75 min 6.3% NaOH. To also detect combined sugars all samples were hydrolysed with 0.09 N HCl at 100°C for 20 h.

6.3.11 Excretion of amino acids

Cultures of *Chl. chlorochromatii* were lyophilized and stored at 4°C until analysis. Concentrations of dissolved free amino acids (DFAA) were analysed by HPLC after ortho-phthaldialdehyde derivatisation according to Lindroth and Mopper (1979) using 2 injections per sample. Dissolved combined amino acids (DCAA) were hydrolysed with 6 N HCl at 155°C for 1 h and analysed as DFAA.

6.3.12 Utilization of amino acids, peptone, and acetate

In order to determine if amino acids and other substrates promote or inhibit growth of *Chl. chlorochromatii*, a substrate test was conducted. Peptone (from caseine; free of tryptophane and vitamins), acetate and the following L-amino acids were used: alanine, arginine, glycine, lysine, phenylalanine, proline, serine, threonine, ornithine, valine, histidine, asparagine, aspartate, glutamate, glutamine, isoleucine, leucine, metionine, tryptophane, tyrosine, and anoxygenic cysteine. Each substrate was tested in three parallels. First, the substrate was added in 22.5 ml gas-tight screw cap glass tubes, in a concentration of 1 mM or 5 mM, respectively (see Fig. 10). Then the tubes were inoculated with 10% of a culture of *Chl. chlorochromatii*, which previously had been grown in SL10 medium, free of acetate and other organic carbon sources. After incubation over night in the dark, the tubes were kept at 20 °C under illumination of 50 μmol quanta m^{-2} s^{-1}. Growth was determined via optical density measurement at 650 nm at 0 h and 72 h. To determine, which substrates positively or negatively influence growth OD_{650} values were compared to control tubes. Statistical significance was determined by t-testing.

6.4 RESULTS AND DISCUSSION

6.4.1 Oxygen tolerance

Green sulfur bacteria (GSB) and phototrophic consortia have been observed to thrive within oxygenated water layers in their natural habitat and are exposed to oxygen concentrations up to 30 µM, which corresponds to 11% of air saturation (Overmann *et al.* 1998). Nevertheless, GSB are considered as obligately anaerobic organisms (Imhoff 1995). Therefore, we studied the physiological response of *Chl. chlorochromatii* to molecular oxygen. To exclude photooxidative stress and damages, oxygen tolerance was first investigated in the dark and then compared with the same approach, conducted under natural illumination. Surprisingly, the oxygen exposition of the two investigated GSB strains *Chl. chlorochromatii* CaD and *Chl. limicola* DSMZ 245^T did not immediately cause cell death. Whereas the exposure to oxygen in the dark had no negative influence on the viability of *Chl. chlorochromatii* for 120 h, *Chl. limicola* survived significantly longer to oxygen exposition (Fig. 1). This is in line with observations of other *Chlorobiaceae*, where for instance the cell division of *Chl. bathyomarinum*, a GSB that occurs in hot spring of the Pacific Ocean was not negatively influenced during an exposure to oxygen for two weeks (Beatty *et al.* 2005).

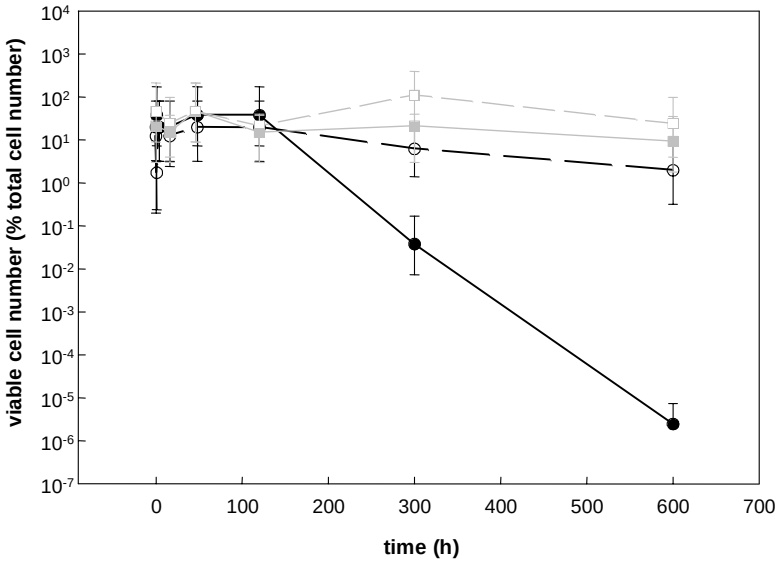

Fig. 1. Viable cell number of *Chl. chlorochromatii* and *Chl. limicola* as percentage of total cell number during incubation in the dark. *Chl. chlorochromatii* oxic = black (-●-); *Chl. chlorochromatii* anoxic = dashed (-○-); *Chl. limicola* oxic = grey (-■-); *Chl. limicola* anoxic = dashed (-□-)

In the light, the proportion of viable cells decreased rapidly during oxygen exposure, whereas the anoxygenic control remained constant up to 150 h (Fig. 2). Similarly, *Chl. limicola* showed a greater oxygen tolerance in the dark. In this context it has been shown that halobacteria show a light-accelerated death under aerobic conditions (Brock and Petersen 1976).

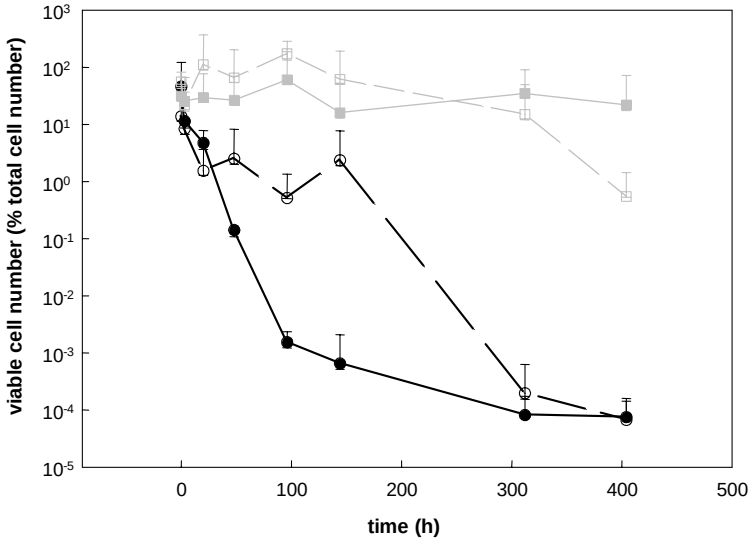

Fig. 2. Viable cell number of *Chl. chlorochromatii* and *Chl. limicola* as percentage of total cell number during incubation in the light. *Chl. chlorochromatii* oxic = black (-●-); *Chl. chlorochromatii* anoxic = dashed (-○-); *Chl. limicola* oxic = grey (-■-); *Chl. limicola* anoxic = dashed (-□-)

These findings show, that even if green sulfur bacteria are obligately anaerobic organisms, they seem to have certain strategies to maintain viability under oxic conditions. Since both strains have been used in the mid-exponential growth phase our results moreover indicate differences in maintenance energy requirement within these GSB. *Chl. thiosulfatophilum* is known to contain glycogen, polyphosphate and polysaccharide as storage compounds (Cole and Hughes 1965, Sirevåg and Ormerod 1977).

The minor tolerance of the epibiont towards oxygen could be a consequence of symbiosis. While *Chl. limicola* is an immotile organism, *Chl. chlorochromatii* has acquired motility due to its association with the flagellated central bacterium in the phototrophic consortium "*C. aggregatum*". If lakes mix, as it naturally occurs in autumn, this acquired motility could allow the associated epibionts to actively retreat into anoxic water layers. In contrast, *Chl. limicola*, is only able to reach anoxic water layers by the means of sinking, which takes more time and therefore might explain its comparatively higher tolerance towards oxygen exposition.

In order to identify possible mechanisms on which the observed oxygen tolerance of *Chl. chlorochromatii* is based, its genome was screened for relevant enzymes, usually involved in oxygen detoxification. Genome annotation of *Chl. chlorochromatii* revealed the presence of the following gene: heme catalase/peroxidase (*katG*), peroxidase, superoxide dismutase (*sodB*), rubredoxin (*rub*), rubrerythrin (*rbr*), cytochrome-*c*-peroxidase, and genes from the cytochrome cbb_3-oxidase (*fixN* and *fixO*). To clarify the phylogenetic relationship of those enzymes, genome analyses of all GSB, which are currently in the sequencing and annotation process, have been made. Figure 3 shows phylogenetic trees for relevant enzymes involved in oxygen detoxification identified in *Chl. chlorochromatii* and all other GSB currently available in genome databases. The overall results are, with the exception of the *katG* gene in the epibiont and the *rub* gene in *Cba. tepidum*, that all green sulfur bacterial sequences are closely related to each other, indicating a common evolutionary origin. If lateral gene transfer had occurred, this can be only the case for the mentioned genes of *Chl. chlorochromatii* and *Cba. tepidum* or to a common ancestor of the *Chlorobiaceae* group.

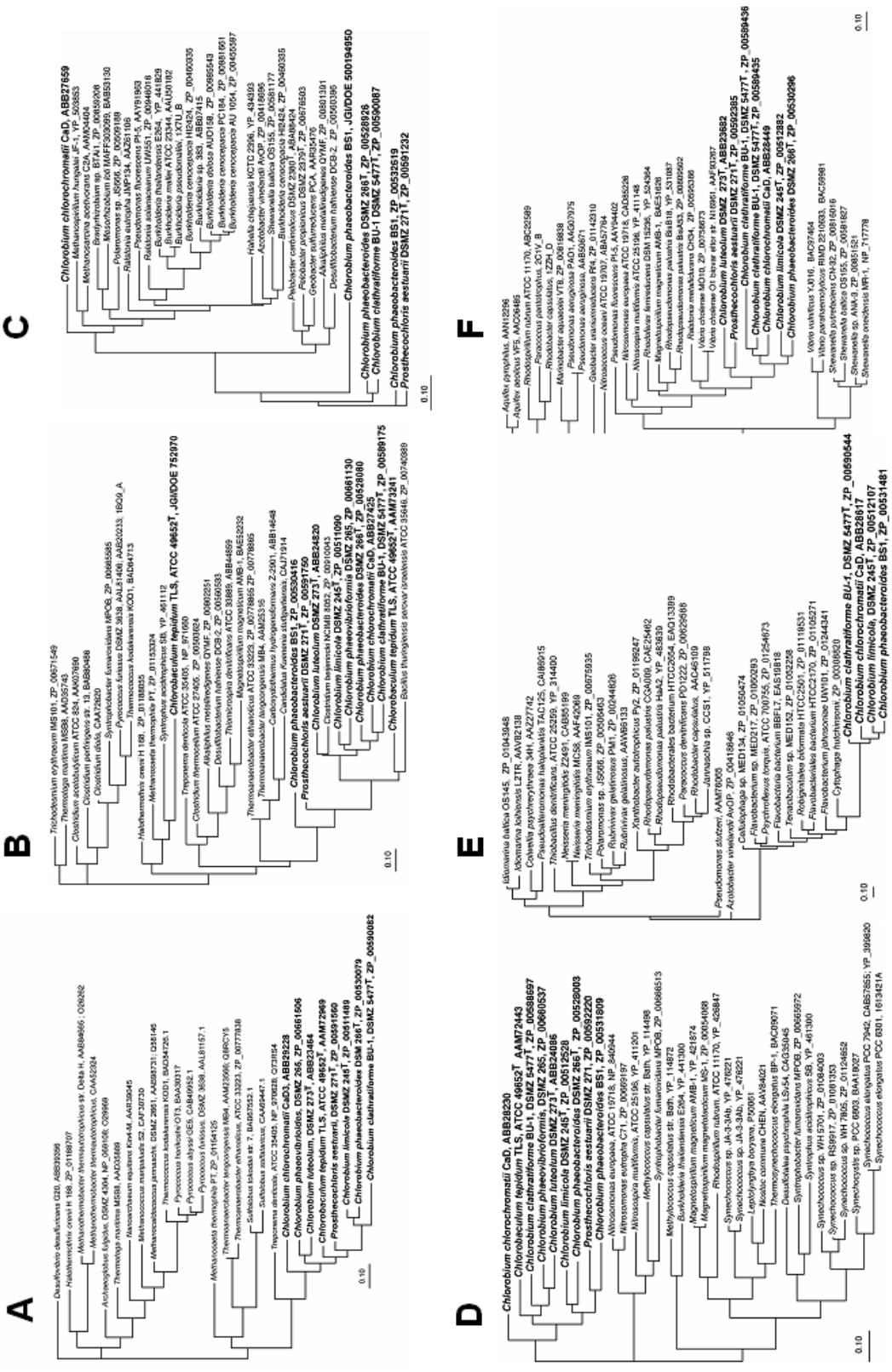

Fig. 3. Maximum likelihood phylogenetic trees of different enzymes involved in oxygen detoxification. The sequences were obtained for all green sulfur bacteria currently available (see experimental procedures) and compared to closest relatives: **A**. peroxidase (400669960) **B**. rubredoxin (rub; 400651240), **C**. heme catalase/peroxidase (katG; 400653690) **D**. superoxide dismutase (sodB; 400659630) **E**. cytochrome-*cbb*$_3$-oxidase (fixO; 400663630) **F**. cytochrome-*c*-peroxidase (400661850). Number indicate JGI gene object ID.

Summarizing the phylogenetic analyses of genes involved in detoxification, the epibiont does not differ from free-living GSB at first glance. However, recently a gene that is not present in any other GSB, econding a RTX-like protein (Vogl *et al.* in prep) has been identified as putuative sybiosis gene. Since RTX-toxins are effector-proteins of various pathogens whereby the RTX-domain mediates the specific binding of the protein to the target cells (Lally *et al.* 1999) this gene is of immediate interest with respect to the cell-cell-interaction in phototrophic consortia. Complementary *in silico* subtractive hybridization revealed 189 unique genes in *Chl. chlorochromatii*. While 147 of those are hypothetical proteins 42 have been assigned function. Some of those genes are likely to play a role in the symbiosis and should be investigated in detail: two of them are involved in membrane fusion (Cag1408 and Cag1409), two are transposases (Cag0682 and Cag1559), and one is involved in glutamate biosynthesis (Cag0668). Especially interesting is Cag1796, an ankyrin, since ankyrins mediate the attachment of integral membrane proteins to the cytoskeleton and are involved amongst others in cell-cell adhesion regulation (Hryniewicz-Jankowska *et al.* 2002). Contrary to the genome composition, the genome size of the epibiont is not distinctive. At 2.6 Mb, the genome of *Chl. chlorochromatii* lies in the range of all other GSB sequenced so far and even surpasses the genome of the model organism for this group (*Cba. tepidum*, 2.154 Mb). In contrast to obligate bacterial symbionts, such as *Buchnera aphidicola* or *Wigglesworthia glossinidia*, it does not show evidence for genome size reduction (Moran 2003, Moran and Plague 2004).

To investigate the activity of oxygen detoxifying enzymes detected in the genome sequences different enzyme assays were conducted. A major portion of cell damage caused via oxidative stress is due to H_2O_2 (Imlay and Linn 1988). The enzyme responsible for the primary detoxification of H_2O_2 into H_2O and O_2 is the catalase. Since the detoxification of H_2O_2 by the catalase is an exothermic reaction, it represents an effective mechanism for detoxification, working independently of additional energy supply (Wang 1955). This makes it an effective mechanism under starvation conditions, as investigated in our experiments. In contrast to *Chl. limicola*, a catalase encoding gene (*katG*) was identified in the genome of *Chl. chlorochromatii*. Qualitative and quantitative catalase assays revealed that *Chl. chlorochromatii* is a catalase positive organism (Fig. 4). In comparison to *B. subtilis*, however, catalase activity of the epibiont is weak. As a facultative anaerobic organism *B. subtilis* almost permanently

relies on this enzyme, whereas the obligately GSB only occasionally need it. This might explain the observed differences in activity. Nevertheless it seems that in *Chl. chlorochromatii* the catalase is constitutively expressed, since the enzyme activity could be measured before oxygen exposition. This was additionally proven by RT-PCR (Fig. 5).

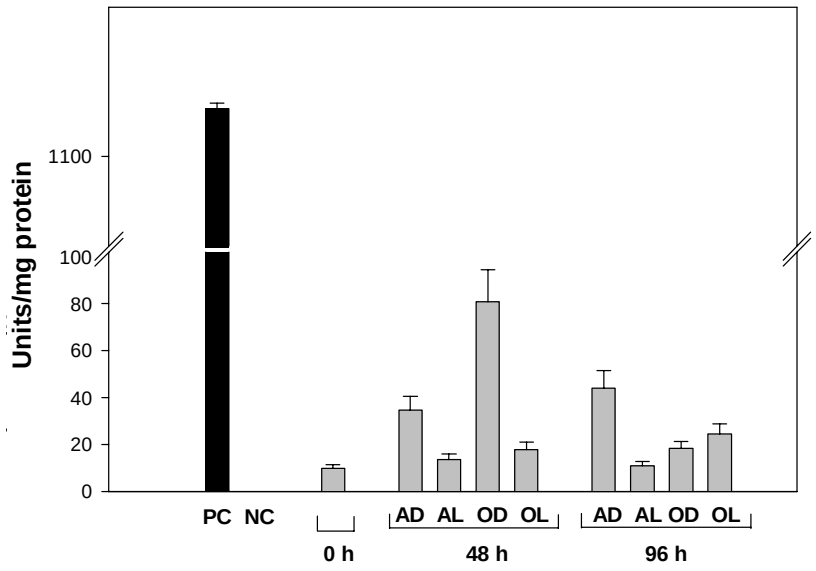

Fig. 4. Catalase assay in *Chl. chlorochromatii*. PC = positive control, NC = negative control, AD = anoxic/dark, AL = anoxic/light, OD = oxic/dark, OL = oxic/light

Rubrerythrin, a non-hem iron protein usually occurs in aerobic or microaerophilic bacteria and seems to represent the terminal unit of a NADH-peroxidase (Coulter *et al.* 1999), which catalyses together with rubredoxin the degradation of H_2O_2. Since genes encoding both proteins could be identified in the genome sequences of *Chl. chlorochromatii* it is likely, that the observed detoxification is additionally mediated by this mechanism.

Another toxic oxygen species is the oxygen superoxide radical ($O_2^{\bullet}$) that harms cells in lower concentrations than H_2O_2 (Storz and Imlay 1999). Superoxide dimutases (SOD) are the enzymes that disproportionate superoxide to hydrogen peroxide and molecular oxygen. A superoxide dismutase-encoding gene could be found in the genome of *Chl. chlorochromatii* as well as in the genome of *Chl. limicola*. We found SOD activity in *Chl. limicola* and *Chl. chlorochromatii*, which is marginally higher in *Chl. limicola* (Fig. 6). Similar to the catalase, the SOD is constitutively expressed as was shown by RT-PCR (Fig. 5). Even if *Chl. limicola* has

been shown to be much more tolerant towards molecular oxygen than *Chl. chlorochromatii*, this fact cannot be explained by a slightly higher activity of SOD and the reason for this remains to be shown.

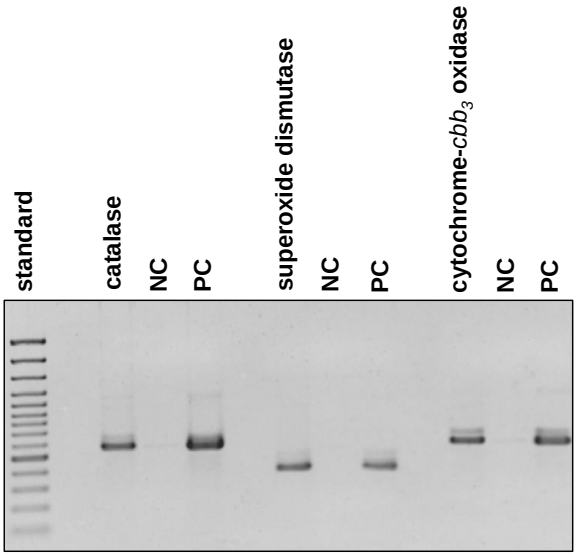

Fig. 5. Detection of cDNA of the isolated *Chl. chlorochromatii* produced by RT-PCR: specific signals on partial gene sequences of superoxide dismutase, catalase and cytochrome-*cbb*$_3$ oxidase show transcription of all three ORFs. Negative control (NC) = RT-PCR with same amount of RNA without reverse transcriptase, positive control (PC) = same conditions as in NC with 50 ng of *Chl. chlorochromatii* DNA.

In order to determine if oxygen can serve as electron acceptor and hence the observed oxygen tolerance can be explained by respiration, oxygen consumption rates of pure cultures of *Chl. chlorochromatii* were determined. In contrast to *B. subtilis*, which served as positive control, no respiration was detectable in the GSB. However, as demonstrated via RT-PCR, the subunit I of the *cbb*$_3$ gene is transcribed (Fig. 5). These contradicting findings indicate, that the *cbb*$_3$-oxidase protein is either not expressed or it is expressed but involved in other functions than respiration. It has been shown that the *cbb*$_3$ oxidase is involved in signal transduction in the PrrBCA two-component system in *Rhodobacter sphaeroides*, regulating the expression of the photosynthetic apparatus depending on the oxygen concentration (Oh and Kaplan 2000). Moreover, the *cbb*$_3$-oxidase is known to have several other regulatory functions under anaerobic conditions concerning the expression of a wide diversity of genes in *R. sphaeroides* (Kaplan *et al.* 2005).

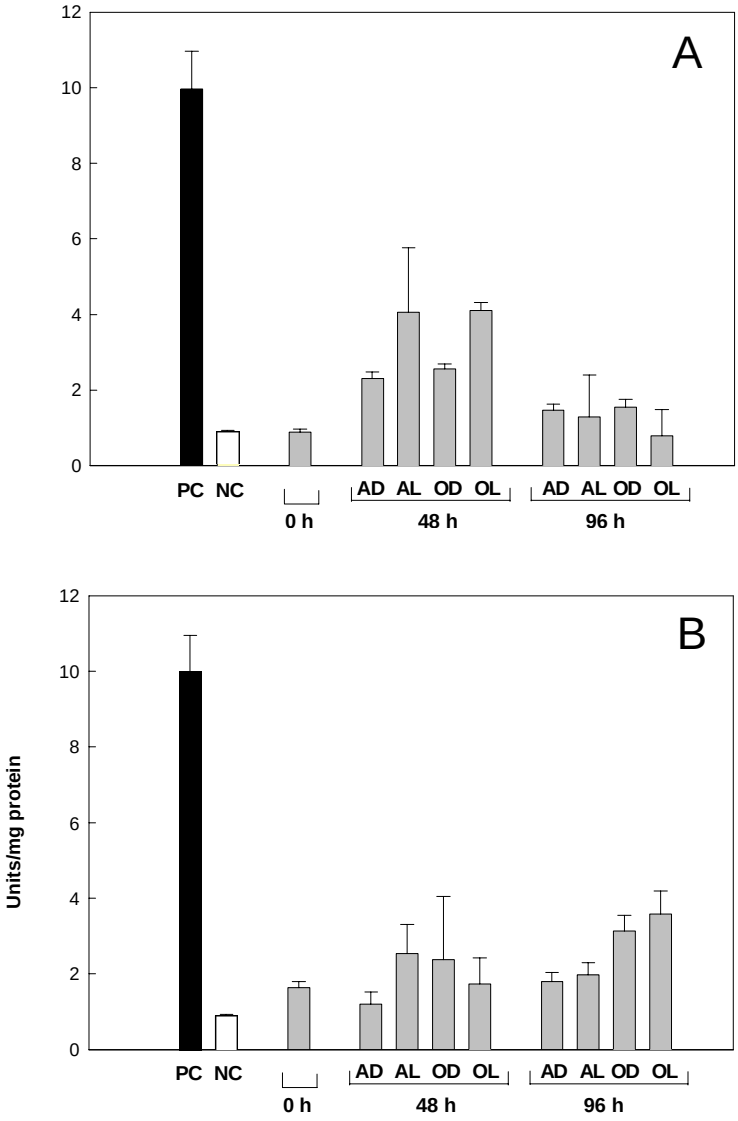

Fig. 6. Superoxide dismutase assay. **A**. *Chlorobium chlorochromatii*. **B**. *Chlorobium limicola.* PC = positive control, NC = negative control, AD = anoxic/dark, AL = anoxic/light; OD = oxic/dark, OL = oxic/light

6.4.2 Composition and content of carotenoids

Carotenoids are lipophilic antioxidants playing a role in oxygen detoxification. Therefore we analysed the carotenoid composition of *Chl. chlorochromatii* in pure culture and in the associated state within "*C. aggregatum*". The carotenoids of *Chl. chlorochromatii* in pure culture were analysed by HPLC and compared to the carotenoid pattern of *Cba. tepidum* (Fig. 7A). In combination with retention time, spectral data and mass determination (Table 2), peak 3 was identified as γ-carotene and peaks 4 and 4′ as two cis/trans isomers of 7,8-

dihydro-γ-carotene. A second cis/trans isomer of γ-carotene could additionally be found in the consortium. Peak 2 was identified as 1′-hydroxy-γ-carotene with an authentic standard. Peak 1 is most like a 1′-hydroxy-γ-carotene glycoside. Trace amounts of other carotenoids not shown here include neurosporene, lycopene and β-carotene. The HPLC trace of Figure 7B demonstrates the different carotenoid composition in *Cba. tepidum* with γ-carotene (gCar), 1′,2′-dihydrochlorobactene (DHChlb) and chlorobactene (Chlb) as prominent carotenoids. All of the carotenoids in *Cba. tepidum* have been previously identified (Takaichi *et al.* 1997).

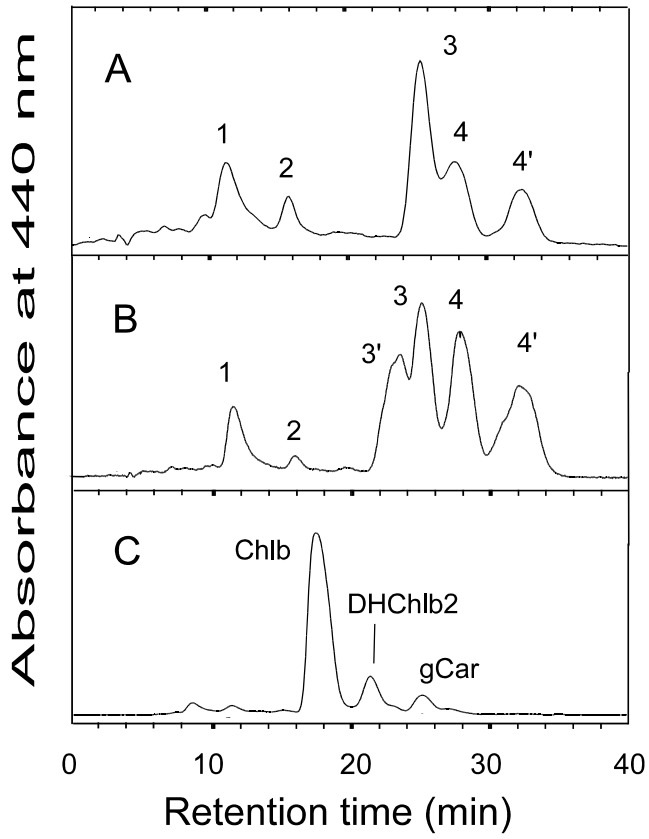

Fig. 7. HPLC separation of carotenoids from *Chlorobium chlorochromatii* (**A**) and "*C. aggregatum*" (**B**). Extracts from *Chlorobaculum tepidum* (**C**) were included for comparison. Peaks are 1, 1′-hydroxy-γ-carotene glycoside (tentatively identified); 2, 1′-hydroxy-γ-carotene: 3, γ-carotene (3′ is a cis isomer) and 4, 7,8,-dihydro-γ-carotene (4′ is a cis isomer).

Carotenoids found in free-living *Chl. chlorochromatii* and the associated epibiont were rather similar. The major differences are a second cis/trans isomer of γ-carotene in the consortium and a generally much higher concentration of the individual carotenoids in the latter (Table 2). This is especially the case for γ-carotene 7,8-dihydro-γ-carotene, which are 8- and 6-fold increased, respectively. Since the enrichment culture of "*C. aggregatum*" contains only one phototrophic bacterium as revealed in former studies (Kanzler *et al.* 2005) we found in this study that the composition as well as the amounts of the carotenoids in the chlorosomes corresponds to those in whole cells, it is most likely that the additional amounts of carotenoids result from higher synthesis activity of *Chl. chlorochromatii*.

With 7,8-dihydro-γ-carotene we found a new carotenoid not identified from any other organism before. In contrast to former presumptions (Vogl *et al.* 2006) chlorobactene, which is known to be the major and characteristic carotenoid of green-coloured GSB (Liaaen-Jensen 1965, Takaichi 1999, Frigaard *et al.* 2004), is not present in the epibiont of "*C. aggregatum*". Its absence is explained by the finding that the gene *crtU*, encoding a desaturase of β-ionone rings (Krubasik 2000) is not present in the genome of *Chl. clorochromatii*, as revealed by genome annotation. In *Cba. tepidum*, the gene product of *crtU* converts γ-carotene into chlorobactene (Frigaard *et al.* 2004). The genome sequences of other GSB were surveyed for *crtU* and this gene was found in all genomes (green and brown-coloured GSB) except for *Chl. chlorochromatii*.

Table 2. Carotenoids of *Chl. chlorochromatii* CaD: spectra, masses, concentrations

		Concentrations (μg/g dry weight)[b]	
Carotenoid[a]	**Absorbance (nm)**	***Chl. chlorochromatii* CaD**	**"*C. aggregatum*"**
1`-HO-γ-carotene-glycoside (P1)	436/461/495	147.9 ± 20.0	237.5 ± 44.4
1´-HO-γ-carotene (P2)	436/461/495	43.8 ± 10.9	63.3 ± 3.8
γ-carotene (P3)	436/461/495	159.6 ± 31.0	473.0 ± 116.1
γ-carotene (consortium)	435/460/495	0	795.2 ± 101.2
7,8-dihydro-γ-carotene (P4)	435/460/495	98.6 ±18.2	650.6 ± 35.0
7,8-dihydro-γ-carotene (P5)	435/460/495	102.3 ± 34.7	506.4 ± 140.9

[a] Numbering (P1-P5) refers to the peaks in Figure 7.[b] Each value represents the average of at least three measurements. Values are given with standard deviation. To simplify qualification all derivatives of fatty acids have been inverted in their corresponding carotenoids.

In analogy to *Cba. tepidum* (Frigaard and Bryant 2004), we can assume in the genome sequence of *Chl. chlorochromatii* that the carotenogenic pathway to lycopene is catalyzed by the gene products of *crtP*, *crtQb* and *crtH* (Fig. 8). From the simultaneous presence of γ-carotene and 7,8-dihydro-γ-carotene, we can conclude that the phytoene desaturase of *Chl. chlorochromatii* works as a combined desaturase producing substantial amounts of neurosporene in addition to lycopene similar to the enzyme of *Rubrivivax gelatinosus* (Harada *et al.* 2001, Stickforth and Dandmann 2007). Furthermore, the cyclase of *Chl. chlorochromatii* is a lycopene mono cyclase (forming less than 1% the dicyclic β-carotene) able to cyclize not only lycopene but also neurosporene. However, the latter carotene modifies almost exclusively the 7,8-dihydro-ψ-end group (see pathway). This is a unique feature of this enzyme in *Chl. chlorochromatii.* Other cyclases (Takaichi *et al.* 1996), preferentially cyclize the end of the neurosporene molecule with the 7,8-double bond. The proposed pathway for the formation of 7,8-dihydro-γ-carotene and other carotenoids in *Chl. chlorochromatii* is shown in Figure 8.

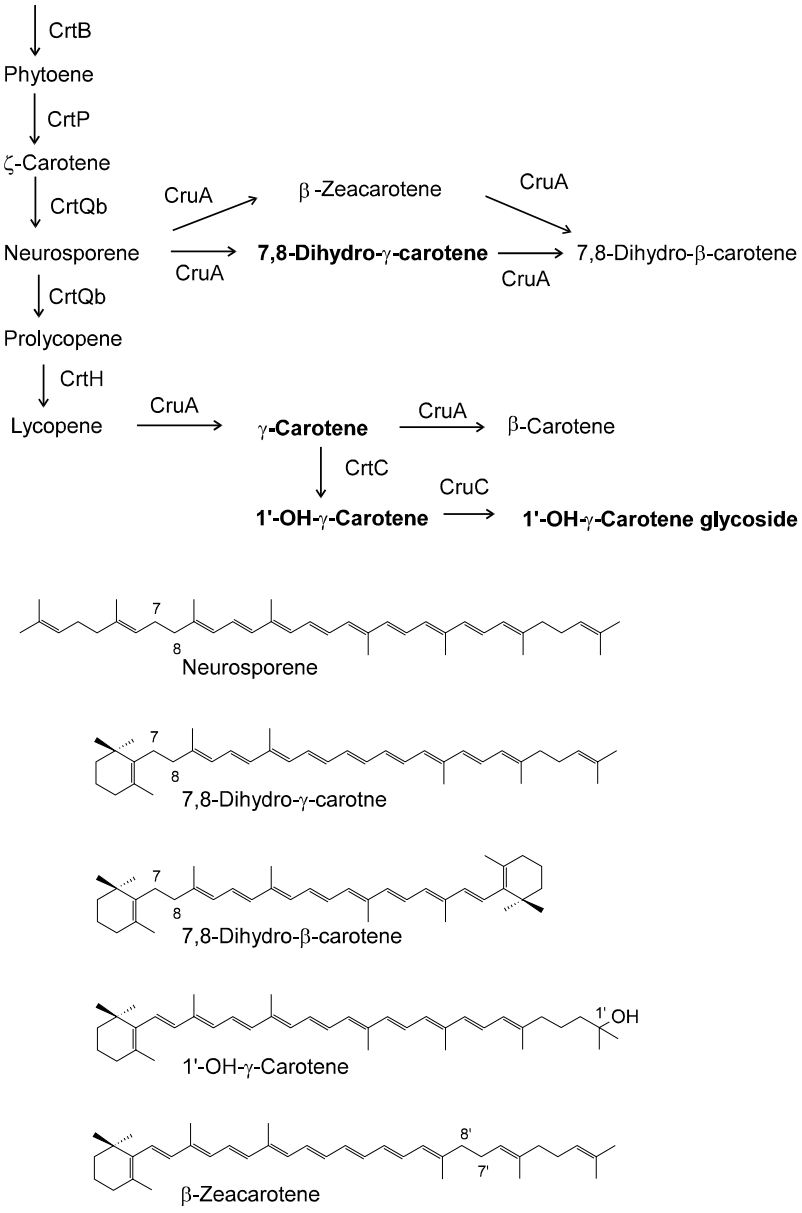

Fig. 8. Proposed carotenogenic pathway in *Chl. chlorochromatii* (top) and structures of intermediates and end products (bottom). Carotenoids in bold indicate the major carotenoids. Genes identified in the genome sequence of *Chl. chlorochromatii* are indicated.

Differences in oxygen tolerance of the investigated GSB might be partially due to their different carotenoid composition. Carotenoids not only detoxify oxygen radicals, but also directly interact with singlet oxygen and dissipate the energy from $^1O_2$ as heat (Foote and Denny 1968). This reaction can only take place if the energy level of the carotenoid is lower than the energy level of the $^1\Delta_g$ state of singlet oxygen. Therefore, the potential of a carotenoid to quench $^1O_2$ increases with the number of conjugated double bonds. While it is assured, that lycopene is the most efficient naturally occurring singlet oxygen quencher

(Conn *et al.* 1991, Di Mascio *et al.* 1989, Edge and Truscott 1999) little is known about the exact efficiency of other carotenoids to detoxify oxygen radicals. The mechanism and rate of scavenging of oxygen radicals has shown to be strongly dependent on the nature of the oxidizing species but much less dependent on the carotenoid structure (Mortensen *et al.* 1997). There is evidence that the ability to quench singlet oxygen increases with the chain length (Edge and Truscott 1999). The optical absorption of a carotenoid is also a good indicator for $^1O_2$ quenching. *Chl. limicola* and *Chl. bathyomarinum*, which both are extremely resistant to oxidative stress (this work, Beatty *et al.* 2005), possess chlorobactene (absorbance at 435, 460 and 490 nm) as their major carotenoid. In 7,8-dihydro-γ-carotene the spectral region of absorbance of this carotenoid about 20 nm blue-shifted as compared to γ-carotene and chlorobactene. Therefore, 7,8-dihydro-γ-carotene is probably less suitable to quench $^1O_2$. This could be one of the reasons for the comparatively lower tolerance of *Chl. chlorochromatii* towards oxygen. To clarify, which carotenoids of *Chl. chlorochromatii* play a role in oxygen detoxification the construction of mutants is required.

To assure that the investigated carotenoids originated exclusively from *Chl. chlorochromatii*, the chlorosomes were extracted from epibiont cells and the carotenoid content of the chlorosomes was compared to the carotenoid content of whole cells. Ultracentrifugation yielded a clearly defined dark green band at about 30% sucrose. In order to prove that this fraction contained the isolated chlorosomes, the absorption spectra of the collected fractions was compared to the *in vivo* absorption spectrum of free and associated epibionts, respectively. All spectra showed two absorption maxima. One, attributed to the soret absorption of Bchl *c*, was found between 455 nm and 461 nm. The other, between 747 nm and 761 nm, corresponded to the absorption maximum of chlorosomal Bchl *c*-aggregates. Due to the absorption of free Bchl *c*, all spectra showed a slight peak at 666 nm. The isolation of the chlorosomes caused a shift in absorption: the soret absorption of Bchl *c* shifted to 455 nm (*in vivo* 460 nm) for the free living epibionts, and to 456 nm (*in vivo* 461 nm) for chlorosomes isolated from associated epibionts, respectively. The Q_y absorption maximum of aggregated Bchl *c* shifted to 751 nm (*in vivo* 761 nm) for the free-living epibionts, and to 747 nm (*in vivo* 761 nm) for chlorosomes isolated from associated epibionts, respectively. However, the absorption spectra of isolated chlorosomes correlated with the spectra of intact cells, indicating successful chlorosome isolation (Fig. 9).

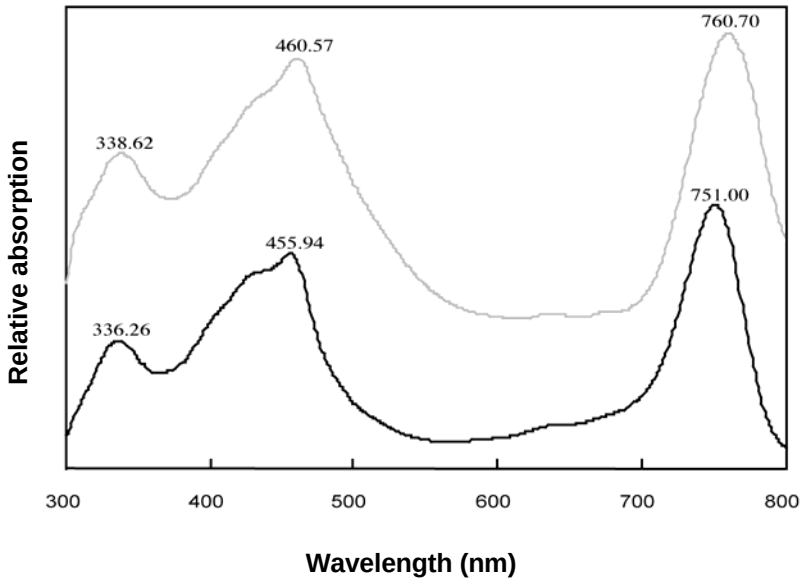

Fig. 9. Absorption spectra of the isolated chlorosomes of an"*Chlorochromatium aggregatum*" enrichment culture in comparison to the corresponding *in vivo* spectra of cells (grey = *in vivo*, black = after isolation of chromosomes).

Successful chlorosome extraction was further confirmed by fluorescence analysis. Since the fluorescence of free Bchl *c*, with a maximum at 666 nm is much stronger than the fluorescence of aggregated Bchl *c* with a maximum at 766 nm, fluorescence analysis can detect damages to the chlorosomes that cause release of Bchl *c*. Fluorescence analysis of the isolated fractions of free and associated living epibionts showed a clear maximum at 766 nm attributed to chlorosomal Bchl *c* aggregates and a second, clearly minor peak at 666 nm, which was caused by the fluorescence of Bchl *c*. This proves that the vast majority of chlorosomes remained intact during the isolation process (Fig. 10).

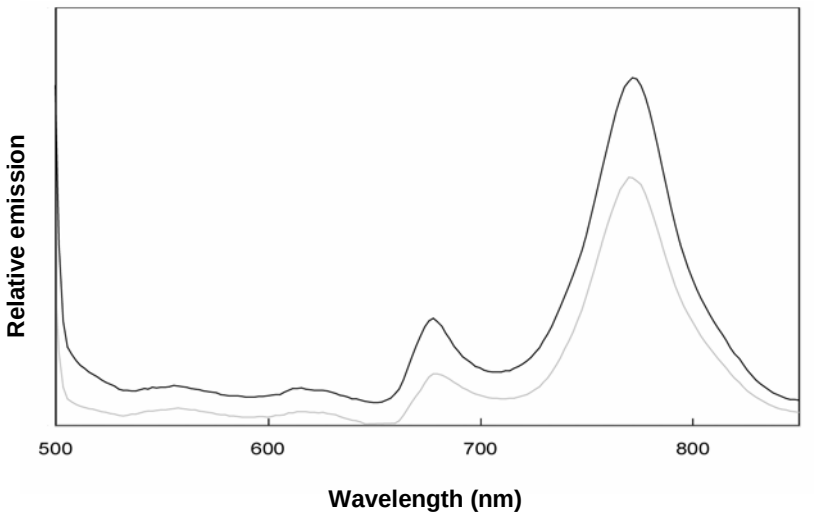

Fig. 10. Emission spectra of the chlorosome fractions of *Chlorobium chlorochromatii* (grey) and "*Chlorochromatium aggregatum*" enrichment culture (black).

As a further, independent approach to assure the isolated chlorosomes to be intact, electron microscopy was conducted. REM using negative contrast revealed that chlorosomes derived from free-living epibiont as well as those derived from associated epibionts were intact, exhibiting a smooth surface. We observed a tendency of chlorosomes to aggregate, which is probably due to their attachment to the membrane (Fig. 11).

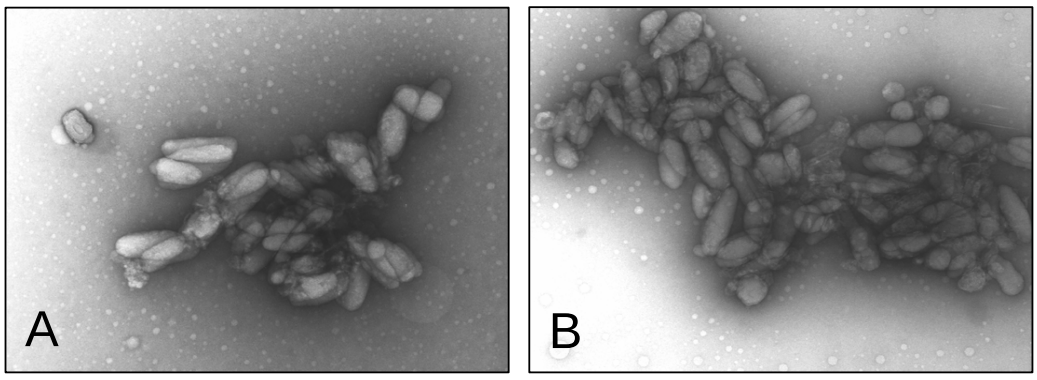

Fig. 11. REM electron microscopy. **A.** Chlorosomes extracted from *Chlorobium chlorochromatii* **B.** Chlorosomes isolated from a "*Chlorochromatium aggregatum*" enrichment culture. Chlorosomes are visibly intact, indication that extraction procedure has no influence on their stability.

6.4.3 Exchange of carbohydrate substances

In order to elucidate if *Chl. chlorochromatii* excretes metabolites, which can be used by the central rod and therefore help to sustain the symbiosis, we analyzed carbohydrates and amino acids in supernatants of epibiont cultures. An overview of the amino acids excreted by *Chl. chlorochromatii* is shown in table 3. The major amino acid excreted is glutamate. In former experiments it has been shown that 2-oxoglutarate is not taken up as a substrate by the epibiont (Vogl *et al*. 2006). While the green sulfur bacterial epibiont in consortia grows photolithoautotrophically, intact consortia incorporate 2-oxoglutarate (Glaeser and Overmann 2003) and growth of the enrichment cultures is improved when supplemented with 2-oxoglutarate. 2-oxoacids, in particular 2-oxoglutarate and 2-oxo-3-methylvalerate, are known to be excretion products of other green sulfur bacteria (Sirevåg and Ormerod 1970). In this context it is remarkable that glutamate is part of the 2-oxoglutarate pathway and the two compounds can easily be interconverted. The fact that the epibiont is capable of fixing nitrogen may play an important role within this context, since glutamate is formed by 2-oxoglutarate and nitrate and hence the 2-oxoglutarate uptake of the central rod might be controlled by the epibiont.

Table 3. Concentrations of amino acids excreted by *Chl. chlorochromatii.*

Amino acid	Exponential growth phase (nM)		Stationary growth phase (nM)	
	DFAA[a]	THAA[b]	DFAA	THAA
Asp	4058.5	8767.1	929.5	1979.4
Glu	13111.5	19694.5	3805.5	4939.8
Asn	239.5	0.0	59.7	0.0
His	189.4	315.9	23.0	206.2
Ser	201.7	1414.6	409.7	1184.1
Arg	163.4	411.2	300.9	179.9
Gly	0.0	1606.0	79.3	645.3
Thre	97.5	1973.3	59.8	2669.1
Ala	46.8	233.1	0.0	165.5
Tyr	116.6	3488.2	282.9	1083.1
Met	0.0	0.0	0.0	0.0
Val	398.7	703.3	1434.8	717.1
Phe	338.1	697.9	78.2	263.6
Ile	118.8	421.7	56.2	179.8
Leu	135.3	697.8	121.4	385.9

The total values for the exponentially grown culture are 19215.9 nM for DFAA and 40424.8 nM for THAA; total values for the culture in the stationary growth phase are 7641.0 nM for FAA and 14599.0 nM for THAA. [a] Dissolved amino acids; [b] total hydrolyzed amino acids.

An overview of the carbohydrates excreted by *Chl. chlorochromatii* is shown in table 4. Interestingly, large amounts of high energetic sugars are excreted. The amount of carbon within the cells was 13.07 mg C for the exponentially grown culture (OD_{650} = 0.42) and 18.73 mg C for the stationary grown culture (OD_{650} = 0.6) (calculation according to Overmann 1989; 250 mg C/l at OD_{650} = 1). The amount of total carbon excreted was 15.01 mg for the exponentially grown culture and 29.74 mg for the stationary culture. This implies an increase of biomass of 5.66 mg while 14.73 mg carbon is excreted into the medium. It is likely, that the central rod uses at least some of those carbohydrates within the symbiosis. However, which excretion products are taken up by the central rod still remains to be investigated.

Table 4. Composition of carbohydrates excreted by *Chl. chlorochromatii.*

	Exponential growth phase (µgC)	Stationary growth phase (µgC)
Fructose	6.50	14.35
Galactose	5.09	303.92
Glucose	305.16	709.84
Xylose	45.60	203.17
Ribose	9.79	31.05
Galacturonic acid	7.65	39.48
Glucoronic acid	5.48	4.71

The total value for the exponentially grown culture is 385.26. For the culture in the stationary growth phase the sum is 1306.51.
Measurements have been conducted in three parallels. Standard deviation is always < 10%.

Substrate utilization tests of *Chl. chlorochromatii* showed that after three days of incubation, only the epibiont cultures supplemented with peptone and acetate showed a significantly higher OD_{650} than the control tubes without any organic carbon source ($p < 0.05$). Alanine, glycine, lysine, threonine, tyrosine, valine and cysteine inhibited growth ($p < 0.05$), whereas the remaining samples did not have any significant impact on the growth of *Chl. chlorochromatii* (Fig. 12). This matches with the fact that all these amino acids are excreted by *Chl. chlorochromatii* (except of lysine and cysteine, for which excretion has not been determined). One remaining question in this context is the minimal inhibitory concentration of these substrates. As demonstrated previously, the growth inducing effect of peptone and acetate on *Chl. chlorochromatii* could be confirmed. It is known that these substrates are photoassimilated in the presence of hydrogen sulfide and hydrogen carbonate (Vogl *et al.* 2006). Usually, peptone only consists of amino acids and peptides, which occur from peptolytic cleavage with pepsine. But no one of the tested amino acids increases growth of *Chl. chlorochromatii*. Therefore, the constituents of peptone that promote growth remain to be identified.

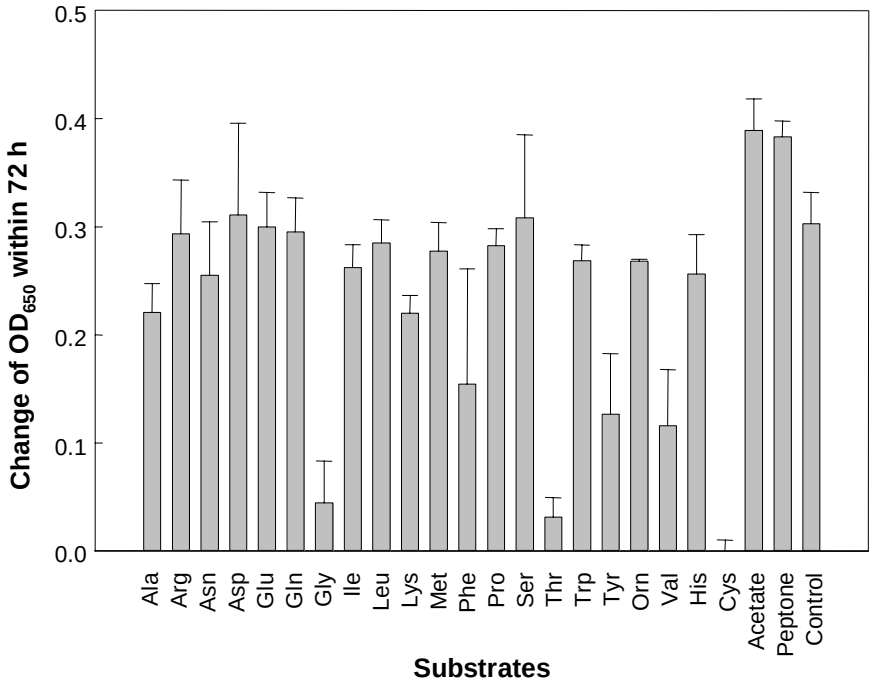

Fig. 12. Substrate test of *Chlorobium chlorochromatii*. The effect of peptone (0.05%), acetate (3 mM) and L-amino acids on the growth of the epibiont of „*C. aggregatum*" has been tested. Names of amino acids are given in standard abbreviations. Alanine, argingine, glycine, lysine, phenylalanine, proline, serine, threonine, ornithine, valine, and histidine was added in a final concentration of 5 mM; asparagine, aspartate, glutamate, glutamine, isoleucine, leucine, metionine, tryptophane, tyrosine, and cysteine was added in a final concentration of 1 mM.

6.5 CONCLUSIONS

In this study we obtained new insights into the physiology of the symbiotic GSB *Chl. chlorochromatii*, which help to further explain the physiological basis in the phototrophic consortium "*C. aggregatum*". We could show, that the obligately anaerobic GSB *Chl. limicola* and *Chl. chlorochromatii* are able to survive under oxygen exposition and constitutively express relevant enzymes for oxygen detoxification. Most of the genes encoding those enzymes share a common phylogenetic origin, suggesting that the oxygen tolerance is not unique to the two investigated strains but a general feature of GSB, which so far was overseen. We suppose that comparatively lower tolerance towards oxygen of the epibiont might be a physiological adaptation to its symbiotic lifestyle. We found 7,8-dihydro-γ-carotene in *Chl. chlorochromatii* as a novel carotenoid in nature. We propose, that the presence of this carotenoid together with the lack of chlorobactene accompanied by new carotenoid metabolism of the epibiont might be one reason for the comparatively lower oxygen

tolerance. It remains to be proven, if this is an adaptation to the symbiosis. The next step should now be an investigation of the physiology of GSB a with respect to molecular oxygen. However, to elucidate further physiological properties which explain the basis of the symbiosis in phototrophic consortia, our results need to be compared not only with other GSB but also with epibionts of other phototrophic consortia, as it is now possible with a new green phototrophic consortia isolated in enrichment culture from Lake Dagow (Pfannes *et al.* in press, *Environ Microbiol*)

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7 UNPUBLISHED DATA

7.1 ITS1-PHYLOGENY OF THE CENTRAL BACTERIUM

7.1.1 Introduction

In bacteria, the 16S and 23S rRNA genes are separated by an internal transcribed spacer (ITS1), which is transcribed together with the ribosomal genes (ITS1) (see Fig. 3, Chapter 4). This intergenic spacer exhibits much greater sequence variability in sequence, lengths, and in number of alleles per genome, which renders it a suitable target for microbial typing and identification purposes (Barry *et al.* 1992, Dasen *et al.* 1994, Gürtler and Stanisich 1996). Besides the ITS1, which usually carries one or two genes for tRNAs, the ITS2 spacer region separates the 23S rRNA and 5S rRNA genes. In contrast to the ITS1, however, little sequence information is available on the intraspecies variability of ITS2 (Maiwald *et al.* 2000). Sequencing of the ITS regions therefore provides valuable targets for subsequent investigations of the diversity and physiology of the central bacterium of phototrophic consortia. In addition, fluorescence *in situ* hybridization (FISH) probing of ITS transcripts can be used as a selective and culture-independent analysis of the phsiological state of the central bacterium in intact consortia employing ITS-FISH activity staining. This method serves as a measurement of physiological activity by monitoring rapid changes in the intracellular levels of precursor rRNA that occur during a response to growth stimulation by appropriate substrates (Oerther *et al.* 2000, Schmid *et al.* 2001).

7.1.2 Experimental Procedures

Sequencing of the ITS1 region was accomplished by primer walking using the custom designed four primers CR-ITS-42f, CR-ITS-90f, CR-23S-575r, and CR-23S-632r (Pfannes *et al.* 2007, Chapter 4). In order to recover the most closely related sequences, the BLAST algorithm as implemented in the GenBank database was employed (Altschul *et al*. 1997). The corresponding sequences were recovered from the GenBank database and ITS sequences were aligned using the program Clustal X (Thompson *et al*. 1997). Alignments were adjusted manually by using the program GeneDoc (Nicholas and Nicholas 1997). The tRNAs were

identified using the program tRNAscan-SE, version 2.21 (Lowe and Eddy 1997). Secondary structure prediction for putative tRNA genes was done by application of the tRNA-Scan software (Lowe and Eddy 1997). From the alignments, phylogenetic trees were calculated with the Phylip Maximum Likelihood program dnaML for ITS sequences (Felsenstein 1989). Designed olionucleotide probes were labeled employing the ULYSIS Alexa Fluor nucleic acid labelling kit (Molecular Probes, Karlsruhe).

7.1.3 Results and Discussion

In the course of this study both, the ITS1 and the ITS2 spacer regions of the central rod of "*C. aggregatum*" have been sequenced (Pfannes *et al.* 2007, Chapter 4). The 662 bp-long ITS1 region was found to contain two tRNA genes, $tRNA^{Ile}$ and $tRNA^{Ala}$. The presence of tRNA genes within the ITS1 spacer regions is a common but not universal feature of rRNA operons (Krawiec and Riley 1990, Jinks-Robertson and Nomura 1987). Thus, the presence and organisation of tRNAs in the central bacterium does not mirror its unique *rrn* tandem operon stucture (Pfannes *et al.* 2007, Chapter 4). Comparison of the sequences of over 30 clones obtained from the central bacterium of *„C. aggregatum"* showed no sequence ambiguity. Therefore the sequence divergence of *rrn* operons in this bacterium must be very small.

Phylogenetic tree reconstruction based on the ITS1 sequences of *„C. aggregatum"* and its closest relatives confirms the isolated position of the central rod within the *Betaproteobacteria* (Fig. 1). Branching pattern and clustering of ITS1 sequence types were consistent with those of *rrs*-phylotypes (Kanzler *et al*. 2005, Chapter 3). Independently from the data set analysed and treeing method used, the central bacterium of *„C. aggregatum"* forms a stable, isolated line of decent within the *Comamonadaceae*.

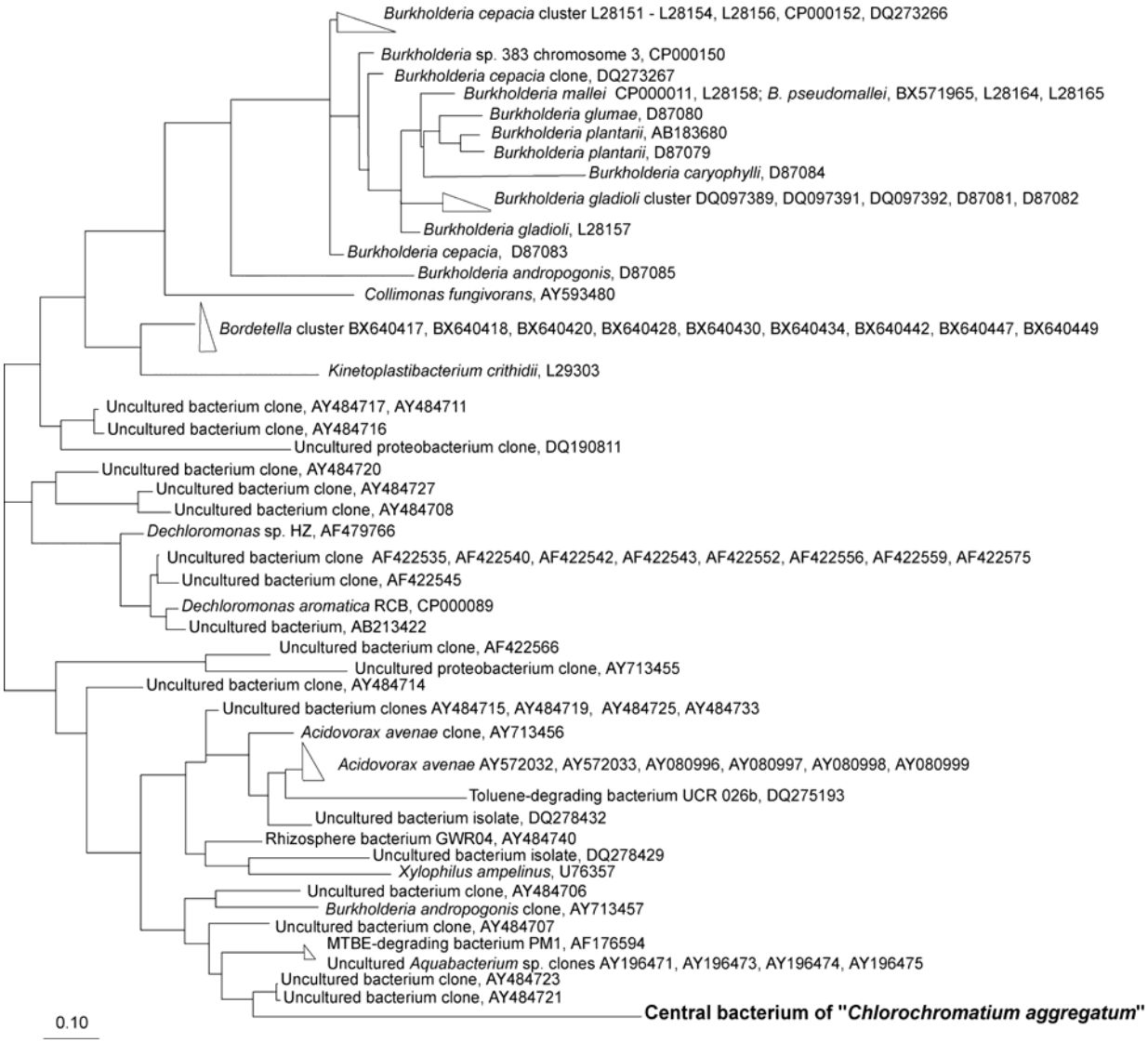

Figure. 1. Phylogenetic tree showing the relationship of the central bacterium of *„C. aggregatum"* and the closely related *Betaproteobacteria* based on the DNA sequences of its 16S-23S rRNA spacer (ITS1) region. The maximum likelihood method was used. The bar represents 10% estimated sequence divergence.

So far, the central bacterium of the phototrophic consortium *„C. aggregatum"*, has escaped all cultivation attempts, in contrast to its epibiont. Therefore, future physiological analyses of the central bacterium still rely on the availability of culture-independent molecular tools. The half-life of the ITS1 transcript present in the precursor RNA of the *rrn* operon is significantly shorter than that of the more stable 16S rRNA. Cellular concentrations of the ITS1 transcripts thus represent a suitable indicator for the momentary activity of the cells, and can be quantified by FISH using specific oligonucleotide probes (Oerther *et al.* 2000, Schmid *et al.* 2001). Since enrichment cultures of *„C. aggregatum"* contain eight other accompanying chemotrophic bacteria, specificity of ITS1-probes, which have been designed in this work, is an important prerequisite for their application during ITS-FISH (Table 1). Based on sequence analysis, the pronounced sequence differences of the ITS1-region of the central bacterium to all sequences in the database render the ITS1-region a suitable target for the *in situ* monitoring of the activity of the central rods in the enrichment culture and in their natural environment. The availability of these probes thus will allow the identification of physiological requirements of the central bacterium like potential substrates and optimum growth conditions in future research.

Table 1. Fluorescently labeled probes against the ITS1

Probe	Sequence (5′-3′)	T_m (°C)
ITS1-4	AAG TTC CTT TGT TCG CCC	53.8
ITS1-12	AGC CTC TTC GGC TAG CAT	55.9
ITS1-21	CGA CCA TCT CCG TCA GGT	58.3

*Numbers indicate 5′- end according to *E. coli* numbering.

7.2 EPIBIONT SEQUENCES – MICROMANIPULATION

7.2.1 Introduction

Phylogenetic analyses of epibionts of phototrophic consortia provide a first insight into the evolution of symbiosis in green sulfur bacteria. When 16S rRNA gene sequences were determined for phototrophic consortia from different freshwater environments in Europe and North America, a total of 19 different phylotypes of epibionts were detected (Glaeser and Overmann 2004). None of these epibiont 16S rRNA gene sequences have so far been found in any free-living green sulfur bacterium, indicating that phototrophic consortia do not represent random associations of bacteria, which are formed just by chance, but rather comprise unique types of bacteria specifically adapted to symbiosis. Based on previous phylogenetic analysis of 16S rRNA genes, neither the epibionts of different types of phototrophic consortia are monophyletic (Glaeser and Overmann 2004) nor the central bacteria (Pfannes *et al.* 2007). Thus, the ability to form symbiotic associations either arose independently from different ancestors or, alternatively, has been present in a common ancestor prior to the radiation of green sulfur bacteria and the transition to a free-living state in independent lineages. This raises the question, whether a coevolution of epibionts and central bacteria has occurred. Recently, with the identification of the 16S rRNA sequences of the central bacterium of "*Chlorochromatium aggregatum*" and a novel consortium, sequence information became available for the first time for the chemotrophic partner bacterium in phototrophic consortia (Kanzler *et al*. 2005, Pfannes *et al.* 2007). Additionally four candiate sequences for an additional central bacterium were obtained (Pfannes *et al.* 2007). To facilitate future coevolution studies, at least three complete "sets" of phototrophic consortia, comprising sequence information for both partner bacteria within the consortium are necessary. Therefore, the corresponding epibiont sequences were obtained by micromanipulation in this work.

7.2.2 Experimental Procedures

Water samples were obtained from the chemocline of Lake Dagow in September 2006. Photorophic consortia were mechanically separated from the chemocline microbial community using a micromanipulator connected to an inverted microscope as described

previously (Fröhlich and König 1999, Fröstl and Overmann 2000, Glaeser and Overmann 2004). Batches of at least 50 consortia of same morphology and colour were collected and directly subjected to PCR amplification. 16S rRNA gene fragments that were 456 bp long were amplified by employing the primers GC/341f (Muyzer *et al.* 1993) and GSB/822r (Overmann *et al.* 1999) and subsequently sequenced.The corresponding central rods have been identified with the primers developed for the amplification of *Betaproteobacteria* with a rare tandem *rrn* operon structure as desrcibed in Pfannes *et al.* 2007.

7.2.3 Results and Discussion

The study of consortia in several lakes worldwide (Pfannes *et al.* 2007) led to the discovery of novel sequence types, which potentially represent those of central bacteria of phototrophic consortia. Within this study the central bacterium sequence has been identified from a novel green-coloured consortium from German Lake Dagow. Moreover, four candidate sequences of a brown-coloured type of consortium have been identified (Pfannes *et al.* 2007). Here, the two 16S RNA sequences of the corresponding epibionts could be determined by micromanipulation and subsequent PCR amplification. Both are sequences, that have not been identified by former sampling of Lake Dagow. The first epibiont sequence is derived from a green consortium morphologically resembling "*C. aggregatum*"; the second sequence was obtained from the dominating consortium in the chemocline of Lake Dagow, ressembling a consortium formerly described as "*Pelochromatium roseum*" (Table 1) (Overmann *et al.* 1998, Glaeser and Overmann, 2004).

This thesis provides for the first time sequence information of several complete "sets" of phototrophic consortia, including sequence information for both partner bacteria within the consortium. Together, these sequences can now be used to directly compare their phylogeny and hence will provide the unique opportunity to assess for the first time the process of the coevolution of a bacteria-bacteria-symbiosis. For this purpose it is now essential that of the achieved candidate sequences the correct one is allocated to the central bacterium of "*P. roseum*", since a minimal of three sets of consortia are the prerequisite for the performance of coevolution studies.

Table 2. Corresponding sequences of epibionts from newly identified phototrophic consortia from Lake Dagow obtained by micromanipulation

>**brown epibiont** (*"Pelochromatium roseum"*)
TGAGGAATATTGCGCAATGGGCGAAAGCCTGACGCAGCAACGCCGCGTGGATGATGAAGTTCTTCGGAATGTA
AAGTCCTTTTGTAGAGGAAGAATATCCTGGTTTACCGGGACTGACGGTACTCTACGAATAAGCCACGGCTAACT
CTGTGCCAGCAGCCGCGGTGATACAGGGGTGGCAAGCGTTGTCCGGATTTACTGGGTGTAAAGGGTGCGCAGG
CGGGCCGATAAGTCGGGGGTTAAATCCATGTGCTTAACACATGCATGGCTTCCGATACTGTCGGCCTAGAGTCT
CGAAGAGGAAGATGGAATTTCCGGTGTAACGGTGGAATGTGTAGATATCGGAAAGAACACCAGTGGCGAAGG
CAGTCTTCTGGTCGAGCACTGACGCTCAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAAACGATG

>**green epibiont** (*"Chlorochromatium aggregatum"*)
TGAGGAATATTGCGCAATGGGCGAAAGCCTGACGCAGCAACGCCGCGTGGATGATGAAGTTCTTCGGAATGTA
AAGTCCTTTTGTAGAGGAAGAATATCCCGGTTTACCGGGAATGACGGTACTCTGCGAATAAGCCACGGCTAACT
CTGTGCCAGCAGCCGCGGTGATACAGGGGTGGCAAGCGTTGTCCGGATTTACTGGGTGTAAAGGGTGCGCAGG
CGGAATAATAAGTCGGGGGTTAAATCCATGTGCTTAACACATGCACGGCTTCCGATACTGTTTTTCTAGAGTCTC
GAAGAGGAAGATGGAATTTCCGGTGTAACGGTGGAATGTGTAGATATCGGAAAGAACACCAGTGGCGAAGGC
AGTCTTCTGGTCGAGTACTGACGCTCAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATG

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8 DISCUSSION

8.1 THE CENTRAL BACTERIUM OF "*CHLOROCHROMATIUM AGGREGATUM*"

This work was dedicated to characterize in detail both symbiotic partner bacteria in the phototrophic consortium "*Chlorochromatium aggregatum*" in detail. Due to the low cell numbers of central bacteria, contributing only about 0.07% to the available cultures, very little information of the central rod-shaped bacterium of "*C. aggregatum*" was available at the beginning of this thesis. Its phylogenetic position had not been determined precisely. Likewise, no molecular experiments could be conducted due to the lack of sequence information. Therefore, the establishment of a suitable method for the separation of genomic DNA of the central bacterium from DNA of the accompanying bacteria was the crucial step in this study. For this purpose, the chemotaxis of "*C. aggregatum*" towards sulfide (Fröstl and Overmann 1998) was exploited as a rapid means to selectively enrich intact consortia. With this approach the central bacteria could be enriched sufficiently to identify the partial 16S rRNA sequence via PCR, DGGE and subsequent FISH analyses (Kanzler *et al.* 2005, Chapter 3).

Since chemotaxis enrichments yielded numbers of consortia far too low for the preparation of genomic DNA of the central bacterium, a large-scale purification method for its genomic DNA based on CsCl-bisbenzimidazole equilibrium density centrifugation was established. Real-time PCR showed, that in comparison to the original enrichment culture of "*C. aggregatum*", genomic DNA of the central bacterium was enriched 150-fold (Kanzler *et al.* 2005, Chapter 3). Nevertheless, the 16S rRNA gene sequence of the central bacterium could not be detected by PCR-DGGE with eubacterial primers. This is in line with data from another study, in which a detection limit for DNA on DGGE gels of 9% was determined (Straub *et al.* 1998). Ultimately, the development of a method to reproducibly grow intact "*C. aggregatum*" in a monolayer biofilm on the walls of large culture flasks (Pfannes *et al.* 2007, Chapter 4) and subsequent CsCl-bisbenzimidazole equilibrium density gradient centrifugation turned out to be the best method for recovery of a sufficient quantity of

genomic DNA for genome sequencing. Compared to the usual enrichment cultures of "*C. aggregatum*" this new combined approach allowed an enrichment of genomic DNA from the central bacterium by approximately 600-fold. Additionally, this approach yielded high quality DNA for genome sequencing as well as for further genome analyses (Pfannes *et al.* 2007, Chapter 4).

In previous FISH-analyses of the "*C. aggregatum*" enrichment culture, the central bacterium could be identified as a member of the *Betaproteobacteria* (Fröstl and Overmann 2000). In this study its precise phylogenetic position has been determined. Analyses of the 16S rRNA (Kanzler *et al.* 2005, Chapter 3) and the ITS1 (Chapter 7) revealed that the central bacterium represents a so far isolated phylogenetic lineage within the family *Comamonadaceae* and clusters with the genera *Rhodoferax* and *Polaromonas* (Glöckner *et al.* 2000). Its closest cultured relative is *Rhodoferax ferrireducens* DSMZ 15236^T with 94.7% sequence similarity.

Additional information about the phylogenetic position of the central bacterium is provided by the mol% G+C content, which was indirectly determined by density gradient centrifugation (Kanzler *et al.* 2005, Chapter 3). With 55.6% it is consistent with the values identified for other *Comamonadaceae*, spanning a range between 52% and 70% (Hiraishi *et al.* 1991, Irgens *et al.* 1996, Jung *et al*. 2004, Willems *et al.* 1991). Besides its function as an additional proof of the phylogenetic categorization of the central bacterium, the mol% G+C content serves as a useful tool in the assembly of the central bacterial genome.

The majority of relatives of the central rod are not-yet-cultured and were found in low-temperature aquatic environments, or aquatic environments containing pollutants like monochlorobenzene and tetrachloroethene, or hydrocarbons (Alfreider *et al.* 2002, Brinkmeyer *et al.* 2003, Finneran *et al.* 2003, Hiraishi *et al*. 2001, Jung *et al*. 2004, Watanabe *et al.* 2000). So far, very little is known about the physiology of the central bacterium of phototrophic consortia. It has been shown, that phototrophic consortia exibit a chemotactic response towards 2-oxoglutarate (Fröstl and Overmann 1998, Glaeser and Overmann 2004), which is taken up by them (Glaeser and Overmann 2003b). Recently, the epibiont of "*C. aggregatum*" could be isolated in pure culture and was found to be incapable of using 2-oxoglutarate (Vogl *et al.* 2006, Chapter 5). Taken together, these findings suggest that 2-oxoglutarate is utilized by the central bacterium.

Most of the cultivated relatives of the central bacterium have no conspicuous physiological properties. Along with the large phylogenetic distance to other known bacteria, physiological properties hence cannot be inferred from its phylogenetic position. Therefore an analysis of the genome sequence and culture-independent physiological studies were a substantial part of this work. Enriched genomic DNA from the central bacterium was used to analyse the regions flanking its known 16S rRNA gene sequence. In this course, the sequence of a complete rRNA operon was obtained by a series of specific PCR amplifications, cloning and sequencing. It was found, that two *rrn* operons occur in a tandem arrangement, in which the 5S rRNA of the first operon (*rrnA*) is separated from the latter (*rrnB*) by a very short intergenic region of only 195 bp, named interoperon spacer (IOS). To date, tandem operons with an interoperon spacer of ≤ 1000 bp have been found in only 5.9% of sequenced bacterial genomes (Pfannes *et al.* 2007, Chapter 4). Most notably, of all 31 available genomes of *Betaproteobacteria*, only the closest relative of the central bacterium, *Rhodoferax ferrireducens*, exhibits a tandem arrangement of *rrn* operons. Harbouring two *rrn* operons with identical sequences, it best matches with the *rrn* operon organisation observed in the central bacterium. This additionally mirrors the determined phylogenetical affiliation of the central bacterium.

The fact that the tandem *rrn* operon structure was found only once among the *Betaproteobacteria*, namely in the closest relative of the central bacterium of "*C. aggregatum*", was exploited to develop a specific PCR method for the detection of rRNA sequences of central bacteria in other types of phototrophic consortia and its relatives (Pfannes *et al*. 2007, Chapter 4). New betaproteobacterial sequences encompassing the *rrnA-rrnB* interoperon region could be obtained using DNA extracts from chemocline environments worldwide. All these sequences, which had been recovered from the bacterial communities in different years, were closely related to each other and formed a novel, distinct subcluster within the phylum *Betaproteobacteria*. 74% of the phylotypes exhibited a sequence divergence of < 3%, indicating that chemoclines of freshwater lakes exhibit an unexpected microdiversity. So far, virtually nothing is known about the microdiversity patterns of chemocline microbial communities in lacustrine (Overmann *et al.* 1999, Casamayor *et al.* 2000) and marine (Madrid *et al.* 2001, Sass *et al.* 2001, Vetriani *et al.* 2003) habitats. These findings indicate, however, that chemoclines of freshwater lakes may provide a multitude of ecological niches not only for

phylogenetically distant and physiologically different groups like sulfate-reducing bacteria, methanogenic archaea, purple and green sulfur bacteria or sulfur oxidizing *Gamma-* or *Epsilonproteobacteria* (Overmann *et al.* 1999, Vetriani *et al.* 2003) but also for phylogenetically much closely related groups of aquatic bacteria. It remains to be elucidated whether the considerable microdiversity is limited to the newly discovered subcluster of *Betaproteobacteria* or also occurs among the other phylogenetic groups present in the chemocline.

The novel subcluster also encompassed the sequence of the central bacterium of "*C. aggregatum*". Therefore, a method which permits a selective retrieval of 16S rRNA gene sequences of the chemotrophic partner bacterium in other phototrophic consortia was established. Based on the phylogenetic results it was hypothesized that, if different phylotypes of central bacteria occur in different phototrophic consortia, they may exhibit a tandem arrangement of their *rrn* operons similar to the central bacterium of "*C. aggregatum*". This hypothesis could be confirmed (Pfannes *et al*. 2007, Chapter 4). According to this, it was speculated that a short IOS is a feature of the chemotrophic central bacterium in phototrophic consortia since the IOS of the central bacterium of "*C. aggregatum*" is with 195 bp the shortest among all clones of our libraries and with the exception of *Bacillus subtilis* subsp. *subtilis* strain 190, the second shortest in all bacteria. Specific targeting of environmental clones with short IOS sequences of the tandem *rrn* operons by FISH indeed resulted in the identification of two novel phylotypes of central bacteria in addition to the symbiont of "*C. aggregatum*". Interestingly, our comprehensive phylogenetic analysis demonstrated that the three known phylotypes of central bacteria are members of a newly discovered subcluster *Betaproteobacteria* with a tandem *rrn* operon organisation. However, they do not form a distinct group with the new subcluster of the *Betaproteobacteria*. The current phylogeny of the chemotrophic partner bacteria therefore reveals a polyphyletic origin of these bacteria (Pfannes *et al*. 2007, Chapter 4), similar to the phylogeny of their phototrophic counterparts (Glaeser and Overmann 2004). This suggests that the ability to form a symbiotic association with green sulfur bacteria either arose independently from different ancestors or, alternatively, may have been present in a common ancestor prior to the radiation of the newly discovered subcluster of *Betaproteobacteria* and a transition to the free-living state in independent lineages.

To elucidate further properties that help to explain the basis of the symbiosis in

phototrophic consortia and to get insights into their coevolution, not only independent comparisons for the epibionts and the central bacteria are required, but also for pairs of epibionts and central rods which together form a consortium. In this regard, substantial progress has been made in the course of this study not only with the establishment of a molecular screening tool for central bacteria in phototrophic consortia, but also with the isolation of a new green phototrophic consortium in enrichment culture and the yield of corresponding central bacterial- and epibiont sequences from novel phototrophic consortia (Pfannes *et al.* 2007, Chapter 4 and Chapter 6).

8.2 THE EPIBIONT OF "*CHLOROCHROMATIUM AGGREGATUM*"

Using 16S rRNA-based methods, the epibionts of phototrophic consortia were previously identified as green sulfur bacteria (GSB) (Fröstl and Overmann 2000, Tuschak *et al*. 1999). In the associated state, they grow photoautotrophically like their free-living GSB relatives (Glaeser and Overmann 2003a). However, until recently, nothing was known about their physiology in the non-associated state. This was changed with the isolation of the epibiont of "*C. aggregatum*" as the first epibiont of phototrophic consortia in pure culture (Vogl *et al.* 2006, Chapter 5). Its isolation proves that the organism is capable of growing free-living like other GSB. Thus, the association is not obligatory, one crucial issue in the understanding of the bacterial association in phototrophic consortia.

The phylogenetic analysis of the 16S rRNA gene of the epibiont placed the new isolate in the phylum of the GSB. Similar to the central bacterium, whose closest relative had 94.7% sequence similarity (Kanzler *et al.* 2005, Chapter 3), no close relationship was found to any GSB strain cultured so far. With 96.6%, the highest sequence similarity was found to the 16S rRNA gene sequence of *Chlorobium phaeobacteroides* III. Based on several novel properties and the phylogenetic analysis, the epibiont was described as a novel species, named *Chlorobium chlorochromatii* strain CaD (Vogl *et al.* 2006, Chapter 5). In pure culture, cells of *Chl. chlorochromatii* are immotile, anaerobic, photolithoautotrophic and only grow with sulfide as electron donor. Cells are Gram-variable upon staining and KOH-string test (Bartholomew 1962, Gregersen 1978), but electron micrographs revealed that cells exhibit a typical Gram-negative cell wall structure (Vogl *et al.* 2006, Chapter 5). The hydrophobicity index of free epibiont cells was shown to be 8.6% in contrast to *Amoebobacter purpureus*,

which has a hydrophobicity index of 96% and aggregates due to cell-to-cell adhesion (Overmann and Pfennig 1992). It is therefore unlikely that *Chl. chlorochromatii* cells have a sufficiently hydrophobic cell surface for the aggregation of the consortia to be based on hydrophobic interactions alone. The optimal growth temperature for *Chl. chlorochromatii* was shown to be 25 °C and optimum pH was 7.0 to pH 7.3. In comparison with free-living species of GSB, which mostly exhibit a pH optimum of 6.8 (Overmann 2001), the pH optimum of the epibiont thus is slightly shifted towards the alkaline range, which may reflect an adaptation to the symbiosis with the central bacterium.

The major amino acid excreted by *Chl. chlorochromatii* is glutamate (Pfannes *et al.* in prep., Chapter 6). Glutamate is involved into the 2-oxoglutarate pathway and these two compounds can easily be inconverted. As the epibiont is able to fix nitrogen and glutamate is formed by 2-oxoglutarate and ammonium, it is possible that nitrogen compounds of the epibiont influence the metabolism of the central bacterium. Former studies revealed the metabolic coulping of the central bacterium and the epibiont. While the green sulfur bacterial epibiont in consortia grows photolithoautotrophically, growth of consortia depends on the addition of exogenous 2-oxoglutarate (Fröstl and Overmann 1998, Glaeser and Overmann 2003b). Since 2-oxoglutarate is not taken up as a substrate by the epibiont (Vogl *et al.* 2006, Chapter 5) it is likely that it is used by the central bacterium. In "*Pelochromatium roseum*" it was likewise shown that 2-oxoglutarate is only incorporated by the consortia when light and sulfide are present. This indicates that the incorporation of 2-oxoglutarate by the central rod is regulated by the metabolic state of the epibionts (Glaeser and Overmann 2003b). Out of 110 different carbon substrates and amino acids tested, only acetate and peptone are photoassimilated (Vogl *et al.* 2006, Chapter 5, Pfannes *et al.* in prep., Chapter 6). This limited range of organic substrates utilized for photomixotrophic growth is consistent with the very limited physiological flexibility of other *Chlorobiaceae*.

Although the epibiont of "*C. aggregatum*" is comparatively more demanding than known green sulfur bacteria with respect to growth conditions, its overall physiology is not unusual for this bacterial group. However, it was revealed that this strain has distinct properties in comparison to its free-living relatives with respect to ultrastructure, oxygen tolerance, pigment composition and genome composition (Vogl *et al.* 2006, Chapter 5, Pfannes *et al.* in prep., Chapter 6). These differences are likely to play a role in the symbiotic

relationship of the epibiont and the central bacterium. Based on previous work, several lines of evidence indicate that a specific signal exchange occurs between the epibionts and the central bacterium in intact phototrophic consortia. First, cell division of epibionts and the central bacterium proceed in a highly coordinated fashion (Overmann *et al.* 1998). Moreover, phototrophic consortia exhibit a scotophobic response in which epibionts function as light sensors, whereas the central bacterium confers motility to the entire cell aggregate (Fröstl and Overmann 1998). Besides, incorporation of 2-oxoglutarate of intact consortia most likely is mediated by the central bacterium, as mentioned in the previous section (Glaeser and Overmann 2003b). Furthermore, differences in ultrastructure between the epibiont and free-living GSB have been revealed by electron microscopy, indicating that signal exchange between the bacterial partners in phototrophic consortia causes an intracellular sorting of chlorosomes and the formation of specific structures at the point of cell-cell-contact. Hence, conspicuous changes in the cellular morphology of the epibiont are specific for the symbiotic state (Vogl *et al.* 2006, Chapter 5).

Differences between *Chl. chlorochromatii* and their free-living counterparts have also been observed regarding oxygen tolerance. In their habitat, the chemocline of stratified freshwater lakes, GSB are exposed to oxygen concentrations up to 30 µM, which corresponds to 11% of air saturation (Overmann *et al.* 1998). Whereas aerotolerant organisms have efficient protective mechanisms such as enzyme systems and antioxidants to minimize interactions with O_2 (Holland *et al.* 1987), strict anaerobes are supposed to have no such protective adaptations (Frigaard 1997). However, in this study it was revealed, that *Chl. chlorochromatii* and *Chl. limicola*, a free-living GSB, tolerate exposure to molecular oxygen (Pfannes *et al.* in prep., Chapter 6). These two strains do not have identical responses to oxygen exposure. *Chl. limicola* survived significantly longer under oxygen exposition in comparison to the epibiont of "*C. aggregatum*". The minor tolerance of the epibiont towards oxygen could be a consequence of symbiosis. While *Chl. limicola* is an immotile organism, *Chl. chlorochromatii* has aquired motility due to its association with the flagellated central bacterium. If water layers of lakes are mixed, as it naturally occurs in autumn, this acquired motility could allow the associated epibionts to actively retreat into anoxic layers. In contrast, *Chl. limicola* is only able to reach anoxic water layers by the means of sinking, which needs more time and therefore might explain its comparatively higher tolerance towards oxygen

exposition. To clarify this hypothesis, the physiological responses of more GSB to molecular oxygen need to be investigated. It has already been shown that in *Chl. bathyomarinum*, a GSB that occurs in hot spring of the Pacific Ocean, the viability is not immediately negatively influenced by exposure to oxygen (Beatty *et al.* 2005). Phylogenetic analyses, which have been conducted to explore the origin of the observed oxygen tolerance in GSB, revealed that all GSB sequenced to date posses several genes that encode enzymes relevant for oxygen detoxification (e.g. peroxidase, catalase, superoxide dismutase). Most of these genes share a common phylogenetic origin, indicating that the oxygen tolerance is not unique to the two investigated strains, but a general feature of green sulfur bacteria (Pfannes *et al.* in prep., Chapter 6). In accordance with this, it was shown, that the tested GSB show activity for some oxygen detoxifying enzymes and that these enzymes are constitutively expressed (Pfannes *et al.* in prep., Chapter 6).

Similar to other representatives of the green-coloured GSB, the epibiont of "*C. aggregatum*" contains BChl *c* (Vogl *et al.* 2006, Chapter 5). In contrast, *Chl. chlorochromatii* is characterized by a distinct carotenoid composition in comparison to other green-coloured GSB (Pfannes *et al.* in prep., Chapter 6). Chlorobactene is typically the dominant carotenoid of green-coloured GSB (Liaaen-Jensen *et al*. 1964, Wahlund *et al.* 1991) whereas brown-coloured species mainly contain isorenieratene and β-isorenieratene (Liaaen-Jensen 1965, Overmann *et al.* 1992), but other derivatives are also found (Schmidt 1980, Takaichi *et al.* 1997). However, *Chl. chlorochromatii* is the only representative of its group that lacks chlorobactene and instead synthesizes γ-carotene (Pfannes *et al.* in prep., Chapter 6). This is in line with the finding that the gene *crtU*, which is involved in converting γ-carotene into chlorobactene (Frigaard *et al.* 2004), is not present in the genome of *Chl. chlorochromatii*. Another major difference is the presence of 7,8-dihydro-γ-carotene, a new carotenoid not identified from any other organism before, that was found to be present in *Chl. chlorochromatii* (Pfannes *et al.* in prep., Chapter 6). In 7,8-dihydro-γ-carotene, the spectral region of absorbance of this carotenoid is about 20 nm blue-shifted as compared to γ-carotene and chlorobactene. It is well known, that carotenoids can act as antioxidants by quenching singlet oxygen or photosensitizer triplet states (Edge and Truscott 1999). For this reason it is likely, that the observed differences in carotenoid composition might be a reason for the lower oxygen tolerance of the epibiont in comparison to free-living GSB.

The isolation of *Chl. chlorochromatii* into pure culture facilitated genome sequencing of the first epibiont of a phototrophic consortium (Vogl *et al.* 2006, Chapter 5). Besides *Chl. chlorochromatii*, seven other green sulfur bacteria have been prepared for genome sequencing within this study. This enables not only analyses within one genome, but also comparisons among each other, as it has been done for relevant genes in oxygen detoxification (Pfannes *et al.* in prep., Chapter 6). With 2.6 Mb, the genome of *Chl. chlorochromatii* lies in the range of all other green sulfur bacteria sequenced so far. In contrast to obligate bacterial symbionts, such as *Buchnera aphidicola* or *Wigglesworthia glossinidia*, it does not show evidence for genome size reduction (Moran 2003, Moran and Plague 2004). So far, all GSB have 2-3 Mb genomes that encode 1750-2800 genes. Pairwise comparisons revealed that all of the strains share a common set of approximately 1500 genes (Bryant and Frigaard 2006). A complex analysis of all these genomes may help to answer the outstanding question of which genes are necessary and specific for symbiosis and hence greatly improve our understanding of the genomic basis of the symbiosis in "*C. aggregatum*". Recent work has focused on the identification of symbiosis-specific genes in the epibiont employing suppression subtractive hybridization (Vogl *et al*. in prep.). Three different genomic fragments could be recovered which are unique to the genome of *Chl. chlorochromatii* strain CaD. One of those is of immediate interest with respect to the cell-cell-interaction in phototrophic consortia since it encodes a putative RTX-like protein, which is involved in mediating the specific binding of the protein to the target cells (Lally *et al.* 1999) and therefore is likely to play a role in attachment. Preliminary complementary *in silico* subtractive hybridization revealed 189 unique genes in *Chl. chlorochromatii*, of which some are likely to be involved in the interaction within the consortium and therefore should be investigated in more detail.

As more genomic sequence data become available for other green sulfur bacteria – and soon, for the first time also for a central bacterium of a phototrophic consortium – (Kanzler *et al.* 2005, Chapter 3) comparative bioinformatics will certainly catalyse advances in knowledge of their metabolism, gene regulation and physiology and hence allow a deeper understanding of the symbiosis in phototrophic consortia.

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Publications

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