Ecological and Phylogenetic Studies on Purple Sulfur Bacteria Based on Their *pufLM* Genes of the Photosynthetic Reaction Center

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GENERAL INTRODUCTION

BACKGROUND

In the Archean eon, at least 3.5 billion years ago, bacterial ancestors became capable of converting electromagnetic energy into chemical energy for cellular maintenance and growth, a mode of life wich is referred to as phototrophy. In the process called photosynthesis solar light is converted into chemical energy via a membrane bound chlorophyll-based electron transport chain, and then used in biomass production. Photosynthesis is the most important biological process on earth, today (Bryant & Frigaard 2006). Due to its importance for life on earth and its long and successful evolution in earth history photosynthesis was subject of innumerous scientific studies concerning first occurrence, evolution processes, working mechanisms, biochemistry and ecological importance e.g. (Madigan & Jung 2008, Xiong & Bauer 2002, Xiong *et al.* 2000, Yurkov & Beatty 1998, Imhoff *et al.* 1998b, Blankenship 1992, Overmann *et al.* 1991, Deisenhofer *et al.* 1985). Generally referred to as a starting point of photosynthesis research are the famous experiments and findings of Joseph Priestley, Jan Ingenhousz and Antoin-Laurent Lavoisier at the end of the 18th century. Priestley discovered that green plants "renew" the air consumed by a candle or animal. Ingenhousz realized that this process was light dependent and stated plants are absorbing CO_2, whereas Lavoisier discovered the "active" compound in the air and called it oxygen. In 1804, Nicolas-Théodore de Saussure demonstrated that water is as necessary as CO_2 for plants growth. At this point, the general chemical equation of photosynthesis was outlined (Fig.1a).

$$\text{a) } CO_2 + 2H_2O \xrightarrow{h\nu} (CH_2O) + H_2O + 2O$$

$$\text{b) } CO_2 + 2H_2S \xrightarrow{h\nu} (CH_2O) + H_2O + 2S$$

$$\text{c) } CO_2 + 2H_2A \xrightarrow{h\nu} (CH_2O) + H_2O + 2A$$

Figure 1: a) photosynthesis equation for oxygenic Cyanobacteria and plants, b) analogous for anoxygenic purple sulfur bacteria, c) universal for all photosynthetic organisms; H_2A= universal hydrogen and electron donor

Although several questions in the broad field of photosynthesis are still unresolved, our knowledge tremendously increased during the last almost 250 years of photosynthesis

research. At present two different types of photosynthesis, namely oxygenic and anoxygenic photosynthesis, are known. Both eukaryotic, such as plants, algae, diatoms and dinoflagellates, and prokaryotic organisms, i.e., cyanobacteria, carry out the oxygenic photosynthesis, which relies on two coupled chlorophyll-based photosystems (PSI and PSII) (Table I-1). Water is used as electron donor and molecular oxygen is produced as a by-product. With the advent of oxygenic photosynthesis, approx. 2.5 billion years ago, oxygen started to accumulate, changing the Precambrian Earth and forming the basis for the development of more complex organisms with aerobic metabolism (Xiong & Bauer 2002, Raymond *et al*. 2002). With the exception of deep-sea hydrothermal vent and subsurface communities, the primary production by oxygenic photosynthetic organisms supports all recent ecosystems (Raymond *et al*. 2002).

In anoxygenic photosynthesis oxygen is not formed. It is believed that this phototrophic way of life was the ancestor of the oxygenic photosynthesis (Xiong & Bauer 2002, Xiong *et al*. 2000). Anoxygenic photosynthesis is only found in a physiological, heterogeneous group of prokaryotes, characterized by the possession of only one photosystem (PSI or PSII), which use reduced sulfur compounds, hydrogen or a number of small organic molecules (acetate and pyruvate) as electron donor (Table I-1). It is believed that the oxygenic photosynthesis was preceded by anoxygenic photosynthesis, which evolved approx. 3.5 billion years ago (Xiong *et al*. 1998, Blankenship 1992). The role of anoxygenic photosynthetic bacteria is believed to have been more prominent in ancient times, and in recent times their contribution to the global primary production is low. However, they are still significant in certain aquatic environments, where their contribution to the primary production may reach up to 83% (Overmann & Garcia-Pichel 2006, Gemerden & Mas 1995).

Due to their relatively simple mechanisms and structures, anoxygenic phototrophic bacteria have been used as model systems to understand the fundamentals of the light-driven processes, the architecture of the photosynthethic units, the genetics of structural and regulatory components and insights into evolution (Gest & Blankenship 2004).

Table 1: Lineages of phototrophic prokaryotes, their preferred growth modes, pigments, reaction center types, and CO_2-fixation pathways; GNSB= green non sulfur bacteria, GSB= green sulfur bacteria, PNSB= purple nonsulfur bacteria, PSB= purple sulfur bacteria, AAPB= aerobic anoxygenic phototrophic bacteria, PRCB= proteorhodopsin containing bacteria, BChl= Bacteriochlorophyll, Chl= Chlorophyll, PBS= Phycobilisomes, ICM= intracytoplasmic membrane, HPP= Hydroxypropionate pathway, CC= Calvin cycle, rTCA= reductive tricarboxylic acid cycle

Taxon			**Preferred growth mode**	**Light harvesting**	**Photochemical reaction**	**CO_2-fixation**
Chloroflexi	GNSB		anoxygenic photoorganoheterotroph	Chlorosomes BChl c carotenoids	Type II reaction center	HPP
			aerobic chemoorganoheterotroph	-	-	-
Chlorobi	GSB		anoxygenic photolithoautotroph	Chlorosomes BChl c/d/e carotenoids	Type I reaction center	rTCA
Helio-bacteria			anoxygenic photoorganoheterotroph	BChl g carotenoids	Type I reaction center	-
Acido-bacteria		„Cand. Chlor-acidobacterium thermophilum“	anoxygenic photoorganoheterotroph?	Chlorosomes BChl a/c	Type I reaction center	?
Alpha-proteo-bacteria	PNSB		anoxygenic photoorganoheterotroph	ICM BChl a/b carotenoids	Type II reaction center	CC
			aerobic chemoorganoheterotroph	-	-	-
	AAPB		aerobic chemoorganoheterotroph	BChl a	Type II reaction center	-
Beta-proteo-bacteria	PNSB		anoxygenic photoorganoheterotroph	ICM BChl a/b carotenoids	Type II reaction center	CC
			aerobic chemoorganoheterotroph	BChl a	Type II reaction center	-
	AAPB		aerobic chemoorganoheterotroph	BChl a	Type II reaction center	-
Gamma-proteo-bacteria	PSB	Chromatiaceae Ectothiorhodo-spiraceae	anoxygenic photolithoautotroph	ICM BChl a/b carotenoids	Type II reaction center	CC
	AAPB		aerobic chemoorganoheterotroph	BChl a	Type II reaction center	-
Cyano-bacteria and relatives			oxygenic photolithoautotroph	Thylacoids PBS Chl a/b/d	Type I and II reaction center	CC
Archaea		Halobacteria	aerobic chemoorganoheterotroph	Purple membrane Bacterio-rhodopsin	Bacterio-rhodopsin	-
Proteo-bacteria	PRCB		aerobic chemoorganoheterotroph	Purple membrane Proteo-rhodopsin	Proteo-rhodopsin	-

ANOXYGENIC PHOTOTROPHIC BACTERIA

The term “anoxygenic phototrophic bacteria” encompasses bacteria that are able to convert radiation into chemical energy but release no oxygen. Anoxygenic phototrophic bacteria employ two distinct mechanisms to use light energy and are found in several phylogenetic lineages (Table I-1). The most prominent way is bacteriochlorophyll-based and

occurs in Alpha-, Beta-, and Gammaproteobacteria (purple bacteria), Chlorobi (green sulfur bacteria), Chloroflexi (green nonsulfur bacteria), Acidobacteria ("*Candidatus* Chloracidobacterium thermophilum"), and Firmicutes (Heliobacteria). Some Archaea, e.g. *Halobacterium* and Alphaproteobacteria like *Pelagibacter ubique* and relatives of the SAR11 cluster use a light-driven proton pump consisting of retinal resembling pigments, bacteriorhodopsin and proteorhodopsin, respectively. For the sake of completeness, it should be mentioned that few Cyanobacteria, e.g. *Oscillatoria limnetica*, at special circumstances perform anoxygenic photosynthesis, as well. (Madigan *et al.* 2003)

Besides this common theme of using light energy, anoxygenic phototrophic bacteria are extremely heterogeneous especially the bacteriochlorophyll carrying Eubacteria. This heterogeneity is demonstrated by great variations in morphology, radiation capturing pigments, habitats, phylogeny, and physiology. High versatility occurs not only between the higher taxa but also between single species of the same genus. In contrast to the oxygenic cyanobacteria and plants, anoxygenic phototrophic bacteria possess just one photosynthetic reaction center. The bacteriochlorophyll containing bacteria are distinguishable according to their types of reaction centers. Purple bacteria and Chloroflexi possess a type II reaction center similar to photosystem II of Cyanobacteria and plants while Chlorobi, "*Candidatus* Chloracidobacterium thermophilum" and heliobacteria carry a photosystem I of cyanobacteria and plants resembling type I reaction center.

The physiology of anoxygenic phototrophic bacteria is very flexible but can be very limited to certain groups or species. Photosynthesis may encompass the reduction of CO_2 into organic molecules, a mode of growth defined as photoautotrophy which is found in many but not all anoxygenic phototrophic bacteria performed via different pathways (Table I-1). Moreover, many anoxygenic phototrophic bacteria also use light energy to synthesize biomass from small organic compounds, which is called photoheterotrophy. In addition to the strictly phototrophic way of life the aerobic purple bacteria use anoxygenic photosynthesis in addition to their chemotrophic metabolism (Table I-1). Variability is also found in oxygen tolerance. For example, Chlorobi and Heliobacteria are strictly anaerobic, while some phototrophic purple bacteria are strictly aerobic. Heliobacteria solely possess BChl *g* and grow photoheterotrophically. Chlorobi have Chlorosomes, a special light harvesting complex that enables growth at light intensities of moon light, were found in 80 m depth of the Black Sea and tolerate highest sulfide concentrations of phototrophic bacteria. The gliding Chloroflexi isolated from a hot spring need temperatures above 50 °C albeit can grow fully aerobically. This physiological versatility enabled anoxygenic phototrophic bacteria to conquer almost all

environments on earth especially aquatic ones. A very versatile and conspicuous group of anoxygenic phototrophic bacteria in all kinds of aquatic environments are the purple bacteria.

ANOXYGENIC PHOTOTROPHIC PURPLE BACTERIA

Anoxygenic phototrophic purple bacteria are an important group of photosynthetic prokaryotes. They primarily inhabit aquatic environments, but several terrestrial members are known as well (Madigan & Jung 2008). At present, nearly 50 genera of anoxygenic phototrophic purple bacteria are described. All phototrophic purple bacteria possess one photosynthetic apparatus, which resembles the photosystem II of cyanobacteria and plants. BChl *a* and *b* together with various carotenoids of the spirilloxanthin, rhodopinal, spheroidene or okenone series are their major photosynthesis pigments. Besides their bacteriochlorophyll-based ability to use electromagnetic radiation for maintenance and growth, anoxygenic phototrophic purple bacteria are extremely heterogeneous on basis of morphological, physiological and molecular data. According to their specific features, there are currently three groups of anoxygenic phototrophic bacteria established: (1) purple sulfur bacteria (PSB) (next paragraph), (2) purple nonsulfur bacteria (PNSB), and (3) aerobic anoxygenic phototrophic purple bacteria. Further, anoxygenic photosynthesis has been demonstrated in methylotrophic bacteria and rhizobia (Giraud & Fleischman 2004, Shimada 1995). Phylogenetic analysis mainly based on the 16S rRNA gene demonstrated the splitting of PNSB into Alpha- and Betaproteobacteria, whereas the aerobic anoxygenic phototrophic purple bacteria are divided in Alpha-, Beta-, and Gammaproteobacteria. The PSB are located in the Gammaproteobacteria only. All anoxygenic phototrophic purple bacteria groups are interspersed with non-phototrophic relatives.

PSB are photoautotrophic Eubacteria (see below), whereas in PNSB the photoheterotrophic metabolism is dominating. Photosynthesis in both, PSB and PNSB, occurs only under anoxic conditions and pigment synthesis in these organisms is repressed by oxygen. However, several species, especially of the PNSB, are well equipped for chemotrophic metabolism and growth in the dark. In spite of the fact, that PSB and PNSB often co-inhabit the same environment, visible blooms as known for PSB, have not been described for PNSB (Overmann 2001). The photosynthetic apparatus of anoxygenic phototrophic purple bacteria is located in more or less extended systems of intracytoplasmic membranes. The group of purple nonsulfur bacteria is by far the most diverse group of the phototrophic purple bacteria. This diversity is reflected amongst others in greatly varying morphology, internal structure, carotenoid composition, utilization of carbon sources and electron donors (Imhoff 1995). The

name purple nonsulfur bacteria is slightly misleading because most PNSB tolerate sulfide in concentrations less than 0.5 mM and are even capable to use sulfide as electron donor.

Anoxygenic phototrophic purple bacteria have been successfully isolated from a variety of extreme habitats, e.g. hot, cold, acidic, alkaline, and hypersaline. In their indigenous habitats anoxygenic phototrophic purple bacteria are seen as primary producers (the photoautotrophs) and detoxifiers, as they eliminate toxic sulfide into less toxic substances.

Within the anoxygenic phototrophic bacteria the aerobic anoxygenic phototrophic purple bacteria have an exceptional position. In contrast to PSB and PNSB, aerobic anoxygenic phototrophic purple bacteria are primarily found in oxic habitats, e.g. the pelagic realms of open oceans. These bacteria are able to perform a photophosphorylation, but the main part of their energy needed is retrieved by chemotrophic metabolism. They are neither able to grow photoautotrophically nor capable of using BChl *a* for anaerobic growth and their pigment synthesis occurs in presence of oxygen (Yurkov & Beatty 1998). They have been isolated from such various aquatic environments as marine waters and sediments, freshwater microbial mats and hot springs (Yurkov & Beatty 1998). Aerobic anoxygenic phototrophic purple bacteria were first discovered in the late 1970s, but only nowadays their ecological importance became evident (Kolber *et al.* 2001).

PURPLE SULFUR BACTERIA

The purple sulfur bacteria (PSB) are unicellular, Gram-negative bacteria, represented by 28 genera in two families within the gammaproteobacterial order Chromatiales. They use hydrogen sulfide as electron donor in photosynthesis, which is the analogon to water in oxygenic photosynthesis, (Fig. I-1) and in general tolerate concentrations of 1-2 mM H_2S. The two families can be easily distinguished by their ability to store highly refractive globules of elemental sulfur (S^0) as an intermediate product either inside (Chromatiaceae, 24 genera) or outside (Ectothiorhodospiraceae, 4 genera) the cell-membrane. A clear distinction is also possible based on differences in quinone, lipid and fatty acid composition (Imhoff 2005b), and Ectothiorhodospiraceae species preferably grow at alkaline conditions.

Many PSB exceed a length of 1 µm and some of them are the biggest bacteria known with cell-sizes of up to 50 µm in length (e.g., *Thiospirillum*). A high variability of morphological traits is found. Coccoid (e.g., *Thiococcus*), rod shaped (e.g., *Marichromatium*) and spiral cell forms (e.g., *Ectothiorhodspira*) as well as platelets (e.g., *Thiopedia*) are known in this group and used for taxonomic classification. Motility and buoyancy is achieved by either mono- or bi-polar flagella or gas vesicles, with the exception of some non-motile *Thiocapsa* species.

The most important and selective environmental factors for PSB in the aquatic habitats are anoxic conditions, the presence of hydrogen sulfide and illumination (Imhoff 2006). PSB inhabit almost all aquatic habitats in which the given characteristics are found. They have been found in marine, brackish and freshwater habitats like coastal habitats, lagoons, lakes and other stagnant water bodies including waste water treatments, ponds and ditches. Also, more extreme environments like alkaline, hypersaline salt lakes and hot springs are inhabited by species of this group. Additional to strictly freshwater dependent species, salt-tolerant and salt-dependent, halophilic species are known. The most halophilic eubacterium is the purple sulfur bacterium *Halorhodospira halophila* in the Ectothiorhodospiraceae which is able to grow at salt-saturation (Anton *et al.* 2000). The open oceans in general are devoid of PSB. However, copepods and sponges were shown to form possible micro-habitats for these phototrophic bacteria.

Phototrophic members of the Chromatiales are generally mesophilic organisms. *Thermochromatium tepidum* as well as *Marichromatium gracile* biotype *thermosulfidiphilum* form exceptions with optimum growth at temperatures of 50°C and 44°C, respectively (Serrano *et al.* 2009, Madigan 1986). Under appropriate conditions, PSB form colorful, visible blooms in their natural habitats. The color of blooms and cell suspensions are caused by photosynthetic pigments and range from purple-violet to purple-red, red, orange-brown, yellowish brown, brownish red, brown and green. Common to all PSB is the presence of BChl *a* or *b* as well as of various carotenoids of the spirilloxanthin, okenone or rhodopinal groups (Imhoff 2005b). While BChl *a*-containing cells display absorption maxima near 800 nm and between 815-960 nm, BChl *b*-containing species absorb long-wave light at 835-850 nm and between 1010-1040 nm. The photosystem type II-like photosynthetic apparatus is located in intracytoplasmic membranes (ICM) of the vesicular (BChl *a*-containing Chromatiaceae), tubular (BChl *b*-containing Chromatiaceae) or lamellar (Ectothiorhodospiraceae) type.

Many PSB species display a high versatility in physiology. Reduced sulfur compounds like hydrogen sulfide or thiosulfate, reduced iron, molecular hydrogen (H_2) or small organic compounds serve as electron donors. All PSB are able to grow photolithoautotrophically, and use the Calvin cycle (=reductive pentosephosphate cycle) for CO_2 fixation. However, metabolically versatile species can also grow photoorganoheterotrophically using a number of small organic compounds (e.g., acetate or pyruvate) as electron donor and carbon source. In addition to their phototrophic capabilities, several members of this group show chemotrophic growth under micro-oxic to oxic conditions in the dark as well as the ability to fix molecular nitrogen (Imhoff 2005b). However, some Chromatiaceace species, e.g. *Chromatium okenii*, *Chr. weissei*, *Chr. buderii*, *Allochromatium warmingii*, *Thermochromatium tepidum*, *Thiospirillum jenense*,

Thiococcus pfennigii, and the gas vesicle containing species of *Lamprocystis* and *Thiodictyon*, are metabolically specialized. These are strictly limited to anaerobic conditions and obligatory photosynthetic.

DIVERSITY AND ECOLOGY OF PHOTOTROPHIC PURPLE SULFUR BACTERIA

Since the discovery of anoxygenic phototrophic purple bacteria in the 19th century plenty newly described species have been studied and isolated from a wide variety of planktonic and benthic environments.

First studies of PSB were conducted in mixed enrichment cultures since the 19th century, e.g., by Ehrenberg 1836, Lancaster 1873, Winogradsky 1888 (Overmann & Garcia-Pichel 2006, Imhoff 2006, Gest & Blankenship 2004). Laboratory growth and study of mixed PSB cultures were performed in the so-called “Winogradsky columns”. At the beginning, studies of PSB were limited to descriptive microscopic analysis due to the lack of a suitable medium for growth and maintenance of pure cultures. Only with the use of a suitable synthetic growth medium created by Pfennig in the 1960s, isolation and study of PSB in pure cultures became possible. Still in recent studies, the Pfennig’s medium and slight variations of it are used with great success in culture-dependent studies. Comparison of metabolic and physiological traits in pure-cultures in combination with molecular methods, e.g. DNA-DNA hybridization and ribosomal gene analysis led to the description of more than 50 purple sulfur bacteria species in 28 different genera.

Purple sulfur bacteria are widely distributed in nature and inhabit all kinds of stagnant water bodies. They are restricted to the anoxic parts of waters and sediments that receive light and in which sulfide is present. Under appropriate conditions, PSB form colorful and visible blooms, which often represent almost pure cultures. In these natural environments, the sulfide-oxidizing PSB often live in close proximity with sulfur-reducing organisms. This natural ecological community is a miniature S-cycle in itself and is called sulphuretum. (Trüper 1970, Suckow 1966, Baas-Becking 1925)

The most important properties that determine the development and distribution of phototrophic bacteria in nature are the availability of light, the concentrations of sulfide and oxygen, which form countercurrent gradients in most of the natural habitats (Imhoff 2006). Available light intensities have to be sufficient for phototrophic growth. In a certain range, PSB respond to changing light intensities by increasing or decreasing their specific photosynthesis pigment content and by migration in case of motile species. Further, the quality of light influences the growth and selection of different PSB species. The spectral

composition of light available is considerably different between pelagic and benthic habitats. While in water the potential light penetration is many meters, in sediments the development of phototrophic bacteria is restricted to the uppermost few millimeters due to strong light absorption (Taylor 1964). In the sediment, the availability of light severely limits the development of phototrophic bacteria and they often form thin, colored layers below a surface layer of cyanobacteria, e.g., "Farbstreifensandwatt" (Imhoff 1992, Pfennig 1989). Infrared radiation penetrates into the sediments and favors the growth of bacteria that use BChl *b*, which displays an absorption maximum in the far infrared. Species containing this pigment, e.g., *Thiococcus* and *Thioflavicoccus* as well as *Halorhodospira halochloris* and *Hlr. abdelmalekii*, are particularly well adapted to sediments, which are not permanently covered with water (Imhoff 2006). In the pelagic environments, due to the quick absorbance of IR radiation by water, BChl *a* containing species are selected over the BChl *b* containing species in the upper layers. In the deeper layers of water, the light consists mainly of blue to yellow-green wavelength and the use of all bacteriochlorophylls for light harvesting is limited. Therefore, the role of carotenoids (absorption maxima at 450– 550 nm) in light harvesting increases with water depth. Especially PSB species with okenone as carotenoid have an advantage in deeper water layers because of the efficient light absorption of this pigment and dominate the communities in the pelagic environment (Gemerden & Mas 1995).

Beside light, the amount of available sulfide is a selective parameter for the group of anoxygenic phototrophic bacteria as well as for different species. In general, the intracellular storage of elemental sulfur by Chromatiaceae gives these bacteria a clear advantage over those bacteria, which store elemental sulfur outside the cells (green sulfur bacteria, purple nonsulfur bacteria and Ectothiorhodospiraceae). Further, differences in hydrogen sulfide concentrations favor different PSB species, depending on their tolerance level. For example, *Marichromatium* species tolerate rather high amounts of hydrogen sulfide (4-8 mM). One representative, *Marichromatium gracile* biotype *thermosulfidiphilum* tolerates even up to 16 mM, while other species and genera tolerate only lower concentrations. Growth of *Thiopedia rosea*, for example, is inhibited by sulfide concentrations above 0.6 mM.

Chromatiaceae are basically anaerobic bacteria and photosynthetic metabolism depends on oxygen-deficient conditions, but some of the species are considerably tolerant to oxygen. Many purple sulfur bacteria are even able to make use of the oxygen and perform respiration (Kämpf & Pfennig 1980, Kondratieva *et al.* 1975). In particular, *Thiocapsa roseopersicina* has a highly flexible metabolism with the ability of respiration and photosynthesis, and is even able to grow under chemolithotrophic conditions during prolonged dark periods (Imhoff 2001, Schaub & van Gemerden 1994, de Witt & van Gemerden 1990a, de Witt & van

Gemerden 1990b). A different strategy to cope with oxic conditions is followed by *Marichromatium purpuratum*, which has adapted to anoxic niches in oxic environments even in the open ocean. It has been isolated from a strictly oxygen-demanding marine sponge, a tunicate species of the genus *Didemnum*, and from the gut of marine copepods (Proctor 1997, Imhoff 1992, Imhoff & Trüper 1980, Imhoff & Trüper 1976).

Further physico-chemical parameters which influence the community composition are, e.g., pH-value, salt concentration and differences in temperature. Communities in alkaline habitats like soda-lakes are dominated by the alkaliphilic Ecothiorhodospiraceae. Depending on the salt concentrations found, typical representatives are the extreme halophilic species *Halorhodospira halophila* and *Hlr. halochloris* in hypersaline environments and different *Ecothiorhodospira* species, e.g., *Ect. variabilis*, *Ect. mobilis*, *Ect. vacuolata* in slightly to moderately halophilic environments (Caumette 1993). In less alkaline hypersaline environments, additionally halophilic Chromatiaceae, namely *Thiohalocapsa halophila* and *Halochromatium salexigens*, are found (Caumette *et al.* 1994, Caumette 1993).

In general, PSB are mesophilic organisms. In hot springs PSB are represented by *Thermochromatium tepidum*. Psychrophilic, obligatory cold-adapted species have not been described, yet. However, presence of Chromatiaceae in sea ice has been indicated (Petri & Imhoff 2001).

By culture-dependent studies, the most diverse PSB communities were observed in marine habitats. Members of the genera *Marichromatium*, *Halochromatium*, *Thiorhodovibrio*, *Rhabdochromatium*, *Isochromatium*, *Thiorhodococcus* and *Thiohalocapsa* are generally regarded as truly marine and halophilic species and form a monophyletic branch based on 16S rRNA gene phylogenetic analysis (Imhoff 2001). *Allochromatium*, *Thiocapsa*, *Thiococcus*, *Thiocystis*, *Thiopedia* and *Thiospirillum* species are freshwater inhabitants, but tolerate salt up to a certain concentration and have been detected in marine and brackish environments regularly. In contrast, *Thiolamprovum pedioforme* , *Chromatium weisseii* and *Chromatium okenii* have been detected only in freshwater habitats.

Over many years, most of the diversity analyses of PSB were conducted by culture-based methods using Pfennig's medium (Martínez-Alonso *et al.* 2005, Vethanayagam 1991, Imhoff & Trüper 1976, Trüper 1970, Pfennig 1961) and taxonomy was based mainly upon morphological and physiological characteristics. Based on the ribosomal gene phylogeny introduced by Woese (Woese 1987), taxonomy of PSB was revised and many species and genera were reclassified and re-arranged (Imhoff *et al.* 1998b). With the establishment of culture-independent methods, e.g., cloning experiments, 16S rRNA genes were used for

diversity studies of heterotrophic bacteria. However, specific 16S rRNA gene primers for all phototrophic purple bacteria do not exist due to the wide distribution of this physiological group in the different subphyla of the proteobacteria. Specific 16S rRNA gene primers designed for the Chromatiaceae covered only 67% of all known species. The position of the primers also led to a short fragment length of only about 400 base pairs (bp) with little phylogenetic information, which was mainly used in DNA fingerprinting methods, i.e., DGGE and TTGE (Bosshard *et al.* 2000, Overmann *et al.* 1999, Coolen & Overmann 1998). Further, the reliable deduction of the physiological trait of photosynthesis is not possible based on 16S rRNA gene sequences due to the close phylogenetic proximity of purely chemotrophic relatives. The use of general bacterial 16S rRNA gene primers often underestimates the diversity of phototrophic bacteria especially of those that are only present in rare numbers.

Functional gene approaches combine phylogenetic information with indications of physiological traits. For the physiological trait of phototrophy genes included in photosynthesis process are most suitable. The combination of phylogenetic and physiological information was successfully demonstrated for the *fmoA* gene specifically occurring in green sulfur bacteria and the recently described "*Candidatus* Chloracidobacterium thermophilum" (Alexander & Imhoff 2006). Photosynthetic unit forming (*puf*) genes coding for the photosynthetic reaction center type II structural proteins are essential to all phototrophic purple sulfur bacteria and are used for functional gene approaches on phototrophic purple bacteria.

PHOTOSYNTHETIC UNIT FORMING GENES

In phototrophic purple bacteria and Chloroflexaceae the conversion of radiation energy into chemical energy takes place in the intracytoplasmic membrane-bound photosynthetic apparatus. The structural components that bind pigments are the antenna and reaction center proteins. The major light absorbing pigments in purple bacteria are bacteriochlorophylls *a*, *b* and the carotenoids. The pigments are non-covalently bound to integral membrane proteins. Together they form the light-harvesting antenna complexes and the phaeophytin/quinone (type II) reaction centers. The antenna complexes rapidly and efficiently transfer the absorbed solar radiation to the photosynthetic reaction center, where the primary energy conversion reactions of photosynthesis take place. The combination of antenna complexes with the reaction center forms the photosynthetic unit.

In purple bacteria the reaction center consists of three subunits, L (light), M (medium) and H (heavy) (Fig. 2). The L- and M-subunits are integral membrane proteins with five α-helical transmembrane domains, each. The heavy subunit is a non-pigmented apoprotein with only one transmembrane domain and a cytoplasmic domain, which shows evidence for facilitating proton uptake from the cytoplasm (Ädelroth *et al.* 2001, Cheng *et al.* 2000, Abresch *et al.* 1998). Subunits L and M both harbor associated pigments, namely the so-called "special pair" bacteriochlorophylls, accessory bacteriochlorophylls and bacteriophaeophytin. Both subunits provide the environment for electron flow in the reaction center. However, it was demonstrated that the electron flow in the light-driven process takes place only in the "L branch" (Lancaster & Michel 1996, Barber & Andersson 1994).

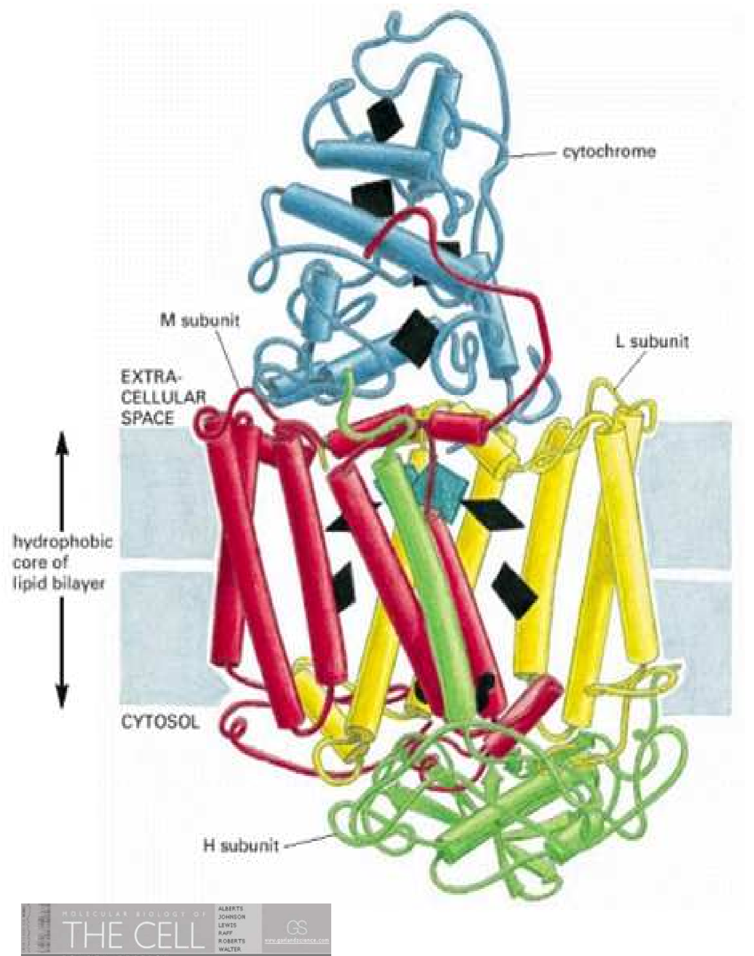

Figure 2: Three-dimensional structure of the photosynthetic reaction center type II of *Blastochloris viridis*
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Light is harvested by the pigments in the antenna complexes and transferred to the reaction centers, where the excitation of a photochemically active "special pair" of bacteriochlorophylls leads to charge separation initiating a cyclic electron transport as shown in Figure 3 (Lengeler *et al.* 1999). Two types of antenna complexes are known in purple bacteria. The light-harvesting complex 1 (LH-1) is the core antenna associated with the reaction center (RC) and present in all purple bacteria (Overmann & Garcia-Pichel 2006). The light-harvesting complex 2 (LH-2) are peripheral antennas, which surround the RC-LH-1 core unit and are present in most, but not all purple bacteria (e.g. BChl *b*-containing purple bacteria). Up to eight peripheral antenna complexes can be present depending on environmental factors such as light conditions.

In purple bacteria most genes encoding for structural and functional components of the photosynthesis process are organized in a single gene cluster of about forty-thousand nucleotides, the "photosynthetic gene cluster" (Beatty 1995). Within this cluster, most genes are grouped into several operons as transcriptional units of genes, e.g., Bchl bio-synthesis (*bch*), carotenoid biosynthesis (*crt*) and structural components of the photosynthetic core unit (*puf* and *puhA*). The *puf* (photosynthetic unit forming) operon is found in anoxygenic phototrophic bacteria of the Alpha-, Beta-, and Gammaproteobacteria and the *Chloroflexaceae*. Up to date, five different *puf* operon types are known (Tuschak *et al.* 2005) (Fig. I-4). They differ in number and arrangement of the *puf* genes. In all purple bacteria, the *puf* operon contains the core motive *pufBALM* encoding for the reaction center subunits L and M as well as the light-harvesting α- and β-proteins (Masuda *et al.* 1999, Hunter *et al.* 1991, Bauer *et al.* 1988). The *puhA* gene encodes for the H-subunit of the reaction center but is not included in the *puf* operon. In most bacteria, additional *puf* genes are found, varying in type and number (Fig. 4). *pufQ* is thought to be involved in BChl synthesis (Fidai *et al.* 1995). *puf*X is upstream of *pufM* and appears to mediate peptide interaction in the RC-LH1 core complex and to facilitate electron transfer from the reaction center to the cytochrome bc1 complex (Francia *et al.* 2002, Parkes-Loach *et al.* 2001). The *pufC* gene encodes the reaction center bound tetraheme cytochrome that serves as electron donor for the oxidized reaction center after photon excitation (Nitsche and Dracheva 1995).

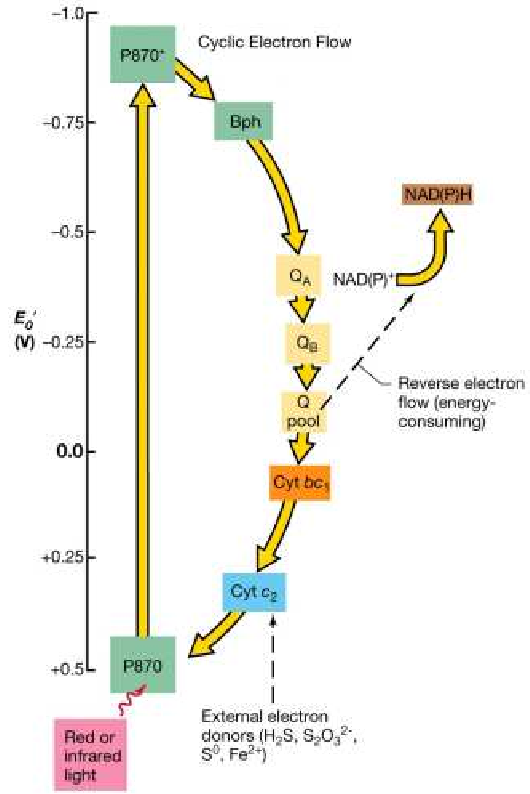

Figure 3: Cyclic electron flow in the anoxygenic photosynthesis of purple bacteria; Bph= bacteripheophytine, Q_A/Q_B= intermediate quinones, Q-pool= intramembrane quinone-pool, Cyt= cytochrome, P870= „special pair" of bacteriochlorophyll, P870*= excited „special pair" of bacteriochlorophyll

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Since the genes *pufL* and *pufM* are essential for phototrophic growth and were demonstrated to be present in all puf operon types known today, these genes have been preferred targets in molecular studies of purple bacteria (Zeng & Jiao 2007, Karr *et al.* 2003, Achenbach *et al.* 2001, Nagashima *et al.* 1997). The majority of the studies on the pufL and/or pufM genes of anoxygenic phototrophic bacteria focused on PNSB (Alpha- and Betaproteobacteria) and aerobic anoxygenic phototrophic purple bacteria (Cho *et al.* 2007, Allgaier *et al.* 2003, Beja *et al.* 2002, Overmann *et al.* 1991). In contrast, studies on *pufLM* of PSB are scarce. At the beginning of this thesis (2006), only a handful PSB *pufLM* sequences were available in public databases. Selective detection of phototrophic purple bacteria was demonstrated for PNSB and aerobic anoxygenic phototrophic purple bacteria in the mentioned studies. However, phylogenetic information of environmental clone sequences is limited due to the lack of evenly phylogenetically distributed reference sequences and the discussion of possible horizontal gene transfer of photosynthesis genes in purple bacteria (Zeng & Jiao 2007, Nagashima *et al.* 1997).

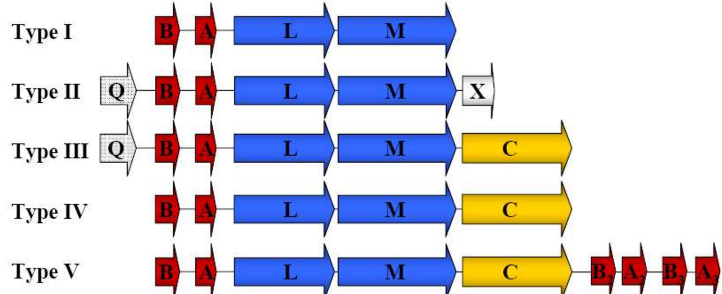

Figure 4: Different types of puf operons reported for purple bacteria (Tuschak *et al.* 2005)

AIMS OF THE STUDY

At the beginning of this thesis, ecological studies on phototrophic purple bacteria especially on purple sulfur bacteria had primarily been conducted by isolation procedures. However, culture dependent methods always show only a small fraction of bacteria sampled which was demonstrated by comparison with more sensitive culture independent methods. Due to their paraphyletic characters and the close phylogenetic relationship to non-phototrophic bacteria, ribosomal genes are not suitable for the analysis of phototrophic purple bacteria communities in their natural environment. Molecular methods using *pufL* and/or *pufM* genes for natural community analysis circumvents the occurring problems in the preceding methods. At the beginning of the thesis, environmental studies using *pufL* and/or *pufM* had focused only on purple nonsulfur and aerobic anoxygenic phototrophic proteobacteria. Specific studies on PSB were lacking and only a handful of *pufLM* sequences were available in the public databases.

The objective of this thesis was to establish a molecular method using *pufLM* genes for the specific detection of PSB communities in environmental samples and its application in ecological studies. The following topics were addressed:

1. A principal aim was to test the suitability of *pufLM* genes as phylogenetic marker. Therefore, detailed phylogenetic and sequence analyses of *pufLM* sequences of PSB were conducted and tree topologies were compared with 16S rRNA gene phylogeny. To ensure reliability of phylogenetic analyses a *pufLM* reference database for PSB was created using type strains and environmental isolates obtained in this study (Chapter I).
2. Another major topic of this thesis was to analyze environmental communities of phototrophic purple sulfur bacteria in different habitats as well as changes in their community composition under varying conditions. Community composition changes were studied with particular respect to salinity and temperature changes using the example of a highly dynamic coastal environment (Chapter II). The search for possible new phototrophic *pufLM* containing bacterial groups was conducted using the example of an extreme environment with a completely unknown anoxygenic phototrophic bacteria community (Chapter III).
3. A further objective was the application of *pufLM* genes as a distinctive criterion in polyphasic taxonomy of phototrophic purple sulfur bacteria (Chapter IV).

ORGANIZATION OF THE THESIS

This thesis is divided into different chapters approaching the different topics of the study. After the general introduction, Chapter I deals with the establishment of a *pufLM* reference database for purple sulfur bacteria (PSB) and the suitability of these genes as phylogenetic marker for the PSB. In Chapter II and III environmental phototrophic purple bacteria communities are studied using the *pufLM* genes. While in Chapter II the impact of salinity and temperature changes on a coastal phototrophic proteobacterial community is studied, in Chapter III a novel lineage of phototrophic proteobacteria was detected in a Chilean hypersaline lake. In Chapter IV *pufLM* gene sequences support the differentiation of a newly isolated strain from known species in a polyphasic taxonomy approach of a species description.

The different chapters are presented in form of manuscripts for publication, followed by a general discussion as well as cited literature. The manuscripts are published or submitted as the following titles:

Chapter I: Phylogenetic Relationship of phototrophic purple sulfur bacteria according to *pufL* and *pufM* genes

Chapter II: Impact of temperature and salinity changes on purple sulfur bacteria communities from a coastal lagoon of the Baltic Sea analyzed by *pufLM* gene libraries

Chapter III: Unique communities of anoxygenic phototrophic bacteria in saline lakes of Salar de Atacama (Chile). Evidence for a new phylogenetic lineage of phototrophic Gammaproteobacteria from *pufLM* gene analyses

Chapter IV: A new species of *Thiohalocapsa*, *Thiohalocapsa marina* sp. nov., from an Indian marine aquaculture pond

CHAPTER I

PHYLOGENETIC RELATIONSHIP OF PHOTOTROPHIC PURPLE SULFUR BACTERIA ACCORDING TO *PUFL* AND *PUFM* GENES

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ABSTRACT

The phylogenetic relationship of purple sulfur bacteria (PSB) of the Chromatiales (Gammaproteobacteria) was analyzed based on photosynthetic gene sequences of the *pufL* and *pufM* genes and the results compared to phylogenetic trees and grouping of the 16S rRNA gene. Primers for *pufL* and *pufM* genes were constructed and used to successfully amplify the *pufLM* genes of members of 16 genera of Chromatiales. In total, *pufLM* and 16S rRNA gene sequences of 66 PSB strains were analyzed, including 29 type strains and 28 new isolates. The inferred phylogenetic trees of *pufLM* and 16S rRNA genes reflect a largely similar phylogenetic development suggesting coevolution of these essential genes within the PSB. It is concluded that horizontal gene transfer of *pufLM* genes within the PSB is highly unlikely, which contrasts the situation in other groups of anoxygenic phototrophic bacteria belonging to Alphaproteobacteria and Betaproteobacteria. In consequence, *pufLM* phylogeny is in good agreement to current taxonomic classification of PSB. A phylogenetic classification of PSB to the genus level is possible based on their *pufL* or *pufM* sequences, in many cases even to the species level. In addition, our data support a correlation between Puf protein structure and the type of internal photosynthetic membranes (vesicular, lamellar or tubular).

INTRODUCTION

Purple sulfur bacteria (PSB) represent a physiological group of unicellular Gram-negative bacteria phylogenetically classified in the Gammaproteobacteria within the order Chromatiales (Imhoff 2001). They are ubiquitously distributed but restricted to aquatic habitats characterized by adequate light conditions, low oxygen tension and moderate sulfide concentrations. Under optimal conditions in nature PSB form visibly colored blooms.

The colors are due to the large amounts of carotenoids and bacteriochlorophylls (Bchl *a* or Bchl *b*) which are located in internal membranes. The most characteristic feature of all PSB is their ability to perform anoxygenic photosynthesis under anoxic conditions mainly using reduced sulfur compounds like H_2S or thiosulfate as electron donors. Anoxygenic phototrophy via bacteriochlorophylls is not restricted to the PSB but is also found in purple nonsulfur bacteria (PNSB), aerobic anoxygenic photosynthetic bacteria (AAPB), green nonsulfur bacteria (*Chloroflexaceae*), green sulfur bacteria (*Chlorobiaceae*) and *Heliobacteriaceae,* under certain conditions also in some cyanobacteria. PSB are clearly separated from all other

anoxygenic photosynthetic bacteria, the known gammaproteobacterial AAPB *Congregibacter litoralis* KT71 and strain HTCC2080 (Cho *et al.* 2007), inclusively. PSB are taxonomically treated as the Chromatiales with the *Chromatiaceae* and the *Ectothiorhodospiraceae* families, which can be distinguished by storage of elemental sulfur as intermediate during photosynthesis inside or outside the cells.

Functional gene approaches can support phylogenetic considerations concluded from the 16S rRNA gene approach. This has already been demonstrated for the FMO protein in green sulfur bacteria (Alexander *et al.* 2002). Concerning anoxygenic phototrophic bacteria, genes of the photosynthetic apparatus which are located on a photosynthetic gene cluster were used as targets in environmental studies (Beja *et al.* 2002). Part of this photosynthetic gene cluster is the *puf* (photosynthetic unit forming) operon containing genes coding for the photosynthetic reaction center type II structural proteins (Puf). *puf* coded reaction center proteins are found in anoxygenic photosynthetic bacteria of the Alpha-, Beta-, and Gammaproteobacteria and the *Chloroflexaceae.* Up to now five different *puf* operon types are known (Tuschak *et al.* 2005). They differ in number and arrangement of the *puf* genes. Since the *puf*L and *pufM* genes do not differ in the different operon types, they were preferred targets for our studies. They code for the light and medium polypeptide chains of the photosynthetic reaction center, respectively (Fathir *et al.* 1997, Belanger *et al.* 1988).

In the past years, several studies have been conducted on *puf*L and/or *pufM* genes of anoxygenic phototrophic bacteria (Jiao *et al.* 2007, Karr *et al.* 2003, Achenbach *et al.* 2001, Nagashima *et al.* 1997). These studies focussed on purple nonsulfur bacteria (Alpha- and Betaproteobacteria) and especially on aerobic anoxygenic photosynthetic bacteria (Salka *et al.* 2008, Cho *et al.* 2007, Allgaier *et al.* 2003, Beja *et al.* 2002). However, genetic information on *puf* genes belonging to photosynthetic members of the purple sulfur bacteria, the Chromatiales still is sparse. Therefore, the present study focussed on *puf*L and *pufM* analysis of PSB and analyzed the suitability of *pufLM* gene sequences as phylogenetic markers of the PSB. The *pufLM* and 16S rRNA gene sequences of 66 PSB strains were phylogenetically analyzed.

MATERIALS AND METHODS

RECOMMENDED ABBREVIATIONS OF PURPLE BACTERIA GENERA NAMES:

Alc. - *Allochromatium*, *Blc.* - *Blastochloris*, *Chr.* - *Chromatium*, *Hch.* - *Halochromatium*, *Mch.* - *Marichromatium*, *Rba.* - *Rhodobacter*, *Tch.* - *Thermochromatium*, *Tco.* – *Thiococcus*, *Tcs.* - *Thiocystis*, *Tfc.* - *Thioflavicoccus*, *Thc.* - *Thiohalocapsa*, *Trc.* - *Thiorhodococcus*

CULTIVATION OF BACTERIA

In general, cultivation of PSB was performed following the instructions given by Imhoff (2005b). Bacterial strains from our culture collection and environmental isolates (Table I-1) were grown in liquid culture (50 or 100 ml screw cap bottles) using freshly prepared Pfennig`s medium amended with 0.5 ml or 1 ml acetate-solution (NH_4-acetate/Mg-acetate (2.5 g each/100 ml).

Depending on the species 0-10% of NaCl was added to the medium (medium for marine isolates contained 2% NaCl). After inoculation, bottles were stored in the dark for several hours. Afterwards, bottles were placed into the light of a tungsten lamp (500 – 700 lx) and incubated at 25°C until turbidity was visible. In order to maintain the amount of sulfide (1 mmol final concentration) in a nearly steady state Na_2S^- were supplied to the medium when necessary. “Feeding” of the cultures and preparing of the feeding solution occurred as described in Imhoff (2005b). Fed cultures were treated as freshly inoculated cultures. The need for feeding was checked using lead (II) acetate paper (Merck). Well grown cultures were directly used for analyses and afterwards stored at 4°C or frozen in liquid nitrogen.

Table I-1: PSB strains analyzed in this study, their group affiliation and accession numbers of the corresponding *pufLM* and 16S rRNA genes

group	species	strain no.	16S rRNA acc. no.	*pufLM* acc. no.
I	*Thiococcus pfennigii*	DSM 226[T]	Y12373[§]	FN257141[p]
I	*Thiococcus pfennigii*	DSM 227	FN293056[p]	FN257142[p]
I	*Thiococcus pfennigii*	DSM 228	FN293055[p]	FN257143[p]
I	*Thioflavicoccus mobilis*	ATCC 700959[T]	AJ010126[§]	FN257144[p]
II	*“Lamprocystis purpurea“* = *Chromatiaceae* bacterium	ML1	AJ006212[a]	AY177752[k]
II	*Thiohalocapsa halophila*	DSM 6210[T]	AJ002796	FN257151[p]
II	*Thiohalocapsa marina*	JA142[T]	AM491592[b]	FN257154[p]
III	*Halochromatium roseum*	DSM 18859[T]	AM283535[c]	FN257161[p]
III	*Halochromatium salexigens*	DSM 4395[T]	X98597[§]	FN257159[p]
III	*Halochromatium* sp.[1]	MTK6IM088	FN293083[p]	FN257173[p]
III	*Halochromatium* sp.[1]	MTK2IM023	FN293080[p]	FN257169[p]
III	*Halochromatium* sp.[1]	MTK8IM030	FN293082[p]	FN257174[p]
III	*Halochromatium* sp.[1]	MTK5IM027	FN293081[p]	FN257171[p]
III	*Halochromatium* sp.[1]	MTK1IM127	FN293079[p]	FN257175[p]
III	*Halochromatium* sp.[1]	MTK2IM039	FN293068[p]	FN257170[p]
IV	*Thiorhodovibrio winogradskyi*	DSM 6702[T]	Y12368[§]	FN257136[p]
V	*Thiocapsa marina*	DSM 5653[T]	FM178270[p]	FN257140[p]
V	*Thiocapsa roseopersicina*	DSM 217[T]	Y12364[§]	FN257146[p]
V	*Thiocapsa rosea*	DSM 235[T]	FM178269[p]	FN257147[p]
V	*Thiocapsa pendens*	DSM 236[T]	AJ002797[§]	FN257145[p]
V	*Thiocapsa* sp.[2]	MTRDDF081	FN293073[p]	FN257163[p]
V	*Thiocapsa* sp.[2]	MTRDDF078	FN293074[p]	FN257164[p]
V	*Thiocapsa* sp.[3]	MTPP2IF162	FN293070[p]	FN257166[p]
V	*Thiocapsa* sp.[4]	MTWDM061	FN293078[p]	FN257178[p]
V	*Thiocapsa* sp.[4]	MTWDM010	FN293076[p]	FN257176[p]
V	*Thiocapsa* sp.[5]	MTCH3IM012	FN293065[p]	FN257182[p]
V	*Thiocapsa* sp.[6]	MTV2IF083	FN293075[p]	FN257180[p]
VI	*Thiolamprovum pedioforme*	DSM 3802[T]	FM178271[p]	FN257152[p]
VI	*Chromatiaceae* bacterium[3]	MTPP2IF163	FN293071[p]	FN257165[p]
VII	*Allochromatium warmingii*	DSM 173[T]	Y12365[§]	FN257132[p]
VII	*Allochromatium vinosum*	DSM 180[T]	FM178268[p]	FN257131[p]
VII	*Allochromatium* sp.[7]	MT6010	FN293054[p]	FN257155[p]
VII	*Allochromatium* sp.[5]	MTCH3IM013	FN293062[p]	FN257181[p]
VII	*Allochromatium* sp.[5]	MTCH3IM086	FN293063[p]	FN257186[p]
VIII	*Thermochromatium tepidum*	DSM 3771[T]	M59150[d]	D85518[l]
IX	*Thiorhodococcus mannitoliphagus*	ATCC BAA-1228[T]	FM178272[p]	FN257139[p]
IX	*Thiorhodococcus minor*	DSM 11518[T]	FN293057[p]	FN257138[p]
IX	*Thiocystis violacea*	DSM 208	FN293060[p]	FN257149[p]
X	*Thiocystis violacea*	DSM 207[T]	FN293059[p]	FN257148[p]
XI	*Thiorhodococcus drewsii*	DSM15006[T]	FM178273[p]	FN257137[p]
XI	*Thiorhodococcus kakinadensis*	DSM 18858[T]	AM282561[q]	AM944094[r]
XII	*Chromatiaceae* bacterium[4]	MTWDM004	FN293061[p]	FN257179[p]
XIII	*Thiocystis gelatinosa*	DSM 215[T]	FN293058[p]	FN257189[p]
XIII	*Thiocystis minor*	DSM 178[T]	Y12372[§]	FN257150[p]
XIV	*Chromatium weissei*	DSM 5161[T]	FN293053[p]	FN257133[p]
XV	*Thiobaca* sp.[2]	MTRDDF079	FN293072[p]	FN257162[p]

XVI	*Marichromatium gracile*	DSM 203[T]	X93473[§]	FN257134[p]
XVI	*Marichromatium purpuratum*	DSM 1591[T]	AJ224439[§]	FN257135[p]
XVI	*Marichromatium bheemlicum*	ATCC BAA-1316[T]	AM180952[e]	AM944099[m]
XVI	*Marichromatium* sp.[8]	MTKK6IM001	FN293069[p]	FN257167[p]
XVI	*Marichromatium* sp.[9]	MTCH2IM059	FN293064[p]	FN257188[p]
XVI	*Marichromatium* sp.[5]	MTCH3IM047	FN293085[p]	FN257184[p]
XVI	*Marichromatium* sp.[1]	MTK6IM015	FN293087[p]	FN257172[p]
XVI	*Marichromatium* sp.[4]	MTWDM034	FN293077[p]	FN257177[p]
XVI	*Marichromatium* sp.[1]	MTK2IM017	FN293086[p]	FN257168[p]
XVI	*Marichromatium* sp.[5]	MTCH3IM033	FN293084[p]	FN257183[p]
XVI	*Marichromatium* sp.[5]	MTCH3IM049	FN293066[p]	FN257185[p]
XVI	*Marichromatium* sp.[5]	MTCH3IM052	FN293067[p]	FN257186[p]
XVII	*Ectothiorhodospira vacuolata*	DSM 2111	X93478[g]	FN257157[p]
XVII	*Ectothiorhodospira variabilis*	WN22[T]	AM943121[s]	FN257153[p]
XVII	*Ectothiorhodospira shaposhnikovii*[†]	DSM243[T] /ATCC31751	M59151[f]	AF018955[n]
XVII	*Ectothiorhodospira haloalkaliphila*	ATCC51935	FN293052[p]	FN257156[p]
XVII	*Ectothiorhodospira imhoffii*	JA319	AM902494	AM944100[o]
XVII	*Ectothiorhodospira mobilis*	DSM237	X93481[g]	FN257158[p]
XVIII	*Halorhodospira halophila*	DSM 244[T]	CP000544[h]	CP000544[h]
XVIII	*Halorhodospira halophila*	H	FN293051[p]	FN257160[p]
outgroup	*Chloroflexus aggregans*	DSM9485[T]	AAUI01000026[i]	AAUI01000013[i]
outgroup	*Chloroflexus aurantiacus*	DSM635[T]	CP000909[j]	CP000909[j]

Footnotes: small letters denote the references for the sequences; a (Coolen & Overmann 1998), b (Kumar *et al*. 2009), c (Kumar *et al*. 2007c), d (Woese, C. R.; direct submission), e (Kumar *et al*. 2007a), f (Woese, C.R. direct submission), g (Imhoff *et al*. 1998b), h (Copland et al. direct submission WGS (whole genome sequence)), i (Copland et al. direct submission WGS), j (Copland et al. direct submission WGS), k (Zeng & Jiao 2007), l (Fathir *et al*. 1997), m (Anil Kumar direct submission), n (Gingras, G., direct submission), o (Anil Kumar, direct submission), p (this study), q (Kumar *et al*. 2007b), r (Anil Kumar, direct submission), s (Gorlenko *et al*. 2009); [§]updated 16S rRNA sequences during this study; numerals denote origin of the new environmental isolates: 1 saltern near Kakinada, indian east coast, 2 pond near Kiel, Germany, 3 pond in Hyderabad, India, 4 Wadden Sea sediment near Büsum, Germany, 5 sandy rock pool near Trivendrum, indian southwest coast, 6 Crocodile lake near Visakhapatnam, India, 7 old culture bottle, Kiel, Germany, 8 sandy rockpool near Kanyakumari southern India, 9 green colored rock pool near Trivendrum indian southwest coast; [†]16S rRNA gene sequence derived from strain DSM244[T] and *pufLM* sequences are from strain ATCC31751

ISOLATION OF NEW ENVIRONMENTAL STRAINS

In order to obtain pure cultures of environmental isolates agar shake dilution series were used and performed as described by Imhoff (Imhoff 2005b). The first agar tube was inoculated with 100 µl of an environmental sample and the inoculated test tubes were treated as described for liquid cultures (see cultivation of bacteria). Dilution series were repeated until purity was achieved (at least 4 times). Pure isolates were transferred into liquid medium. Purity of isolates was checked microscopically and acknowledged by high sequence qualities of 16S rRNA gene and *pufLM* genes.

New isolates were obtained from sampling locations in India and Germany and are found in several PSB groups (Table I-1). Marine isolates obtained from Indian east coast

habitats were identified as *Halochromatium* spp. and *Marichromatium* spp. Indian east coast freshwater isolates as well as one isolate obtained from a polluted freshwater pond of the city of Hyderabad (India) belong to the genus *Thiocapsa*. A second isolate (strain MTPPIF163) obtained from this pond is distinct from all available sequences and most closely related to *Thiolamprovum pedioforme* DSM 3802^T (16S rRNA gene similarity 96%). Marine Indian west coast isolates are most closely related or identical to *Marichromatium gracile* DSM 203^T whereas two isolates are affiliated to the genus *Allochromatium* with *Alc. vinosum* DSM 180^T as next relative type strain. Isolates received from marine and freshwater samples from Germany belong to the *Marichromatium* and the *Thiocapsa* groups. Additionally, isolate MTWDM004 obtained from the Wadden Sea is next related to *Thiorhodococcus kakinadensis* $JA130^T$ and *Marichromatium gracile* DSM 203^T with 16S rRNA gene sequence similarities of 96% to both strains. Based on 16S rRNA gene sequence, the freshwater pond isolate MTRDDF079 is 99% similar to the freshwater bacterium *Thiobaca trueperi* DSM 13587^T.

DNA EXTRACTION, PRIMER, PCR AND SEQUENCING

DNA extraction was performed by mechanical disruption of the cells using a bead mill (type MM200, Retsch, Germany). 1 ml of a freshly grown culture was centrifuged in a 1.5 ml tube (10 min; 8,000 x g). Supernatant was discarded and the cell pellet was dissolved in 200 µl DNA-free water. Zirconia/Silica beads with 0.1 mm in diameter (0.8 g) were filled into the tubes followed by bead mill treatment for 6 min with a frequency of 30 Hz/s. Finally the suspensions were centrifuged for 10 min at 10,000 x g. The extracted DNA was directly used for PCR or stored at -20°C until analysis.

PCR amplification of the *puf*L and *pufM* genes was performed using a modified primer set based on primers previously published by Nagashima et al. (Nagashima *et al.* 1997). The forward primer sequence (67F) is 5'- TTC GAC TTY TGG RTN GGN CC-3' and the reverse primer sequence (781R) is 5'-CCA KSG TCC AGC GCC AGA ANA-3'. The *pufLM* fragment length was ~1.5 kb. 16S rRNA genes were amplified using the primers 27F (Brosius *et al.* 1978) and 1492R (Suzuki & Giovannoni 1996).

PCR for amplification of the *puf* genes and the 16S rRNA gene was conducted using puReTaq Ready-To-Go PCR beads (GE Healthcare, USA) in a final volume of 25 µl. Differing conditions for 16S rRNA gene amplification are given in brackets. 3 to 5 µl (1 to 2 µl) of template and 1 µl of each primer with a concentration of 10 µM were applied. PCR was performed using the following conditions: initial denaturation step at 94°C for 2 min, primer annealing at 55°C (50°C) for 40 sec, elongation step at 72°C for 1.5 min (1 min.) and

denaturation at 94°C for 40 sec. Overall, 35 (30) cycles were performed. Before cooling down the PCR to 8°C, final annealing at 50°C (42°C) for 40 sec. and a final elongation step at 72°C for 5 min was conducted.

Sequencing was performed using the BigDye Terminator v1.1 Sequencing Kit (Applied Biosystems, USA) in a 3730-DNA-Analyzer (Applied Biosystems, USA) as specified by the manufacturer. Sequencing of the *pufLM* gene fragment was made using the PCR primers (67F and 781R). 16S rRNA gene amplificates were sequenced with the primers 342f (Lane 1991), 790f (5`-GATACCCTGGTAGTCC-3`) and 534r (Muyzer *et al.* 1993). Sequences obtained during this study were deposited in the EMBL database and were assigned accession numbers as given in Table I-1. Sequences were edited using the Seq Man II program (DNASTAR, USA).

PHYLOGENETIC ANALYSIS

With regard to reliability and comparability of the phylogenetic trees, only 16S rRNA gene and *puf*L and *pufM* sequences derived from the same strain with a minimum length of ~1400 bp and ~700 bp for each *puf* gene, respectively, were taken into account. In order to achieve a comparability of the *pufLM* nucleotide and amino acid tree the non-coding region between the *puf*L and *pufM* genes was not included into phylogenetic analyses. In cases where several sequences of identical strains were available in the databases, these were carefully compared and the best sequence was used for the analyses.

puf nucleotide sequences were converted into amino acid sequences using BioEdit version 7.0.1 (Thompson *et al.* 1997) and manually refined. The *puf* nucleotide sequence alignment was obtained by reconverting the amino acid sequence alignment. The corresponding 16S rRNA gene sequences were aligned using the integrated aligner function implemented in the ARB program package (Ludwig *et al.* 2004) and manually refined with respect to secondary structure information. The evolutionary models GTR+I+G and WAG+I+G used for phylogenetic analyses of nucleotide and amino acid sequences were determined using the program modelgenerator version 0.85 Keane T.M., et al. (2004) ModelGenerator: amino acid and nucleotide substitution model selection. National University of Ireland, Maynooth, Ireland]. Phylogenetic trees were calculated using the PhyML Online program (Guindon *et al.* 2005). *Chloroflexus aggregans* DSM 9485^{T} and *Chloroflexus aurantiacus* DSM635^{T} were used as outgroup species. Nucleotide and amino acid sequence similarities were obtained using the DNAdist and Protdist program of the Phylip package (Felsenstein 1989), respectively using “similarity table” option with default parameters. The hydropathy

plot was created using the Kyte and Doolittle algorithm (Kyte & Doolittle 1982) (scan window size 20) implemented in the BioEdit program package (Hall 1999).

RESULTS

PRIMER SYSTEM

Due to bacterial whole genome and metagenomic sequencing projects, the number of *puf* gene sequences - covering the whole *pufLM* gene - available in the database significantly increased over the past few years. Based on the available *pufLM* gene sequences the previously published primer set by Nagashima et al. (1997) exhibited several mismatches to the target sequences. Therefore, the primer sequences were suitably modified to cope with all available sequences. The modified primer system (67F/781R) reproducibly amplified *pufLM* genes of all purple sulfur bacteria (Table I-1) used in this study. Optimal annealing temperature for amplifying a PSB *pufLM* gene fragment was 55°C. It should be emphasized that the used primers also amplify *pufLM* genes of purple nonsulfur bacteria, aerobic anoxygenic photosynthetic bacteria and *Chloroflexus aurantiacus* (data not shown) and thus represent a universal primer system for these genes.

GENETIC ANALYSIS OF THE PSB STRAINS

In the present study, the phylogenetic relationship of 66 PSB strains was analyzed according to *pufLM* gene sequences and compared to the phylogeny based on 16S rRNA genes. 16S rRNA gene sequences of previously analyzed strains were repeated and refined whenever necessary and the corresponding EMBL database entries were updated. The datasets cover 16 out of 25 described genera of the PSB. The majority of the 16S rRNA and *pufLM* gene sequence data derived from strains of our culture collection and include 29 validly described PSB type strains. (Table I-1)

PUF GENE SEQUENCES

Nucleotide sequences of *pufL* and *pufM* as well as the deduced amino acid sequences obtained during this study formed a coherent cluster together with available database sequences. Compared to 16S rRNA gene sequences *pufL* and *pufM* genes had a wider range of variation. The overall similarity range of nucleotide sequences of *pufLM* for the studied PSB

was between 63.8-100% (amino acid similarities 62.4- 100%). Nucleotide sequence similarities of the Bchl *a* containing *Chromatiaceae* were between 71.5-100% (77.9-100% of amino acids). Bchl *b* possessing *Chromatiaceae* had sequence similarities of 63.8–71.4% (nucleotides) and 64.3-68.9% (amino acids) to the Bchl *a* containing *Chromatiaceae*. Nucleotide and amino acid similarities between the Bchl *a* harboring *Chromatiaceae* and *Ectothiorhodospiraceae* ranged from 66.5–77.4% and 71.3–77.5%, respectively. Bchl *b*-possessing *Chromatiaceae* and the analyzed *Ectothiorhodospiraceae* exhibited similarities of 66.7–70.3% for the DNA and 62.4–64.8% for the amino acid sequences to each other. Within the different groups of the *Chromatiaceae* as given in Table I-1, DNA similarities ranged from 86.0-100%, amino acid similarities varied from 92.2-100%. *Ectothiorhodospiraceae* species showed sequence similarities varying from 80.8-100% (amino acids) and 87.5-100% (nucleotides) for the genus *Ectothiorhodospira*, whereas the two *Halorhodospira halophila* strains exhibited 92.8% (nucleotides) and 97.4% (amino acids) sequence similarities to each other. Corresponding sequences of *Ectothiorhodospira* species to *Halorhodospira halophila* strains were 71.6–75.6% (nucleotides) and 71.2–74.6% (amino acids) similar to each other. Nucleotide similarities between the gammaproteobacterial AAPB and the PSB show a range from 62.2-65.6% for the Bchl-b possessing PSB, 68.3-74.7% for the Bchl-a containing *Chromatiaceae* and 67.1%-72.9% for the *Ectothiorhodospiraceae*.

According to *pufLM* nucleotide sequence similarities of less than 86.0% *Chromatiaceae* species were found in different groups. This corresponds to 16S rRNA gene sequence similarities of $\leq$ 95.0-96.0%. The situation is similar for the *Ectothiorhodospiraceae species*, where groups are distinguished at similarity values less than 75.6% according to *pufLM* nucleotide sequences.

Though the deduced amino acid sequences of both *pufL* and *pufM* genes of PSB have highly conserved structures, a detailed sequence analysis revealed some interesting aspects. Hydropathy plots of all analyzed *pufL* and *pufM* proteins revealed the characteristic presence of five alpha helices, as was first reported for these proteins of *Blc. viridis*, *Rba. sphaeroides* and *Tch. tepidum* (Fathir *et al.* 1997, Drews & Golecki 1995, Allen *et al.* 1987). At a closer look, the PufLM proteins showed several distinctive features. Most remarkable were several insertions of amino acids in certain PSB. Insertions of several amino acids occurred between the helices A and B of the PufL protein. *Tch. tepidum* had a known 8 amino acid long insertion (Fathir *et al.* 1997) between position 59 and 60 (*Blc. viridis* numbering). At the same position, the phylogenetically defined groups as shown in Table I-1 representing species and strains of *Thiocapsa* (group V), *Allochromatium* (group VII), *Thiorhodococcus* A (group IX), *Tcs. violacea* DSM 207^T (group X), *Thiorhodococcus* B (group XI), isolate MTWDM004 (group XII), *Thiocystis* (group XIII), *Chr. weissei* DSM 5161^T (group XIV), *Marichromatium* (group XVI) exhibit a five

amino acid insertion that is similar to that of *Tch. tepidum* (Table I-1). A four amino acid insertion at the same position was found for the Bchl *b*-possessing *Chromatiaceae* (group I). This insertion is identical in all Bchl *b*-containing strains but harbors completely different amino acids (suppl. Fig. I-1) as compared to the inserted amino acids of other *Chromatiaceae*. In addition, Bchl *b*-containing *Chromatiacea*e have an additional leucine after PufL position 75. An extra amino acid (serine) is also located at the end of PufL in all strains of group I, II (except *Thc. marina*) and III. All *Ectothiorodospira* species have an alanine at that position. Both *Halorhodospira halophila* strains exhibit a three amino acid elongation (Trp - Gly - Gly) at that position.

Insertions within the PufM are limited to one position and to a few species only. Non-motile *Halochromatium* strains (but not the motile *Hch. salexigens*) have a three amino acids insertion between position 32 and 33. At this position, the *Thiobaca*-like isolate MTRDDF079 possesses an additional glutamine and the Bchl *b*-containing species contain an extra glycine.

In addition to these insertions several substitutions of amino acids occur. All PSB (except species of *Thiorhodovibrio,* group IV) possess a serine and a tryptophan at PufL positions 72 and 73. In *Thiorhodovibrio* species aspartic acid and valine occur at these positions. Another example of a signature amino acid is the cysteine at PufM position 15 present only in members of the *Marichromatium* group.

Despite the foregoing differences suitable for differentiation, crucial parts of the sequences are highly conserved. Noticeably, none of the mentioned insertions occur in membrane spanning regions of the proteins. In the membrane spanning alpha helices of both proteins all important proline residues acting as helix turn motives are located at the same positions in all PSB. Histidine residues serving as ligands for the special pair of bacteriochlorophyll and the accessory bacteriochlorophylls are also conserved (suppl. Figs. I-1 and I-2) in both genes of all analyzed strains including the Bchl *b*-containing ones. In addition, the tryptophan residue at PufM position 130 acting as ligand for the bacteriophaeophytin is conserved in all strains.

PHYLOGENETIC ANALYSIS OF *PUFLM* GENES

pufLM gene sequence based trees exhibit a clear distinction of the PSB into major groups as resolved by 16S rRNA gene analyses, regardless whether amino acid or DNA sequence alignments were used. Species of the *Chromatiaceae* and the *Ectothiorhodospiraceae* formed clearly separate clades, with two clearly distinguished lines in the latter, *Ectothiorhodospira* species and the extremely halophilic *Halorhodospira halophila* strains.

In general, the phylogenetic trees based on *pufL* and *pufM* gene sequences, respectively, reflect the topology of *pufLM* sequence based trees. Therefore, only results of the *pufLM* DNA and amino acid tree are presented. Substantial variations between the inferred *pufLM* DNA and deduced amino acid trees as well as differences between the 16S rRNA gene phylogenetic tree and the corresponding *pufLM* tree are pointed out.

Most remarkably, *pufLM* based phylogenetic analyses lead to phylogenetic clades within the *Chromatiaceae* containing the same strains as seen according to the 16S rRNA gene based phylogeny (groups I-XVIII, Table I-1). This grouping is strongly supported by high bootstrap values (Figs. I-1, I-2 and I-3). The overall topology and the arrangement of the PSB groups to each other revealed some differences between the *pufLM* and the 16S rRNA gene based trees, e.g. the positions of the groups of *Halorhodospira and Ectothiorhodospira*. However, within these two groups tree topology of *pufLM* corresponds exactly to 16S rRNA gene tree topology.

Phylogenetic analyses of the *pufLM* genes of *Chromatiaceae* revealed several analogies to the 16S rRNA gene data. Certain groups formed clusters based on both 16S rRNA gene and *pufLM* gene sequences, such as the salt dependent species of the genera *Halochromatium* and *Thiohalocapsa* (groups III + II). Moreover, inside the *Halochromatium* clade the branch topology exactly corresponds to that of the 16S rRNA phylogenetic tree (group III, Figs. I-2 and I-3). A second cluster that always occurred in all phylogenetic analyses harbours *Thiocapsa* and *Thiolamprovum* species and relatives (groups V + VI). The *Thiocapsa* group includes 12 sequences. While the results of the *pufLM* trees (especially the amino acid tree) indicated a division of the *Thiocapsa* group into two subclades, in the 16S rRNA tree this was less well-defined. Subclade one harbors validly described species of *Tca. marina* and *Tca. pendens*. Subclade two is represented by the species *Tca. rosea* and *Tca. roseopersicina*. According to the phylogenetic results based on 16S rRNA gene analyses and supported by data on *pufLM* genes, *Thermochromatium tepidum* and *Chromatium weissei* are next related to the *Allochromatium* and the *Thiocystis* group, respectively. However, at the amino acid level *Thermochromatium tepidum* is more closely related to the new *Thiobaca*-like isolate MTRDDF079 than to *Allochromatium*. Members belonging to the *Allochromatium* group (group XII) showed the same topology in the inferred *pufLM* trees but differ slightly in comparison with the topology in the 16S rRNA gene phylogenetic tree. *pufLM* trees show that the isolates MTCH3IM013 and MTCH3IM086 are in close phylogenetic vicinity to *Alc. vinosum* while in the 16S rRNA inferred tree they are located between *Alc. vinosum* and *Alc. warmingii*. The *Thiocystis* group consisting of *Tcs. minor* and *Tcs. gelatinosa* (group XIII, Fig. I-2) forms a distinguishable group with a quite similar topology within all inferred phylogenetic trees. *Thiocystis violacea* DSM 207^{T}

formed a distinct and separate branch with a close relationship to members of the *Thiorhodococcus* group I and does not cluster with the other *Thiocystis* species. The two groups of members belonging to the genus *Thiorhodococcus* as seen by 16S rRNA gene sequences were also recognized by *pufLM* phylogenetic analyses. The *Thiorhodococcus* clade A (group IX) harbors *Trc. minor*, *Trc. mannitoliphagus* and *Tcs. violacea* DSM 208 whereas *Trc. drewsii* and *Trc. kakinadensis* were located in *Thiorhodococcus* clade B (group XI). Group XVI represents the coherent and robust clade of *Marichromatium* species highly supported by both 16S rRNA gene and *pufLM* phylogeny. On 16 rRNA gene sequence level, *Mch. purpuratum* and *Mch. gracile* are closely related (> 99% similar to each other and 98% to *Mch. bheemlicum*) reflected also by tree topology. Based on *pufLM* sequences (AA and nucleotide), however, the okenone-containing *Mch. purpuratum* separates from the spirilloxanthin-series carotenoids containing species, *Mch. gracile* and *Mch. bheemlicum*, by sequence similarity and tree topology. A quite distinct and outstanding group of the *Chromatiaceae* harbors the genera of the Bchl *b*-containing *Chromatiacea*e, namely *Tco. pfennigii* and *Tfc. mobilis* (group I), well separated by long branches and well established by high bootstrap values. This separate position of Bchl *b*-containing *Chromatiaceae* is also seen in trees based on 16S rRNA gene sequence (Fig. I-3).

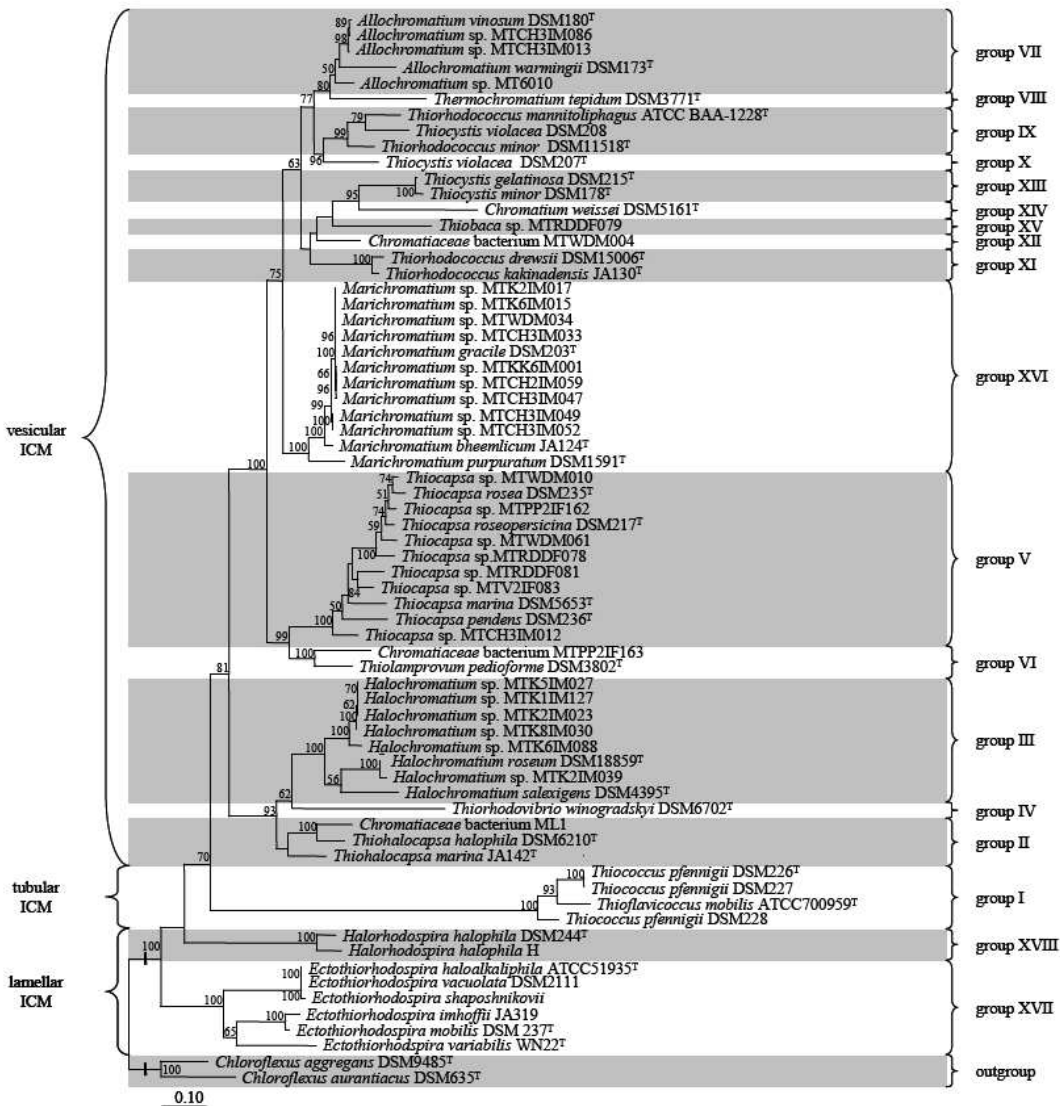

Figure I-1: Bootstrapped maximum likelihood based phylogenetic pufLM tree of nucleotide sequences of PSB based on their pufLM gene sequences. Bootstrap values of 50 or higher are given at the nodes. Brackets on the left define the groups of vesicular, tubular and lamellar ICM. ICM: intracytoplasmic membrane. Brackets on the right define groups within the Chromatiaceae (I-XVI) whose members sharing nucleotide similarities ≥ 86% (≥ 81% for the Ectothiorhodospiraceae (XVII-XVIII) which equal to ≥ 95% similarity of their corresponding 16S rRNA gene and thus concurrently represent the genus level on PufLM.

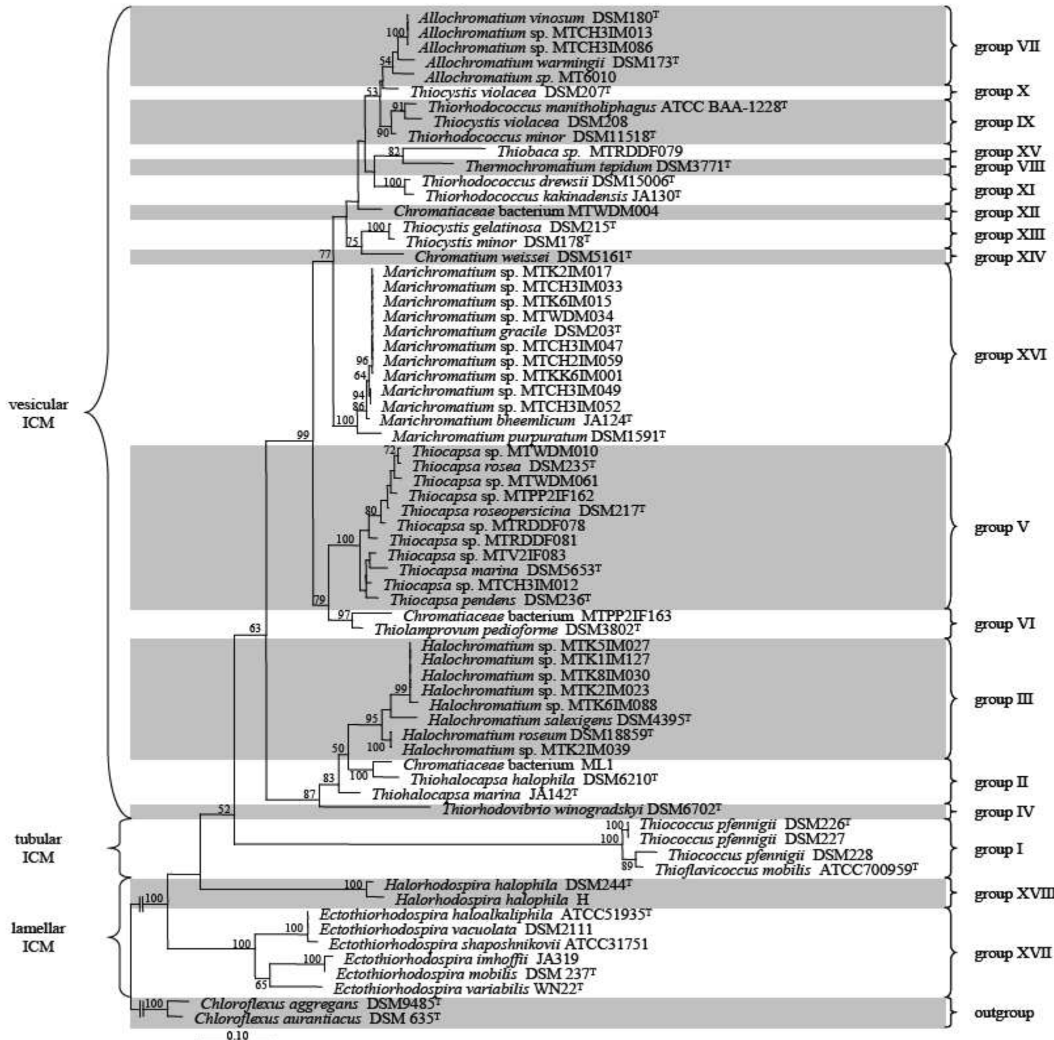

Figure I-2: Bootstrapped maximum likelihood based phylogenetic PufLM tree of deduced amino acids sequences of PSB based on their pufLM gene sequences. Bootstrap values of 50 or higher are given at the nodes. Brackets on the left define the groups of vesicular, tubular and lamellar ICM. ICM: intracytoplasmic membrane. Brackets on the right define groups within the Chromatiaceae (I-XVI) whose members sharing nucleotide similarities ≥ 86% (≥ 81% for the Ectothiorhodospiraceae (XVII-XVIII) which equal to ≥ 95% similarity of their corresponding 16S rRNA gene and thus concurrently represent the genus level on PufLM.

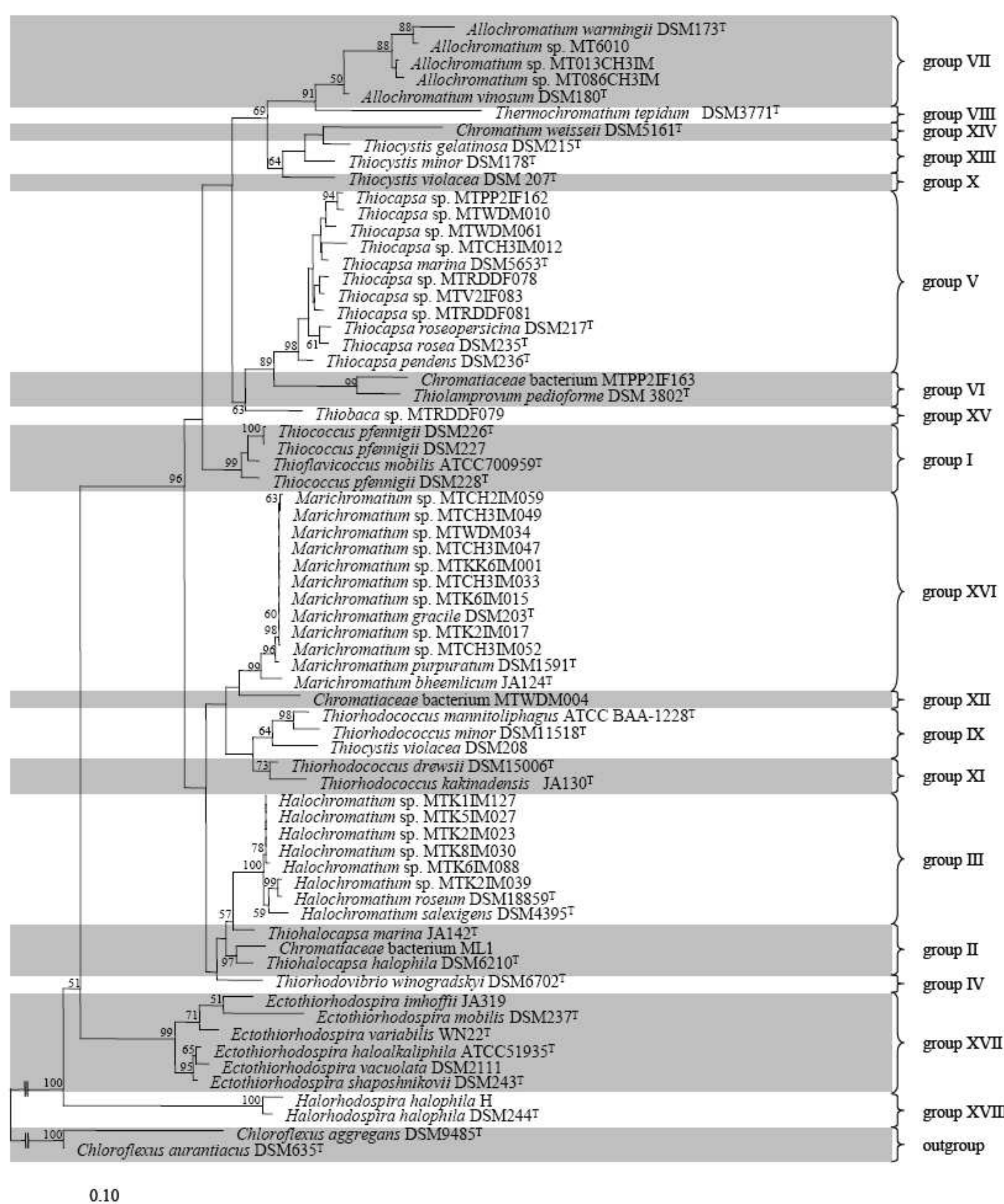

Figure I-3: Bootstrapped maximum likelihood based phylogenetic 16S rRNA gene tree of PSB. Bootstrap values of 50 or higher are given at the nodes. Brackets on the right define groups within the *Chromatiaceae* (I-XVI) whose members sharing nucleotide similarities ≥ 86% (≥ 81% for the *Ectothiorhodospiraceae* (XVII-XVIII) which equal to ≥ 95% similarity of their corresponding 16S rRNA gene and thus concurrently represent the genus level on PufLM.

PHYLOGENETIC AND 16S rRNA GENE SEQUENCE ANALYSES OF PSB

As demonstrated earlier (Imhoff 2001, Imhoff *et al.* 1998b, Imhoff & Süling 1996) and completely supported by the present data, species characterized as members of the *Chromatiaceae* and *Ectothiorhodospiraceae* form distinct groups based on their 16S rRNA gene sequences. The *Ectothiorhodospiraceae* are divided into two well separated groups containing *Ectothiorhodospira* species (group XVII) and *Halorhodospira* species (group XVIII), respectively. Within the *Chromatiaceae* 16 groups were recognized (Fig. I-3), which in general represent the different genera of *Chromatiaceae* and are supported by high bootstrap values. Among the few exceptions the most obvious is the distinction of two clades within the genus *Thiorhodococcus* (groups IX and XI). Furthermore the genus *Thiocystis* is divided into two groups (groups X and XIII). Indeed the type strain *Thiocystis violacea* DSM 207^T, though closely related to other representatives of this genus, forms a distinct branch. This fact is also strongly supported by the *puf* gene tree analyses (see above and Fig. I-2). Interestingly, *Thiohalocapsa* species (group II) and *Halochromatium* species (group III) show a tendency to form a halophilic branch within the *Chromatiaceae*. *Thiolamprovum* (group VI) and *Thiocapsa* (group V) are robust sister groups consisting of non-motile cells and classified as mainly freshwater species (Imhoff 2001, Guyoneaud *et al.* 1998).

DISCUSSION

pufLM genes are essential in photosynthesis of purple sulfur bacteria and therefore may represent important conservative markers for phylogenetic analyses. Because of the limited number of available sequence data, we have analyzed *pufLM* genes of a large number of strains available in our strain collection (including 29 type strains) and in addition of several new isolates. Analyses contained 66 PSB strains of 16 PSB genera from all over the world, adapted to various environmental conditions such as hypersaline, marine, brackish or fresh water habitats. Primary structure of the PufLM proteins and their encoding nucleotide sequences showed distinct sequence signatures down to species specific level.

In general, a high sequence conservation of *puf* was observed both with regard to the amino acid sequences and also the corresponding DNA sequences. Furthermore, hydropathy plots revealed five alpha helices and the corresponding interhelical structures with conserved amino acid motives. These regions were highly conserved in all studied PSB. Regarding the resulting structure of the membrane-spanning protein, conserved motives may represent

structure elements, which can be regarded as crucial in order to maintain functionality of the proteins. Correspondingly, highest conservation of the *pufL* and *pufM* gene sequences is observed at the binding sites of the cofactors involved in the photochemical reaction. Additionally, highly conserved motives were found within regions of the five alpha helices possibly indicating a functionally relevant structure element. Further, interhelical conserved regions are dominated by the amino acid proline responsible for the helix turns. The conserved structure of the entire PufL and PufM proteins, within the alpha helices, in interhelical regions and at cofactor binding sites indicates the importance of the structure of PufLM proteins as important factors for the functionality of the PSB photosynthetic reaction center.

In spite of the overall conserved feature of the proteins there are amino acid/nucleotide sequence similarity differences of up to approximately 40%. Within the defined groups belonging to the *Chromatiaceae* (Table I-1), nucleotide similarities of the *puf* genes always exceeded 86% (81% in *Ectothiorhodospiraceae*). Environmental isolates obtained during this study specifically supported these findings. Those isolates sharing *pufLM* gene sequence similarities of >86% to any of the described species also revealed high 16S rRNA gene sequence similarities (>95 %) and can be reliably assigned to one of the defined groups (Table I-1). On the contrary, isolates showing *pufLM* gene sequence similarities of less than 86% (e.g. strains MTWDM004 and MTRDDF079) could not be identified as belonging to any of the known groups and may thus represent new species or even genera. In this sense, *pufLM* sequences give confidence that an isolate originally assigned to "*Lamprocystis purpurea",* strain ML1 (Overmann *et al.* 1991) has been misclassified. Strain ML1 only shows 16S rRNA gene sequence similarity of 94% to the type strain of *Lcs. purpurea* DSM4197^T which clearly supports the false classification, even at the genus level. This isolate revealed nucleotide sequences of *pufLM* most similar to *Thc. marina* JA142^T and *Thc. halophila* DSM6210^T strains with sequence similarities of PufLM of 87% to 90% and of the 16S rRNA gene of 97% and 98%.

Despite the overall nucleotide similarity values, the amino acid sequences exhibited recurrent insertions and/or substitutions at distinct positions. These signatures are very useful in differentiation of the PSB groups. For example, all non-motile *Halochromatium* species share an additional three amino acids at *pufM* position 33 and *Ectothiorhodospiraceae* share a glycine at *pufM* position 230 while all *Chromatiaceae* possess a serine at that position. The amino acid insertions after *pufL* position 59 provide the possibility to distinguish a PSB from all other *puf* possessing bacteria, because these insertions only occur in gammaproteobacterial PSB. Furthermore, EVGPA insertions at this position are defining the Bch *b*-harboring genera

Thiococcus and *Thioflavicoccus*. Our findings demonstrate that nucleotide sequence similarities together with specific signature amino acids are suitable properties in a polyphasic taxonomy of PSB as suggested by Imhoff and Caumette (Imhoff & Caumette 2004). Overall, *pufL* and *pufM* gene sequence based phylogenetic analyses revealed stable groups (Table I-1, Fig. I-1) which are in good agreement with the currently recognized genera. Two exceptions were found in the genera *Thiocystis* and *Thiorhodococcus* which are separated into two distinct groups, each. Both of these exceptions were also found in phylogenetic trees of the 16S rRNA gene and therefore support a possible reclassification of these bacteria.

A distinct positioning of *Thiococcu*s and *Thioflavicoccus* within the *Chromatiaceae* was found in all trees and corresponds to their possession of Bchl *b* in the reaction center instead of Bchl *a* present in other *Chromatiaceae*. In addition to the different bacteriochlorophyll this group also differs by their tubular internal membrane system compared to the vesicular system in other *Chromatiacea*e. The separate clustering of the *Ectothiorhodospiraceae* species possessing a lamellar internal membrane system further demonstrates coherence between the Puf protein structure, bacteriochlorophyll structure and type of internal membrane system. A correlation between protein structure and membrane type was already proposed earlier (Drews & Golecki 1995) and it could be demonstrated that a mutant of *Rba. sphaeroides* lacking LH2 forms tubular membranes whereas the wild type possesses vesicular internal membranes [19]. It was also demonstrated that the functionality of *puf* gene products is necessary for ICM formation in *Rsp. rubrum* (Hessner *et al.* 1991). Interestingly, single signature nucleotides may correlate with the type of internal membranes. All *Ectothiorhodospiraceae* share a glycine at PufM position 230 while all *Chromatiaceae* possess a serine at that position. Furthermore Bchl b-containing PSB harbor phenylalanine at PufM position 71 while all other PSB have leucine or isoleucine there.

A separate grouping of salt-dependent genera (*Thiococcus, Thioflavicoccu*s, *Thiorhodovibrio*, *Thiohalocapsa*, *Halochromatium*, *Marichromatium* and *Thiorhodococcus*) as was demonstrated earlier on the basis of 16S rRNA gene sequence-based trees (Imhoff 2001) was confirmed in the *pufLM* trees. *pufLM* phylogeny demonstrated a close relationship between *Thiorhodovibrio*, *Thiohalocapsa* and *Halochromatium* and formation of distinct branches for the group around *Thiococcus* and *Thioflavicoccu*s and another one for *Marichromatium* species (Figs. I-1 to I-3). This study demonstrated an overall high consistence of tree topologies of the two *puf* genes and the 16S rRNA gene and proves a coherent evolution of these genes and the suitability of *puf* genes as phylogenetic markers for the purple sulfur bacteria.

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CHAPTER II

IMPACT OF TEMPERATURE AND SALINITY CHANGES ON PURPLE SULFUR BACTERIA COMMUNITIES FROM A COASTAL LAGOON OF THE BALTIC SEA ANALYZED BY *PUFLM* GENE LIBRARIES

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY

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ABSTRACT

Coastal habitats are characterized by large variations in physical and chemical parameters over short time periods and microorganisms living in this dynamic environment have to cope with these changes. The impact of temperature and salinity variations on a community of purple sulfur bacteria (PSB) of a Baltic Sea lagoon was studied under standardized culture conditions. For the first time changes in the community composition of phototrophic bacteria were specifically analyzed based on the photosynthetic reaction center genes *pufL* and *M* by RFLP and cloning experiments. The approach using *pufLM* proved to be a powerful tool in studying the impact of environmental factors on the PSB communities. The PSB community inhabiting the Baltic Sea lagoon appeared to be well adapted and was accompanied by purple non-sulfur bacteria and aerobic anoxygenic phototrophic Alpha- and Gammaproteobacteria. Major components of the PSB community of the brackish lagoon affiliated to PSB genera and species known as marine, halophilic or salt-tolerant. Most abundant phylotypes in the original sample as well as in the experiments were found along the salinity gradient from freshwater conditions up to 7.5% NaCl or were salt-dependant but did not develop in the absence of salt. Phylogenetic analysis identified members of the PSB genera *Marichromatium*, *Allochromatium*, *Halochromatium*, *Thiorhodocococcus*, *Thiocapsa*, *Thiorhodovibrio*, and *Thiohalocapsa*. In addition, several phylotypes not closely associated with known species of PSB were found. A dramatic shift in the community occurred at elevated temperatures of 41°C and 44°C when *Marichromatium gracile* was most prominent which was not detected at lower temperatures.

INTRODUCTION

Purple sulfur bacteria (PSB) typically develop in all kinds of aquatic habitats with low or no oxygen tensions that are reached by sunlight and contain reduced sulfur compounds (H_2S, elemental sulfur, thiosulfate). They are characterized by performing anoxygenic photosynthesis and a photoautotrophic way of life (Imhoff 2003b). Phylogenetically PSB are classified into two families within in the gammaproteobacterial order of the *Chromatiales*, the *Chromaticeae* and the *Ectothiorhodospiraceae* (Imhoff 2006, Imhoff 2005b). In dynamic coastal habitats PSB inhabit the upper sediments layers from where they can colonize the water column under appropriate conditions. In these habitats, in enclosed lagoons, mangrove soils, estuaries, salt marshes and others, they have to cope among others with strong changes of salinities, temperatures and light conditions.

The salt and temperature relations preferred by PSB show some differences among the various species. All known PSB are mesophilic bacteria having temperature optima between 20-35°C with the exception of the moderately thermophilic *Thermochromatium tepidum* and the slightly thermophilic *Marichromatium gracile* strain SW26 with best growth at 50°C and 44°C , respectively (Serrano *et al.* 2009, Imhoff *et al.* 1998b, Fathir *et al.* 1997).

With respect to their salt-relations freshwater species, salt-tolerant species and salt-dependent marine or halophilic purple sulfur bacteria are known. Typical freshwater PSB are species of *Lamprocystis*, *Thiolamprovum* and the large *Chromatiaceae* like *Chromatium okenii* and *Thiospirillum jenense*. Though salt-relations of the studied pure cultures also characterize *Allochromatium* species as freshwater species, they have been found in both freshwater and saltwater habitats. Salt-dependent PSB are represented by species of the genera *Marichromatium*, *Halochromatium*, *Thiorhodococcus*, *Thiorhodovibrio*, *Thiohalocapsa*, *Halorhodospira* and *Ectothiorhodospira*. A genus that contains salt-dependent as well as salt-tolerant freshwater species is *Thiocapsa*.

Both, temperature optima and the need of salt for these PSB species are known pretty well from pure culture experiments but almost nothing is known about the behavior of natural communities of PSB according to changes of temperature and salinity.

In the present study, we studied the influence of temperature and salinity on the composition of a PSB community originating from a Baltic Sea coastal lagoon at the Kiel Fjord, Germany. In the experimental setup, the environmental mixed community of PSB was inoculated into defined media and incubated under controlled growth conditions. The *pufLM* gene fragment of the PSB was used for analyzing changes of the PSB community. The 16S rRNA gene cannot resolve the composition of environmental communities of PSB which are related to a number of non-phototrophic Gammaproteobacteria (Martínez-Alonso *et al.* 2005, Fowler *et al.* 1984). The *pufLM* genes encode for the light and medium subunits of the photosynthetic reaction center. They are essential for PSB and represent specific and selective target genes for these bacteria. Previously, we have established a PSB *pufLM* sequence database that demonstrated congruence of 16S rRNA and pufLM gene phylogeny and makes possible the classification of environmental PSB on basis of the *pufLM* sequences (Tank *et al.* 2009). The great advantage of *pufLM* gene analyses is the combined information on physiological properties and phylogenetic relationship which allows a rapid and specific identification of PSB originated from environmental communities (Tank *et al.* 2009, Achenbach *et al.* 2001).

MATERIALS AND METHODS

SAMPLE ORIGIN AND SAMPLING

The experiment was conducted using a mixed water and sediment sample from a coastal lagoon of the Baltic Sea. The lagoon is located at the mouth of the Kiel fjord nearby the village of Stein (54° 25’ 07” N latitude and 10° 16’ 35” O longitude) and is characterized by changes in environmental conditions throughout the year. At the time of sampling (June 2008) most parts of the lagoon were covered by a thin water layer. Small cloudy spots of pinkish-reddish color were observed above the sediment at some locations. The original mud and water sample had a temperature of 23.5°C, a salinity of 2%, a pH value of 8.2 and a slight smell of hydrogen sulfide.

EXPERIMENTAL SET UP

The duration of the temperature and the salinity series experiments was 30 days and occurred in special test tubes (Schott AG, Mainz, Germany) that were sealed with a screw-capped septum. The test tubes were filled with ~ 35 ml freshly prepared Pfennig’s medium (pH 7.2) (Imhoff 2003b) and had a 1 ml headspace of N_2. The medium used for the temperature treatments contained 2% NaCl. The salinity series was conducted within a salinity range from 0 – 12% NaCl with values of S1=0%, S2=0.5%, S3=1%, S4=1.5%, S5=2%, S6=3%, S7=3.5%, S8=4%, S9=5%, S10=7.5%, S11=10%, S12=12% NaCl. Additionally, stock media for all corresponding treatments were prepared separately to refill the tubes after sampling. The test tubes were inoculated with 1 ml of a well mixed environmental sample and sealed. Immediately after inoculation air in the headspace was replaced by N_2 gas. The inoculated tubes were illuminated from perpendicular above with a 40 W tungsten lamp (OSRAM, Munich, Germany) and a light intensity of approx. 700 lx. Light and dark cycles were 14 and 10 hours, respectively and the light phase started at 6 a.m. in the morning. The salinity treatments were incubated at an average temperature of 23.5°C (+/- 3°). The temperature series was conducted in a temperature organ (VLM GmbH, Bielefeld, Germany) within a temperature range from 12-53°C over a series of 14 test tubes placed in a metal block. Temperatures in the individual tubes were recorded to be T1=13°C, T2=19°C, T3=21°C, T4=23°C, T5=26°C, T6=29°C, T7=31°C, T8=33°C, T9=36°C, T10=39°C, T11=41°C, T12=44°C, T13=46°C, T14=52°C, all (+/- 1°C). Temperature was measured once a day using a digital thermometer. Growth within the experimental tubes was monitored visually every day. Samples were taken from the treatments 24 hours after inoculation and

thereafter at day 6, 9, 13, 17, 20, 23, and 30, in total 8 samplings were done. Before sampling the tube content was manually mixed in order to achieve a homogenous liquid. 2 ml were taken out of each tube and 1 ml portions were centrifuged at 8,000 x g for 10 min, the supernatant was discarded and the pellet was stored at -20°C until further processing. The loss of media within the test tubes caused by sampling was simultaneously replaced by the corresponding stock medium. Anaerobic headspace of the experimental tubes and the stock media was assured by venting the bins' headspaces with N_2 for 30 s directly after sampling.

DNA EXTRACTION

DNA extraction was performed by mechanical disruption of the cells using a bead mill (type MM200, Retsch GmbH, Haan, Germany). The frozen cell pellet was resuspended in 200 µl DNA-free water. Zirconia/silica beads 0.1 mm in diameter (0.8 g) were filled into the tubes followed by bead mill treatment for 6 min with a frequency of 30 Hz. Finally, the suspensions were centrifuged for 10 min at 12,000 × g. The extracted DNA was directly used for PCR or stored at −20 °C until analysis.

PRIMERS

PCR amplification of the *pufL* and *pufM* genes was performed using primers previously published by Tank et al.(2009) with 5'-TTCGACTTYTGGRTNGGNCC-3' as forward primer sequence (pufL67F) and 5'-CCAKSGTCCAGCGCCAGAANA-3' as reverse primer sequence (pufM781R). The *pufLM* fragment length was approx. 1.5 kb. This universal primer system amplifies not only purple sulfur bacteria *pufLM* genes but also *pufLM* genes of purple non-sulfur bacteria, aerobic anoxygenic phototrophic Proteobacteria and *Chloroflexaceae*.

PCR CONDITIONS

PCR for amplification of the *pufLM* genes was conducted using puReTaq Ready-To-Go PCR beads (GE Healthcare, Piscataway, NJ, USA) in a final volume of 25 µl. 3 µl of template and 1 µl of each primer with a concentration of 10 µM were applied. PCR was performed using the following conditions: initial denaturation step at 94°C for 2 min, primer annealing at 55°C for 40 s, elongation step at 72°C for 1.5 min and denaturation at 94°C for 40 s. Overall, 35 cycles were performed. Before cooling down the PCR to 8°C, final annealing at 50°C for 40 s and a final elongation step at 72°C for 5 min was conducted.

RFLP ANALYSIS

First the *pufLM* nucleotide fragments were amplified using PCR conditions stated above. The *pufLM*-PCR product was digested with Alu I, Hinf I, and Msp I (New England Biolabs, Frankfurt, Germany) in three separate approaches. Each approach contained 7 µl PCR product, 1 µl enzyme (≙1 U enzyme), 2.5 µl buffer II, and 14.5 µl DNA-free water. The DNA fragment digestion was performed at 37°C for 20 min followed by a 30 min enzyme inactivation at 72°C and finally cooled down to 4°C. The complete approach (25 µl) of the obtained restriction fragments was separated on a 2.5% agarose gel (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) with 100 V for approx. 45 min and visualized with ethidium bromide under UV light (312 nm). Documentation occurred with the intas gel imaging system (Intas Science Imaging Instruments GmbH, Göttingen, Germany). The restriction fragment lengths were estimated by using X length standard (Roche Deutschland Holding GmbH, Kulmbach, Germany). The banding patterns were converted into a presence/absence matrix for comparison in a cluster analysis using the program PAST.

CLONING

On basis of the RFLP results experiments of salinity and temperature treatments were chosen to generate clone libraries for detailed PSB community analyses. All *pufLM*-PCR products were extracted using the Qiagen Gel extraction Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's conditions. The purified PCR products were directly used for cloning experiments. Cloning was performed with TOPO TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA, USA) and as specified by the manufacturer. From each cloning experiment 96 randomly picked colonies were amplified by performing a M13 PCR following the manufacturer's instructions. PCR products of the correct fragment length (approx. 1.6 kb) were prepared for sequencing; while other clearly differing PCR products were discarded.

SEQUENCING AND SEQUENCE ANALYZES

Sequencing was performed using the BigDye Terminator v1.1 Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) in a 3730-DNA-Analyzer (Applied Biosystems, Carlsbad, CA, USA) as specified by the manufacturer. Sequencing of the *pufLM* gene fragment was made using the cloning vector primers T3 and T7. Sequences were edited using the SeqMan II program (DNASTAR, Madison, WI, USA). Clone sequences obtained during this

study were deposited in the EMBL database and assigned the accession numbers FN869918 - FN869956. Search for chimeric sequences was conducted manually and by using the Bellerophone programme (Huber *et al.* 2004).

PHYLOGENETIC ANALYSIS

Edited and quality checked *pufLM* nucleotide clone sequences and the reference sequences were aligned by converting the nucleotide sequences into amino acid sequences using the in BioEdit version 7.0.1 implemented clustalX (default parameter) program (Hall 1999). After manual refining of the amino acid alignment, the *pufLM* nucleotide sequence alignment was obtained by reconverting into nucleotides. The *pufLM* nucleotide clone sequences were merged into OTUs (operational taxonomic units) using the program package mothur (Schloss *et al.* 2009) using default settings and a cutoff of 98% nucleotide sequence similarity. The non-coding region between the *pufL* and *pufM* genes was not included into phylogenetic and similarity analyses. OTUs were determined for each clone library individually. Representative *pufLM* nucleotide sequences of each OTU were also chosen using the mothur program package. Representative OTU sequences sharing nucleotide sequence similarities ≥98% detected in different treatments were assigned to one phylotype. Based on previous studies (Tank *et al.* 2009) sequences sharing ≥86.0% nucleotide sequence similarity to known PSB species and type strains where available, were assigned to genera or equivalent phylogenetic groups.

Reference *pufLM* nucleotide sequences used in phylogenetic calculations and their accession numbers are listed in supplementary Table II-1. In cases where several reference sequences of identical strains were available in the databases, these were carefully compared and the best sequence was used for the analyses.

The evolutionary model GTR+I+G used for phylogenetic analyses of nucleotide sequences were determined using the program modelgenerator version 0.85 (Keane *et al.* 2004). Phylogenetic trees were calculated using the PhyML program desktop version 2.4.4 (Guindon & Gascuel 2003). *Chloroflexus aggregans* DSM9485^{T} and *Chloroflexus aurantiacus* DSM635^{T} were used as outgroup species.

RESULTS

THE HABITAT

The community of PSB studied originated from a Baltic Sea coastal lagoon at the Kiel Fjord, Germany. The main organic input was entrained by the prevailing currents in the form of decaying macroalgae and seaweed. Reed that delimits the lagoon from the shore side and sea-gull droppings also were important sources of organic material. Degradation of the organic matter consumed the oxygen and larger amounts of sulfide were produced in the top layers of the sediment and released into the shallow water (approx. 10-30 cm depth) under calm conditions. Under such conditions and in particular after warm and sunny periods, blooms of PSB have been regularly observed in the lagoon's water over the past years.

The environmental sample was inoculated into PSB specific media with salt concentrations from 0-12% NaCl and incubated at room temperature for salt-dependent experiments and at temperatures between 12-53°C and 2% NaCl for the temperature-dependent experiments. Bacterial growth as indicated by development of reddish color and turbidity was first observed five days after inoculation in both, salinity and temperature series. Fastest development was found in those tubes which represented salinity and temperature values closest to those of the original environmental sample (2% salinity and 23.5°C). The less the natural conditions were met, the longer it took until growth of PSB was observed. After 14 days of incubation all tubes of the temperature experiment were equally well grown up to 44°C and the turbidity remained constant until the end of the experiment after 30 days. No growth occurred in temperature treatments higher than 44°C. A clear and constant turbidity in tubes of the salinity treatments was noticed after 20 days from 0-7.5% NaCl. A hardly visible pink-reddish turbidity was observed at 10% NaCl, whereas no growth occurred at 12% NaCl within 30 days. However, 42 days after inoculation a weak pinkish turbidity also appeared in the latter.

COMPARISON OF THE COMMUNITY COMPOSITION UNDER VARYING CONDITIONS USING RFLP

In order to compare changes of the PSB communities within the different temperature and salinity treatments over time, we employed RFLP analyses after 6, 13, 20 and 30 days of inoculation. After 20 days of inoculation almost all treatments provided sufficient cell material to conduct RFLP experiments. Three different enzymes (MspI, AluI, HinfI) were used for

RFLP analyses of the *pufLM* genes. A number of approx. 20 bands was found for each treatment and enzyme with a strong decrease at temperatures of 41°C and 44°C and at salinities >7.5% NaCl. The banding patterns were converted into a presence/absence matrix of the corresponding bands and this matrix was used for cluster analysis.

Temperature treatments at 13°C, 21°C, 26°C, 31°C, 36°C, 41°C and 44°C were chosen for RFLP comparison. The cluster analysis exhibited high similarities of approx. 90% for treatments at 13°C, 21°C, 26°C 31°C and 36°C (Fig. II-3). A few bands occurring at 26°C that were not observed at the other temperatures caused the slight separation of this treatment. Treatments at 41°C and 44°C showed completely different banding patterns in number and occurrence. Based on these findings the enrichments at 13°C, 26°C, 41°C and 44°C were chosen for further analysis using cloning libraries.

Treatments S1=0%, S3=1%, S4=2%, S5=3.5%, S7=5% and S9=7.5% of the salinity experiment were chosen for RFLP and cluster analysis. The dendrogram (Fig. II-3) visualizes the high similarities (>90%) of the communities after incubation at 0-5% NaCl and significant differences to those incubated at 7.5% NaCl. Within the range of 0-5% NaCl, three subgroups were visible in the RFLP results. One subgroup was represented by the freshwater treatment, the second included the 1% and 2% treatments and the third the treatments at 3.5% and 5% NaCl (Fig. II-3). Based on these results, the treatments at 0, 2, 5 and 7.5% NaCl were chosen as representatives for the cloning experiments.

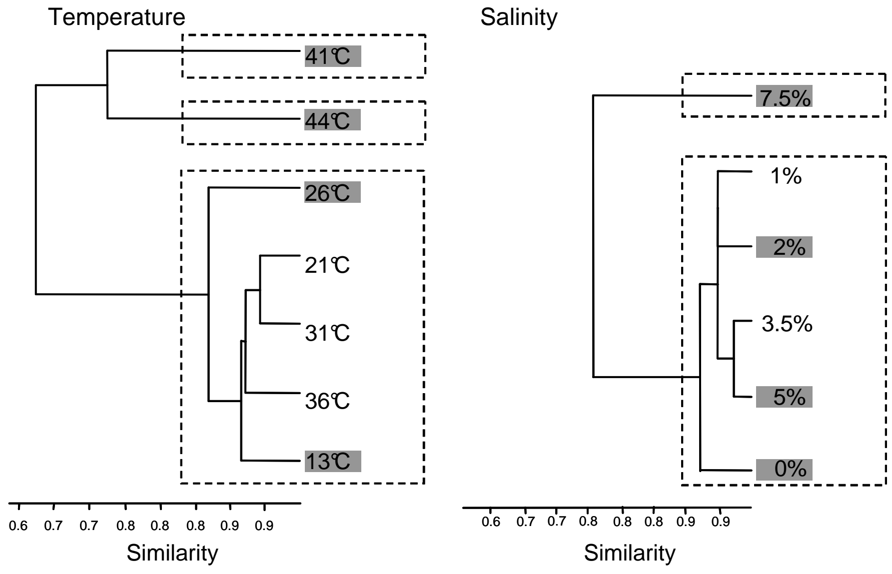

Figure II-3: RFLP cluster analyses of selected temperature and salinity treatments after 20 days of incubation. Treatments chosen for detailed analyses by clone library construction are highlighted in grey

PUFLM CLONING EXPERIMENTS

After quality checking, 482 *pufLM* nucleotide sequences obtained from the cloning experiments were used for a detailed analysis and were arranged in 39 phylotypes (PTs, Table II-2). These phylotypes comprised purple sulfur bacteria (20 PTs), purple non-sulfur bacteria (2 PTs) and aerobic anoxygenic phototrophic bacteria of the Alpha- (11 PTs) and Gammaproteobacteria (6 PTs). The use of a PSB specific medium apparently provided highly selective enrichment conditions for members of the PSB community. Non-PSB representatives were mainly found in the original sample and only rarely in the enrichments. One important exception was phylotype 34 distantly related to *Jannaschia* which was present in most experimental treatments (Table II-1b) but not detected in the original sample.

PHYLOGENETIC ANALYSIS

The classification of the *pufLM* nucleotide sequences obtained from all nine cloning experiments based on phylogenetic calculations and sequence similarities quarried a highly diverse community of phototrophic bacteria inhabiting this brackish lagoon. High bootstrap values support the robustness of the inferred phylogenetic tree as well the high number of distinct phylotypes. The inferred phylogenetic tree, consisting of all 39 phylotypes and reference *pufLM* nucleotide sequences, showed a clear and robust separation into anoxygenic phototrophic Gamma- and Alphaproteobacteria (Figs. II-1a and II-1b).

Table II-2: Number of *pufLM* nucleotide sequences obtained from the original sample and selected temperature and salinity treatments, corresponding OTUs[†] and rarefaction analysis

treatments	original sample (T0)	13 °C	26 °C	41 °C	44 °C	0 %	2 %	5 %	7.5 %
no. *pufLM* sequences	72[1]/90[2]	34/38	43/51	57/60	38/41	37/57	48/51	42/43	50/54
no. OTUs	14[a]/26[b]	9/12	6/9	6/9	4/6	7/10	10/12	8/9	9/11
rarefaction[§]	14[#]/17[*]	9/10	6/7	n.p.	n.p.	n.p.	10/12	8/8	9/11
rarefaction[§] [%]	82.4	90.0	85.7	n.p.	n.p.	n.p.	83.3	100.0	81.8

[1] after quality and chimera check for purple sulfur bacteria, [b] after quality and chimera check for all *pufLM* nucleotide sequences, [a] obtained from purple sulfur bacteria related *pufLM* nucleotide sequences using a threshold of ≥98.0 % nucleotide similarities, [b] obtained from all *pufLM* nucleotide sequences using a threshold of ≥98.0 % nucleotide similarities, [§] Rarefaction analysis of purple sulfur bacteria related pufLM nucleotide sequences, [#] detected OTUs, [*] predicted OTUs, n.p. not possible to determine, [†] operational taxonomic unit

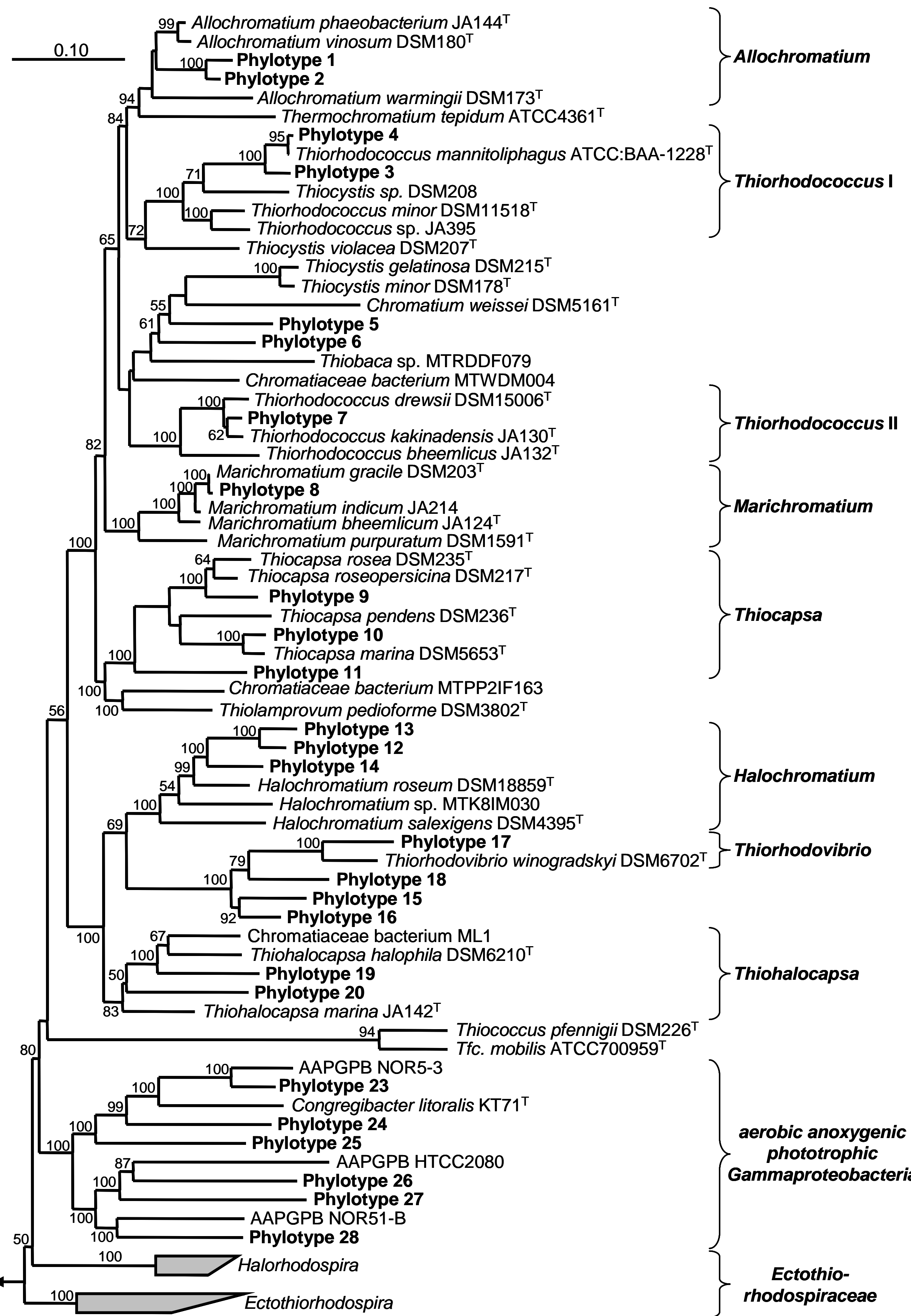

Figure II-1a: Phototrophic Gammaproteobacteria part of a 100 times bootstrapped maximum likelihood phylogenetic *pufLM* nucleotide tree. Bootstrap values ≥50 a given at nodes.

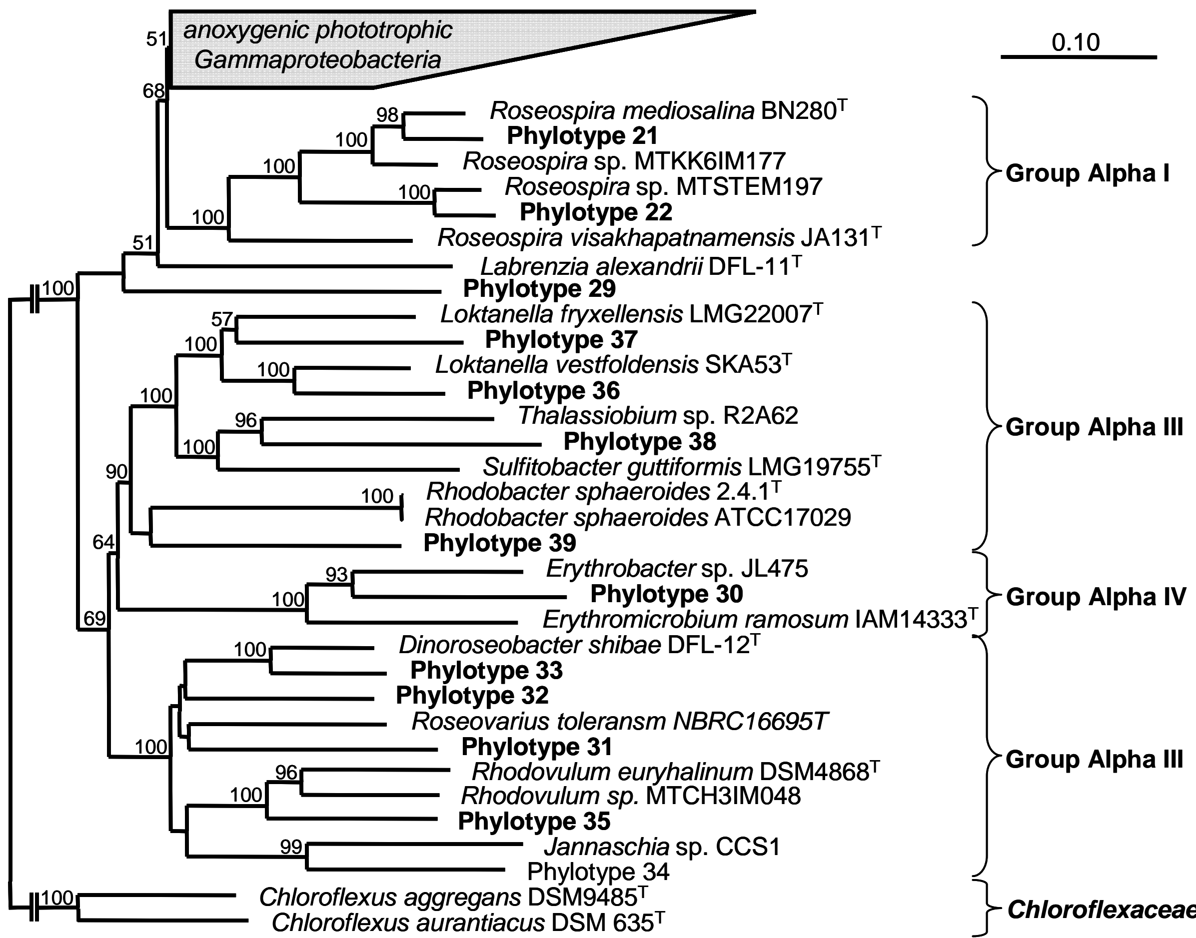

Figure II-1b: 100 times bootstrapped maximum likelihood phylogenetic *pufLM* nucleotide tree of phototrophic Proteobacteria with the uncollapsed part of the phototrophic Alphaproteobacteria. Bootstrap values ≥50 are given at nodes.

According to similarity and phylogenetic analyses 20 phylotypes were identified as *Chromatiaceae* and 15 out of these could be assigned to known genera of the *Chromatiaceae* with respect to a threshold of ≥86% *pufLM* nucleotide sequence similarity as suggested by Tank et al. (Tank *et al.* 2009) (Table II-1a). Several of these phylotypes associate to known halophilic representatives of PSB of the genera *Halochromatium* (PT 12-14), *Thiorhodovibrio* (PT 17) and *Thiohalocapsa* (PT 19 and 20). Other phylotypes were closely associated with *Allochromatium* (PT 1 and PT 2), with the salt-dependent *Thiorhodococcus* I (PT 3 and PT 4), with *Thiorhodococcus* group II (PT 7), with the marine *Marichromatium gracile* (PT 8) and with *Thiocapsa* (PT 9, PT 10 and PT 11). None of the phylotypes was assigned to freshwater PSB such as *Thiolamprovum*, *Lamprocystis, Thiobaca* or *Chromatium weissei* or to Bchl-*b* harboring species of *Thiococcus* and related genera. Neither in the environmental sample nor in any of the experimental conditions *pufLM* nucleotide sequences of salt-loving *Ectothiorhodspiraceae* representatives was obtained.

Corresponding to the benchmark of 95% *pufLM* sequence similarity supposed by Zeng et al. (2007) for separation of species, phylotypes 3, 4, 7, 8 and 10 were classified as representatives of *Thiorhodococcus mannitoliphagus* (PTs 3 and 4), *Thiorhodococcus kakinadensis*

(PT 7), *Thiocapsa marina* (PT 10) and *Marichromatium gracile* (PT 8) (Tables II-1a and II-1b). Altogether 5 phylotypes shared *pufLM* nucleotide similarities of less than 86% to known species and therefore cannot safely be assigned to known genera (Fig. II-1a, Table II-1a). Phylotypes 5 and 6 were associated with *Thiocystis gelatinosa* DSM215^T (nucleotide sequences similarities of 84.0% and 84.9%). Phylotypes 15, 16 and 18 were phylogenetically associated with the truly marine *Thiorhodovibrio winogradskyi* (nucleotide sequences similarities of 83.4, 84.6 and 85.6).

In addition to the PSB, 19 phylotypes (PTs 21-39) showed highest sequence similarities to anoxygenic phototrophic Alphaproteobacteria and aerobic anoxygenic phototrophic Gammaproteobacteria. Nucleotide sequence similarities of these phylotypes to known species varied from ~71.3% to 84.0% with only a few exceptions and therefore could not be clearly assigned to genera. Most of these phylotypes were obtained from the environmental clone library and were assigned to groups Alpha I (PT 21, PT 22), Alpha III (PT 29 and PT 31-39) and Alpha IV (PT 30) and to the aerobic anoxygenic phototrophic Gammproteobacteria (PT 23-28), respectively. Using the ≥86% *pufLM* nucleotide sequence similarity benchmark as proposed by Tank et al. (2009), only 3 of these phylotypes could be assigned to genera. The sequence of phylotype 21 was highly similar (92.6%) to that of the salt-dependent purple non-sulfur bacterium *Roseospira mediosalina* BN280^T. Phylotype 33 shared *pufLM* nucleotide sequence similarities of 89.1% to the aerobic purple Alphaproteobacterium *Dinoroseobacter shibae* DFL-12^T of the Alpha III group and PT 36 was related to *Loktanella* (86.8%). In addition, phylotype 23 showed 83.0% similarity to the type strain of the so only far validly described aerobic anoxygenic phototrophic Gammaproteobacterium *Congregibacter litoralis* KT71^T but was much more similar (94.3%) to a new isolate from this group, NOR5-3.

COMPOSITION OF THE ENVIRONMENTAL COMMUNITY OF PSB ACCORDING TO *PUFLM* CLONE SEQUENCES

The highest number of phylotypes was present in the original sample. 14 phylotypes of purple sulfur bacteria and 12 phylotypes of other anoxygenic phototrophic Proteobacteria ("non-PSB") were identified (Table II-1a-b and II-2). Five from 20 phylotypes from purple sulfur bacteria were represented only by single sequences (<1%) and 3 phylotypes found in the original sample were not found in any of the experimental treatments. In turn, 6 phylotypes not detected in the original sample showed up in several of the experiments, 4 as single clones and 2 developed at temperatures above 40°C exclusively. This is a clear hint towards a high diversity in the environmental sample with a number of phylotypes being

present as minor components below the detection limit of the method. Almost 80% of the retrieved clone sequences belong to 6 phylotypes, which are adapted to growth within a wide range of salt concentrations and temperatures (PTs 1, 2, 3, 12, 14, 15). These phylotypes were classified as related to *Allochromatium* species (PTs 1 and 2), to *Trc. mannitoliphagus* (PT 3), to *Halochromatium roseum* (PTs 12 and 14) and distantly related to *Trv. winogradskyi* (PT15) (Fig. II-1a, Table II-1a).

NON-PSB TO *PUFLM* CLONE SEQUENCES OF THE ENVIRONMENTAL SAMPLE

The majority of non-PSB phylotypes were only found in the original sample (12 out of 19) and 10 of these were represented by single *pufLM* nucleotide sequences. Only two phylotypes namely PT 37 related to *Loktanella fryxellenis* and PT 38 related to *Sulfitobacter guttiformis* occurred two and six times, respectively, but were not found in any other clone library. This is certainly due to the experimental conditions using a PSB-specific medium composition that is expected to select against purple non-sulfur bacteria and aerobic anoxygenic phototrophic Proteobacteria. Nonetheless a few phylotypes of non-PSB not detected in the original sample were found in enrichments and most interestingly, phylotype 34, which is related to *Jannaschia*, was found in all treatments (except at 44°C).

THE EFFECT OF TEMPERATURE

Surprisingly, temperature had a dramatic effect upon the community of PSB. The community was most diverse at 13°C where 9 PTs were detected, 6 of which represented approx. 90% of the sequences (PT 12 26%, PT 15 24% and PTs 1 and 2 21%, PT 3 9% and PT 14 12%). The composition at 26°C was significantly changed. Two *Allochromatium* PTs (PT 1 and PT 2: 76%) were clearly predominant and with the exception of PT 3 (*Trc. mannitoliphagus* = 12%) all others clearly decreased in abundance. The most dramatic changes of the PSB community occurred in treatments with elevated temperatures at 41°C and 44°C. In addition to the decrease of the number of phylotypes to higher temperatures, these treatments were dominated by one phylotype that was not detected in any of the other clone libraries of this study. Nearly 90% of the *pufLM* clone sequences in both treatments were represented by phylotype 8, which is identical to *Marichromatium gracile* DSM203^{T} (Fig. II-2). Several isolates of this phylotype were obtained from the original sample and had identical *pufLM* sequences to those of PT 8 (data not shown) but no clone sequence of PT 8 could be retrieved from the original sample. In addition to *Marichromatium gracile*, a phylotype related to

Thiocapsa roseopersicina (PT 11) was found only at 44°C and 2 phylotypes related to *Halochromatium roseum* (PTs 12 and 14) were present over the entire temperature range.

THE EFFECT OF SALT

From a total of 14 phylotypes found under different salt treatments, 6 PTs were represented by just a single clone sequence in all the experimental sets and 4 of these were not found in the original sample (PTs 4, 7, 17 and 19). Most abundant phylotypes in the original sample as well as in the salinity treatments were found along the salinity gradient from freshwater conditions up to 7.5% NaCl (PTs 1, 2, 3, 12, and 14) or were salt-dependant but did not develop in the absence of salt (PTs 15 and 16).

Most of these phylotypes were related to marine or halophilic PSB such as *Trc. mannitoliphagus* (PT 3), *Halochromatium roseum* (PTs 12 and 14) and the salt-dependant *Thiorhodovibrio winogradskyi* (PTs 15 and 16). Interestingly, 5 phylotypes that were assigned to salt-dependant groups or species of PSB, namely *Thiohalocapsa* (PTs 19 and 20), *Halochromatium* (PTs 12 and 14) and *Thiorhodococcus* (PT 3) were also detected in the freshwater treatment. This might indicate their possible adaptation to freshwater and brackish water conditions.

Quite interesting, phylotypes related to *Allochromatium vinosum* (PTs 1 and 2) which is considered a freshwater bacterium (though with a reported salt tolerance), were frequent and found from 0-7.5% NaCl. Both phylotypes together represented approx. 20% of the sequences found in the environmental sample, which points to their successful competition not only under the applied experimental conditions but also in the environment. They were most dominant under freshwater conditions (81%) but were replaced by halophilic phylotypes only to a limited extent (46% PT 1 and PT 2 at 2% and 31% at 5% NaCl). They were dominant also at 7.5% NaCl.

Table II-1a: Type strain affiliation and sequence frequencies of purple sulfur bacterial phylotypes according to their *pufLM* nucleotide sequence similarities

			temperature series				salinity series				
phylo-type	**next type strain**	**sim.[c] [%]**	**13°C**	**26°C**	**41°C**	**44°C**	**0%**	**2%**	**5%**	**7.5%**	**orig. sample**
1	*Allochromatium vinosum* DSM180[T]	93.8	3	26	1	-	15	16	7	16	6
2	*Allochromatium vinosum* DSM180[T]	94.1	4	7	2	-	15	6	6	12	8
3	*Trc.*[†] *mannitoliphagus* ATCC BAA-1228[T]	96.9	3	5	-	-	2	10	4	3	4
4	*Trc. mannitoliphagus* ATCC BAA-1228[T]	99.8	-	-	1	-	-	1	-	-	-
5	*Thiocystis gelatinosa* DSM215[T]	84.0[*]	-	-	-	-	-	-	-	-	1
6	*Thiocystis gelatinosa* DSM215[T]	84.9[*]	-	-	-	-	-	4	-	1	4
7	*Trc. kakinadensis* JA130[T]	98.2	-	-	-	-	-	-	-	1	-
8	*Marichromatium gracile* DSM203[T]	100.0	-	-	51	34	-	-	-	-	-
9	*Tca.*[$] *roseopersicina* DSM217[T]	93.6	-	-	-	-	-	-	-	-	1
10	*Thiocapsa marina* DSM5653[T]	96.8	-	-	-	-	-	-	-	-	1
11	*Tca. roseopersicina* DSM5653[T]	86.8	-	-	-	2	-	-	-	-	-
12	*Halochromatium roseum* DSM18859[T]	89.8	9	2	1	1	2	1	7	2	6
13	*Halochromatium roseum* DSM18859[T]	89.0	1	-	-	-	-	-	1	-	1
14	*Halochromatium roseum* DSM18859[T]	90.7	4	2	-	1	1	4	5	5	2
15	*Trv.*[£] *winogradskyi* DSM6702[T]	83.4[*]	8	-	1	-	-	4	10	9	27
16	*Trv. winogradskyi* DSM6702[T]	84.6[*]	-	-	-	-	-	1	2	1	2
17	*Trv. winogradskyi* DSM6702[T]	90.9	1	1	-	-	-	1	-	-	-
18	*Trv. winogradskyi* DSM6702[T]	85.6[*]	1	-	-	-	-	-	-	-	4
19	*Thiohalocapsa halophila* DSM6210[T]	86.4	-	-	-	-	1	-	-	-	-
20	*Thiohalocapsa marina* JA142[T]	85.9	-	-	-	-	1	-	-	-	1

- not detected, [*] uncertain affiliation, [†] *Thiorhodococcus*, [$] *Thiocapsa*, [£] *Thiorhodovibrio*

Table II-1b: Type strain affiliation and sequence frequencies of non-purple sulfur bacterial and aerobic phototrophic proteobacterial phylotypes according to their *pufLM* nucleotide sequence similarities

			temperature series				salinity series				
phylo-type	**type strain affiliation**	**Sim.[c] [%]**	**13°C**	**26°C**	**41°C**	**44°C**	**0%**	**2%**	**5%**	**7.5%**	**orig. sample**
21	*Roseospira mediosalina* BN280[T]	92.6	1	2	-	-	-	-	-	1	-
22	*Roseospira mediosalina* BN280[T]	82.8[*]	-	-	-	-	-	1	-	-	-
23	*Congregibacter litoralis* KT71[T]	83.0[*]	-	-	-	-	-	-	-	-	1
24	*Congregibacter litoralis* KT71[T]	79.1[*]	-	-	-	-	-	-	-	-	1
25	*Congregibacter litoralis* KT71[T]	77.6[*]	-	-	1	-	-	-	-	-	-
26	*Congregibacter litoralis* KT71[T]	71.3[*]	-	-	-	-	-	-	-	-	1
27	*Congregibacter litoralis* KT71[T]	72.1[*]	-	-	-	-	-	-	-	-	1
28	*Congregibacter litoralis* KT71[T]	74.6[*]	-	1	-	-	-	-	-	-	-
29	*Labrenzia alexandrii* DFL-11[T]	71.1[*]	-	-	-	-	-	-	-	-	1
30	*Erm.*[α] *ramosum* IAM14333[T]	78.2[*]	-	-	-	-	-	-	-	-	1
31	*Roseovarius tolerans* NBRC16685[T]	79.0[*]	1	-	-	1	-	-	-	-	1
32	*Dinoroseobacter shibae* DFL-12[T]	80.8[*]	-	-	-	-	-	-	-	-	1
33	*Dinoroseobacter shibae* DFL-12[T]	89.1	-	-	-	-	-	-	-	-	1
34	*Jannaschia* sp. CCS1[#]	79.4[*]	2	5	1	-	17	2	1	3	-
35	*Rdv.*[β] *euryhalinum* DSM4868[T]	83.8[*]	-	-	-	-	1	-	-	-	-
36	*Lok.*[γ] *vestfoldensis* SKA53[T]	86.8	-	-	1	2	-	-	-	-	1
37	*Lok. fryxellensis* LMG22007[T]	79.9[*]	-	-	-	-	-	-	-	-	2
38	*Sfb.*[δ] *guttiformis* LMG19755[T]	73.0[*]	-	-	-	-	-	-	-	-	6
39	*Rba.*[ε] *sphaeroides* 2.4.1[T]	74.9[*]	-	-	-	-	2	-	-	-	-

- not detected, [*] uncertain affiliation, [#] not validly described, [α] *Erythromicrobium*, [β] *Rhodovulum*, [γ] Loktanella, [δ] *Sulfitobacter*, [ε] *Rhodobacter*

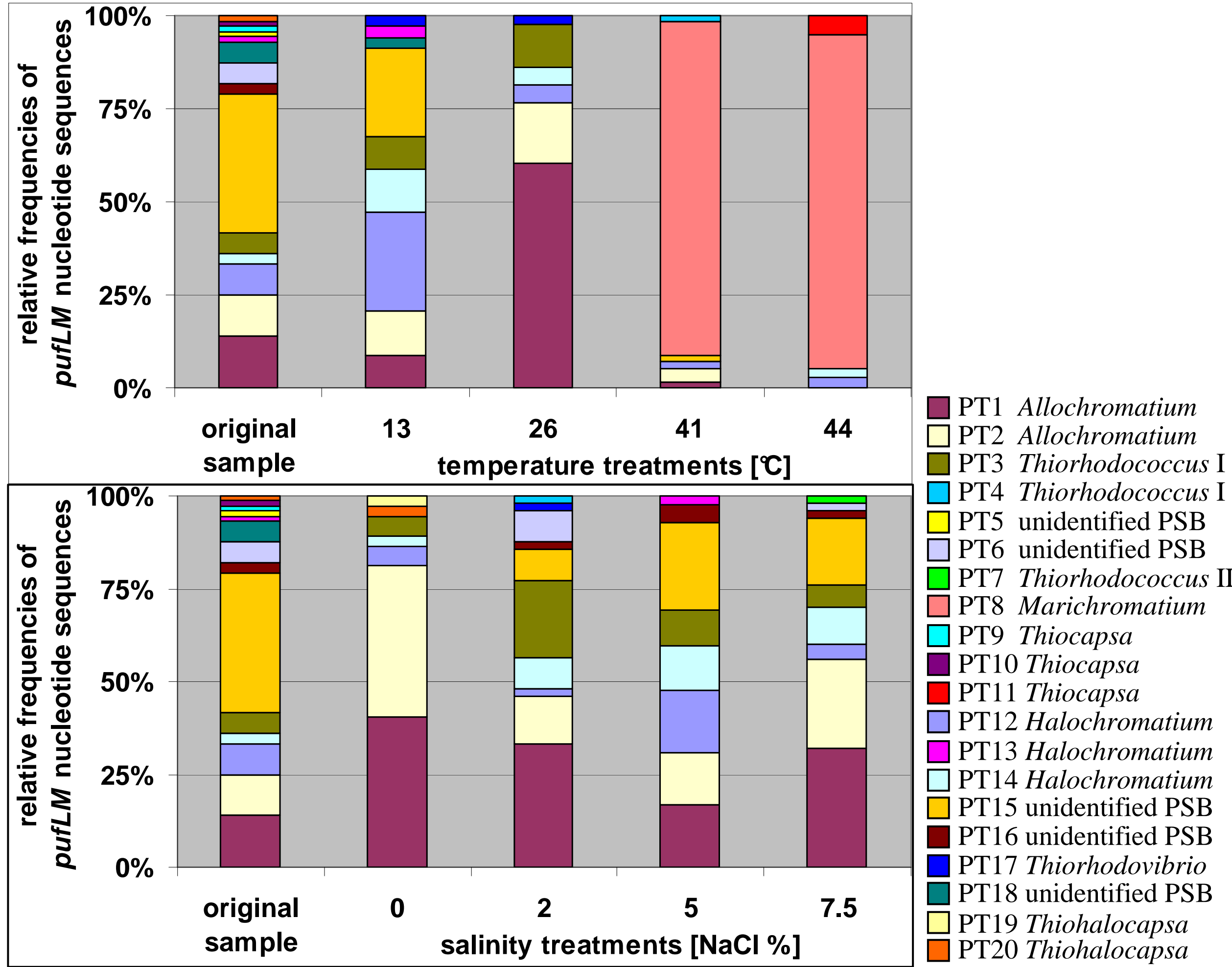

Figure II-2: PSB community composition of selected temperature and salinity treatments revealed from *pufLM* clone libraries, PT=phylotype; PSB=purple sulfur bacteria

DISCUSSION

The present study combined molecular biological techniques with culture experiments to answer questions concerning the composition of purple sulfur bacteria (PSB) communities and changes thereof under variation of temperature and salinity. For the first time an environmental purple sulfur bacteria community was specifically analyzed by using functional genes coding the light and medium polypeptide of the photosynthetic reaction center in order to detect variations in community composition at different temperatures and salinities. The *pufLM* gene was proven as a powerful tool for the analysis of environmental PSB communities.

The properties prevailing in the investigated Baltic Sea lagoon favored the development of PSB and resemble those in other coastal habitats containing PSB found e.g. at Sippewissett salt marsh (East coast USA), at the northern Black Sea coast, at the Danish coast and at Bodden waters of Hiddensee (Trüper 1970, Suckow 1966, Baas-Becking 1925). All of

these marine environments, the habitat analyzed during this study included, fulfill the basic requirements for the development of PSB. Differences in the prevailing conditions through scales of time and space apparently cause significant variations in the PSB community composition in all of these habitats. In these so called sulfureta (Durner *et al.* 1965, Baas-Becking 1925) PSB genera such as *Allochromatium*, *Marichromatium*, *Rhabdochromatium*, *Thiocystis*, *Thiocapsa* or *Ectothiorhodospira* have been repeatedly found (Overmann & Garcia-Pichel 2006, Imhoff 2001, Guyoneaud *et al.* 1996, Trüper 1970, Baas-Becking 1925). Detailed overviews of PSB isolated from marine environments were given by Imhoff (2001, 1988b).

The overall diversity of anoxygenic phototrophic purple bacteria in general found in the sample and the experiments (39 PTs) and of purple sulfur bacteria in particular (20 PTs) was comparable to those found in other studies on anoxygenic phototrophic purple bacteria using molecular techniques (Waidner & Kirchman 2008, Ranchou-Peyruse *et al.* 2006, Martínez-Alonso *et al.* 2005). However, our results demonstrate that the diversity of PSB was not exhaustedly discovered and probably much larger than anticipated, in particular in the case of non-PSB anoxygenic phototrophic bacteria. Corresponding to the benchmarks for PSB genera of 86% *pufLM* as suggested by Tank et al. (2009) and for PSB species of 95% as proposed by Zeng et al. (2007) most of the PSB phylotypes could be assigned to *pufLM* nucleotide sequences of the PSB genera *Allochromatium*, *Halochromatium*, *Marichromatium*, *Thiocapsa*, *Thiohalocapsa*, *Thiorhodococcus*, *Thiorhodovibrio* though only few of them were highly affiliated on a species level. As species the halophilic purple bacteria, *Thiorhodococcus mannitoliphagus*, *Thiorhodococcus kakinadensis*, *Thiocapsa marina*, *Marichromatium gracile* and *Roseospira mediosalina* could be identified (Tables II-1a and II-1b). In contrast to other brackish coastal waters, we found neither members of freshwater PSB genera, e.g. *Lamprocystis*, *Thiolamprovum*, *Thiopedia* or *Thiospirillum* nor *pufLM* sequences of *Ectothiorhodospiraceae* and BChl-*b* containing PSB such as *Thiococcus* (Ranchou-Peyruse *et al.* 2006, Martínez-Alonso *et al.* 2005, Imhoff & Pfennig 2001, Suckow 1966, Baas-Becking 1925).

The phylogenetic analyses showed a distinct classification of the *pufLM* nucleotide sequences into PSB, aerobic anoxygenic phototrophic Alpha- and Gammaproteobacteria and alphaproteobacterial purple non-sulfur bacteria reflecting the phylogeny of the 16S rRNA gene. High bootstrap value (>50%) at almost all nodes of the inferred phylogenetic *pufLM* nucleotide tree support its robustness. This distinctive classification was important to allow application of the *pufLM* gene sequences for detailed analyses of PSB communities and identification of species and genera. This clear phylogenetic separation of *pufLM* nucleotide sequences of Gamma- and Alphaproteobacteria is in contrast to a number of previous studies on the phylogeny of either *pufL*, *pufM* or *pufLM* gene fragments showing the anoxygenic

phototrophic Gammaproteobacteria interspersed within the anoxygenic phototrophic Alphaproteobacteria (Cho *et al.* 2007, Zeng & Jiao 2007, Martínez-Alonso *et al.* 2005, Nagashima *et al.* 1997), but in line with the previous report on the phylogeny of *pufLM* genes in purple sulfur bacteria (Tank *et al.* 2009). The inferred phylogenetic tree of our analyses does not support horizontal gene transfer of the *pufLM* genes which is still under debate (Boucher *et al.* 2003). It is considered that the largely increased number of *pufLM* reference sequences available in the public databases, the use of the entire *pufLM* fragment for the phylogentic calculations, together with improved accuracy in the sequences and alignment preparation led to significantly more robust results.

Our results demonstrated, that the major components of the PSB community of the brackish lagoon were represented by bacteria which develop well at 2% NaCl but also at hypersaline conditions up to 7.5% NaCl. PSB phylotypes affiliated to PSB genera known to contain marine (*Marichromatium*), halophilic (*Halochromatium, Thiorhodovibrio*) or salt-tolerant (*Allochromatium vinosum*) species were dominant in the clone library from the environmental sample.

Altogether the large number of phylotypes related to species requiring elevated salt concentration and having growth optima at salt concentrations exceeding sea water and their frequencies in the clone libraries is quite remarkable. This might point towards elevated salt concentrations in the habitat probably occurring seasonally by high evaporation in the lagoon. However, phylotypes highly affiliated to genera known to contain moderately halophilic species such as *Halochromatium*, *Thiohalocapsa* and *Thiorhodococcus* were also found in the freshwater conditions of the salinity series. Though validly described representatives of these genera are known as strictly salt-dependant (Kumar *et al.* 2007c, Rabold *et al.* 2006, Imhoff 2001, Caumette *et al.* 1997, Caumette *et al.* 1988), their presence also under experimental freshwater conditions, as demonstrated, points out that these bacteria have the potential to succeed at low salt concentrations or even in the absence of salt. Therefore, they should be considered as inhabitants also of brackish coastal water habitats.

Interestingly, phylotypes related to *Allochromatium vinosum* obviously tolerate elevated salt concentrations (Fig. II-4) and can compete even at hypersaline conditions. This is in contrast to properties known from cultures of species of the genus *Allochromatium* (Srinivas *et al.* 2009, Kumar *et al.* 2008, Imhoff 2003b) but in line with the regular observation of these bacteria in marine habitats. Thus our findings emphasize the common occurrence of *Allochromatium* species in coastal marine habitats as well as their potency to enforce against other PSB.

Although the non-PSB community was quite diverse and only single clone sequences were obtained for most phylotypes, the majority could be assigned to marine, halophilic and salt-tolerant species or genera according to *pufLM* nucleotide sequences (Table II-1b). Some were representatives of the salt-dependant genera *Roseospira, Rhodovulum*, *Dinoroseobacter or Congregibacter* (Spring *et al.* 2009, Chakravarthy *et al.* 2007, Srinivas *et al.* 2006, Biebl *et al.* 2005, Guyoneaud *et al.* 2002, Imhoff *et al.* 1998a) which were representatives of alphaproteobacterial purple non-sulfur bacteria and aerobic anoxygenic phototrophic Proteobacteria, respectively.

The temperature experiment revealed dramatic changes in the composition of the community above 40°C. Although the temperature range of all species related to the identified phylotypes were recorded as mesophilic (Imhoff 2003b), the experiment revealed clear differences in the preference for temperatures within the range applied. The fact that a phylotype of *Marichromatium gracile* was predominant at 41°C and 44°C, but was not found under any of the other conditions nor in the original sample points to a so far unrecognized property of this species, which was regularly observed in marine habitats and has been isolated from a number of such habitats (Imhoff 2001). Support for this finding comes from a recent report on the isolation of a *Marichromatium gracile* strain from the German Wadden Sea which showed optimum growth above 40°C (Serrano *et al.* 2009).

Apparently okenone containing PSB related to *Marichromatium purpuratum*, *Thiocapsa marina* or *Thiohalocapsa marina* which were commonly found in other marine habitats (Kumar *et al.* 2009, Caumette *et al.* 2004, Imhoff 2003b) were of minor importance in the investigated lagoon, though sequences related to *Thiocapsa marina* were found in the environmental sample. Because okenone has a light absorption maximum moved towards the blue light and is very efficient in light absorption, the strong illumination and efficient light penetration in the investigated habitat may be in disfavor for these okenone containing phototrophic bacteria (Overmann & Garcia-Pichel 2006, Overmann 2001).

Though some of the unidentified *pufLM* nucleotide sequences they may be related to described species from which *pufLM* sequences presently are not available in the public databases. Because of the congruency of 16S rRNA and *pufLM* gene phylogeny as demonstrated by Tank *et al.* (2009), and the close phylogenetic relationship of *Rhabdochromatium* to *Thiorhodovibrio* according to 16S rRNA gene analyses (Imhoff 2001, Dilling *et al.* 1995, Trüper & Jannasch 1968), phylotypes 15, 16 and 18 might possibly represent members related to *Rhabdochromatium*. *Rhabdochromatium marinum* is common in marine environments, strictly salt dependant and sensitive to freshwater conditions (Imhoff 2001, Dilling *et al.* 1995, Trüper & Jannasch 1968, Baas-Becking 1925) which could be demonstrated

also for phylotypes 15 and 16 (Figs. II-1a and II-2), and has been shown to be part of the phototrophic community in the Kiel fjord before (Petri & Imhoff 2001). In addition, microscopic examination of the original sample did show a morphotype with the characteristics of *Rhabdochromatium marinum* with cells of 2 µm width and 20 µm length (Caumette *et al.* 2004, Dilling *et al.* 1995).

Though the investigated Baltic Sea lagoon appears to be a typical coastal habitat of anoxygenic phototrophic bacteria, the detailed analysis revealed a number of remarkable results. These include the presence of individual strains adapted to elevated temperatures that might be relevant at sunny summer days and give highly selective advantage for those identified as *Marichromatium gracile*. The analysis of *pufLM* clone libraries successfully demonstrated changes of the community of phototrophic sulfur bacteria upon changes of environmental conditions and revealed the dominance of marine, halophilic and salt-tolerant representatives which are well adapted to changing salinities and temperatures. The PSB community was accompanied by purple non-sulfur bacteria as well as aerobic anoxygenic phototrophic Alpha- and Gammaproteobacteria. Quite remarkable a relative to *Jannaschia*, which is known as an aerobic phototrophic Alphaproteobacterium, developed in the experimental conditions set up for purple sulfur bacteria. This should be reason to study the capabilities of *Jannaschia* to perform phototrophic life also under anaerobic conditions.

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CHAPTER III

UNIQUE COMMUNITIES OF ANOXYGENIC PHOTOTROPHIC BACTERIA IN SALINE LAKES OF SALAR DE ATACAMA (CHILE). EVIDENCE FOR A NEW PHYLOGENETIC LINEAGE OF PHOTOTROPHIC GAMMAPROTEOBACTERIA FROM *PUFLM* GENE ANALYSES

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ABSTRACT

Phototrophic bacteria are important primary producers of salt lakes in the Salar de Atacama and at times form visible mass developments within and on top of the lake sediments. The communities of phototrophic bacteria from two of these lakes were characterized by molecular genetic approaches using key genes for the biosynthesis of the photosynthetic apparatus in phototrophic purple bacteria (*pufLM*) and in green sulfur bacteria (*fmoA*). Phototrophic purple bacteria were present in all samples. T-RFLP of the *pufLM* genes indicated high variability of the community composition between the two lakes and subsamples thereof. The communities were characterized by the dominance of a novel, so far undescribed lineage of *pufLM* containing bacteria and the presence of representatives related to known halophilic *Chromatiaceae* and *Ectothiorhodospiraceae*. In addition, the presence of BChl *b*-containing anoxygenic phototrophic bacteria and of aerobic anoxygenic bacteria was indicated. Green sulfur bacteria were not detected in the environmental samples, though a bacterium related to *Prosthecochloris indicum* was identified in an enrichment culture. This is the first comprehensive description of phototrophic bacterial communities in a salt lake of South America made possible only due to the application of the functional *pufLM* genes.

INTRODUCTION

It has been known for long that the strongly saline environment is primarily a domain of prokaryotes and the spectrum of eukaryotic species in highly saline biotopes is rather restricted. The dominant primary producers are halophilic and halotolerant algae and cyanobacteria as well as anoxygenic phototrophic bacteria (Imhoff 2002, Imhoff 2001, Imhoff 1988b, Trüper & Galinski 1986, Imhoff *et al.* 1979). A variety of anoxygenic phototrophic bacteria has been isolated from different hypersaline habitats, such as marine salterns (Caumette 1993, Caumette *et al.* 1991, Caumette *et al.* 1988, Rodriguez-Valera *et al.* 1985), alkaline soda lakes in the Egyptian Wadi Natrun (Imhoff & Trüper 1981, Imhoff *et al.* 1978, Imhoff & Trüper 1977), in Siberia and Mongolia (Bryantseva *et al.* 2000, Bryantseva *et al.* 1999) and from Solar Lake (Sinai) (Caumette *et al.* 1997, Cohen & Krumbein 1977) as reviewed in Imhoff (2001). Also green sulfur bacteria have been observed in various saline environments mainly based on microscopic and macroscopic observations (Oren 1993, Caumette 1993, Giani *et al.* 1989). Species of the genus *Prosthecochloris* were obtained from marine and saline

environments and are recognized as halotolerant and moderately halophilic organisms (Triadó-Margarit *et al.* 2010, Alexander & Imhoff 2006, Imhoff 2003a, Vila *et al.* 2002, Imhoff 2001, Gorlenko 1970).

Highly saline lakes in the extremely aride Atacama Desert located in northern Chile are characterized by high UV-radiation, high salt concentrations and wide diurnal temperature variations. The Salar de Atacama is located at an altitude of 2300 m and is the largest evaporitic basin in Chile (2900 km^2). It has several permanent hypersaline lakes that receive waters from the Andes Range (Risacher & Alonso 1996). Like other hypersaline environments, the studied lakes of Salar de Atacama (Laguna Chaxa and Laguna Tebenquiche) are inhabited by only a few higher organisms like brine shrimp, some copepods, and surrounding macrophytes (Zúñiga *et al.* 1991). Visually, the shallow Laguna Tebenquiche and Laguna Chaxa exhibit the presence of extensive red-purple colored microbial mats on the surface of the lake sediments. Based on low $\delta^{13}C(HCO_3)$ values of -1.38 for Laguna Chaxa measured in previous studies, biological productivity in these lakes is expected to be high (Boschetti *et al.* 2007). However, the content of chlorophyll *a* was shown to be rather low in previous studies (Demergasso *et al.* 2008), leading to the assumption of a considerable impact of anoxygenic phototrophic bacteria on the primary productivity in these habitats. Despite the visual indication, phototrophic bacteria have not been specifically studied and almost nothing is known about the diversity and composition of the communities of anoxygenic phototrophic bacteria in these lakes. Main studies of the microbiology of the Salar de Atacama have been focused on the cultivable diversity. Heterotrophic strains of moderately halophilic bacteria have been analyzed by numerical taxonomy (Valderrama *et al.* 1991, Prado *et al.* 1991) and chemotaxonomic analysis (Marquez *et al.* 1993) and dominated the isolation-based studies (Campos 1997, Ramos-Cormenzana 1993). The only phototrophic bacteria so far described in Laguna Tebenquiche (Salar de Atacama) were oxygenic cyanobacteria represented by *Oscillatoria* (Zúñiga *et al.* 1991). Recently the bacterial diversity in water samples of Laguna Tebenquiche has been studied by ribosomal gene library analysis (Demergasso *et al.* 2008). However, sequences related to anoxygenic phototrophic bacteria were not recovered, except for a single clone related to the aerobic phototrophic purple bacteria of the *Roseobacter* clade (Demergasso *et al.* 2008) with no evidence for any phototrophic potential and activity.

In order to specifically study the communities of phototrophic prokaryotes of these habitats, we used molecular genetic analyses with group specific primers for functional genes (*pufLM, fmoA)* which target phototrophic bacterial communities. Because they represent a physiological group of polyphyletic origin, it is not possible to recover the diversity of phototrophic communities using 16S rRNA gene sequences. The *pufLM* genes encode for the

light (L) and medium (M) subunit of the photosynthetic reaction center type II structural proteins of phototrophic purple bacteria including purple sulfur bacteria, purple nonsulfur bacteria and aerobic anoxygenic phototrophic bacteria, as well as *Chloroflexaceae*. These genes have been used previously to access phototrophic bacteria in environmental samples (Hu *et al.* 2006, Oz *et al.* 2005) and were demonstrated to be suitable phylogenetic markers for purple sulfur bacteria (Tank *et al.* 2009). The *fmoA* gene encodes the monomer of the FMO protein, which binds Bchl *a* and is associated in a trimeric structure (Fenna *et al.* 1974). Its unique occurrence in green sulfur bacteria and the recently described "*Candidatus* Chloracidobacterium thermophilum" (Bryant *et al.* 2007) makes *fmoA* an appropriate target to specifically analyze environmental communities of these bacteria (Alexander & Imhoff 2006).

MATERIAL AND METHODS

STUDY AREA

The Salar de Atacama is a closed saline basin within the pre-Andean depression of the Atacama Desert located at 20" 30'S and 68" 15'W in northern Chile and covers approx. 2900 km^2 (Zúñiga et al., 1991; Demergasso et al., 2004). The Atacama Desert is characterized by extreme aridity and is considered one of the driest places on earth (Rech *et al.* 2006). The average amount of precipitation in the desert and the Salar area reaches < 3 $mm \cdot year^{-1}$ and 25-50 mm $year^{-1}$, respectively. The low precipitation together with an exceptionally high evaporation of 1800– 3200 mm $year^{-1}$ leads to a hyper-arid ecosystem (Boschetti *et al.* 2007, Risacher *et al.* 2003). Solar radiation is high, especially UV-B radiation which is 20% increased compared to at sea level (Cabrera *et al.* 1995).

The saline basin of the Salar de Atacama is covered with a thick halite crust of several hundred meters (Bobst *et al.* 2001). At its edges and in its interior, there are small ponds and a number of shallow lakes with high concentrations of salts which receive streams of fresh sub-surface water. Laguna Chaxa and Laguna Tebenquiche (the largest of these lakes) are two of these hypersaline lakes receiving freshwater from the subsurface (Zúñiga *et al.* 1991). The pH was only slightly alkaline (Table III-1) and despite the shallow character of the lakes low dissolved oxygen concentrations have been measured in this (1.2 mg/L) and in previous studies (0.6 mg/L, (Boschetti *et al.* 2007, Zúñiga *et al.* 1991). Sodium and chloride are the dominating ions, followed by sulfate (Risacher & Alonso 1996).

SAMPLES

Sediment samples were taken from two lakes at the Salar de Atacama, Laguna Tebenquiche and Laguna Chaxa located in the North and in the East of the Salar, respectively. Four sediment samples were taken from Laguna Tebenquiche (23.13S 68.24W; samples SAT1 – SAT5) and six from Laguna Chaxa (23.29S 68.18W, samples FC1 – FC6). The samples contained differently colored bacterial mats with supernatant water. They were homogenized and separated into aliquots. Samples for DNA extraction were stored at -20 °C until analysis.

Table III-1: Samples of Salar de Atacama used in this study, their visual appearance and chemical parameters. Samples of (Flamingo) Lake Chaxa (FC) were taken at 24th of November 2008 at approx. GPS position 23.29S 68.18W); samples of Lake Tebenquiche (SAT) were taken at 22th of November 2008 at approx. GPS position 23.13S 68.24W).

		chemical data			
Sample	**Description**	**pH**	**salinity [PSU]**	**Temp [°C]**	**Redox [mV]**
FC1	orange-red saltcrust	7.32	o	36.0	-34.0
FC2	purple mat	7.55	o	27.0	-48.0
FC3	greenish surface with purple layer beneath	7.64	o	32.0	-53.0
FC4	bacteria mat with gas-enclosures; black beneath	7.86	193	36.0	-67.0
FC5	Saltcrust with pink and green layer	o	o	32.5	o
FC6	pink sand; beneath black sediment	7.99	o	33.7	-74.0
SAT1	red bacteria mat	o	o	o	o
SAT2	pink filamentous mat with leatherskin and gas-enclosures	7.00	178	32.0	o
SAT3	orange, leatherlike surface	o	o	o	o
SAT4	pink filamentous bacteria mat with gas-enclosures	o	o	o	o
SAT5	green mats with gas-enclosures	7.96	30	33.5	o

o – not available

DNA-EXTRACTION

DNA was extracted from 250 mg of homogenized sediment samples with the PowerSoil DNA isolation Kit (MoBio Laboratories, Inc.) following the manufacturer's protocol. Lysis was performed following the alternative protocol including 2 × 10 min heating intervals (70°C) with vortexing before, after and in-between the intervals.

PCR

Amplification of functional genes was performed using puReTaq Ready-To-Go PCR beads (GE Healthcare, USA) in a final volume of 25 µl. For amplification of *pufLM* genes the primers 67F and 781R (Tank *et al.* 2009) were used. 3–5 µl of template (~50 ng DNA for SAT samples and ~100 ng DNA for FC samples) and 1 µl of each primer (10 pmol) were used. PCR was carried out using the following conditions: initial denaturation (94°C for 2 min) followed by 35 cycles of primer annealing (40 s at 55°C), elongation (1.5 min at 72°C), and denaturation (40 s at 94°C), a final primer annealing (1 min at 42°C), and a final extension (5 min at 72°C). In PCR for terminal restriction fragment length polymorphism (T-RFLP) analysis, the annealing temperature was 56°C.

For amplification of *fmoA* genes the primers fmoA_Start_mod (5'-ATT ATG GCT CTN TTC GGC-3'; modified from Alexander *et al.* (2002)) and fmoA_1094r (Alexander *et al.* 2002) were used. Amplification of vector inserts was accomplished with the primers M13f (5'-GTA AAA CGA CGG CCA G-3') and M13r (5'-CAG GAA ACA GCT ATG AC-3'). In both cases (*fmoA* and M13 PCR), an annealing temperature of 50°C was used. The other PCR conditions were as stated above for *pufLM* amplification.

CLONING AND SEQUENCING

PCR products were purified by extraction from a 1% TAE agarose gel using the QIAquick Gel Extraction Kit (QIAGEN, Germany). DNA was eluted in 30µl elution buffer and stored at -20°C until further analysis. DNA was ligated into pCR4-TOPO vector and transformed into One Shot Competent *E. coli* cells using the TOPO TA Cloning Kit (Invitrogen). Inserts were amplified as described above using the M13f/M13r primer set. Insert size was checked by agarose gel electrophoresis.

Sequencing was done with the BigDye Terminator v1.1 sequencing kit (Applied Biosystems) on a 3730 DNA analyzer (Applied Biosystems) as specified by the manufacturer. Clone inserts were sequenced using the vector specific primers T3 and T7 (Invitrogen). Sequences obtained in this study were deposited in the EMBL database (Kulikova *et al.* 2004) and assigned the accession numbers FN813740-FN813767.

PHYLOGENETIC ANALYSES

Sequences were edited using the Seq Man II program (DNASTAR, USA). Closest relatives were determined by comparison with sequences in the National Centre for

Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.* 1990). Sequences posted as possible chimera using the Bellerophon server (http://foo.maths.uq.edu.au/~huber/bellerophon.pl, Huber *et al.* 2004) were further checked for breakpoints and parental clones manually. Sequences with similarity to non-*pufLM* gene sequences as well as putative chimeric sequences were removed from the dataset prior to richness estimation and phylogenetic analysis.

The nucleotide sequences of the *pufLM* genes were converted into amino acid sequences using BioEdit version 7.0.1 (Hall 1999), aligned with the integrated version of Clustal X (Thompson *et al.* 1997), and manually refined. For phylogenetic analysis the corresponding *pufLM* nucleotide sequence alignment was used. Replicate sequences (≥98.0% sequences similarity) were grouped for each library using MOTHUR (Schloss *et al.* 2009). Representative sequences were chosen manually considering maximal sequence length and quality. Only one representative sequence each was used for phylogenetic analysis. Representative sequences with nucleotide similarities ≥98.0% were identified as belonging to a single phylotype. As suggested by Tank *et al.* (2009), *pufLM* clone sequences of ≥86% nucleotide similarity were grouped together in clusters considered equivalent to genera.

For phylogenetic analysis representative clones as well as reference sequences of each phototrophic *pufLM*-containing group including next relatives as determined by BLAST search were used (Suppl. Table III-1). The evolutionary model GTR+I+G used for phylogenetic analyses of nucleotide sequences was determined using the program ModelGenerator, version 0.85 (Keane *et al.* 2004). Phylogenetic trees were calculated with the PhyML program version 3.0 (Guindon & Gascuel 2003). *Chloroflexus aggregans* DSM 9485^T and *Chloroflexus aurantiacus* DSM635^T served as outgroup species. Sequence similarities were calculated as percentage similarity between all aligned sequences used for phylogenetic calculation by the Phylip 3.63 DNADIST program (Felsenstein 1989).

RICHNESS ESTIMATION

Nucleotide sequences with similarities ≥ 98% were grouped using MOTHUR (Schloss *et al.* 2009). The proportion of prokaryotic diversity represented by the clone libraries was estimated by rarefaction analysis combined with nonlinear regression. Rarefaction analysis calculations were performed applying the algorithm described by Hurlbert (1971) with the program aRarefact-Win (http://www.uga.edu/strata/software.html) after removal of putative chimera. Rarefaction curves were plotted and regressions performed using the equation $y = a \cdot (1-e^{-bx^c})$ as published earlier (Thiel *et al.* 2007) where x is the sample size, y the observed

number of phylotypes, *a* is the number of phylotypes to be expected with infinite sample size (i.e. total phylotype richness) (Koellner *et al.* 2004), and *b* and *c* are additional curve fitting parameters. SigmaPlot v10.0 (Systat Software Inc.) was used for plotting and regression analysis. Coverage was calculated as the ratio of obtained number of phylotypes to the expected number of phylotypes *a* as calculated by the regression.

T-RFLP FINGERPRINTING

Restriction digests. PCR products were purified by excision from a 1% agarose gel in TAE buffer, subsequent extraction with the QIAquick Gel Extraction Kit (QIAGEN), and eluted from spin columns with 30 µl elution buffer. The PCR products were digested with three restriction endonucleases AluI, HinfI, and MspI (New England Biolabs). 10 µl of purified PCR product were mixed with 3.5 µl of restriction enzyme master mix containing NEBuffer 4 and 10 U (HinfI; AluI) or 20 U (MspI), respectively and filled up with DNAfree H_2O to a total volume of 25 µl. Restriction reactions were incubated for 6 h at 37°C, followed by 20 min at 65°C to denature the enzyme.

T-RFLP analysis. Restriction products were purified by isopropanol precipitation and re-suspended in 12 µl of Hi-Di™ formamide (Applied Biosystems). For analysis, the re-suspended restriction product was mixed with 1 µl of GeneScan - 1000 [ROX] size standard, and denatured at 95°C for 5 min. T-RF signals were detected by capillary electrophoresis on an ABI PRISM 310 Genetic Analyzer with POP-6™ Polymer in a 30 cm capillary (Applied Biosystems) under the following conditions: injection time 15 sec, injection/electrophoresis voltage 15 kV, gel temperature 60°C, run time 90 min. Electropherograms were analyzed with the program GeneScan v2.0.2 (Applied Biosystems).

Statistics. Peak data of terminal restriction fragments (T-RFs) between 20 and 928 nucleotides (nt) and signal intensities ≥50 (arbitrary units) were exported as tabular data from the genetic analyzer. A data matrix was created from the combined Alu I and Msp I peak data with samples as columns and peak positions (T-RF lengths) as rows. Data from the Hinf I digestion were omitted from the statistics because digestion of sample FC6 with this enzyme was unsuccessful. The area under each peak was used as a measure of T-RF abundance, standardized as percentage of total peak area as described by (Lukow *et al.* 2000). Due to rounding errors and minor variations in size determination, the length of defined T-RFs varied among samples. The variation was empirically determined to be ±0.3% of the fragment length, but at least ±1 nt. This meant a size variation of ±1 nt for fragment lengths form 20 to 499 nt, ±2 nt for fragment lengths form 500 to 833 nt, and ±3 nt for fragment lengths from 834 to

928 nt. T-RFs were aligned within this range from their expected mean length (Lukow *et al.* 2000). Two distance matrices were derived from the peak matrix based on (i) the Bray-Curtis coefficient which compares relative abundances of T-RFs shared between any two samples, and (ii) the Dice coefficient (aka Sørensen coefficient) which is analogue to the Bray-Curtis coefficient but only takes into account binary data (i.e., presence or absence of T-RFs). Non-metric multidimensional scaling (MDS) was applied to ordinate samples in three dimensions according to their distances. The number of three dimensions were chosen in order to keep the stress value for dimensional downscaling below the recommended threshold of 0.1 for an ideal preservation of the original distances between samples (Clarke 1993). For clearness, of these three dimensions only the two with the most conspicuous differences were plotted in the MDS diagrams.

To see whether distances within and between sample groups were connected to the sampling sites, a Mantel test (Mantel 1967) was performed. This test compared each of the T-RFLP-derived distance matrices (based on Bray-Curtis and Dice coefficients, respectively) with a second "distance" matrix listing "0" for any two samples from the same sampling site, and "1" for any two samples not from the same sampling site. Based on the Spearman correlation coefficient it was tested whether sample pairs of the same site had lower T-RFLP-derived distance values than sample pairs from different sites (one-tailed test). Statistical analyses were performed with XLSTAT version 2009.6.02 (Addinsoft).

RESULTS

In this study, we used functional genes as molecular targets to specifically analyze anoxygenic phototrophic bacteria inhabiting two hypersaline lakes of the saline evaporate basin, Salar de Atacama. We thereby took advantage of recently established phylogenetic congruence between the ribosomal genes and genes for specific structural components of the photosynthetic apparatus of green sulfur bacteria (*fmoA*, Alexander & Imhoff 2006, Alexander *et al.* 2002) and of phototrophic purple bacteria (*pufLM*, Tank *et al.* 2009). *pufLM* genes were successfully amplified from 10 out of 11 samples (SAT5 did not yield *pufLM* amplificates), while *fmoA* genes were not amplified from any environmental sample.

PUFLM FINGERPRINTING

In order to get an overview of the diversity of *pufLM*-containing organisms between and within the different sampling locations we employed T-RFLP as a genetic fingerprinting

method. T-RFLP data were visualized by non-metric multidimensional scaling (MDS). (Fig. III-1). According to MDS, the phototrophic bacterial communities of Laguna Chaxa (FC) and Laguna Tebenquiche (SAT) clearly differed from each other. These results were obtained independently of the method used for distance calculation: In the MDS plot based on normalized peak areas (Bray-Curtis coefficient), the separator between FC and SAT phototrophic bacterial communities was the axis of dimension 1 (Fig. III-1a); MDS based on peak presence-absence data (Dice coefficient) separated these communities along the main diagonal (upper right to lower left corner) of the plot (Fig. III-1b).

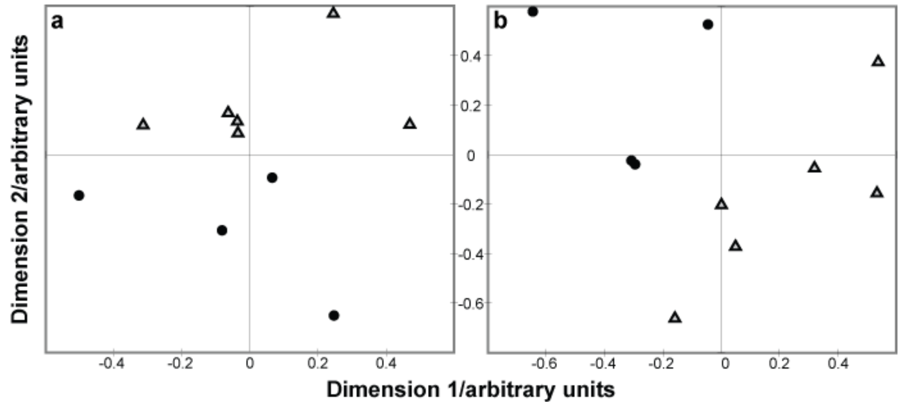

Figure III-1: Non-metric MDS plot based on T-RFLP fingerprinting data of Laguna Chaxa samples FC 1-6 (▲) and Laguna Tebenquiche samples SAT 1-4 (●). Ordination was based on the Bray-Curtis coefficient (a) and the Dice coefficient (aka Sørensen coefficient) (b) derived from the T-RF peak matrix. For clearness, of the three calculated MDS dimensions only the two with the most conspicuous differences were plotted. The stress value for dimensional downscaling was 0.06 (a) and 0.09 (b), respectively. In the two plots, data points of FC and SAT phototrophic bacterial communities are separated by the axis of dimension 1 (a) and by the main diagonal (upper right to lower left corner) (b), respectively. This indicates that phototrophic bacterial communities of the two sampling locations clearly differed from each other.

A highly significant correlation between phototrophic bacterial community composition and sampling location was also indicated by the Mantel test ($p = 0.0028$ and $p = 0.0053$ for the distance matrices based on Bray-Curtis and Dice coefficients, respectively).

MDS plots also showed conspicuous distances between different bacterial populations of the same location (Fig. III-1a, b). This was due to the fact that subsamples of phototrophic communities from each lake differed considerably in both the overall number of peaks and the presence or absence of specific T-RFs (Table III-2). For example, within Laguna Chaxa the number of peaks originating from AluI digestion varied between 8 and 74 (median, 19) for different community samples. Only 2 (2%) of all T-RFs produced with AluI were found in every sample, while 60 peaks (58%) occurred only once in a single sample. Similar results were obtained with the HinfI and MspI digestions (Table III-2).

Table III-2: T-RFLP peak statistics.

Number of peaks found in…	Enzyme		
	Alu I	Hinf I*	Msp I
all samples (min - median - max)	6 - 17 - 74	7 - 28 - 90	4 - 29 - 102
all FC samples (min - median - max)	8 - 19 - 74	18 - 59 - 90	10 - 35.5 - 102
all SAT samples (min - median - max)	6 - 12 - 20	7 - 17 - 28	4 - 12 - 37
every sample (percentage of all peaks)	2 (2%)	0 (0%)	0 (0%)
2-9 samples (percentage of all peaks)	41 (40%)	94 (61%)	80 (53%)
1 sample only (percentage of all peaks)	60 (58%)	61 (39%)	71 (47%)

* HinfI digest not successful with sample FC6

PHOTOTROPHIC PURPLE BACTERIA

Clone sequences were retrieved from 2 selected samples each of Laguna Chaxa (FC2, FC5) and Laguna Tebenquiche (SAT3, SAT4). In total four clone libraries of *pufLM* genes were constructed and 159 clone sequences grouped into 25 different phylotypes (PTs) were analyzed. The number of phylotypes differed greatly between the clone libraries, with values between 1 (SAT3) and 14 (FC2) (Table III-3, Fig. III-3). Rarefaction analyses showed relations of detected to predicted numbers of phylotypes of > 69% for the different clone libraries (Table III-3). Phylogenetic analysis resulted in a tree topology with a clear separation between the three subclasses, Gamma-, Beta- and Alphaproteobacteria, and the outgroup (Fig. III-2). The different lineages within these subclasses (e.g., Chromatiaceae, *Ectothiorhodospira*, *Halorhodospira*, BChl *b*-containing bacteria and the aerobic Gammaproteobacteria) were supported by high bootstrap values (Fig. III-2, data for Alphaproteobacteria are not shown). Based on this phylogenetic analysis, all but one phylotype were assigned to the anoxygenic phototrophic Gammaproteobacteria (Fig. III-2).

Table III-3: Rarefaction analysis and presumed coverage of *pufLM* clone libraries.

clone library	sequences	phylotype obtained	phylotype expected	St. Dev.	R^2	coverage
FC2	68	14	19	0.1523	0.9999	75.0%
FC5	58	8	12	0.4348	0.9984	69.0%
SAT3	5	1	n.d.	n.d.	n.d.	n.d.
SAT4	28	5	7	0.3218	0,9985	71.9%

n.d. not determinable

The composition of the clone libraries demonstrated a highly diverse and variable community of anoxygenic phototrophic bacteria in Salar de Atacama. Each of the subsamples consisted of a distinct phototrophic community and the presence of most of the phylotypes was restricted to only one of the samples. Only three phylotypes were retrieved from multiple samples (*Halochromatium*-1, *Ectothiorhodospira*-1 and Novel-6, Fig. III-2, Table III-4).

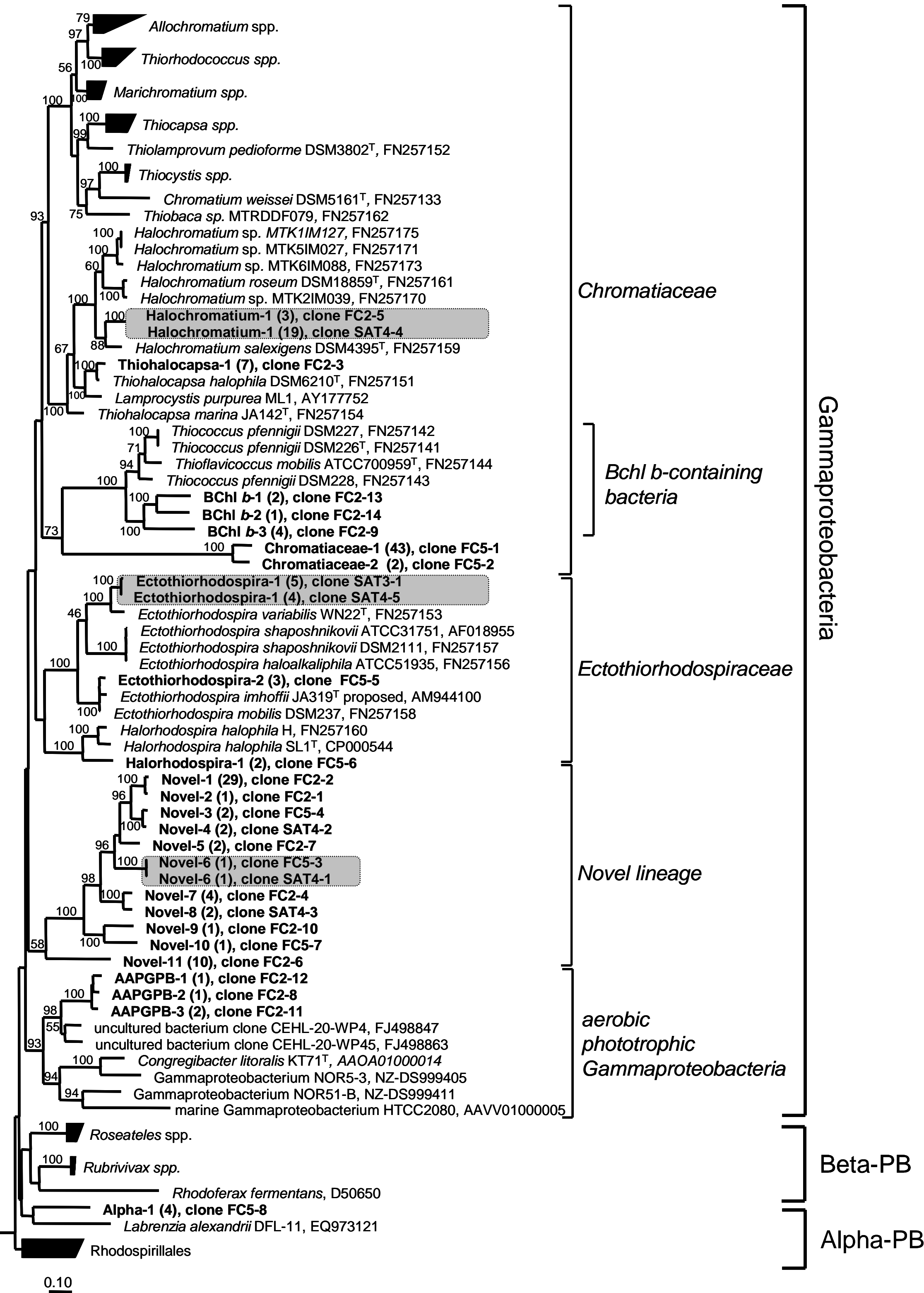

Figure III-2: Partly condensed Maximum Likelihood phylogenetic tree of *pufLM* sequences. Sequences obtained in this study are printed in bold. Behind the phylotype name the number of sequences represented is given in parenthesis followed by the representative clone. Most phylotypes were present in only one of the samples. Only three phylotypes were obtained from more than one sample (highlighted in shaded boxes). Bootstrap values ≥50 are shown at the nodes. The scale bar indicates 0.1 change per nucleotide. Alphaproteobacteria groups without any sequences obtained in this study as well as the Chloroflexaceae sequences used as outgroup were withdrawn from the tree graphically. All database sequences used for phylogenetic calculation are listed in supplementary Table III-1. PB = Proteobacteria.

Almost half of the phylotypes (48%) could not be assigned to any of the known groups and genera but formed a separate, highly supported monophyletic lineage within the Gammaproteobacteria (Novel lineage in Fig. III-2). This novel lineage is highly diverse (11 phylotypes, Novel-1 to -11, Fig. III-2) and prevalent in Salar de Atacama. Representatives were observed in all but one sample. One of the novel lineage phylotypes (Novel-6, Table III-4) was present in both lakes, Laguna Chaxa and Laguna Tebenquiche (Table III-4). Others dominated individual clone libraries (i.e., Novel-1, FC2, 43%, Fig. III-3).

Nearly one third (28%) of the phylotypes inhabiting Salar de Atacama were identified as closely related to genera of the salt-loving *Ectothiorhodospiraceae* as well as to the halophilic *Chromatiaceae* genera *Halochromatium* and *Thiohalocapsa* (Table III-4). Of the three different members of the Ectothiorhodospiraceae present in Salar de Atacama, the *Ectothiorhodospira variabilis* related phylotype was obtained from both Laguna Tebenquiche samples, while relatives of *Ect. mobilis* and of *Halorhodospira halophila* were present in the Laguna Chaxa sample FC5 only. The *Halochromatium salexigens* related phylotype dominated the Laguna Tebenquiche sample SAT4 clone library and was obtained from both lakes. *Thiohalocapsa marina* related bacteria were only present in the Laguna Chaxa sample FC2.

Five phylotypes obtained from Laguna Chaxa samples showed affiliations to the Bchl *b*-containing purple sulfur bacteria of the *Thiococcus*/*Thioflavicoccus* group, but shared sequence similarities with them of only ≤80% (Fig. III-2, Table III-4). For three of them, phylogenetic analysis (Fig. III-2) and signature amino acids (data not shown) clearly supported affiliation to the group of BChl *b*-containing phototrophic Gammaproteobacteria (Tank *et al.* 2009). The other two phylotypes clustered with the *Thiococcus*/*Thioflavicoccus* group more distantly and do not contain signature amino acid insertions and therefore were not assigned to the group of BChl *b*-containing Gammaproteobacteria (Fig. III-2).

Additionally, three phylotypes from Laguna Chaxa sample FC2 clustered with aerobic phototrophic Gammaproteobacteria (Table III-4). They showed closest relation (84-86%) to *pufM* clone sequences retrieved from a hypersaline lake in Tibet (Jiang *et al.* 2010). Due to low sequence similarities to *pufLM* sequences of identified aerobic anoxygenic phototrophic Gammaproteobaceria isolates from the NCBI database (max. 76%) they cannot reliably be assigned to that phylogenetic group.

Only one single clone was found to phylogenetically cluster with the Alphaproteobacteria. However, due to low similarity values of 73% to its next phylogenetic relative, *Labrenzia alexandrii*, and of < 77% to all known sequences, this phylotype could not reliably be assigned phylogenetically (Table III-4).

Table III-4: Cluster of pufLM clone sequences obtained from this study and its affiliation regarding BLAST search.

affiliation and next relatives	Phylotypes	representative sequence (No. of clones)	Lake
Halochromatium *Hch. salexigens* (86%)	Halochromatium-1	FC2-5 (3) SAT4-4 (19)	Chaxa Tebenquiche
Thiohalocapsa *Thc. halophila* (96%)	Thiohalocapsa-1	FC2-3 (7)	Chaxa
BChl *b*-containing bacteria (uncertain) *Thiococcus pfennigii*[T] (79-80%)	BChl *b*-1	FC2-13 (2)	Chaxa
	BChl *b*-2	FC2-14 (1)	Chaxa
	BChl *b*-3	FC2-9 (4)	Chaxa
Chromatiaceae*	Chromatiaceae-1	FC5-1 (43)	Chaxa
	Chromatiaceae-2	FC5-2 (2)	Chaxa
Ectothiorhodospira *Ect. varabilis* (92%)	Ectothiorhodospira-1	SAT3-1 (5) SAT4-5 (4)	Tebenquiche Tebenquiche
Ectothiorhodospira *Ect. mobilis* (97%)	Ectothiorhodospira-2	FC5-5 (3)	Chaxa
Halorhodospira *Hlr. halophila* (85%)	Halorhodospira-1	FC5-6 (2)	Chaxa
Novel lineage*	Novel-1	FC2_2 (29)	Chaxa
	Novel-2	FC2_1 (1)	Chaxa
	Novel-3	FC5_4 (2)	Chaxa
	Novel-4	SAT4_2 (2)	Tebenquiche
	Novel-5	FC2_7 (2)	Chaxa
	Novel-6	FC5_3 (1) SAT4_1 (1)	Chaxa
	Novel-5	FC2-7 (2)	Tebenquiche
	Novel-7 Novel-8	FC2_4 (4) SAT4_3 (2)	Chaxa Tebenquiche
	Novel-9	FC2_10 (1)	Chaxa
	Novel-10	FC5_7 (1)	Chaxa
	Novel-11	FC2_6 (10)	Chaxa
Aerobic anoxygenic phototrophic gammaproteobacteria (uncertain) Tibetan saline lake clones CEHL-20-WP4/-WP45 (84-86%)	AAPGPB-1	FC2-8 (1)	Chaxa
	AAPGPB-2	FC2-11 (2)	Chaxa
	AAPGPB-3	FC2-12 (1)	Chaxa
Alphaproteobacteria (uncertain) *Rubrivivax gelatinosus* (77%)	Alpha-1	FC5-8 (4)	Chaxa

*all next relatives are below 75% sequence similarity.

PHOTOTROPHIC GREEN SULFUR BACTERIA

Though *fmoA* genes could be amplified from enrichment cultures of FC2 (Pfennigs media with 5%, 7.5% and 10% NaCl, respectively, data not shown), they were not detected in the environmental samples. From 96 clone sequences of the enrichment only one phylotype was obtained. BLAST search and phylogenetic analysis revealed close relationship to *Prosthecochloris indicum* strain 2K (due to the lack of phylogenetic information formerly treated as *Ptc. aestuarii* 2K) with *fmoA* nucleotide sequence similarity of 98.2%.

DISCUSSION

The hypersaline lakes in the Atacama Desert represent unique and extreme habitats clearly dominated by various forms of microbial life. Microbiological studies have been performed only occasionally and almost nothing is known about the phototrophic microbial communities in these habitats. Highly diverse communities of anoxygenic phototrophic bacteria were present in both Laguna Chaxa and Laguna Tebenquiche, but significant differences were found between the two lakes according to *pufLM* T-RFLP analysis (Fig. III-1). In addition, clear differences were also obvious between the subsamples of each of the two lakes. Both *pufLM* T-RFLP and clone libraries revealed a high heterogeneity of the phototrophic bacterial communities within the different samples of both lakes. Highly patchy and variable conditions were observed in microhabitats including varying salinity, pH, temperature, and redox values (Table III-1, (Demergasso *et al.* 2008). These variable conditions apparently have major impact on the bacterial communities of the phototrophic bacteria leading to the patchiness observed as different colors and textures of the microbial mats as well as different molecular patterns in this study.

The clear delineation of the phototrophic purple bacteria from hypersaline lakes into phylogenetic groups in this study is based on the recently demonstrated congruence of *pufLM* and 16S rRNA gene phylogenies of the purple sulfur bacteria (Tank *et al.* 2009) and the aerobic Gammaproteobacteria (M. Tank, unpublished data). Accordingly a large number of *pufLM* phylotypes were affiliated with the Gammaproteobacteria, a diverse group of a novel monophyletic lineage of anoxygenic phototrophic Gammaproteobacteria as well as related to Alphaproteobacteria.

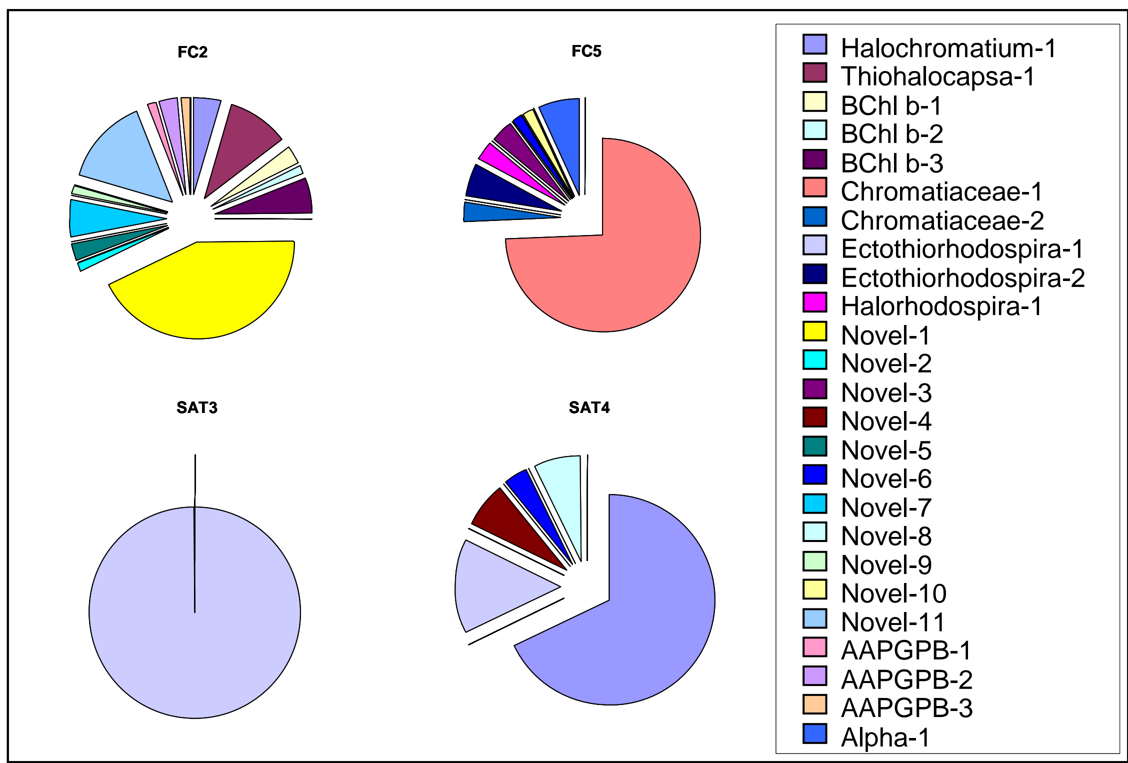

Figure III-3: Relative abundance of *pufLM*-phylotypes in the environmental clone libraries.

The identification of a novel and so far unknown group of phototrophic Gammaproteobacteria which inhabits and possibly dominates the extreme habitats of Salar de Atacama, once more indicates that our knowledge of the diversity of APB is far from complete. This novel lineage is represented by a phylogenetically diverse number of phylotypes possibly reflecting several species and even genera (differentiated at a 86% *pufLM* sequence similarity level as proposed by Tank *et al.* (2009)) and may be specifically adapted to Chilean salt lakes, similar to a group of Bacteroidetes found in Laguna Tebenquiche (Dorador *et al.* 2009, Demergasso *et al.* 2008).

The identification of moderately and extreme halophilic Chromatiaceae and Ectothiorhodospiraceae corresponds to the hypersaline character of the lakes (Imhoff 2001). The next relatives of the halophilic Chromatiaceae, *Halochromatium salexigens* and *Thiohalocapsa halophila*, as well as *Ectothiorhodospira* species are well known as halophilic bacteria tolerating salinities up to 20% while *Halorhodospira halophila* is regarded as extremely halophilic with salt optima between 13% and 23% NaCl (Gorlenko *et al.* 2009, Caumette *et al.* 1994, Imhoff 1993, Imhoff 1992, Caumette *et al.* 1991, Caumette *et al.* 1988). Although most *Ectothiorhodospira* species were isolated from alkaline habitats, others are known to tolerate pH values as low as 7.5 (Gorlenko *et al.* 2009) like the only slightly alkaline pH values prevalent at Salar de

Atacama (Table III-1). The mainly moderately halophilic characteristics of the identified phototrophic purple sulfur bacteria in Salar de Atacama is consistent with previous isolation-based microbiological studies which were dominated by moderately halophilic heterotrophic bacteria while extreme halophiles were not isolated (Ramos-Cormenzana 1993). However, in contrast to those studies, our data indicate also the presence of extremely halophilic phototrophic bacteria related to *Halorhodospira halophila* reflecting variations in habitat salt concentrations known from literature (Demergasso *et al.* 2008) and measured in this study (Table III-1).

It was not surprising to find phylogenetic evidence for the presence of novel phototrophic bacteria possibly related to the BChl *b*-containing Chromatiaceae group of *Thiococcus/Thioflavicoccus* species, which is also pointed out by in-vivo absorption spectra for Laguna Chaxa (data not shown). Members of this group have been isolated from freshwater and marine habitats as well as from alkaline soda lakes (Imhoff & Pfennig 2001, Bryantseva *et al.* 2000, Eimhjellen *et al.* 1967). BChl *b* has an absorption maximum in the infrared part of the spectrum and bacteria with this pigment are particularly well adapted to sediments covered by water not permanently or only by a thin layer. The lakes of Salar de Atacama characterized by shallow water bodies and particularly wet sediments around the lakes might thus be preferred habitats of BChl *b*-containing phototrophic bacteria. Unfortunately, up to date, a *pufLM* sequence of the only moderately halophilic BChl *b*-containing Chromatiaceae known is unavailable for phylogenetic comparison. *Thioalkalicoccus limnaeus* was isolated from Siberian soda lakes and tolerates salt concentrations of up to 6% (Imhoff & Pfennig 2001, Bryantseva *et al.* 2000). Because of the phylogenetic congruency between 16S rRNA and *pufLM* gene in PSB (Tank *et al.* 2009), the three *pufLM* phylotypes from Laguna Chaxa possibly represent *Thioalkalicoccus limnaeus* or relatives and contain BChl *b* as supported by signature amino acids.

The presence of aerobic phototrophic Alpha- and Gammaproteobacteria in Laguna Chaxa displays the coexistence of both aerobic and anaerobic anoxygenic phototrophic bacteria in the bacterial mats covering the shallow lake sediments and in its saline waters. Possibly aerobic anoxygenic phototrophic *Roseobacter*-like bacteria were also detected by ribosomal genes retrieved from Laguna Tebenquiche (Demergasso *et al.*, 2008). However, using ribosomal genes as target led to the detection of only one single phylotype of the *Roseobacter*-clade, a group comprising a number of heterotrophic Alphaproteobacteria of which only some have photosynthetic potential. Therefore, it is not clear whether the identified sequence belongs to a photosynthetically active bacterium. In contrast, the molecular approach using photosynthesis genes specifically targeting anoxygenic phototrophic bacteria demonstrated a rich diversity of anoxygenic phototrophic Proteobacteria including aerobic

anoxygenic phototrophic Gammaproteobacteria in Salar de Atacama. Only few aerobic anoxygenic phototrophic Gammaproteobacteria have been isolated and only one described species (*Congregibacter litoralis*) is known so far. They have been shown to be abundant in marine waters and sediments but were also found in hypersaline habitats as indicated by 16S rRNA gene sequences (Yan *et al.* 2009), including a Chilean salt lake geographically close to Salar de Atacama (EMBL entry no. EF632657). Recently, one isolate was recovered from a Canadian hypersaline spring system (Csotonyi *et al.* 2008). Unfortunately, a *pufLM* sequence of the halophilic isolate is unavailable for comparison and only few *pufLM* sequences of this group are retrievable from the databases. Nonetheless, *pufM* sequences related to those found in Salar de Atacama were also present in a Tibetan hypersaline lake (Jiang *et al.* 2010) indicating a possible preference for high-salt conditions in these bacteria (Fig. III-2).

Phototrophic members of the Alphaproteobacteria apparently represent a rather insignificant group of anoxygenic phototrophic bacteria in Salar de Atacama. The low similarity values further suggest that special forms of so far not recognized aerobic alphaproteobacterial phototrophic bacteria have adapted to the habitats of Salar de Atacama.

Green sulfur bacteria (GSB) are quite likely a minor component of the phototrophic bacterial community in Salar de Atacama, but may develop under appropriate conditions as demonstrated by enrichment cultures (data not shown). However, a natural abundance below the detection limit of molecular methods is indicated by the lack of amplification of *fmoA* genes. Species of the genus *Prosthecochloris* are known as salt-dependent organisms from marine and hypersaline habitats and have been obtained by isolation and molecular methods from different saline habitats (Imhoff & Thiel 2010, Triadó-Margarit *et al.* 2010, Alexander & Imhoff 2006, Vila *et al.* 2002, Gorlenko 1970). Detection of the halotolerant *Prostecochloris indica* (7% salt tolerance) in the Salar de Atacama correlates with the recognition of *Prosthecochloris* species being common as representatives of the Chlorobiaceae in saline and hypersaline habitats (Triadó-Margarit *et al.* 2010, Alexander *et al.* 2002). At the same time they demonstrate low abundance and a possibly quite restricted phylogenetic diversity of this group in the salt lakes of Salar de Atacama.

CONCLUSIONS

The studied hypersaline lakes in Salar de Atacama are unique habitats harboring highly diverse anoxygenic phototrophic bacterial communities including numerous still unknown *pufLM* containing anoxygenic phototrophic bacteria. The communities have significantly different composition in the two lakes as well as in subsamples of each of the lakes. Highly variable conditions such as water variability, salt concentrations, and light conditions apparently shape the community structure in microhabitats of the salt lakes. The great number of yet uncultured and unidentified *pufLM* phylotypes retrieved emphasizes the uniqueness of the studied area as well as the need of further studies on phototrophic bacteria, including culture-dependent approaches. Further, this study demonstrated the great power of molecular methods targeting the photosynthesis-related functional genes *pufLM* in studying natural communities of anoxygenic phototrophic Proteobacteria in environmental samples.

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CHAPTER IV

A NEW SPECIES OF *THIOHALOCAPSA*, *THIOHALOCAPSA MARINA* SP. NOV., FROM AN INDIAN MARINE AQUACULTURE POND

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ABSTRACT

A spherical-shaped, phototrophic, purple sulfur bacterium was isolated in pure culture from anoxic sediment in a marine aquaculture pond near Bheemli (India). Strain JA142^T is Gram-negative and non-motile. It has a requirement for NaCl (optimum of 2% and maximum of 6% w/v NaCl). Intracellular photosynthetic membranes are of the vesicular type. In vivo absorption spectra indicate the presence of bacteriochlorophyll *a* and carotenoids of the okenone series as photosynthetic pigments. Phylogenetic analysis on the basis of 16S rRNA gene sequences showed that strain JA142^T is related to halophilic purple sulfur bacteria of the genera *Thiohalocapsa* and *Halochromatium*, with the highest sequence similarity to *Thiohalocapsa halophila* DSM 6210^T (97.5 %). Morphological and physiological characteristics differentiate strain JA142^T from other species of the genera *Halochromatium* and *Thiohalocapsa*. Strain JA142^T is sufficiently different from *Thiohalocapsa halophila* based on 16S rRNA gene sequence analysis and morphological and physiological characteristics to allow the proposal of a novel species, *Thiohalocapsa marina* sp. nov., with the type strain JA142^T (=JCM 14780^T =DSM 19078^T).

INTRODUCTION

The genus *Thiohalocapsa* was established to separate species of purple sulfur bacteria from other species of the genus *Thiocapsa* based on their halophilic growth response, lack of gas vesicles, large phylogenetic distance and clustering with marine and halophilic strains (Imhoff et al., 1998). At present, the genus *Thiohalocapsa* comprises only one species, *Thiohalocapsa halophila* (Imhoff *et al.* 1998b; originally described as *Thiocapsa halophila* (Caumette *et al.* 1991)).

Strain JA142^T was isolated from photolithoautotrophic enrichments with 2% NaCl (w/v) of anoxic sediment and water (sample properties: pH 7.0, salinity 2% and temperature 30 uC) from a marine aquaculture pond near Bheemli, Visakhapatnam, India (17° 54´ N 83° 27´ E). Purification was achieved by repeated agar-shake dilution series (Pfennig & Trüper 1992, Imhoff 1988a, Trüper 1970)). Polyphasic taxonomic studies and spectral analysis were carried out as described earlier (Kumar *et al.* 2008, Kumar *et al.* 2007b). Utilization of organic compounds as carbon sources/electron donors for phototrophic growth was tested in the presence of yeast extract (0.03 %, w/v) without any additional carbon source/electron donor. The concentrations of these compounds were 1 mM benzoate, 0.1% (v/v) for formic acid,

propionic acid, butyric acid, caproic acid, valeric acid, lactic acid, glycerol, methanol and ethanol and 0.3% (w/v) for the other organic compounds tested. Cells of strain JA142^T were spherical, non-motile, 1.5–2.0 mm in diameter and multiplied by binary fission (Supplementary Fig. IV-S1). Electron photomicrographs of ultrathin sections of the cells revealed a vesicular type of internal membranes. Strain JA142^T was able to grow photolithoautotrophically [anaerobic, light (30 μE m^{-2} s^{-1}), Na_2S $9H_2O$ (2 mM)/ $Na_2S_2O_3$ $5H_2O$ (5 mM) and $NaHCO_3$ (12 mM)]. Phototrophic growth (Table IV-1) in the presence of bicarbonate (12 mM) (photomixotrophy) and Na_2S $9H_2O$ (0.5 mM) was observed with acetate, pyruvate, lactate, fumarate, succinate, glucose and Casamino acids.

Substrates not utilized included formate, propionate, butyrate, malate, fructose, ethanol, propanol, glycerol and crotonate. Photo-organoheterotrophy [anaerobic, light (30 μE m^{-2} s^{-1}), pyruvate (27 mM)], chemolithoautotrophy [aerobic, dark, $Na_2S_2O_3$ $5H_2O$ (5 mM) and $NaHCO_3$ (12 mM)], chemo-organoheterotrophy [aerobic, dark, pyruvate (27 mM)] and fermentative growth [anaerobic, dark, pyruvate (27 mM)] could not be demonstrated. Na_2S $9H_2O$ and $Na_2S_2O_3$ $5H_2O$ were utilized as electron donors under photolithoautotrophic conditions with a minimum concentration of 0.5 mM Na_2S $9H_2O$ and a tolerance of up to 4 mM, while sulfite, elemental sulfur and hydrogen did not support growth.

During oxidation of sulfide, elemental sulfur droplets were stored inside the cells. Na_2S $9H_2O$ and $Na_2S_2O_3$ $5H_2O$ were utilized as sulfur sources by strain JA142^T, while sulfate, sulfite, thioglycolate and cysteine did not support growth.

Ammonium chloride was utilized as a nitrogen source by strain JA142^T, while nitrate, nitrite, glutamate, glutamine, urea and dinitrogen did not support growth. Strain JA142^T is a true marine strain; growth occurs at 1.0–6.0% NaCl (w/v) with an optimum at 2.0% (w/v). The pH range for growth of strain JA142^T is pH 6.5–8.5 with an optimum at pH 7.5. The temperature optimum for growth is 25–30°C (range 25–35°C). Strain JA142^T does not require vitamins for growth. The colour of the phototrophically grown cell suspension is purple–red. The whole-cell absorption spectrum of strain JA142^T exhibited absorption maxima at 395, 509, 584, 803 and 845 nm and a shoulder at 878 nm, confirming the presence of bacteriochlorophyll *a* (Supplementary Fig. IV-S2a), and the absorption spectrum for pigments extracted with acetone exhibited maxima at 462, 488 and 516 nm, indicating the presence of the carotenoid okenone (Supplementary Fig. IV-S2b). DNA was extracted and purified by using the Qiagen genomic DNA extraction kit. The DNA base composition of strain JA142^T

Table IV-1. Differential characteristics between strain JA142^T and species of the genera *Thiohalocapsa* and *Halochromatium* Strain/species: 1, JA142^T; 2, *Thc. halophila*; 3, *Hch. roseum*; 4, *Hch. salexigens*; 5, *Hch. glycolicum*. Data for *Hch. roseum* were taken from Anil Kumar et al. (Kumar *et al*. 2007c); data for the other reference species were taken from Imhoff (2005a). Cells of all taxa studied divide by binary fission. All strains have internal membranes of the vesicular type. Na_2S and thiosulfate are utilized by all strains. Organic substrate utilization was tested in the presence of sulfide and bicarbonate. Propionate, butyrate, ethanol, propanol and crotonate were not utilized by any of the strains. +, Substrate utilized or present; 2, substrate not utilized or absent; (+), weak growth; NR, not reported. *According to absorption spectra, the presence of okenone as major carotenoid is likely.

Characteristic	1	2	3	4	5
Cell shape	sphere	sphere	Rod	Rod	Rod
Motility	-	-	-	+	+
Cell diameter (μm)	1.5-2.0	1.5-2.5	2.0-3.0 x 3.0-5.0	2.0-2.5 x 4.0-7.5	0.8-1.0 x 2.0-4.0
Gas vesicles	-	-	+	-	-
Color of cell suspensions	Purple-red	Purple-red	Purple-pink	Pink, Rose-red	Pink, Pinkish red
Carotenoid group	Okenone?	Okenone	Okenone	Spirilloxanthin	Spirilloxanthin
Mol% G+C of DNA	64.8	65.9-66.6	64	64.6	66.1-66.5
B_{12} requirement	-	-	+	+	-
Chemolithotrophic growth	-	+	-	+	+
pH optimum	7.5	7.0	7.5	7.4-7.6	7.2-7.4
(range)	(6.5-8.5)	(6.0-8.0)	(7-8)	(7.0-8.0)	(6.2-9.0)
Temperature optimum (°C)	25-30	20-30	27	20-30	25-35
NaCl optimum (%, w/v)	2	4-8	1.5-2.5	8-11	4-6
(range)	(1-6)	(3-20)	(1-3)	(4-20)	(2-20)
Substrates photoassimilated:					
Hydrogen	-	+	NR	+	+
Sulfur	-	+	-	+	+
Sulfite	-	+	-	+	+
Formate	-	-	-	-	(+)
Acetate	+	+	-	+	(+)
Pyruvate	+	+	+	+	(+)
Lactate	+	+	-	-	-
Fumarate	+	-	+	-	+
Succinate	+	-	+	-	+
Malate	-	-	+	-	-
Fructose	-	+	-	-	NR
Glucose	+	(+)	-	-	-
Glycerol	-	(+)	-	-	+
Glycolate	-	-	-	-	+
Crotonate	-	-	-	-	-
Valerate	-	-	-	-	NR
Casamino acids	+	-	+	-	(+)

was 64.8 mol% G+C (by HPLC). PCR amplification and 16S rRNA gene sequencing were performed as described previously (Imhoff *et al.* 1998b). Recombinant Taq polymerase was used for PCR, which was started with primers 5'-GTTTGATCCTGGCTCAG-3' and 5'-TACCTTGTTACGACTTCA-3' (*Escherichia coli* positions 11–27 and 1489–1506, respectively). Sequences were obtained by cycle sequencing with the SequiTherm sequencing kit (Biozym) and the chain termination reaction (Sanger *et al.* 1977) using an automated laser fluorescence sequencer (Pharmacia). Nearest relatives and sequence similarities were determined by BLAST search (Altschul *et al.* 1990) and BLAST 2 SEQUENCES alignment

(Tatusova & Madden 1999). 16S rRNA gene sequences of the closest related type strains belonging to the genera *Halochromatium* and *Thiohalocapsa* were newly determined and the corresponding EMBL database entries were updated prior to phylogenetic analysis. 16S rRNA gene sequences of type strains of representative species of the Chromatiaceae and of strain JA142^{T} were aligned using the FASTAlign function of the alignment editor implemented in the ARB software package (http://www.arb-home.de; (Ludwig *et al.* 2004) and refined manually employing secondary structure information. For phylogenetic calculations, the PhyML online version (Guindon *et al.* 2005), MEGA version 4.0 (Kumar *et al.* 2004) and the PHYLIP DNAPARS program implemented in ARB (Ludwig *et al.* 2004) were used. For tree calculation, a character-based method [maximum-likelihood (Felsenstein 1981)], two distancebased methods [neighbour joining (Saitou & Nei 1987) and minimum evolution (Rzhetsky & Nei 1993)] as well as a maximum-parsimony method (Fitch 1977, Fitch 1971, Eck & Dayhoff 1966) were employed. The Tamura–Nei model was determined as the model best suited for phylogenetic calculation using the program ModelGenerator (Keane *et al.* 2006). The maximum-likelihood tree was calculated using the TN93 model, six rate categories, gamma distribution parameter alpha=0.31 and proportion of invariable sites=0.43 as determined by ModelGenerator. For the maximum-likelihood bootstrap analysis, the nonbootstrapped maximum-likelihood tree was used as the starting tree. The neighbour-joining tree was calculated based on distances corrected by the Tamura–Nei nucleotide substitution model, using sites corresponding to the pairwise deletion option, including transition and transversion substitutions, assuming a heterogeneous pattern among lineages and a gamma-distributed substitution rate (alpha=0.31). The maximum-parsimony tree was calculated using the 'more thorough search' option and a randomized sequence order.

The 16S rRNA gene sequence analysis revealed that the new isolate belongs to the family Chromatiaceae and is affiliated to a group of marine and halophilic genera including *Halochromatium, Marichromatium, Thiorhodovibrio, Rhabdochromatium* and *Thiohalocapsa*. Highest 16S rRNA gene sequence similarity was shared with *Thc. halophila* DSM 6210^{T} (97.5 %) and *Halochromatium glycolicum* 6340^{T} (97.2%). Similarity values of <98.7% suggest separation at the species level according to Stackebrandt & Ebers (Stackebrandt & Ebers 2006). Phylogenetic analyses (Fig. IV-1) confirmed a close relationship between strain JA142^{T} and both *Halochromatium roseum* JA134^{T} and *Thc. halophila* DSM 6210^{T}. In all cases, *Halochromatium* species and *Thc. halophila* and JA142^{T} clustered monophyletically. Additionally, in all trees (Fig. IV-1 and Supplementary Fig. IV-S3), *Halochromatium* species formed a tight subcluster, strongly supported by bootstrap analysis, that did not include strain JA142^{T}. The distance-based trees further indicate a separate clustering of *Thc. halophila* and strain JA142^{T}. Detailed

comparison of 16S rRNA gene sequences revealed particular sequence differences in a number of characteristic nucleotide positions of strain JA142^T from both *Halochromatium* species and *Thc. halophila* DSM 6210^T (Table IV-2). Overall sequence similarity as well as signature nucleotides demonstrate a closer relationship of strain JA142^T to *Thc. halophila* compared with *Halochromatium* species (12 nucleotides identical to *Thiohalocapsa* compared with eight identical nucleotides to *Halochromatium*; Table IV-2). However, nine characteristic nucleotides were different from both *Thc. halophila* and *Halochromatium* species, which indicates an intermediate or borderline position between known representatives of the two genera. This view is supported by phylogenetic relationships, as demonstrated by phylogenetic trees constructed by a variety of different methods. All methods used (neighbour-joining, minimum-evolution, maximumlikelihood and maximum-parsimony; Fig. IV-1 and Supplementary Fig. IV-S3) demonstrate the clustering of JA142^T with *Halochromatium* and *Thc. halophila*. Furthermore, all phylogenetic methods strongly support a subcluster of the three known *Halochromatium* type strains that did not include strain JA142^T or K. Sequences of *pufLM* support the association of the new isolate with the *Halochromatium*/*Thiohalocapsa* cluster (not shown). More specifically, they demonstrate a clear relationship to the *pufLM* sequence of *Thc. halophila*, but not those of *Halochromatium* species (M. Tank and J. F. Imhoff, unpublished results). Similarity of the *pufLM* nucleotide sequence of strain JA142^T (approx. 1390 bp) to that of the type strain of *Thc. halophila* was 88%; similarities to several sequences from *Halochromatium* species were 84–85% and to sequences from *Thiorhodovibrio* species were below 80 %. A value of 70% DNA relatedness has been used as a benchmark for separation at the species level for a number of years, and a 16S rRNA gene sequence similarity of 97% was regarded as borderline for requiring DNA–DNA hybridization data, assuming that this value more or less coincides with 70% DNA–DNA relatedness. In their critical analysis, Stackebrandt & Ebers (Stackebrandt & Ebers 2006) carefully compared 16S rRNA gene sequence similarities with DNA–DNA reassociation values from a great number of publications. Their convincing result was that, below 98.5% 16S rRNA gene sequence similarity, there was not a single case where DNA-DNA reassociation was more than 70 %, and these authors argued that, with high-quality sequences (as used in this study), 99% sequence similarity almost excludes reassociation values of 70% or more. They recommended a 16S rRNA gene sequence similarity threshold range of 98.7–99% as the point at which DNA–DNA reassociation experiments should be mandatory for testing the genomic uniqueness of new isolates. Therefore, the 97.5% 16S rRNA gene sequence similarity between strain JA142 ^{T} and *Thc. halophila* DSM 6210^T indicates their separation into different species. This is supported by differences in the G+C content of the two bacteria of 1.1–1.8 mol%, by

different salt responses and by a number of differences in substrate and electron donor utilization, including the ability to grow chemolithotrophically (Table IV-1). Because of the closer association of the novel bacterium with *Thc. halophila* in terms of both sequence information and phenotypic properties, strain JA142T is recognized as a member of a novel species of the genus *Thiohalocapsa*, for which the name *Thiohalocapsa marina* sp. nov. is proposed.

Table IV-2. 16S rRNA signature nucleotides for *Thiohalocapsa* and *Halochromatium* species Positions are given according to the sequence of *E. coli*. Shared nucleotides are highlighted in bold .

E. coli **Position**	***Halochromatium*** **spp. (N=7)**	**Strain JA142^T**	***Thiohalocapsa*** **spp. (N=5)**
144	G	G	A
148	A	G	G
223	G	A	G
250	M = U/G	A	A
269	U	C	U
381	A	A	C
444	G	A	G
454	A	U	A
457	C	U	C
473	U	U	C
490	C	U	C
589	U	C	C
590	G	U	G
653	U	U	C
658	A	C	C
660	G	A	G
745	C	U	C
748	U	G	G
838	U	U	C
839	C	C	U
1001	U	C	C
1007	-	U	U
1010	U	G	G
1021	A	U	U
1022	-	U	U
1256	U	U	C
1257	C	U	U
1265	C	C	A
1424	C	U	U

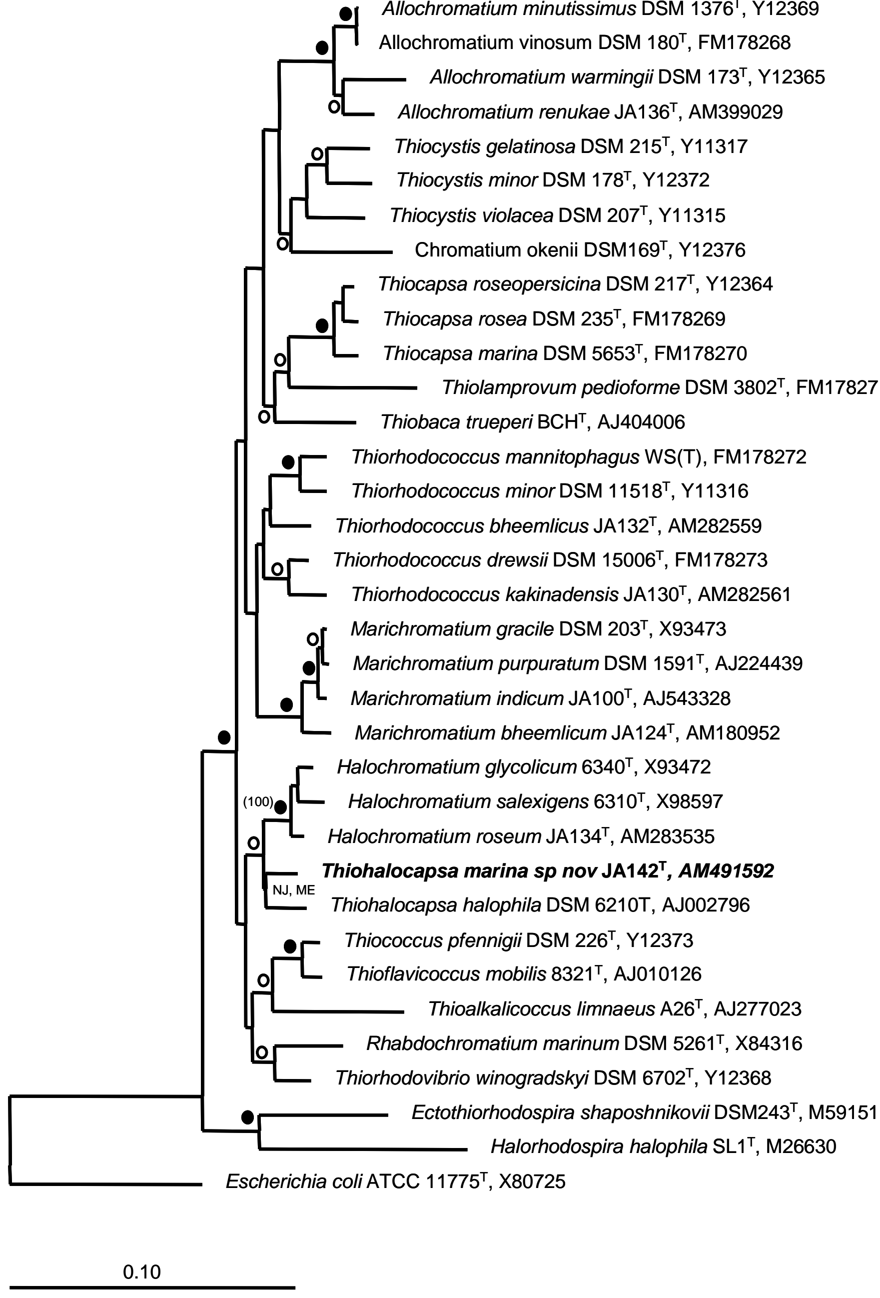

Figure IV-1: Phylogenetic consensus tree based on 16S rRNA gene sequences showing the relationship of strain JA142^T within the family Chromatiaceae. Phylogenetic trees were calculated by the minimum-evolution and neighbour-joining methods as well as by the maximum-parsimony and maximum-likelihood methods. Tree topology of all four methods was compared and shared nodes are marked in the minimum-evolution tree as follows: nodes supported by all four methods are marked by open circles (○); nodes shared by all four methods and supported by bootstrap values .95% or 100% are marked with filled circles [● and (100)●, respectively]; a node supported only by the distance-based methods is indicated by NJ ME. Bar, 0.1 substitutions per alignment position.

DESCRIPTION OF *THIOHALOCAPSA MARINA* SP. NOV.

Thiohalocapsa marina (ma.ri´na. L. fem. adj. marina pertaining to the sea, marine). Cells are spherical, 1.5–2.0 mm in diameter, non-motile and divide by binary fission. Growth occurs under anaerobic conditions in the light under photolithoautotrophic conditions. In addition, several organic substrates can be photoassimilated. Internal photosynthetic membranes are of the vesicular type. Colour of the phototrophically grown cell suspension is purple–red. The in vivo absorption spectrum of intact cells in sucrose exhibits maxima at 395, 509, 584, 803 and 845 nm, indicating the presence of bacteriochlorophyll *a* and carotenoids of the okenone series as photosynthetic pigments. The type strain is mesophilic (30°C), with a pH optimum at 7.5 (range pH 6.5–8.5). Salt is required for growth of the type strain; growth occurs at 1.0–6.0% NaCl (w/v) with an optimum at 2.0% (w/v). Photolithotrophic growth is possible in the presence of bicarbonate (12 mM) and $Na_2S \cdot 9H_2O$ (0.5 mM). A few organic substrates can be photoassimilated in the presence of sulfide and bicarbonate, including acetate, pyruvate, lactate, fumarate, succinate, glucose and Casamino acids. Photo-organoheterotrophy and chemotrophy are not detected. No growth factors are required. The DNA base composition of the type strain is 64.8 mol% G+C (by HPLC). The type strain, JA142^T (=JCM 14780^T =DSM 19078^T), was isolated from a marine aquaculture pond near Bheemli, Visakhapatnam, India.

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GENERAL DISCUSSION

Ecological studies on purple sulfur bacteria (PSB) mainly focused on the isolation and description of new species from many different habitats. After the introduction of the ribosomal gene analysis into ecological microbiology, several 16S rRNA gene based studies were also conducted for PSB. However, the interpretation of the results was rather difficult due to the uncertainty caused by the close phylogenetic proximity of purely non-phototrophic relatives. A specific, highly sensitive and reliable method to analyze diversity of purple sulfur bacteria and variations in the composition of their natural communities was lacking.

pufLM genes encode for the light and medium subunit of the photosynthetic reaction center of all phototrophic purple bacteria including PSB. These genes fulfill the requirements concerning functional gene approaches on natural habitats, as they are present in and essential to all members of the studied group. However, at the beginning of this thesis, knowledge on the phylogenetic information carried by *pufLM* was largely missing, and PSB-related *pufLM* sequences available in public databases were rare. The aim of this thesis was to fill this gap by establishing a reference database of PSB *pufLM* sequences and by investigating the suitability of these genes as phylogenetic markers for the group of PSB. Secondly, the application of this functional gene approach on natural communities of phototrophic purple bacteria including PSB was affected.

Important criteria influencing reliability and robustness in phylogenetic analyses include sequence information, i.e., sequence length and dataset size. In order to gain as much sequence information as possible, a modified primer set based on the primer pair designed by Nagashima *et al.* (1997) was developed. The modified primers match *pufLM* genes of all phototrophic purple bacteria and Chloroflexaceae available in the databases at the beginning of this study (2006). In addition, they cover approx. 80% of the total length of both genes, *pufL* and *pufM* (~1.5 kb). In contrast to most previous studies that focused on a comparably small part (171-600bp) of the *pufM* gene only and therefore comprise rather limited phylogenetic information, e.g., (Zeng & Jiao 2007, Zeng *et al.* 2007, Hu *et al.* 2006, Ranchou-Peyruse *et al.* 2006, Karr *et al.* 2003, Beja *et al.* 2002, Achenbach *et al.* 2001), in all phylogenetic calculations during the present study only sequences of >1400 bp length were used. Due to the co-habitation of different *pufLM*-containing bacteria in their natural environment, importance was attached to the design of a universal primer pair targeting all *pufLM* containing bacteria, i.e. PSB, purple nonsulfur bacteria (PNSB), aerobic anoxygenic

phototrophic proteobacteria (AAPB), and Chloroflexaceae. In previous studies *pufLM* was used on natural communities of mainly PSNB and AAPB (Hu *et al.* 2006, Karr *et al.* 2003, Beja *et al.* 2002). In this thesis, analyses of PSB-specific *pufLM* genes were conducted for the first time.

Over 60 PSB-derived *pufLM* genes mainly obtained from type strains (n=29) and isolates (n=28) together with a handful of database-derived sequences were analyzed in detail (Chapter I). PSB-derived *pufLM* sequences were shown to resemble *pufLM* of PNSB in certain characteristics, e.g., the number of helices and the regions coding for pigment binding sites. Further, coherence between PufLM protein structure and the type of internal membrane system was demonstrated. Comparison of *pufL* and *pufM* gene nucleotide sequence similarities showed *pufL* to be more conserved, possibly reflecting the higher conservation due to electron transfer through the "L-branch" of the reaction center (data not shown). The suitability test of *pufLM* genes as phylogenetic markers for PSB was of great success (Chapter I). In contrast to other studies, which found little analogy between 16S rRNA gene and *pufLM* phylogeny (Ranchou-Peyruse *et al.* 2006, Beja *et al.* 2002, Nagashima *et al.* 1997), the results obtained in this study showed high consistency of both gene phylogenies. Irrespective of using *pufLM* nucleotide or deduced amino acid sequences in phylogenetic analyses, high concordance with the 16S rRNA gene phylogeny was achieved. Furthermore, the results demonstrated that classification of known and unknown PSB up to at least the genus level is possible based on *pufLM* nucleotide sequence. This makes the *pufLM* genes a powerful tool for analyzing PSB communities because the method delivers phylogenetic as well as physiological information. In addition, signature amino acids and nucleotides were revealed for different PSB groups, which make *pufLM* genes a helpful tool in polyphasic taxonomy (Chapter IV) and in classification of uncultured clone sequences (Chapter II and III).

After verification and establishment of a reliable PSB *pufLM* gene database, the newly developed primer pair was successfully employed on natural phototrophic purple bacteria communities. It was possible to follow changes in community composition in experimental set-ups by fingerprinting methods as well as by PCR-based cloning experiments with the new primers. The primers amplified *pufLM* genes of PSB as well as other phototrophic purple bacteria and Chloroflexaceae (data not shown). The primer system was shown to detect both known and unknown species, genera, and even unidentified groups. This supports its suitability as a universal primer pair for detecting *pufLM*-containing bacteria (Chapter II and III). The high diversity of obtained clone sequences necessitated the inclusion of *pufLM* genes from all phototrophic purple bacterial groups into the phylogenetic analysis. The inferred phylogenetic trees showed a clear separation of the phototrophic purple Alpha-, Beta-, and

Gammaproteobacteria, which is antithetic to all *pufLM* based phylogenetic studies published before (Zeng & Jiao 2007, Ranchou-Peyruse *et al.* 2006, Beja *et al.* 2002, Nagashima *et al.* 1997). This congruency with ribosomal gene-based phylogeny indicates a suitability of *pufLM* as a phylogenetic marker for purple nonsulfur bacteria, aerobic anoxygenic phototrophic bacteria and Chloroflexaceae as well. The incongruence between ribosomal and *pufLM* gene based phylogenies obtained in other studies has been explained by the possibility of horizontal gene transfer of genes involved in anoxygenic photosynthesis (Zeng & Jiao 2007, Ranchou-Peyruse *et al.* 2006, Beja *et al.* 2002, Nagashima *et al.* 1997). However, the studies in this thesis indicate congruency and co-evolution between 16S rRNA and *pufLM* gene based phylogenies, allowing the assignment of uncultured phototrophic bacteria to phylogenetic groups and subphyla based on *pufLM* genes. This new knowledge opposing the results obtained in previous studies is most likely owed to the higher amount of phylogenetic information used in this thesis, i.e., sequence length as well as the dataset available.

PUFLM GENES USED IN PSB COMMUNITY ANALYSES

The modified primer pair was successfully applied to environmental communities of phototrophic purple bacteria from samples of a Baltic Sea coastal lagoon and a Chilean salt lake in the Atacama Desert. These two habitats displayed fundamental differences in abiotic conditions shaping highly specific and different phototrophic bacterial communities as determined by *pufLM* clone libraries. Moreover, the studies showed highly variable anoxygenic phototrophic purple bacteria communtities in Salar de Atacama indicating variable microhabitats within the lakes that shape unique and different anoxygenic phototrophic purple bacteria communities as already described for beach sulphureta (Suckow 1966). The sample derived from a Baltic Sea coastal lagoon represents a brackish habitat with moderate temperatures and was inhabited by a highly diverse PSB community of purely Chromatiaceae species, co-existing with purple nonsulfur bacteria and aerobic anoxygenic phototrophic bacteria of the Alpha- and Gammaproteobacteria (Chapter II). The majority of PSB (15/20) were successfully assigned to known genera based on their *pufLM* sequences; while only a minor fraction of the purple nonsulfur bacteria and aerobic anoxygenic phototrophic bacteria (3/19) were classifiable on the genus level. In contrast, Salar de Atacama typifies a slightly alkaline hypersaline environment at 2300 m altitude. It harbors a diverse set of so far unidentified phototrophic bacteria as well as some typical moderately to extremely halophilic PSB of the Chromatiaceae and Ectothiorhodospiraceae, and aerobic anoxygenic phototrophic Gammaproteobacteria. A further difference to the Baltic Sea coastal lagoon is the almost complete lack of alphaproteobacterial purple bacteria in Salar de Atacama. Both habitats favor

the growth of *Halochromatium* and *Thiohalocapsa* but differ with respect to the represented species. Representatives related to the moderately halophilic and motile *Halochromatium salexigens* inhabit the salt lake while members of a novel species related to the marine, gas vesicles containing *Halochromatium roseum* were found in the Baltic Sea coastal lagoon. PSB blooms have been frequently observed in different coastal environments of the "saltmarsh" type, including the Danish and German Baltic Sea coast (Trüper 1970). In general, salt-dependent and salt-tolerant species described from brackish and marine environments dominated the Baltic Sea anoxygenic phototrophic bacteria community studied in this thesis (Chapter II, Ranchou-Peyruse *et al.* 2006, Martínez-Alonso *et al.* 2005, Imhoff 2001, Overmann *et al.* 1992, Trüper 1970). The Chromatiaceae and Ectothiorhodospiraceae detected in the Salar de Atacama resemble typical moderately and extremely halophilic PSB inhabiting hypersaline environments as e.g., marine salterns as well as salt and soda lakes (Gorlenko *et al.* 2009, Imhoff 2001, Caumette 1993, Raymond & Sistrom 1969).

The quality of light is known to influence the growth and selection of different PSB species (Overmann & Garcia-Pichel 2006, Imhoff 2006). In sediments not permanently covered with water, which is a strong absorbing medium of infrared light, the growth of phototrophic bacteria containing BChl *b* is capable due to the absorption maximum in the far infrared (Imhoff 2006). BChl *b* containing Chromatiaceae were detected in the shallow hypersaline lakes in the Salar de Atacama. On the other hand, bacteria containing okenone as carotenoids were only rarely detected in both environments studied. Okenone displays an absorption maximum in the blue-green light-spectrum and is known to be beneficial in deeper pelagic layers but not in sediments (Imhoff 2006).

Just as light-quality influences the growth and selection of specific PSB species, temperature and salinity conditions control the composition of a bacterial community (Overmann & Garcia-Pichel 2006, Imhoff 2006, Imhoff 2001). The Chromatiaceae and Ectothirhodospiraceae identified in the hypersaline habitats of the Chilean salt lake are known as moderately and extremely halophilic organisms, and a halophilic character can also be assumed for the unidentified species e.g., for the aerobic anoxygenic phototrophic Gammaproteobacteria (Chapter III). The PSB community inhabiting the Baltic Sea coastal lagoon was characterized by mesophilic, salt-tolerant and salt-dependent species, specifically well adapted to the changing conditions in salinity and temperature at their natural environment (Chapter II). The community changes were less distinct within presumed natural ranges. Only elevated temperatures of >40°C, which are not expected to occur frequently in their natural habitat, dramatically lowered the diversity and pivotally changed the community composition. Above these temperatures a *Marichromatium gracile* phylotype dominates the clone

libraries, which was not observed at any other conditions or the original environmental sample. Based on isolation studies, *Marichromatium* species are regarded as common and typical members of marine coastal environments (Imhoff 2006, Imhoff *et al.* 1998b) and the *Marichromatium gracile* phylotype was also isolated from the original sample of this study. However, molecular data do not support a considerable abundance in the natural anoxygenic phototrophic bacteria community at *in-situ* temperatures. There is no freshwater influence at the natural habitat other than rain. This was reflected in the lack of typical freshwater PSB species. On the other hand, the community tolerated naturally occurring temporal freshwater conditions. Even species regarded as salt-dependent outlasted freshwater conditions for as long as three weeks, suggesting that the "salt dependency" of these species needs to be reconsidered.

The molecular functional gene approach further quarried additional diversity of so far unidentified phototrophic bacteria. Just like metagenomic studies using small subunit ribosomal genes aroused the awareness of a huge uncultured diversity of bacteria, environmental *pufLM* cloning experiments disclosed a novel lineage of so far uncultured phototrophic bacteria in Salar de Atacama (Chapter III). Equally, the presence of uncultured new genera of PSB was demonstrated in both, the Baltic Sea coastal lagoon and the Chilean salt lake (Chapter II and III). Moreover, the number of *pufLM* sequences identical to described species was very low (n=3) indicating novel species even for phylotypes identified at genus level. An even higher diversity of possible novel purple nonsulfur bacteria and aerobic anoxygenic phototrophic bacteria genera was might be present in the Baltic Sea coastal lagoon community (Chapter II). Further, most of the new isolates from various habitats that were used in the suitability study of *pufLM* genes as phylogenetic marker (Chapter I) likely represent new species or genera, indicating that neither the uncultured nor the cultivable diversity of PSB is fully covered yet.

CONCLUSION

In this thesis, *pufLM* genes were demonstrated as suitable phylogenetic markers for specific analysis of purple sulfur bacteria (PSB) in natural phototrophic purple bacteria communities. Furthermore, a reliable reference database of purple sulfur bacterial *pufLM* gene sequences was created which serves as basis for phylogenetic classification of uncultured *pufLM* containing bacteria. Congruency between *pufLM* and 16S rRNA gene-based phylogenetic tree topologies was demonstrated. Thereby, it is possible to classify PSB at least to the genus level on the basis of *pufLM* gene sequences which is also helpful in polyphasic taxonomy. It is concluded that horizontal gene transfer of *pufLM* genes within the PSB is highly unlikely. A similar suitability of *pufLM* genes as phylogenetic markers for all phototrophic purple bacteria is indicated. Therefore, reconsideration of the question of horizontal gene transfer in phototrophic Proteobacteria is recommended. Future studies using high-throughput and next-generation sequencing techniques will deepen the understanding of the evolution of photosynthesis and possible horizontal gene transfer of photosynthesis genes.

The functional approach using *pufLM* genes on natural anoxygenic phototrophic bacteria communities disclosed a high diversity of both known and so far uncultured *pufLM*-containing phototrophic bacteria. The presence of a possibly novel lineage of phototrophic purple bacteria was demonstrated in the Chilean salt lake Salar de Atacama. Further, uncultured as well as undescribed species and genera of phototrophic Proteobacteria were shown by molecular and culture-based studies demonstrating that our knowledge of the diversity of phototrophic purple bacteria is far from being complete. Different salinity and temperatures selectively favour the growth of different phototrophic bacteria and thus different phototrophic purple bacteria communities were detected in brackish and hypersaline environments. In general, the Baltic Sea coastal lagoon inhabiting PSB community consisted of salt-tolerant and strictly salt-dependant mesophilic species and was shown to be well adapted to the dynamic conditions of varying salinities and temperatures.

Although research on phototrophic purple bacteria has been done for more than 100 years, there are still many open questions on the ecology of anoxygenic phototrophic purple bacteria in natural communities co-existing with different phototrophic bacterial groups. The specific detection and molecular methods of the functional gene approach offer new strategies and possibilities in ecological studies of mixed natural communities of anoxygenic phototrophic purple bacteria and might be of great value in future analyses.

SUMMARY

In this thesis *pufLM* genes were tested for their suitability as phylogenetic markers for purple sulfur bacteria (PSB) and applied in environmental community studies as well as in polyphasic taxonomy. *pufLM* genes encode for the light and medium subunit of the photosynthetic reaction center type II proteins exclusively present in the polyphyletic group of anoxygenic phototrophic purple bacteria and Chloroflexaceae. Congruency between 16S rRNA gene and *pufLM* phylogeny was obtained in this study for PSB which allows the identification of phototrophic purple bacteria based on their *pufLM* gene sequence exclusively. It is concluded that horizontal gene transfer of *pufLM* genes within the PSB is highly unlikely. Additional phylogenetic calculations including representatives of purple nonsulfur bacteria (PNSB) and aerobic anoxygenic phototrophic Proteobacteria (AAPB) showed a clear separation of the phototrophic purple Alpha-, Beta-, and Gammaproteobacteria, which is antithetic to all *pufLM* based phylogenetic studies published before and indicates a suitability of *pufLM* as a phylogenetic marker for PNSB, AAPB and Chloroflexaceae as well. In addition, signature amino acids and nucleotides were revealed for different PSB groups and proved to be a helpful tool in polyphasic taxonomy.

The modified primer pair was successfully applied to environmental communities of phototrophic purple bacteria from a Baltic Sea coastal lagoon and a Chilean salt lake. *pufLM* was further used to monitor changes in community composition at different salinity and temperature conditions via RFLP and cloning experiments. The Baltic Sea coastal lagoon inhabited a highly diverse PSB community of mesophilic salt-tolerant and salt-dependent Chromatiaceae species, co-existing with PNSB and alpha- and gammaproteobacterial AAPB. They were demonstrated to be specifically well adapted to the naturally occurring salinity and temperature variations. However, at elevated temperatures (>40°C) PSB diversity was distinctively lower and the community compostion completely changed. The Chilean Salar de Atacama typifies a slightly alkaline hypersaline environment and was inhabited by a diverse set of so far unidentified phototrophic bacteria, some typical moderately to extremely halophilic PSB of the Chromatiaceae and Ectothiorhodospiraceae and gammaproteobacterial AAPB.

The molecular functional gene approach quarried a high diversity of so far unidentified bacteria. Equally, the presence of cultured and uncultured possible new genera and species of PSB was demonstrated indicating that neither the uncultured nor the cultivable diversity of PSB is fully covered, yet.

ZUSAMMENFASSUNG

In der vorliegenden Arbeit wurden die *pufLM* Gene auf ihre Eignung als phylogenetische Marker für Schwefelpurpurbakterien (PSB) untersucht und zur Analyse natürlicher Bakteriengemeinschaften sowie in der polyphasichen Taxonomie angewendet. *pufLM* Gene kodieren für die leichte und mittlere Untereinheit des photosynthetischen Reaktionszentrums Typ II und kommen ausschließlich in der polyphyletischen Gruppe der anoxygenen phototrophen Purpurbakterien und Chloroflexaceae vor. Für die PSB konnte eine hohe Übereinstimmung der Phylogenien ihrer 16S rRNA und *pufLM* Gene gezeigt werden, was eine Identifizierung von PSB anhand ihrer *pufLM* Gene erlaubt. Horizontaler Gentransfer der *pufLM* Gene innerhalb der PSB wird als sehr unwahrscheinlich erachtet. Mittels zusätzlicher phylogenetischer Berechnungen mit Vertretern von Nichtschwefelpurpurbakterien (PNSB) und aeroben anoxygenen phototrophen Proteobakterien (AAPB) konnte eine eindeutige Trennung in phototrophe Alpha-, Beta-, and Gammaproteobakterien gezeigt werden. Dieses steht allerdings im Gegensatz zu allen bisherigen *pufLM* basierten phylogenetischen Analysen, deutet aber auf eine Eignung von *pufLM* als phylogenetischer Marker auch für PNSB, AAPB und Chloroflexaceae hin. Des weiteren konnten für die verschiedenen PSB-Gruppen Aminosäure- und Nukleotidsequenz Signaturen aufgezeigt werden, die nutzbringend in der polyphasischen Taxonomie eingesetzt werden konnten und können.

Das modifizierte *pufLM* Primer Paar wurde erfolgreich auf natürliche Gemeinschaften phototropher Purpurbakterien einer Küstenlagune der Ostsee und eines chilenischen Salzees angewendet. Darüberhinaus wurden mittels *pufLM* durch RFLP und Klonierungsexperimente die Veränderugen in der Gemeinschaftszusammensetzung bei unterschiedlichen Salzgehalten und Temperaturen verfolgt. Die Ostsee Lagune beherbergt eine sehr vielfältige PSB-Gemeinschaft mesophiler, salztoleranter und salzabhängige Chromatiaceae, die mit PNSB und AAPB der Alpha- und Gammaproteobakterien coexistieren. Es konnte gezeigt werden, daß sie besonders gut and die natürlich vorkommenden Salz- und Temperaturschwankungen angepaßt sind. Bei Temperaturen jenseits der 40°C ist die PSB Diversität deutlich geringer und die Gemeinschaftszusammensetzung ändert sich grundlegend. Der Salar de Atacama in Chile repräsentiert ein leicht alkalisches hypersalines Habitat, daß typisch moderat bis extrem halophile PSB der Chromatiaceae und Ectothiorhodospiraceae, gammaproteobakterielle AAPB und eine große neue Gruppe unidentifizierter phototropher Bakterien aufweist.

Die Nutzung der funktionellen Gene *pufLM* als Zielgene förderte eine hohe Diversität von bis jetzt unidentifizierten Bakterien zu Tage. Die Präsens von kultivierten und unkultivierten möglichen neuen Gattungen und Arten deutet auf eine noch nicht erschöpfend entdeckte Diversität von PSB hin.

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INDIVIDUAL SCIENTIFIC CONTRIBUTION TO MULTIPLE-AUTHOR PUBLICATIONS

The chapters of this thesis are published (Chapter I, III and IV) or submitted (Chapter II) papers of multiple authorships. This list serves as a clarification of personal contributions on each publication.

Chapter I:
Phylogenetic Relationship of phototrophic purple sulfur bacteria according to *pufL* and *pufM* genes
Marcus Tank, Vera Thiel and Johannes F. Imhoff
International Microbiology (Vol. 12 (3): 175-185)

Isolation and culture of the strains, modification of primers, amplification and phylogenetic analysis of the *pufLM* genes was done by Marcus Tank under supervision of Johannes F. Imhoff. Phylogenetic analysis of the 16S rRNA genes was done by Vera Thiel. Evaluation of data and preparation of the manuscript was done by Marcus Tank. All co-authors contributed to the manuscript by critical revision.

Chapter II:
Impact of temperature and salinity changes on purple sulfur bacteria communities from a coastal lagoon of the Baltic Sea analyzed by pufLM gene libraries
Marcus Tank, Johannes F. Imhoff
submitted as research paper for Applied and Environmental Microbiology (Status: in review)

Sampling, experimental work as well as evaluation of the data, phylogenetic analysis and preparation of the manuscript was done by Marcus Tank under the supervision of Johannes F. Imhoff. All co-authors contributed by critical revision of the manuscript.

Chapter III:

Unique communities of anoxygenic phototrophic bacteria in saline lakes of Salar de Atacama (Chile). Evidence for a new phylogenetic lineage of phototrophic Gammaprotoebacteria from pufLM gene analyzes

Vera Thiel, Marcus Tank, Sven C. Neulinger, Linda Gehrmann,
Cristina Dorador and Johannes F. Imhoff

FEMS Microbiology Ecology (Vol.74 (3):510–522 (December 2010))

Sampling was done by Andrea Gärtner, Cristina Dorador, and Johannes F. Imhoff. Experimental work was done by Linda Gehrmann under the supervision of Vera Thiel and Marcus Tank. Phylogenetic analysis was conducted by Marcus Tank. Evaluation of data and preparation of the manuscript was done by Vera Thiel in close collaboration with Marcus Tank. All co-authors contributed by critical revision of the manuscript.

Chapter IV:

A new species of *Thiohalocapsa*, *Thiohalocapsa marina* sp. nov., from an Indian marine aquaculture pond

P. Anil Kumar, T. N. R. Srinivas, V. Thiel, M. Tank, Ch. Sasikala,
Ch. V. Ramana and J. F. Imhoff

International Journal of Systematic and Evolutionary Microbiology (Vol. 59: 2333–2338)

Sampling was done by P. Anil Kumar and T. N. R. Srinivas. Isolation and culture experiments were done by P. Anil Kumar and T. N. R. Srinivas under the supervision of Ch. Sasikala and Ch. V. Ramana. Molecular studies and phylogenetic analysis was done by Vera Thiel (16S rRNA) and Marcus Tank (*pufLM*). Evaluation of the molecular data was done by Marcus Tank and Vera Thiel. Preparation of the manuscript was done by P. Anil Kumar in collaboration with Vera Thiel and Marcus Tank. All co-authors contributed by critical revision of the manuscript.

LIST OF CONFERENCE CONTRIBUTIONS

Part of this thesis has been presented as poster or oral presentation at following symposia:

- **Marcus Tank, Vera Thiel and Johannes F. Imhoff**: *Phylogenetic Relationship of Purple Sulfur Bacteria according to pufL and pufM genes*. Poster presentation at the annual meeting of the 'Vereinigung für Allgemeine und Angewandte Mikrobiologie, VAAM', March 2009 in Bochum, Germany
- **Marcus Tank, Vera Thiel and Johannes F. Imhoff**: *Suitability of pufL and pufM Genes as Phylogenetic Markers for Purple Sulfur Bacteria*. Oral presentation at the13th International Symposium on Phototrophic Prokaryotes, ISPP, August 2009 in Montreal, Canada
- **Vera Thiel, Marcus Tank, Cristina Dorador and Johannes F. Imhoff**: *Anoxygenic phototrophic bacteria of Salar de Atacama, Chile: A functional gene approach*. Poster presentation at the 13th International Symposium on Phototrophic Prokaryotes, ISPP, August 2009 in Montreal, Canada
- **Marcus Tank, Martina Blümel and Johannes F. Imhoff:** *Influence of salinity and temperature on a Purple Sulfur Bacteria Community using pufLM gene analysis*. Oral presentation at the annual meeting of the 'Vereinigung für Allgemeine und Angewandte Mikrobiologie, VAAM', March 2010 in Hannover, Germany
- **Vera Thiel, Marcus Tank, Sven C. Neulinger, Linda Gehrmann, Cristina Dorador and Johannes F. Imhoff:** *Anoxygenic phototrophic bacterial communities of Salar de Atacama, Chile: a functional gene approach using fmoA and pufLM genes*. Poster presentation at the annual meeting of the 'Vereinigung für Allgemeine und Angewandte Mikrobiologie, VAAM', March 2010 in Hannover, Germany

ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt habe und, dass sie nach Form und Inhalt meine eigene Arbeit ist. Sie wurde keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt und entstand unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft. Dies ist mein einziges und bisher erstes Promotionsverfahren. Des Weiteren erkläre ich, dass ich Zuhörer bei der Disputation zulasse.

____

(Marcus Tank)

APPENDIX – CHAPTER I

Supplementary Figure I-1: Alignment of deduced PufL amino acid sequences of purple sulfur bacteria of the defined groups

```
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```

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PAHMLGITFFFTTCLALAMHGSLILSVTNPAKGEPVKTGEHENTFFRDLIGYSVGALGIH
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>Thiocapsa marina DSM5653T
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~XATLFFAILGTLLIVWGATLGPN~
~~PELQTFNIWQINIAPPDLSYG~LGMAPLDQGGLWQFITVCAIGAFVSWALREVEIARK
LGIGFHVPFAFAFAIGAYITLVVIRPILMGAWGHGFPYGILSHLDWVSNVGYQYLHFHYN
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>Thiocapsa sp. MTCH3IM012
~~~~~~XLGTLLIVWGATLGPT~
~~PELQTYNIWQISIAPPDLSYG~LGMAPLDQGGLWQFITICAIGAFVSWALREVEIARK
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PAHMLAITFFFTTCLALAMHGSLILSVTNPKKGEPVKTGEHENTFFRDLIGYSIGALGIH
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>Thiocapsa pendens DSM236T
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PAHMLAITFFFTTCLALAMHGSLILSVTNPAKGEPVKTGEHENTFFRDLIGYSIGALGIH
RLGLFLALSAVFWSAVCIVISGP~FWTRGWPEWWNWWLELPLW~~~~*
>Chromatiaceae bacterium MTPP2IF163
~~~~~~XLGTLLIVWGAALGPT~
~~~~~~~FNIWQINIAPPDLSYG~LGMAPLNEGGLWQIITICAIGAFVSWMLREVEICRK
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RLGLFLALAAVFWSAVCIVISGP~FWTRGWPEWWNWWLELPLW~~~~*
>Thiolamprovum pedioforme DSM3802T
~~~~~~~~~~~~~~~~~~~~XLFDFWVGPFYVGFFGVSAFFFALLGTLLIVWGAALGPT~
~~~~~~~FNIWQINIAPPDLSYG~LGMAPLTEGGLWQIITFCAIGAFVSWALREVEICRK
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PAHMIAITFFFTTCLALAMHGSLILSVTNPAKGEPVGTGEHENTFFRDLVGYSIGALGIH
RLGLFLALAAVFWSAVCIVISGP~FWTRGWPEWWNWWLELPLW~~~~*
>Thiobaca sp. MTRDDF079
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~AFLFALLGTLLIVWGAALGPT~
~~~~~~~WNLWQISIAPPDLSYG~LGMAPLTEGGLWQIITICAIGAFVSWALREVEICRK
LGIGFHVPFAFSFAIGAYLVLVFFRPLLMGAWGHGFPYGILSHLDWVSNTGYQFLHFHYN
PGHMLGISFFFINALALSMHGSLILSVVNPQKGEPVKTGEHENTYFRDIVGYSIGALAIH
RLGLFLALSAVFWSAVCMILSGP~FWTRGFPEWWNWWPQLPIW~~~~*
>Halochromatium sp. MTK2IM023
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~IFFATLGTLLIIWGAAIGPT~
~~~~~~~WNPWEINIAPPDLSYG~LNMAPLTEGGLWQFITICAIGAFVSWALRQVEIARK
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PAHMLAITFFFTNALALALHGSLILSMTNPQKGEPVKTGEHENTFFRDFVGYSIGALGIH
~~~~~~~

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>Halochromatium sp. MTK5IM027
~~~~~~XGTLLIIWGAAIGPT~
~~~~~~~WNPWEINIAPPDLSYG~LNMAPLTEGGLWQFITICAIGAFVSWALRQVEIARK
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PAHMLAITFFFTNALALALHGSLILSMTNPQKGEPVKTGEHENTFFRDFVGYSIGALGIH
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>Halochromatium sp. MTK1IM127
~~~~~~XGTLLIIWGAAIGPT~
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>Halochromatium sp. MTK8IM030
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PAHMLAITFFFTNALALALHGSLILSMTNPQKGEPVKTGEHENTFFRDFVGYSIGALGIH
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>Halochromatium sp. MTK6IM088
~~~~~~LGTLLIIWGAAIGPT~
~~~~~~~WNPWEINIAPPDLSYG~LNMAPLTEGGLWQFITICAIGAFVSWALRQVEIARK
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>Halochromatium roseum DSM18859T
~~~~~~LGTLLIIWGAAMGPT~
~~~~~~~WNPWEINIAPPDLSYG~LGMAPLMEGGLWQFITICAIGAFVSWALRQAEIARK
LGMGLHVPIAFGFAILAYVTLVVIRPVLLGAWGHGFPYGIFSHLDWVSNVGYQYLHFHYN
PAHMLAITFFFTNALALALHGSLILSMTNPQKGEPVKTGEHENTFFRDLVGYSIGALGIH
RLGLFLAINAAFWSAVCIVLSGP~FWTRGWPEWWNWWLELPFWS~~~*
>Halochromatium sp. MTK2IM039
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~XLTIFFATLGTLLIIWGAAMGPT~
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LGMGLHVPIAFGFAILAYVTLVVIRPVLLGAWGHGFPYGIFSHLDWVSNVGYQYLHFHYN
PAHMLAITFFFTNALALALHGSLILSMTNPQKGEPVKTGEHENTFFRDLVGYSIGALGIH
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>Halochromatium salexigens DSM4395T
~~~~~~ATLGTLLIIWGAAMGPT~
~~~~~~~WNPWQISIAPPDLSYG~LNMAPLTEGGLWQFITFCAIGAFVSWALRQAEIARK
LGMGLHVPIAFGFAILAYVTLVVIRPVLLGAWGHGFPYGIFSHLDWVSNVGYQYLHFHYN
PAHMLAITFFFTNALALALHGSLILSMTNPAKGEPVKTGEHENTFFRDLVGYSIGALGIH
RLGLFLAINAAFWSAVCIVISGP~FWTRGWPEWWNWWLELPFWS~~~*
>Chromatiaceae bacterium ML1
MAMLSFEKKYRVRGGTLVGGDLFDFWVGPFYVGFFGVTSVFFATLGTLLIIWGAVIGPT~
~~~~~~~QNIWRINIAPPDLSYG~LMMAPLTEGGLWQIIITICAIGAFVSWALRQVEISRK
LGIGLHVPFAFAFAILAYVTLVVIRPVLMGAWGHGFPYGIFSHLDWVSNVGYQYLHFHYN
PAHMLAITFFFTNALALALHGSLILSMVNPQEGEPVKTAEHENTFFRDLVGYSIGALAIH
RLGLFLAINAAFWSAVCIVLSGP~FWTKGWPEWWNWWLQLPFWS~~~*
>Thiohalocapsa halophila DSM6210T
~~~~~~LGTLLIIWGAAIGPT~
~~~~~~~WNVWRINIAPPDLSYG~LMMAPLTEGGLWQIIITICAIGAFVSWALRQVEISRK
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PAHMLAITFFFTNALALALHGSLILSMVNPREGEPVKTSEHENTFFRDFIGYSIGALAIH
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>Thiohalocapsa marina JA142T
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~FFGVTTVFFATLGTLLIIWGAAIGPT~
~~~~~~~WNIWRINIAPPDLSYG~LGMAPLTEGGLWQIIITICAIGAFVSWALRQVEISRK
LGMGLHVPFAFSFAILAYVTLVVIRPVLLGAWGHGFPYGIFSHLDWVSNVGYQYLHFHYN
PAHMLAITFFFTNALALGLHGSLILSVANPQKGEPVKTGEHENTFFRDLVGYSIGALGIH
RLGLFLAINAAFWSAVCIVLSGP~FWTRGWPEWWNWWLELPLW~~~~*
>Thiorhodovibrio winogradskyi DSM6702T
~~~~~~XLGTLLIILGAALGPT~
~~~~~~~WNIWQISIAPPDLDVG~LGVAPLKEGGLWQFITICAIGAFVSWALRQVEIARK
LGMGLHVPFAFAFAILAYITLVVIRPVLMGAWGHGFPYGIFSHLDWVSNVGYQTLHFHYN
PAHMLAISFFFTNCLALAMHGSLILSVTNPRDGEAVKTDEHENTVFRDIVGYSIGALGIH
RLGVFLAINAAFWSAVCILLTGP~FWTRGWPEWWMWWPNLPIW~~~~*
~~~~~~~

>Thiococcus pfennigii DSM226T
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~XLFXLTGIFLIITGAIYSWP~
~~EVGPAPNIFQINIAPPDISYGLLQIPPFGEGLVWQLITICAAGAFISWALREVEIARK
LGIGYHVPIAFSFAIFAYLAATVIRPILLGSWGHGLTYGILGHLDWTNNIGFQQLNYHYH
PGHMLAISFFFASAFALSLHGGIILSVTNPKKGEVVKTAEHENTFFRDVLGYSVGALGIH
RLGLFLALSAGFWCAVLDFTTGT~FWTRPWPEWWTWWLKFPWWS~~~*
>Thiococcus pfennigii DSM227
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~XLFILTGIFLIITGAIYSWP~
~~EVGPAPNIFQINIAPPDISYGLLQIPPFGEGLVWQLITICAAGAFISWALREVEIARK
LGIGYHVPIAFSFAIFAYLAATVIRPILLGSWGHGLTYGILGHLDWTNNIGFQQLNYHYH
PGHMLAISFFFASAFALSLHGGIILSVTNPKKGEVVKTAEHENTFFRDVLGYSVGALGIH
RLGLFLALSAGFWCAVLDFTTGT~FWTRPWPEWWTWWLKFPWWS~~~*
>Thioflavicoccus mobilis ATCC700959T
~~~~~~XLTGIFLITIGAVYSWP~
~~EVGPAPNIFQINIAPPDISYGLLQIPPFGEGLVWQLITICAAGAFISWALREVEIARK
LGIGYHIPIAFSFAIFAYLAATVIRPILLGSWGHGLTYGILGHLDWTNNIGFQQLNYHYH
PGHMLAISFFFASAFALSLHGGIILSVTNPKKGEVVKTAEHENTFFRDVLGYSVGALGIH
RLGLFLALSAGFWCAVLDFTTGT~FWTRPWPEWWTWWLKFPWWS~~~*
>Thiococcus pfennigii DSM228
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~XYDXLFVLTGIFLIVVGAVYSWP~
~~EVGPAPNIFQINIAPPDISYGLLQVPPFGEGLVWQLITICAAGAFISWALREVEIARK
LGIGYHIPIAFSFAIFAYLAATVIRPILLGSWGHGLTYGILGHLDWTNNIGFQQLNYHYH
PGHMLAISFFFASAFALSLHGGIILSVTNPKKGEVVKTAEHENTFFRDVLGYSIGALGIH
RLGLFLALTAGFWCAVLDFTTGT~FWTRPWPEWWTWWLKFPWWS~~~*
>Ectothiorhodospira haloalkaliphila ATCC51935T
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~VLTAFFALLGTLLIVWGAAMGPT~
~~~~~~~WNIWQISINPPDLSYG~LGFAPLTEGGLWQMITICALGAFVSWALRQAEIARK
LGMGLHIPIAFSVAVFAYFTLVVIRPVLLGAWGHGFPYGIMSHLDWVSNTGYQFLHFHYN
PAHMLAIAFFFTTALALSLHGGLILSATNPKKGEPVKTPEYEDTFFRDVVGYSIGALGIH
RLGLFLALSAAFWSAVCIVISGP~FWTQSWPEWWNWWLDLPIWA~~~*
>Ectothirohodospira vacuolata DSM2111
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~VLTAFFALLGTLLIVWGAAMGPT~
~~~~~~~WNIWQISINPPDLSYG~LGFAPLTEGGLWQMITICALGAFVSWALRQAEIARK
LGMGLHIPIAFSVAVFAYFTLVVIRPVLLGAWGHGFPYGIMSHLDWVSNTGYQFLHFHYN
PAHMLAIAFFFTTALALSLHGGLILSATNPKKGEPVKTPEYEDTFFRDVVGYSIGALGIH
RLGLFLALSAAFWSAVCIVISGP~FWTQSWPEWWNWWLDLPIWA~~~*
>Ectothiorhodospira shaposhnikovii ATCC31751
MSMLSFEKRYRVRGGTLIGGDLFDFWVGPFYVGFFGVLTAFFALLGTLLIVWGAAMGPT~
~~~~~~~WNIWQISINPPDLSYG~LGFAPLTEGGLWQMITICALGAFVSWALRQAEIARK
LGMGLHLPIAFSVAVFAYFTLVVIRPVLLGAWGHGFPYGIMSHLDWVSNTGYQFLHFHYN
PAHMLAIAFFFTTALALSLHGGLILSATNPKKGEPVKTPEYEDTFFRDVVGYSIGALGIH
RLGLFLALSAAFWSAVCIVISGP~FWTQSWPEWWNWWLDLPIWA~~~*
>Ectothiorhodospira variabilis WN22T
~~~~~~LGTLLIVWGAAMGPT~
~~~~~~~WNIWQINIAPPDLSYG~LGLAPLAEGGLWQFITICAIGAFVSWALRQVEICNK
LGMGYHVPIAFGVAIFAYVALVVIRPVLLGAWGHGFPYGIMSHLDWVSNVGYQFLHFHYN
PAHMLAITFFFTTALALSLHGGLILSVTNPKKGEAVKTPEYEDTFFRDTVGYSIGALGVH
RLGLFLALNAAFWSAVCIILSGP~FWTRSWPEWWNWWLNLPIWA~~~*
>Ectothiorhodospira imhoffii JA319
~~~~~~XLGTLLIVWGAAMGPT~
~~~~~~~WNIWQISINPPDLSYG~LGLAPLDEGGLWQFITMCAIGAFVSWGLRQVEICNK
LGMGYHVPIAFGVAVFAYVTLVVFRPILLGAWGHGFPYGIMSHLDWVSNVGYQFLHFHYN
PAHMLAITFFFTTALALSLHGGLILSVTNPKKGQAVKTPEYEDTFFRDTIGYSIGALGIH
RLGLFLALNAAFWSAVCIIISGP~FWTRSWPEWWNWWLDLPIWA~~~*
>Ectothiorhodospira mobilis DSM237
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~XGFTAVFALLGTLLIVWGAAMGPT~
~~~~~~~WNIWQISINPPDLSYG~LGLAPLDEGGLWQFITMCAIGAFVSWGLRQVEICNK
LGMGYHVPIAFGVAVFAYVTLVVFRPILLGAWGHGFPYGIMSHLDWVSNVGYQFLHFHYN
PAHMLAITFFFTTALALSLHGGLILSVTNPKKGQAVKTPEYEDTFFRDTIGYSIGALGIH
RLGLFLALNAAFWSAVCIIISGP~FWTRSWPEWWNWWLDLPIWA~~~*
>Halorhodospira halophila DSM244T
MAMLDFEKKYRVRGGTLLGGDLFDFWIGPFYVGIFGVLTAIFAVLGTVLIIYGASQD~T~
~~~~~~~FNLWQISIAPPDLSYG~LALAPMMEGGLWQIITVCALGAFIAWALRQAEISKK
LGMGYHVPVAFAVAILAYATLVVFRPLLMGAWGHGFPYGILSHLDWVSNVGYQYLHFHYN
PAHMLAVTFFFTTTLALALHGGLILSVTNPKKGESVKTAEHENTFFRDVVGYSIGSLGIH
RLGLFLALNAAFWSAVCIIISGP~FWTRGWPEWWNWWLNVPIWSWGG*
>Halorhodospira halophila H

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~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~XLGTVLIIYGASQD~T~
~~~~~~~FNLWQISIAPPDLSYG~LALAPMMEGGLWQIITVCALGAFITWALRQAEISKK
LGMGYHVPVAFAVAILAYATLVVFRPLLMGAWGHGFPYGILSHLDWVSNVGYQYLHFHYN
PAHMLAVTFFFTTTLALALHGGLILSVTNPKKGEPVKTAEHENTFFRDVIGYSIGSLGIH
RLGLFLALNAGFWSAVCIIISGP~FWTRGWPEWWNWWLNVPIWSWGG*
>Blastochloris viridis ATCC19567T
MALLSFERKYRVRGGTLIGGDLFDFWVGPYFVGFFGVSAIFFIFLGVSLIGYAASQGPT~
~~~~~~~WDPFAISINPPDLKYG~LGAAPLLEGGFWQAITVCALGAFISWMLREVEISRK
LGIGWHVPLAFCVPIFMFCVLQVFRPLLLGSWGHAFPYGILSHLDWVNNFGYQYLNWHYN
PGHMSSVSFLFVNAMALGLHGGLILSVANPGDGDKVKTAEHENQYFRDVVGYSIGALSIH
RLGLFLASNIFLTGAFGTIASGP~FWTRGWPEWWGWWLDIPFWS~~~*
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Supplementary Figure I-2: Alignment of deduced PufM amino acid sequences of purple sulfur bacteria of the defined groups

>Allochromatium vinosum DSM180T
MPEYQNIFTTVQVRAPAYPGVPLPKGSLPRIGK~~~PIFSYWAGKIGDAQIGPIYLGFTG
TLSIIFGFMAIFIIGFNMLASVDWNIIQFVKHFFWLGLEPPAPQYGLTI~PPLSEGGWWL
MAGFFLTMSILLWWVRTYKRAEALGMSQHLSWAFAAAIFFYLSLGFIRPVMMGSWAEAVP
FGIFPHLDWTAAFSIRYGNLYYNPFHMLSIAFLYGSALLFAMHGATILAVSRFGGDREID
QITDRGTAAERAA~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>Allochromatium sp. MT086CH3IM
MPEYQNIFTTVQVRAPAYPGXPLPKGSLPRIGK~~~PIFSYWAGKIGDAQIGPIYLGFTG
TLSIIFGFMAIFIIGFNMLASVDWNIIQFVKHFFWLGLEPPAPQYGLTI~PPLSEGGWWL
MAGFFLTMSILLWWVRTYKRAEALGMSQHLSWAFAAAIFFYLSLGFIRPVMMGSWAEAVP
FGIFPHLDWTAAFSIRYGNLYYNPFHMLSIAFLYGSALLFAMHGATILAVSRFGGDREID
QITDRGTAAERAAX~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>Allochromatium sp. MT013CH3IM
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TLSIIFGFMAIFIIGFNMLASVDWNIIQFVKHFFWLGLEPPAPQYGLTI~PPLSEGGWWL
MAGFFLTMSILLWWVRTYKRAEALGMSQHLSWAFAAAIFFYLSLGFIRPVMMGSWAEAVP
FGIFPHLDWTAAFSIRYGNLYYNPFHMLSIAFLYGSALLFAMHGATILAVSRFGGDREID
QIX~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>Allochromatium warmingii DSM173T
MPEYQNIFTTVQVRSPAYPGVPLPKGSLPRIGK~~~PIFSYWVGKIGDAQIGPIYLGFTG
TLSIIFGFFALFIIGFNMLASVDWNIIQFVKHFFWLALEPPAPQYGLSI~PPLSEGGWWL
MAGGFLTASIMLWWVRTYKRAEALGMSQHLSWAFAAAIFFYLSLGFIRPVMMGSWAEAVP
FGIFPHLDWTAAFSIRYGNLYYNPFHMLSIAFLYGSALLFAMHGATILAVSRFGGDREID
QITDR~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>Allochromatium sp. MT6010
MPEYQNIFTAVQVRSPAYPGVPLLKGSLPRIGK~~~PIFSYWVGKIGDAQIGPIYLGFTG
TLSIIFGFFALFIIGFNMLASVDWSIIQFVKNFFWLGLEPPAPQYGLSI~PPLSEGGWWL
MAGFFLTMSILLWWVRTYKRAEALGMSKHLSWAFAAAIFFYLSLGFIRPVMMGSWAEAVP
FGIFPHLDWTAAFSIRYGNLYYNPFHSLSIAFLYGSALLFAMHGATILAVSRFGGDREX~
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~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>Thermochromatium tepidum DSM3771T
MPEYQNIFTAVQVRAPAYPGVPLPKGNLPRIGR~~~PIFSYWLGKIGDAQIGPIYLGLTG
TLSIFFGLVAISIIGFNMLASVHWDVFQFLKHFFWLGLEPPPPQYGLRI~PPLSEGGWWL
IAGLFLTLSILLLWVRTYKRAEALGMSQHLSWAFAAAIFFYLVLGFIRPVMMGSWAKAVP
FGIFPHLDWTAAFSIRYGNLYYNPFHMLSIAFLYGSALLFAMHGATILSVSRFGGDREID
QITHRGTAAEGAALFWRWTMGFNATMESIHRWAWWCAVLTVITAGIGILLSG~TVVDNWY
LWAVKHGMAPAYPEVVTAVNPYETAAEVMQ*
>Thiorhodococcus mannitoliphagus ATCC BAA-1228T
MAEYQNIFNSVQVRSPAYPGVPLPKGHLPRIGN~~~PIFSYWLGKIGDAQIGPIYLGFTG
TLSLIFGFAALFIIGFNMLASVDWSVIQFLKHFFWLALEPPAPQYGLTI~PPLSEGGWWL
IAGGFLTASILLWWVRTYKRAEALGMSQHLSWAFAAAIFFYLSLGFIRPLMMGSWAEAVP
FGVFPHLDWTAAFSIRYGNLYYNPFHMLSIAFLYGSALLFAMHGATILAVSRFGGDREID
QITDX~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>Thiocystis violacea DSM208
MPEYQNIFNTVQVRSPAYPGVPLPKGSLPRIGN~~~PIFSYWLGKIGDAQIGPIYLGFTG
TLSLIFGVAALFIMGFNMLASVDWSVIQFVKHFFWLALEPPAPQYGLSI~PPLSEGGWWL
IAGGFLTASILLWWVRTYKRAEALGMSQHLSWAFAAAIFFYLSLGFIRPIMMGSWAEAVP
FGVFPHLDWTAAFSIRYGNLYYNPFHMLSIAFLYGSALLFAMHGATILAVSRFGGDREID
QITDRGTAAERAX~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>Thiorhodococcus minor DSM11518T
MPEYQNIFNSVQVRSPAYPGVPLPKGHLPRIGN~~~PIFSYWLGKIGDAQVGPIYLGFTG
TLSLIFGFTAIFIIGFNMLASVDWSIIQFVKHFFWLGLEPPAPQYGLSI~PPLSEGGWWL
MAGFFLTMSILLWWVRTYKRAEALGMSQHLSWAFAAAIFFYLSLGFIRPIMMGSWAEAVP
FGIFPHLDWTAAFSIRYGNLYYNPFHMLSIAFLYGSALLFAMHGATILAVSRFGGDREID
QITDRX~~~~~~
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>Thiocystis violacea DSM207T
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MPEYQNIFNSVQVRSPAYPGVPLPRGSLPRIGK~~~PIFSYWLGKIGDAQIGPIYLGFTG
TLSLIFGFVALFIIGFNMLASVDWSVIQFVKHFFWLGLEPPAPQYGLSI~PPLSEGGWWL
MTGFFLTMSIILWWVRTYKRAEALGMSQHLSWAFAAAIFFYLSLGFIRPVMMGSWAEAVP
FGIFPHLDWTAAFSIRYGNLYYNPFHMLSIAFLYGSALLFAMHGATILAVSRFGGDREID
QITDRGTAAERAAX~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>Thiocystis gelatinosa DSM215T
MPEYQNIFNSVQVRSPAYPGVPLPKGSLPRIGK~~~PIFSYWLGKIGDAQIGPIYLGFAG
TLSLIFGFFAIEIIGFNMLASVDWSPIEFVRNLFWLALEPPAPQYGLSI~PPLSEGGWWL
MAGFFLTASILLWWVRTYKRAEALGMSQHLSWAFASAIFFYLCLGFIRPIMMGSWAEAVP
FGIFPHLDWTAAFSIRYGNLYYNPFHMVSIAFLYGSALLFAMHGATILAVSRYGGDREID
QIVDRGTAAERA~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>Thiocystis minor DSM178T
MPEYQNIFNSVQVRSPAYPGVPLPKGSLPRIGK~~~PMFSYWLGKIGDAQIGPIYLGFAG
TLSLIFGFFAIEIIGFNMLASVDWNPIEFVRNLFWLALEPPAPQYGLSI~PPLSEGGWWL
MAGFFLTASILLWWVRTYKRAEALGMSQHLSWAFASAIFFYLCLGFIRPVMMGSWAEAVP
FGIFPHLDWTAAFSIRYGNLYYNPFHMVSIAFLYGSALLFAMHGATILAVSRYGGDR~~~
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>Chromatium weissei DSM5161T
MPEYQNIFNSVQVRSPAYPGVPLPKGSLPRLGK~~~PLFSYWAGKIGDAQIGPLYLGFTG
TLSLIFGFIALEIIGLNMLASVDWSPIELVKNLFWLALEPPAPSYGLSI~PPLSEGGWWI
MAGVFLTASIILWWVRTYKRAAALGMSQHLSWAFAAAIFFYLSLGFIRPVMMGSWAEAVP
FGIFPHLDWTAAFSIRYGNLYYNPFHMLSIAFLYGSALLFAMHGATILAVSRFGGDREID
QILX~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>Chromatiaceae bacterium MTWDM004
MPEYQNIFNGVQVRSPAYPGVPLPKGHLPRIGK~~~PIFSYWLGKIGDAQVGPIYLGFTG
TLSVIFGFVAIFIIGFNMLATVDWSIIEFVKNFFWLALEPPAPQYGLSI~PPLSEGGWWL
MAGFFLTMSILLWWVRTYKRAEALGMSQHLSWAFAAAIFFYLSLGFIRPIMMGSWAEAVP
FGIFPHLDWTAAFSIRYGNLYYNPFHMLSIAFLYGSALLFAMHGATILSVSRFGGDREID
QVX~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>Thiorhodococcus drewsii DSM15006T
MPEYQNIFTSVQVRSPAYPGVPLPKGHLPRIGK~~~PIFSYWLGKIGDAQIGPLYLGFTG
TLSLIFGFVALFIIGFNMLASVDWNVIQFVKHFFWLALEPPAPQYGLSI~PPLSEGGWWL
MAGFFLTMAIILWWVRTYKRAEALGMNQHLSWAFASAIFFYLVLGFIRPVMMGSWAEAVP
FGIFPHLDWTAAFSIRYGNLYYNPFHMLSIAFLYGSALLFAMHGATILSVSRFGGDREID
QIVDRGX~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>Thiorhodococcus kakinadensis JA130T
MPEYQNIFTSVQVRSPAYPGVPLPKGHLPRIGK~~~PIFSYWLGKIGDAQFGPLYLGFTG
TLSLIFGFVALFIIGFNMLASVDWNIIQFVKHFFWLALEPPAPQYGLSI~PPLSEGGWWL
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FGIFPHLDWTTAFSLKYGNLYYNPFHMLSIAFLYGSALLFAMHGATVLAVGRYGGEREID
QITDRGTX~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>Ectothiorhodospira imhoffii JA319
MAEYQNIFTRVQVQAEPEAGVPIEGGKRERIGK~~~PTLVYWFGKIGSAQLGPIYLGWTG
LLSIVFGLTAILIIGFNFLAQVNYDVIQFIRQLFWLGLEPPQPEYGLGI~PPLQEGGWWL
MAGFFLTTSLLLWWVRMYRRARALELGTHVAWAFASAIFLFLCLGFFRPLLMGDWSEAVP
FGIFPHLDWTTAFSLKYGNLYYNPFHMLSIAFLYGSALLFAMHGATVLAVGRYGGEREIE
QIT~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>Ectothiorhodospira mobilis DSM237
MAEYQNIFNRVQVQAEPEAGVPIERGNRERIGK~~~PTLVYWFGKIGSAQLGPIYLGWTG
LLSIVFGLTAILIIGFNFLAQVNYDVIQFIRQLFWLGLEPPQPEYGLGI~PPLQEGGWWL
MAGFFLTTSLLLWWVRMYRRARALELGTHVAWAFASAIFLFLCLGFFRPVLMGDWSEAVP
FGIFPHLDWTTAFSLKYGNLYYNPFHMLSIAFLYGSALLFAMHGATVLAVGRYGGEREIE
QITDRX~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>Halorhodospira halophila DSM244T
MAEYQNIFTRVQVRGPTDPGVELPAADWPRTKG~~~STHSWLLGKIGDAQVGPIYLGTTG
VMSILFGLLSIVIIGMNMLASVDWSPIEWIRQFFWVALEPPPPEYGLSL~PPLNDGGWWL
IAGFTLTLSILLWWVRTYNRARALGLGTHVAWAFAAAIFLFLAIGFIWPVLMGSWAKSVP
FGIFPHLDWTTAFSLRYGNLYYNPFHMLSIVFLFGSALLFAMHGATILAAGRYNAEREID

QITDRGTAAERSALFWRWTMGFNATMESIHRWGYWFAILCVITGGIGILLTG~TVVENWY
LWGVHHGIAPEYPEFFTPAVDPAAGGTE*~~
>Halorhodospira halophila H
MAEYQNIFTRVQVRGPTDPGVELPAADWPRTKG~~~ATHSWLLGKIGDAQVGPIYLGTTG
VMSILFGIVSIVIIGMNMLASVDWSPLEFIRQFFWVALEPPPPEYGLSL~PPLNDGGWWL
IAGFTLTLSVLLWFARTYNRARALGLGTHVAWAFAAAIFLFLAIGFIWPVLMGSWAKSVP
FGIFPHLDWTTAFSLRYGNLYYNPFHMLSIVFLFGSALLFAMHGATILAAGRYNAEREIE
QITDRGX~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
>BltVir20
```

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MADYQTIYTQIQARGP~HITVSGEWGDNDRVGK~~~PFYSYWLGKIGDAQIGPIYLGASG
IAAFAFGSTAILIIILFNMAAEVHFDPLQFFRQFFWLGLYPPKAQYGMGI~PPLHDGGWWL
MAGLFMTLSLGSWWIRVYSRARALGLGTHIAWNFAAAIFFVLCIGCIHPTLVGSWSEGVP
FGIWPHIDWLTAFSIRYGNFYYCPWHGFSIGFAYGCGLLFAAHGATILAVARFGGDREIE
QITDRGTAVERAALFWRWTIGFNATIESVHRWGWFFSLMVMVSASVGILLTG~TFVDNWY
LWCVKHGAAPDYPAYLPATPDPASLPGAPK*
```

APPENDIX – CHAPTER II

Supplementary Table II-1: Accession numbers of reference *pufLM* nucleotide sequences used in phylogenetic analyses

strain name	strain number	accession number
AAPGPB*	HTCC2080	AAVV01000005
AAPGPB*	NOR5-3	NZ_DS999405
AAPGPB*	NOR51-B	NZ_DS999411
Allochromatium phaeobacterium	JA144^T	FM208081
Allochromatium sp.	MT6010	FN257155
Allochromatium sp.	MTCH3IM013	FN257181
Allochromatium sp.	MTCH3IM086	FN257187
Allochromatium vinosum	DSM180^T	AB011811
Allochromatium warmingii	DSM173^T	FN257132
Chloroflexus aggregans	DSM9485^T	AAUI01000013
Chloroflexus aurantiacus	J-10-fl^T	CP000909
Chromatiaceae bacterium	MTCANM188	FN984733
Chromatiaceae bacterium	MTHHDM183	FN984735
Chromatiaceae bacterium	MTCANM191	FN984734
Chromatiaceae bacterium	ML1	AY177752
Chromatiaceae bacterium	MTPP2IF163	FN257165
Chromatiaceae bacterium	MTWDM004	FN257179
Chromatium weissei	DSM5161^T	FN257133
Congregibacter litoralis	KT71^T	AAOA01000014
Dinoroseobacter shibae	DFL-12^T	CP000830
Ectothiorhodospira haloalkaliphila	ATCC51935	FN257156
Ectothiorhodospira imhoffii	JA319	AM944100
Ectothiorhodospira mobilis	DSM237	FN257158
Ectothiorhodospira shaposhnikovii	DSM2111	FN257157
Ectothiorhodospira shaposhnikovii	ATCC31751	AF018955
Ectothiorhodospira variabilis	WN22^T	FN257153
Erythrobacter sp.	JL475	DQ484037
Erythromicrobium ramosum	IAM14333^T	AB010873
Halochromatium roseum	DSM18859^T	FN257161
Halochromatium salexigens	DSM4395^T	FN257159
Halochromatium sp.	MTK2IM023	FN257169
Halochromatium sp.	MTK2IM039	FN257170
Halochromatium sp.	MTK5IM027	FN257171
Halochromatium sp.	MTK6IM088	FN257173
Halochromatium sp.	MTK8IM030	FN257174
Halochromatium sp.	MTK1IM127	FN257175
Halorhodospira halophila	H	FN257160
Halorhodospira halophila	SL1^T	CP000544
Jannaschia sp.	CCS1	CP000264
Labrenzia alexandrii	DFL-11^T	EQ973121
Loktanella fryxellensis	LMG22007^T	DQ915716
Loktanella vestfoldensis	SKA53^T	AAMS01000002
Marichromatium bheemlicum	JA124^T	AM944099
Marichromatium gracile	DSM203^T	FN257134
Marichromatium indicum	JA214	AM944098

Marichromatium purpuratum	DSM1591^{T}	FN257135
Rhodobacter sphaeroides	2.4.1^{T}	CP000143
Rhodobacter sphaeroides	ATCC 17029	CP000577
Rhodospirillaceae bacterium	MTSTEM197	FN984737
Rhodovulum euryhalinum	DSM4868^{T}	AF486825
Rhodovulum sp.	MTCH3IM048	FN984739
Roseospira mediosalina	BN280^{T}	FN984736
Roseospira sp.	MTKK6IM177	FN984738
Roseospira visakhapatnamensis	JA131^{T}	FM208080
Roseovarius tolerans	NBRC16695^{T}	DQ915720
Sulfitobacter guttiformis	LMG19755^{T}	DQ915723
Thalassiobium sp.	R2A62	NZ_GG697169
Thermochromatium tepidum	ATCC4361^{T}	D85518
Thiobaca sp.	MTRDDF079	FN257162
Thiocapsa marina	DSM5653^{T}	FN257140
Thiocapsa pendens	DSM236^{T}	FN257145
Thiocapsa rosea	DSM235^{T}	FN257147
Thiocapsa roseopersicina	DSM217^{T}	FN257146
Thiocapsa sp.	MTRDDF081	FN257163
Thiocapsa sp.	MTRDDF078	FN257164
Thiocapsa sp.	MTPP2IF162	FN257166
Thiocapsa sp.	MTWDM010	FN257176
Thiocapsa sp.	MTWDM061	FN257178
Thiocapsa sp.	MTV2IF083	FN257180
Thiocapsa sp.	MTCH3IM012	FN257182
Thiococcus pfennigii	DSM226^{T}	FN257141
Thiococcus pfennigii	DSM227	FN257142
Thiococcus pfennigii	DSM228	FN257143
Thiocystis gelatinosa	DSM215^{T}	FN257189
Thiocystis minor	DSM178^{T}	FN257150
Thiocystis violacea	DSM207^{T}	FN257148
Thiocystis violacea	DSM208	FN257149
Thioflavicoccus mobilis	ATCC700959^{T}	FN257144
Thiohalocapsa halophila	DSM6210^{T}	FN257151
Thiohalocapsa marina	JA142^{T}	FN257154
Thiolamprovum pedioforme	DSM3802^{T}	FN257152
Thiorhodococcus bheemlicus	JA132^{T}	FM208083
Thiorhodococcus drewsii	DSM15006^{T}	FN257137
Thiorhodococcus kakinadensis	JA130^{T}	AM944094
Thiorhodococcus mannitoliphagus	ATCCBAA-1228^{T}	FN257139
Thiorhodococcus minor	DSM11518^{T}	FN257138
Thiorhodococcus sp.	JA395	FN356026
Thiorhodovibrio winogradskyi	DSM6702^{T}	FN257136

* aerobic anoxygenic phototrophic Gammaproteobacteria

APPENDIX – CHAPTER III

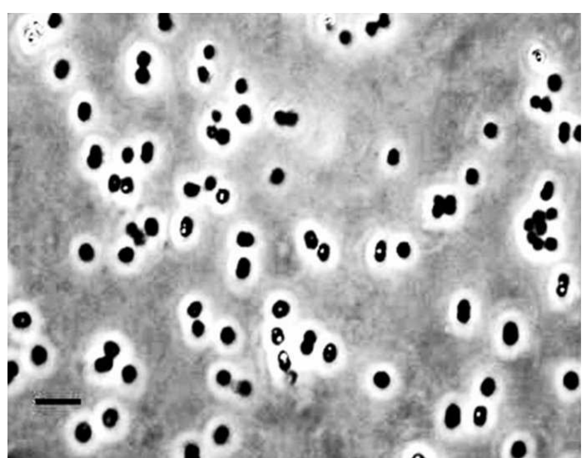
Supplementary Table III-1: Database sequences used for phylogenetic analysis.

Organism	strain	Phylum	Acc. No.
Acidiphilium acidophilum	ATCC27807^T	Alphaproteobacteria	AB013379
Acidiphilium angustum	ATCC 35903	Alphaproteobacteria	AB005219
Acidiphilium cryptum		Alphaproteobacteria	AB005220
Acidiphilium multivorum		Alphaproteobacteria	AB005221
Acidiphilium rubrum	ATCC 35905	Alphaproteobacteria	AB005218
Acidiphilium sp.	T25	Alphaproteobacteria	AB005224
aerobic Gammaproteobacterium	NOR51-B	Gammaproteobacteria	NZ_DS999411
aerobic Gammaproteobacterium	NOR5-3	Gammaproteobacteria	NZ_DS999405
Allochromatium phaeobacterium	JA144^T	Gammaproteobacteria	FM208081
Allochromatium vinosum	DSM180	Gammaproteobacteria	AB011811
Allochromatium warmingii	DSM173^T	Gammaproteobacteria	FN257132
Blastomonas natatoria	DSM3183^T	Alphaproteobacteria	AB012060
Bradyrhizobium sp.	ORS278	Alphaproteobacteria	CU234118
Bradyrhizobium sp.	BTAi1	Alphaproteobacteria	CP000494
Chloroflexus aggregans	DSM9485^T	Chloroflexi	CP001337
Chloroflexus aurantiacus	J-10-fl^T	Chloroflexi	CP000909
Chromatium weissei	DSM5161^T	Gammaproteobacteria	FN257133
Citromicrobium sp.	JL354	Alphaproteobacteria	DQ484039
Congregibacter litoralis	KT71	Gammaproteobacteria	AAOA0100001
Dinoroseobacter shibae	DFL 12	Alphaproteobacteria	CP000830
Ectothiorhodospira haloalkaliphila	ATCC51935	Gammaproteobacteria	FN257156
Ectothiorhodospira imhoffii	JA319^T	Gammaproteobacteria	AM944100
Ectothiorhodospira mobilis	DSM237	Gammaproteobacteria	FN257158
Ectothiorhodospira shaposhnikovii	ATCC31751	Gammaproteobacteria	AF018955
Ectothiorhodospira shaposhnikovii	DSM2111	Gammaproteobacteria	FN257157
Ectothiorhodospira variabilis	WN22^T	Gammaproteobacteria	FN257153
Erythrobacter litoralis	IAM 14332	Alphaproteobacteria	AB010981
Erythrobacter longus	MBIC2294	Alphaproteobacteria	AB035570
Erythrobacter sp.	JL475	Alphaproteobacteria	DQ484037
Erythrobacter sp.	MBIC3017	Alphaproteobacteria	AB015709
Erythrobacter sp.	MBIC3031	Alphaproteobacteria	AB015710
Erythromicrobium ramosum	IAM14333	Alphaproteobacteria	AB010873
Halochromatium roseum	H^T	Gammaproteobacteria	FN257161
Halochromatium salexigens	DSM4395^T	Gammaproteobacteria	FN257159
Halochromatium sp.	MTK1IM127	Gammaproteobacteria	FN257175
Halochromatium sp.	MTK6IM088	Gammaproteobacteria	FN257173
Halochromatium sp.	MTK5IM027	Gammaproteobacteria	FN257171
Halochromatium sp.	MTK2IM039	Gammaproteobacteria	FN257170
Halorhodospira halophila	SL1^T	Gammaproteobacteria	CP000544
Halorhodospira halophila	H	Gammaproteobacteria	FN257160
Jannaschia sp.	CCS1	Alphaproteobacteria	CP000264
Labrenzia alexandrii	DFL-11	Alphaproteobacteria	EQ973121
Lamprocystis purpurea	ML1	Gammaproteobacteria	AY177752
Loktanella fryxellensis	LMG22007	Alphaproteobacteria	DQ915716
Loktanella vestfoldensis	LMG22003	Alphaproteobacteria	DQ915717

Loktanella vestfoldensis SKA53	SKA53	Alphaproteobacteria	AAMS01000002
Marichromatium bheemlicum	JA124^T	Gammaproteobacteria	AM944099
Marichromatium indicum	JA214	Gammaproteobacteria	AM944098
Marichromatium purpuratum	DSM1591^T	Gammaproteobacteria	FN257135
Marichromatium sp.	MTK2IM017	Gammaproteobacteria	FN257168
marine Gammaproteobacterium	HTCC2080	Gammaproteobacteria	AAVV01000005
Methylobacterium chloromethanicum	CM4	Alphaproteobacteria	CP001298
Methylobacterium extorquens	PA1	Alphaproteobacteria	CP000908
Methylobacterium populi	BJ001	Alphaproteobacteria	NC_010725
Methylobacterium radiotolerans	JCM2831	Alphaproteobacteria	CP001001
Methylobacterium sp.	Apr-46	Alphaproteobacteria	NC_010511
Methylocella silvestris	BL2	Alphaproteobacteria	ABLP01000002
Porphyrobacter neustonensis	DSM9434	Alphaproteobacteria	AB011073
Porphyrobacter sanguineus	IAM12620	Alphaproteobacteria	AB011074
PorphyrobacterTepidarius	MBIC3363	Alphaproteobacteria	AB020599
Rhodobacter aestuarii	JA296^T	Alphaproteobacteria	FM208068
Rhodobacter azotoformans	IFO 16436	Alphaproteobacteria	AB062783
Rhodobacter blasticus	ATCC 33485	Alphaproteobacteria	D50649
Rhodobacter capsulatus	BEC404	Alphaproteobacteria	Z11165
Rhodobacter changlensis	JA139^T	Alphaproteobacteria	FM208070
Rhodobacter megalophilus	JA194^T	Alphaproteobacteria	FM208072
Rhodobacter ovatus	JA234^T	Alphaproteobacteria	FM208073
Rhodobacter sphaeroides	2.4.1^T	Alphaproteobacteria	CP000143
Rhodobacter sphaeroides	ATCC 17025	Alphaproteobacteria	CP000661
Rhodoferax fermentans		Betaproteobacteria	D50650
Rhodomicrobium vannielii	MBIC2956	Alphaproteobacteria	AB010830
Rhodomicrobium vannielii	MC-3	Alphaproteobacteria	D50652
Rhodopseudomonas faecalis	gc	Alphaproteobacteria	AB498849
Rhodopseudomonas palustris	TUT3625	Alphaproteobacteria	AB498870
Rhodopseudomonas palustris	TUT3612	Alphaproteobacteria	AB498859
Rhodopseudomonas palustris	TUT3611	Alphaproteobacteria	AB498858
Rhodopseudomonas palustris	ATCC17005	Alphaproteobacteria	AB498853
Rhodopseudomonas palustris	BisA53	Alphaproteobacteria	CP000463
Rhodopseudomonas palustris	BisB18	Alphaproteobacteria	CP000301
Rhodopseudomonas palustris	CGA009	Alphaproteobacteria	BX572597
Rhodopseudomonas rhenobacensis	RB	Alphaproteobacteria	AB251423
Rhodopseudomonas sp.	TUT3624	Alphaproteobacteria	AB498869
Rhodopseudomonas sp.	TUT3623	Alphaproteobacteria	AB498868
Rhodopseudomonas sp.	TUT3630	Alphaproteobacteria	AB498863
Rhodopseudomonas sp.	TUT3615	Alphaproteobacteria	AB498861
Rhodopseudomonas sp.	TUT3601	Alphaproteobacteria	AB498850
Rhodospirillum centenum	SW	Alphaproteobacteria	CP000613
Rhodovulum kholense	JA297^T	Alphaproteobacteria	FM208076
Rhodovulum sulfidophilum		Alphaproteobacteria	AB020784
Roseateles depolymerans	61A	Betaproteobacteria	AB028938
Roseateles depolymerans	CCUG 52219^T	Betaproteobacteria	AM773545
Roseateles depolymerans	CCUG 52220	Betaproteobacteria	AM773546
RoseatelesTerrae	CCUG 52222^T	Betaproteobacteria	AM773547
Roseiflexus castenholzii	DSM13941	Chloroflexi	CP000804
Roseiflexus sp.	RS-1	Chloroflexi	CP000686
Roseivivax halodurans	NBRC16685^T	Alphaproteobacteria	DQ915718
Roseivivax halotolerans	NBRC16686^T	Alphaproteobacteria	DQ915719
Roseobacter denitrificans	OCh114^T	Alphaproteobacteria	CP000362
Roseobacter litoralis	MBIC 2685^T	Alphaproteobacteria	AB016990

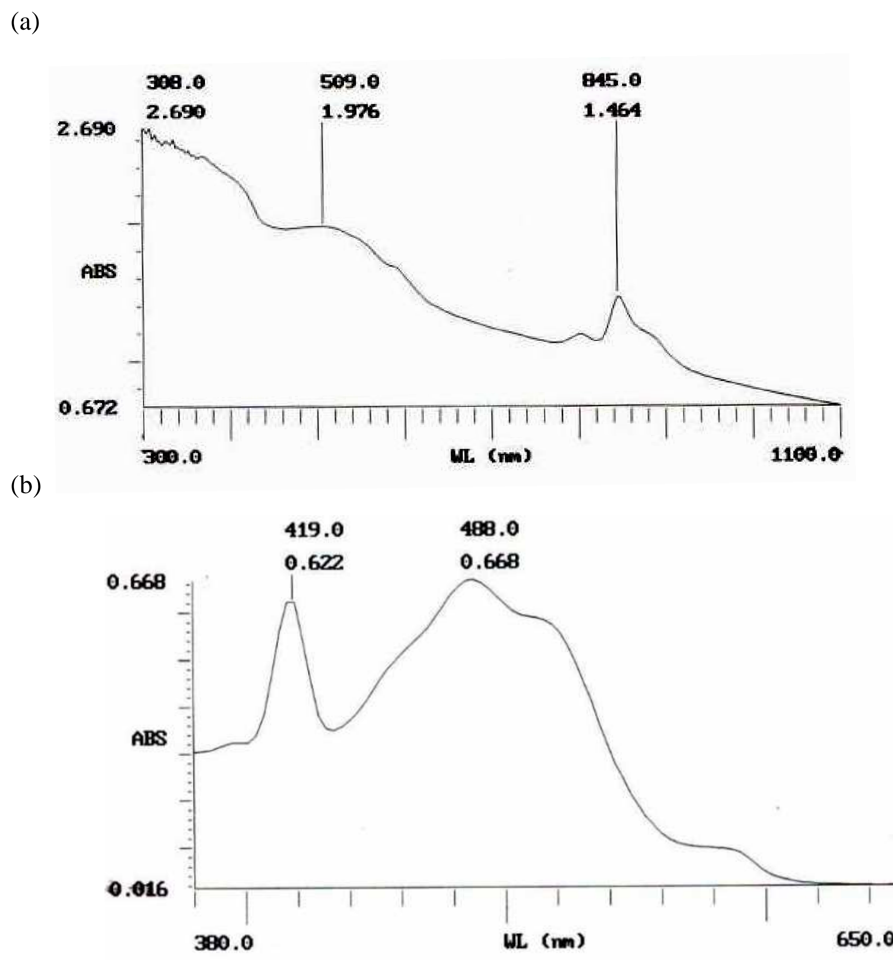
RoseococcusThiosulfatophilus	DSM 8511	Alphaproteobacteria	AY064410
RoseovariusTolerans	NBRC16695^{T}	Alphaproteobacteria	DQ915720
Rubritepida flocculans	H8	Alphaproteobacteria	AY064409
Rubrivivax gelatinosus	ATCC17011^{T}	Betaproteobacteria	D16822
Rubrivivax gelatinosus	S1	Betaproteobacteria	AY234384
Sphingomonas sp.	PB229	Alphaproteobacteria	AY853585
Sphingomonas sp.	PB56	Alphaproteobacteria	AY853583
Sphingomonas sp.	PB180	Alphaproteobacteria	AY853584
Sphingomonas ursincola	DSM 9006	Alphaproteobacteria	AB031016
Thalassiobium sp.	R2A62	Alphaproteobacteria	NZ_GG697169
Thalassobacter stenotrophicus	CECT5294	Alphaproteobacteria	DQ915724
ThermochromatiumTepidum	ATCC4361^{T}	Gammaproteobacteria	D85518
Thiobaca sp.	MTRDDF079	Gammaproteobacteria	FN257162
Thiocapsa marina	DSM5653^{T}	Gammaproteobacteria	FN257140
Thiocapsa pendens	DSM236^{T}	Gammaproteobacteria	FN257145
Thiocapsa rosea	DSM235^{T}	Gammaproteobacteria	FN257147
Thiocapsa roseopersicina	T.ork	Gammaproteobacteria	AJ544223
Thiocapsa roseopersicina	DSM217^{T}	Gammaproteobacteria	FN257146
Thiococcus pfennigii	DSM228	Gammaproteobacteria	FN257143
Thiococcus pfennigii	DSM227	Gammaproteobacteria	FN257142
Thiococcus pfennigii	DSM226^{T}	Gammaproteobacteria	FN257141
Thiocystis gelatinosa	DSM215^{T}	Gammaproteobacteria	FN257189
Thiocystis minor	DSM178^{T}	Gammaproteobacteria	FN257150
Thiocystis violacea	DSM208	Gammaproteobacteria	FN257149
Thioflavicoccus mobilis	ATCC700959^{T}	Gammaproteobacteria	FN257144
Thiohalocapsa halophila	DSM6210^{T}	Gammaproteobacteria	FN257151
Thiohalocapsa marina	JA142^{T}	Gammaproteobacteria	FN257154
Thiolamprovum pedioforme	DSM3802^{T}	Gammaproteobacteria	FN257152
Thiorhodococcus mannitoliphagus	ATCC BAA-1228^{T}	Gammaproteobacteria	FN257139
Thiorhodococcus minor	DSM11518^{T}	Gammaproteobacteria	FN257138
uncultured bacterium	CEHL-20-WP4	Gammaproteobacteria	FJ498847
uncultured bacterium	CEHL-20-WP45	Gammaproteobacteria	FJ498863

APPENDIX – CHAPTER IV

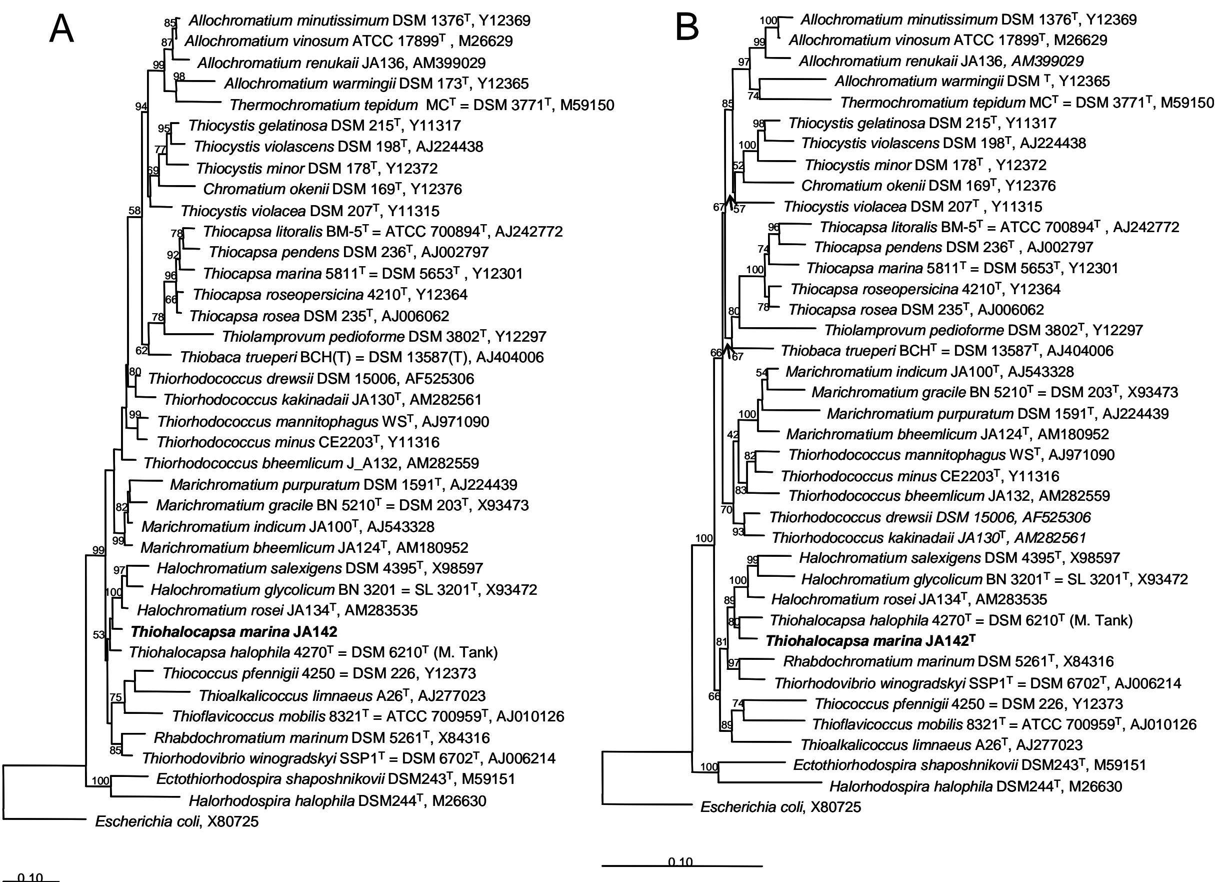


Supplementary Figure IV-S1: Phase-contrast micrograph of cells of strain JA142^T. Bar, 5µm.

(a)



Supplementary Figure IV-S2: Whole-cell absorption spectrum of strain JA142^T (a) and acetone spectrum of extracted pigments (b).



Supplementary Figure IV-S3: Phylogenetic trees based on 16S rRNA gene sequences showing the relationship of strain JA142^T within the family *Chromatiaceae*. The trees were calculated by the maximum likelihood method (A) and the neighbor-joining method (B) respectively. Numbers at nodes represent percent bootstrap values of 100 (ML) and 1000 (NJ) replicates done respelctively. Only bootstrap values above 50 are shown. The bar represents 0.1 substitutions per alignment position.

APPENDIX – V: POSTER PRESENTATIONS

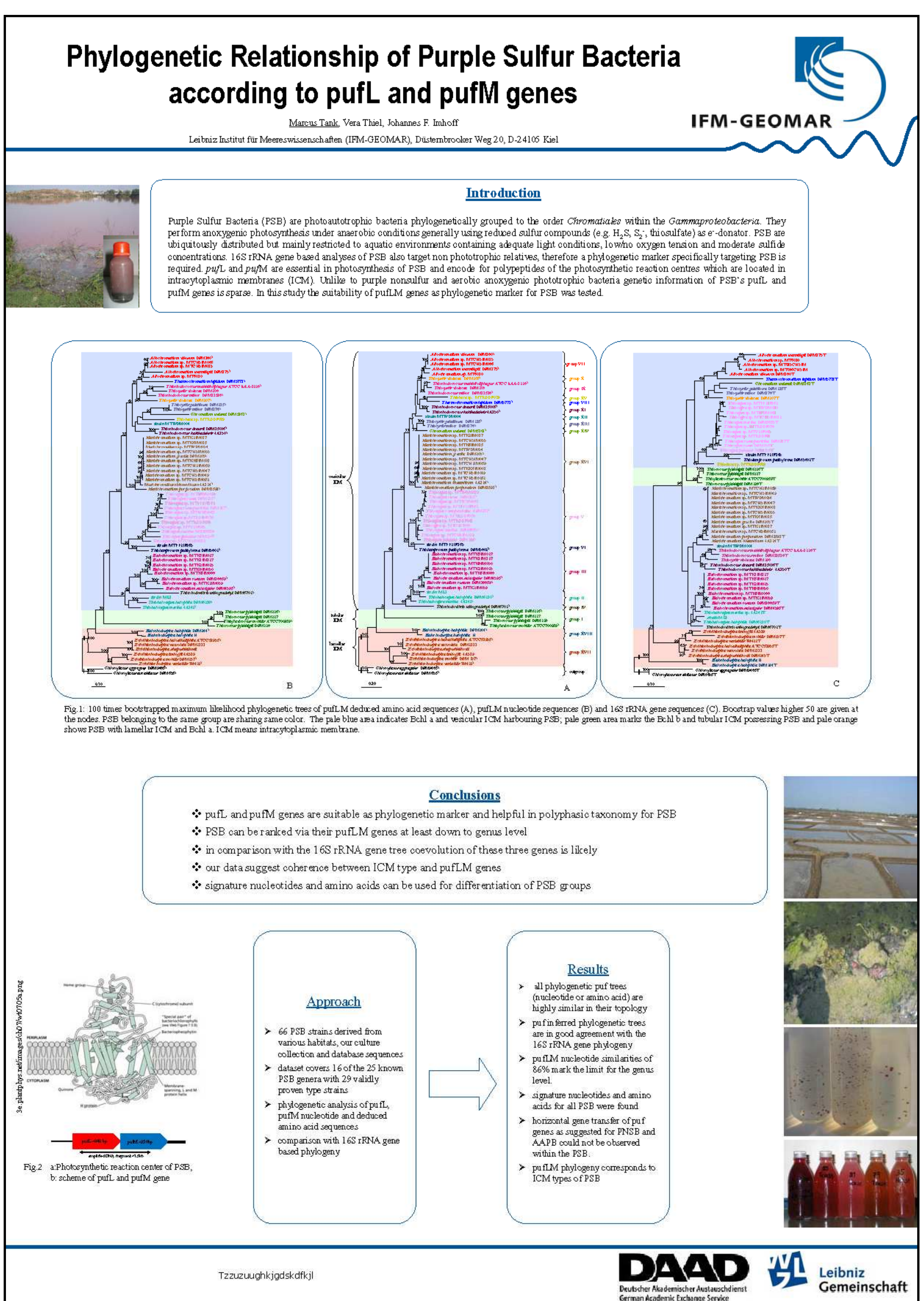


Figure V-1: Poster presented at the annual meeting of the 'Vereinigung für Allgemeine und Angewandte Mikrobiologie, VAAM', March 2009 in Bochum, Germany

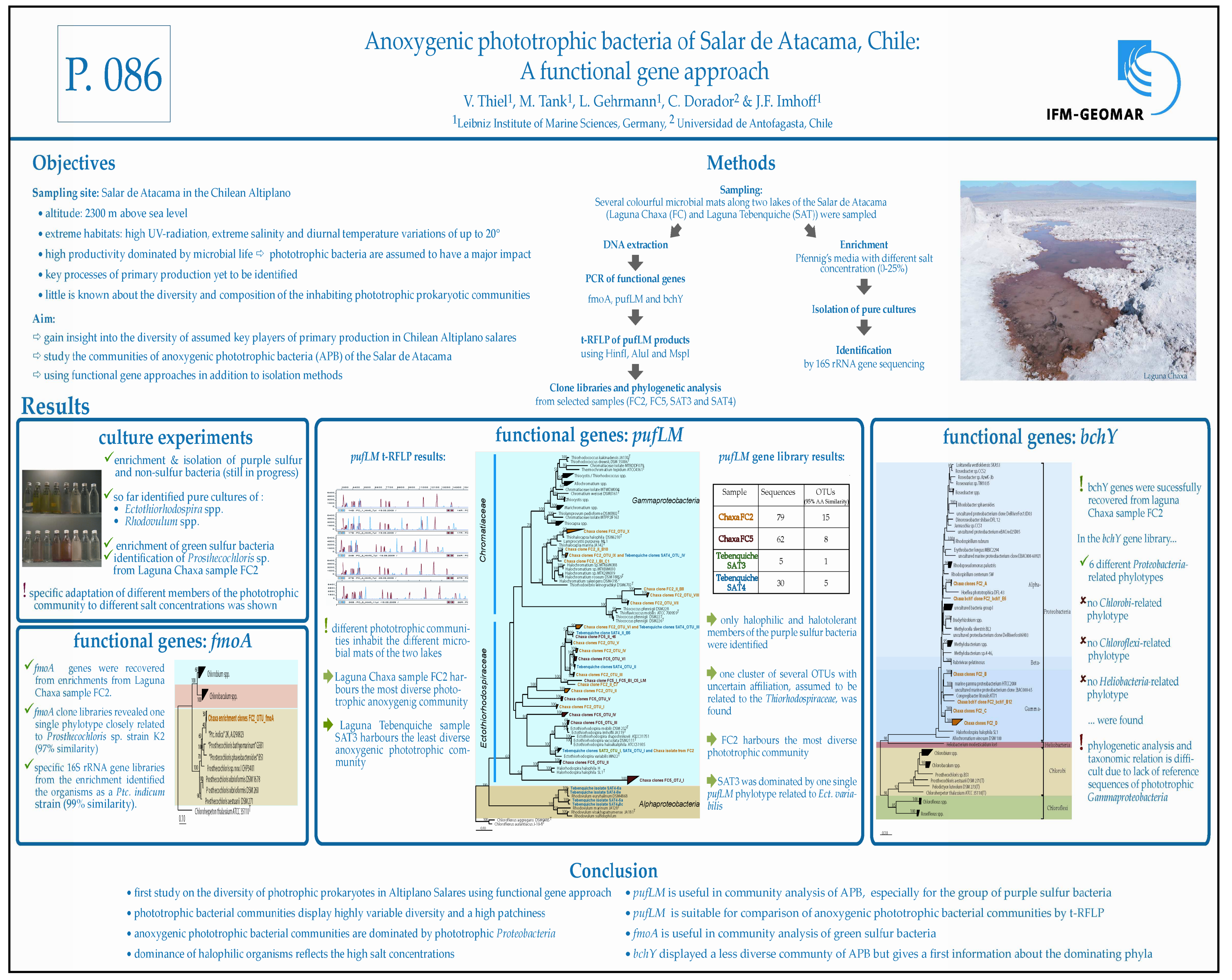


Figure V-2: Poster presented at the13th International Symposium on Phototrophic Prokaryotes, ISPP, August 2009 in Montreal, Canada

Anoxygenic phototrophic bacterial communities of Salar de Atacama, Chile: a functional gene approach using *fmoA* and *pufLM* genes

Vera Thiel[1], Marcus Tank[1], Sven C. Neulinger[1], Linda Gehrmann[1], Cristina Dorador[2] and Johannes F. Imhoff[1]
[1]Leibniz Institute of Marine Sciences, Germany, [2] Universidad de Antofagasta, Chile

IFM-GEOMAR

Geographic region and sampling sites

Bolivia
Salar de Atacama
Chile
Argentina
Laguna Tebenquiche
Laguna Chaxa

L. Tebenquiche: 23.1261S 68.2432W
L. Chaxa: 23.2887S 68.1772W

Characteristics

- hypersaline (up to 200 PSU), receive fresh water from sub-surface
- temperature: 27-36°C
- pH 7-8
- low to no dissolved oxygen
- altitude: 2300 m above sea level
- low atmospheric pressure (-35%)
- extreme habitats:
  - high UV-radiation
  - extreme salinity
  - diurnal temperature variations of up to 20°

Allochromatium
Thiorhodococcus mannitoliphagus, ATCC:BAA-1228T, FN257139
Thiocystis violacea, DSM208=4341, FN257149
Thiorhodococcus minor, DSM11518T, FN257138
Marichromatium
Thiocapsa
Thiolamprovum pedioforme DSM3802[T], FN257152
Thiocystis gelatinosa DSM215[T], FN257189
Thiocystis minor, DSM178T=5710T, FN257150
Chromatium weissei, DSM5161T, FN257133
Thiobaca sp., MTRDDF079, FN257162
Halochromatium
Thiohalocapsa
Lamprocystis purpurea, ML1, AY177752
Thiohalocapsa marina, JA142T, FN257154
Thiococcus / Thioflavicoccus
unidentified Chile clones group I
unidentified Chile clones group II
Chromatiacea
Ectothiorhodospira
Halorhodospira
Ectothiorhodospiraceae
unidentified Chile cluster IV
unidentified Chile cluster V
Chile clone FC2BID3puf,
unidentified Chile cluster III
Chile clone FC2pufIIC7,
Chile clone FC5BIC5puf,
Chile clone FC2BID6puf,
unidentified phototrophic Gammaproteobacteria
unidentified Chile cluster I
uncultured bacterium CEHL-20-WP4, FJ498847
uncultured bacterium CEHL-20-WP45, FJ498863
Aerobic Gammaproteobacteria
aerobic phototrophic Gammaproteobacteria
Gammaproteobacteria
Roseateles
Rubrivivax
Rhodoferax fermentans D50650
Rubritepida flocculans H8, AY064409
Roseococcus thiosulfatophilus DSM 8511, AY064410
Rhodospirillum centenum SW, CP000613
Chile cluster VI
Labrenzia alexandrii, DFL-11, EQ973121
Rhodospirillales
Rhizobiales
Rhizobiales II
Sphingomonadales
Rhodobacterales I
Rhodobacterales II
Chloroflexus
Roseiflexus
Alphaproteobacteria
0.10

Figure 1: Partly condensed phylogenetic tree of *pufLM* sequences obtained in this study and from database entries. Groups containing clone sequences obtained in this study are printed in red. Clone sequences sharing more than 86% nucleotide similarity were grouped to clusters.

Aims

In order to gain insight into the diversity of assumed key players of primary production in Chilean Atacama Desert salares, we studied the communities of anoxygenic phototrophic bacteria (APB) of the Salar de Atacama using the functional genes *pufLM* and *fmoA*, which are unique for red and green anoxygenic phototrophic bacteria, respectively.

Results & Conclusions

pufLM T-RFLP fingerprinting showed that the anoxygenic phototrophic bacterial communities of hypersaline lakes in Salar de Atacama differ in diversity and composition between and within the lakes. Highly variable conditions such as salt concentration forming micro-habitats apparently shape the community structure in the Salar de Atacama.

The anoxygenic phototrophic bacterial community is mainly composed of unidentified phototrophic proteobacteria as well as of halophilic purple sulfur bacteria such as *Halochromatium* spp., *Thiohalocapsa* spp., *Ectotiorhodospira* spp. and *Halorhodospira* sp. (Fig. 1).

No *fmoA* genes were detected in any of the environmental samples. Therefore, green sulfur bacteria (*Chlorobiaceae*) are assumed to be a rather minor component of the phototrophic bacterial community. However, they may develop under appropriate conditions as seen in enrichment cultures (data not shown).

The studied hypersaline lakes are unique habitats harboring a highly diverse set of many still unknown *pufLM* containing anoxygenic phototrophic bacteria and thus are of highest interest for further studies. The great number of yet uncultured and unidentified *pufLM* phylotypes retrieved emphasizes the uniqueness of the studied area as well as the need of further studies on phototrophic bacteria, including isolation-based studies.

Approach

sediment samples taken of different sites at Laguna Tebenquiche and Laguna Chaxa

whole genome DNA extraction

gene amplification (*pufLM, fmoA*)

comparison of diversity and composition of the amplified *pufLM* genes by terminal restriction fragment polymorphism (T-RFLP)

cloning and sequencing of selected PCR products

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Figure V-3: Poster presented at the annual meeting of the 'Vereinigung für Allgemeine und Angewandte Mikrobiologie, VAAM', March 2010 in Hannover, Germany