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Variability of photosynthesis genes in purple bacteria and molecular determinants of their spectral properties

Von der Fakultät für Mathematik und Naturwissenschaften
der Carl von Ossietzky Universität Oldenburg
zur Erlangung des akademischen Grades eines
Doktors der Naturwissenschaften
angenommene

Dissertation

von
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geboren am 12. Dezember 1969 in Recklinghausen

Oldenburg, September 2003

Erstreferent: Prof. Dr. Heribert Cypionka

Korreferent: Prof. Dr. Jörg Overmann

Tag der Disputation: 17.12.2003

Für meine Eltern

Für Judit

List of Abbreviations

[32P]dCTP	deoxy cytidine triphosphate, labled with radioactive phosphor ^{32}P
aa	amino acids
ATCC	American type culture collection
ATP	adenosine triphosphate
bp	base pairs
bch	bacteriochlorophyll biosynthesis genes
BChl	bacteriochlorophyll
crt	carontenoid biosynthesis genes
DSM / DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen
EDTA	ethylendiamine tetraacetic acid
iPCR	inverse polymerase chain reaction
LH1	light harvesting complex 1 (core antenna)
LH2, LH3	light harvesting complexes 2 and 3 (peripheral antennae)
MOPS	3-(N-morpholino)propanesulfonic acid
NIR	near infrared
nt	nucleotides
orf	open reading frame
PCR	polymerase chain reaction
PDB	protein database
PS I, PS II	photosystem I and II
puc	structural and regulatory genes for the peripheral antenna
puf	**p**hotosynthetic **u**nit **f**orming genes (reaction center + light harvesting core antenna)
puh	structural gene for the reaction center H subunit (**p**hotosynthetic **u**nit **H** protein)
Q_A, Q_B	acceptor quinones A and B in the purple bacterial reaction center
Q_y	long wavelength transition of bacteriochlorophylls
RC	reaction center
SDS	sodium dodecyl sulfate
SSC	sodium chloride-sodium citrate
TBE	Tris-boric acid-EDTA
TE	Tris-EDTA
Tris	Tris(hydroxymethyl)aminomethane

Abbreviations of genus names

Acp.	*Acidiphilium*
Alc.	*Allochromatium*
Amb.	*Amoebobacter*
Blc.	*Blastochloris*
Blm.	*Blastomonas*
Cfx.	*Chloroflexus*
Ery.	*Erythrobacter*
Ect.	*Ectothiorhodospira*
Hlr.	*Halorhodospira*
Psp.	*Phaeospirillum*
Rbc.	*Rhodobacter*
Rbi.	*Rhodobium*
Rbl.	*Rhodoblastus*
Rcy.	*Rhodocyclus*
Rdv.	*Rhodovulum*
Rps.	*Rhodopseudomonas*
Rsb.	*Roseobacter*
Rss.	*Roseospirillum*
Rsp.	*Rhodospirillum*
Rst.	*Roseateles*
Rvi.	*Rubrivivax*
Thc.	*Thermochromatium*

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Chapter I

Introduction

1 Photosynthesis

Sunlight is the primary source of energy for life on earth. The biological conversion of solar energy into chemical energy is performed in the process of photosynthesis. Photosynthesis is carried out by both eukaryotic and prokaryotic organisms. Eukaryotic phototrophs such as plants (including algae, diatoms and dinoflagellates) and the prokaryotic cyanobacteria carry out oxygenic photosynthesis. These organisms employ two coupled chlorophyll-based photosystems (PS II and PS I), and use water as an electron donating substrate. Molecular oxygen evolves as a by-product of water splitting. The oxygen in our today's atmosphere derives from this process and is essential for aerobic metabolism.

In anoxygenic photosynthesis oxygen is not formed. Anoxygenic photosxnthesis is performed by a heterogeneous group of phototrophic bacteria. These bacteria employ only one type of bacteriochlorophyll-based photosystems, which do not allow the use of water as electron donor. Instead electron donors are required with a lower standard redox potential such as molecular hydrogen, reduced sulfur compounds (e.g. H_2S) or simple organic substrates (OVERMANN & GARCIA-PICHEL, 2000). This mode of photosynthesis is believed to be more ancient and is thought to be the precursor of oxygenic photosynthesis

(XIONG *et al.*, 1998; BLANKENSHIP, 1992). Their contribution to the global primary production plays a minor role today (OVERMANN & GARCIA-PICHEL, 2000), but anoxygenic phototrophic bacteria might have been more prominent in ancient times and they are still significant in certain aquatic environments. Where light reaches anoxic interfaces of stratified environments such as freshwater and salt lakes, littoral sediments, lagoons, hot springs, marine water-bodies and also soils anoxygenic phototrophic bacteria are ubiquitous (YURKOV & BEATTY, 1998; CASTENHOLZ & PIERSON, 1995; MADIGAN & ORMEROD, 1995; VAN GEMERDEN & MAS, 1995). In such ecosystems these bacteria can come to mass development and form dense macroscopic visible blooms or microbial mats, where they can contribute significantly to the fixation of carbon and cycling of sulfur (OVERMANN, 1997; VAN GEMERDEN & MAS, 1995; LENGELER *et al.*, 1999).

Because of the relatively simple mechanisms and structures involved, anoxygenic phototrophic bacteria are well suited as model systems for the understanding of the fundamentals of the light driven processes, the architecture of the photosynthetic apparatus, the genetics behind structural and regulatory components and insights into evolutionary aspects.

2 Anoxygenic phototrophic bacteria

Anoxygenic phototrophs are distributed in four lineages of the domain Bacteria (Fig. 1) (OVERMANN & GARCIA-PICHEL, 2000). They encompass (1) the purple bacteria (purple sulfur and purple non-sulfur bacteria), (2) the green sulfur bacteria, (3) the gliding green filamentous bacteria and (4) the gram-positive heliobacteria. All these bacteria carry out bacteriochlorophyll-based photosynthesis. In addition the halobacteria of the domain Archaea carry out a particular type of anoxygenic photosynthesis. However, the latter encompasses a unique, retinal-based bacteriorhodopsin photoreaction (OVERMANN & GARCIA-PICHEL, 2000).

According to their 16S rRNA, the four lineages of anoxygenic phototrophic bacteria are only distantly related. Within each lineage phototrophic species are intermixed with non-photosynthetic relatives, except for the green sulfur bacteria, which form a coherent group (OVERMANN & GARCIA-PICHEL, 2000; STACKEBRANDT *et al.*, 1996; WOESE, 1987). If other genetic makers of these bacteria are considered, such as genes of the conserved photosynthetic apparatus, the interpretation of interrelationships becomes difficult. It is

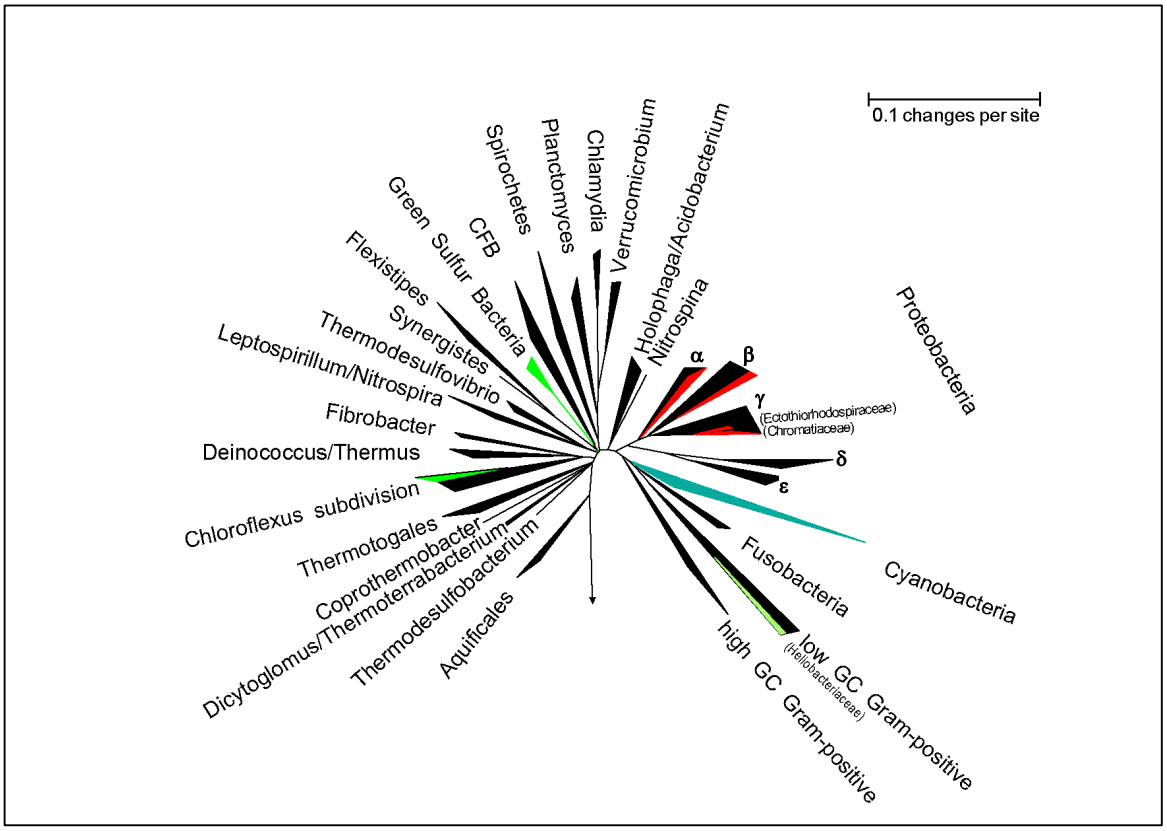

Figure 1: Phylogenetic tree based on 16S rRNA sequences of the domain Bacteria (adopted from OVERMANN & GARCIA-PICHEL, 2000). Phyla (or portion of phyla) with phototrophic members are shown in colour. (*light green*) Bacteria containing chlorosomes as light-harvesting antenna. (*red*) Bacteria containing antenna complexes within the cytoplasmic membrane and quinone/pheophytin-type reaction centers. *(medium green*) Gram-positive phototrophic bacteria with FeS-type reaction centers. (*turquoise*) Bacteria containing the two types of reaction centers. Width of coloured wedges indicates the phylogenetic diversity

commonly believed that all photosynthetic apparatuses share a common ancestor (OLSON, 2001; BLANKENSHIP, 1992). However, the distribution of the components involved (e.g. different types of reaction center, antennae, bacteriochlorophylls) do not follow clear evolutionary lines in the phyla (OLSON, 1998; BLANKENSHIP, 1992).

Photosystems are the structural units that provide the environment for the pigment-based photochemical reactions and consist of reaction centers and light harvesting antenna complexes. Two fundamental types of photosystems are distributed among the various groups of phototrophs (OVERMANN & GARCIA-PICHEL, 2000; BLANKENSHIP, 1992). They are distinguished by the chemical nature of early electron acceptors of the reaction centers (BLANKENSHIP, 1992). Type I reaction centers are characterized by the presence of iron-sulfur clusters as intermediate electron acceptors, whereas type II reaction centers employ pheophytins and quinones. Type I reaction centers are found in green sulfur bacteria and heliobacteria and are similar to the PS I of oxygenic phototrophs. The purple and the green

filamentous bacteria (*Chloroflexus* group) employ type II reaction centers, which resemble the PS II of oxygenic phototrophs (OLSON, 1998). Several types of associated light-harvesting antenna complexes exist as well. Green sulfur and filamentous bacteria harvest light via unique chlorosomes, in which bacteriochlorophylls are densely packed in aggregates with few proteins (OLSON, 1998; BLANKENSHIP *et al.*, 1995a). Purple bacteria employ antennae, where bacteriochlorophylls are non-covalently bound in protein complexes (ZUBER & COGDELL, 1995). Heliobacteria possess also protein-based antennae, which however appear to be part of a single antenna-reaction center complex (AMESZ, 1995).

The pigments are the key elements that allow absorption and transfer of energy. Different types of bacteriochlorophylls (BChls) are employed by the various phototrophic bacteria. Generally, the BChls differ in their substituents on various parts of the porphyrin ring and their esterifying alcoholic side chains (BROCK *et al.*, 1994; SCHEER, 1991). Most purple bacteria possess BChl *a*, while some species use BChl *b*. In green sulfur and green filamentous bacteria BChl *a* is present in the reaction center, whereas BChl *c*, *d* or *e* are found in the chlorosomes. Heliobacteria use exclusively BChl *g* in their photosystem. The bacteriochlorophylls play the dominant role in light harvesting and the photochemical primary reaction. In addition various carotenoids are present which have light-harvesting capabilities, too, but also protective functions to prevent photooxidation (FRASER *et al.*, 2002). For light absorption, bacteriochlorophylls and carotenoids possess extensive systems of conjugated double bonds in form of circular tetrapyrrols and isoprenoid units respectively (BROCK *et al.*, 1994; SCHEER, 1991).

Based on interrelationships of the individual components of the photosynthetic apparatus, an independent and linear evolution of the four lineages of anoxygenic phototrophic bacteria after diversification from a common ancestor seems unlikely. The origin and evolution of photosynthesis is still subject of controversy (RAYMOND *et al.*, 2002; OLSON, 2001; XIONG *et al.*, 2000; BLANKENSHIP, 1992). Gene duplications and/or selective loss of photosynthesis genes in species that derived from a common ancestor might have occurred (OLSON, 2001; BLANKENSHIP, 1992; WOESE, 1987). Lateral transfer of genes has also been invoked to explain such inconsistencies (IGARASHI *et al.*, 2001; NAGASHIMA *et al.*, 1997; NAGASHIMA *et al.*, 1993; BLANKENSHIP, 1992; DICKERSON, 1980) and evidence is growing that horizontal gene transfer indeed played a pivotal role for photosynthesis evolution (RAYMOND & BLANKENSHIP, 2003; RAYMOND *et al.*, 2002).

3 Purple bacteria

Among the anoxygenic photosynthetic bacteria the purple bacteria form the most diverse group, based on their phenotypic, metabolic and phylogenetic properties (STACKEBRANDT *et al.*, 1996; IMHOFF, 1995; PFENNIG & TRÜPER, 1989). Specialized photosynthetic membranes are common to all purple bacteria and harbour their photosynthetic apparatus. These "purple" membranes are continuous intracytoplasmatic invaginations continuous to cytoplasmic membrane and often form lamellar, vesicular or stack structures (ZUBER & BRUNISHOLZ, 1991). Due to species specific mixtures of bacateriochlorophyll with a variety of carotenoid pigments the colour shadings of cultures reach from purple-violet, pink, red, brown, to yellowish and green (PFENNIG & TRÜPER, 1989). Metabolically, the purple bacteria cover a wide range of capabilities ranging from photosynthetic to chemotrophic growth modes. In terms of photosynthesis they utilize the widest range of wavelengths of all photosynthetic organisms.

All members of the purple bacteria are Proteobacteria. The Proteobacteria are divided into five subclasses (α, β, γ, δ and ε), of which the δ and ε subclass contain only non-photosynthetic species (Fig. 1) (OVERMANN & GARCIA-PICHEL, 2000; STACKEBRANDT *et al.*, 1996). For traditional taxonomical reasons (PFENNIG & TRÜPER, 1989) the phototrophic members are divided into (1) the purple sulfur bacteria with the families *Chromatiaceae* and *Ectothiorhodospiraceae* and (2) the purple non-sulfur bacteria which are also referred to as *Rhodospirillaceae*.

The purple sulfur bacteria are all members of the γ Proteobacteria. Characteristic for them is the presence of elemental sulfur, which derives from the oxidation of sulfide. *Chromatiaceae* deposit sulfur globules inside, whereas *Ectothiorhodospiraceae* deposit sulfur outside of the cells. Most members of the *Chromatiaceae* are obligate phototrophic anaerobes and metabolically rather specialized. The *Ectothiorhodospiraceae* and some *Chromatiaceae* exhibit more metabolic flexibility (OVERMANN & GARCIA-PICHEL, 2000). However, purple sulfur bacteria are metabolically restricted in contrast to the more versatile purple non-sulfur bacteria. Interestingly, especially those species with limited metabolic flexibility form dense blooms under natural conditions (OVERMANN & GARCIA-PICHEL, 2000).

The purple non-sulfur bacteria are classified into the two subclasses α and β of the Proteobacteria. Members of both groups vary considerably in their morphology, physiology and pigmentation (IMHOFF, 1995; PFENNIG & TRÜPER, 1989). On the basis of 16S rRNA analysis the α subclass was divided further into four subgroups (α-1 to α-4),

whereas the β Proteobacteria form a more coherent group (STACKEBRANDT *et al.*, 1996). However classification schemes are currently subject to change, due to the growing availability of molecular data. Most purple non-sulfur bacteria are faculative phototrophs (preferably photoheterotrophic), and many are also capable of various chemotrophic growth modes, including aerobic respiration (IMHOFF, 1995).

Taxonomically related to the purple non-sulfur bacteria are a physiological group of aerobic anoxygenic phototrophic bacteria. Based on 16S rRNA analyses they are affiliated with the α Proteobacteria. These bacteria perform anoxygenic photosynthesis only in the presence of oxygen and are incapable of autotrophic growth (BEATTY, 2002; YURKOV & BEATTY, 1998). Only recently, their importance in natural environments became evident (KOLBER *et al.*, 2001).

3.1 Photochemical processes in purple bacteria

Purple bacteria harvest light via their membrane bound antenna complexes and funnel the energy towards the reaction center, where the primary photosynthetic reaction takes place (Fig. 2). There are two possible branches of electron transfer in the (hetero)dimeric reaction center complex (L and M subunit) but for yet unknown reasons only the cofactors of the "L branch" are active in the light driven process (LANCASTER & MICHEL, 1996; BARBER & ANDERSSON, 1994). The excitation of a photochemically active "special pair" of bacteriochlorphylls leads to charge separation, which initiates a cyclic transport of electrons (LENGELER *et al.*, 1999). An immediate reduction of the special pair BChls prevents the backflow of electrons and is facilitated either directly by a soluble cytochrome c_2 or in most cases via a reaction center bound tetraheme cytochrome (NITSCHKE & DRACHEVA, 1995). In a cascade of transfers, electrons are translocated via accessorial BChls and bacteriopheophytins to a quinone molecule Q_A. From this Q_A electrons are transferred further to a Q_B molecule. After a second photon has triggered this series of events again, a doubly reduced Q_B picks up two protons from the cytoplasm. This reduced Q_BH_2 molecule is replaced by a new quinone (Q-cycle), leaves the reaction center and functions as a mobile charge carrier (OKAMURA & FEHER, 1995). Protons and electrons are transferred to the membrane bound cytochrome bc_1 complex. Here the actual energetic potential is generated by charge separation over the membrane. Both, protons and electrons, are translocated outside of the cell via the cytochrome and iron-sulfur components of the complex. The protons are released to the periplasm, whereas the electrons are transferred to a mobile, periplasmic cytochrome c_2. The electron cycle is

finally closed when this soluble cytochrome fills the electron gap of the primary electron donor, the reaction center bound tetraheme cytochrome (ORTEGA *et al.*, 1999). The generated proton motif force finally drives membrane potential dependent processes like the generation of ATP by ATP synthetase (HU *et al.*, 1998).

3.2 Structure of the pigment binding proteins and architecture of the purple bacterial photosynthetic apparatus.

The structural components of the photosynthetic apparatus that bind pigments are the antenna and reaction center proteins. In purple bacteria the reaction center consists of three subunits (Fig. 2 and 3) (LANCASTER & MICHEL, 1996; LANCASTER *et al.*, 1995). The L- and the M-subunit are integral membrane proteins with five α-helical transmembrane domains each. Both harbour the reaction center associated pigments, the special pair BChls, accessory BChls and bacteriopheophytins and provide the environment for electron

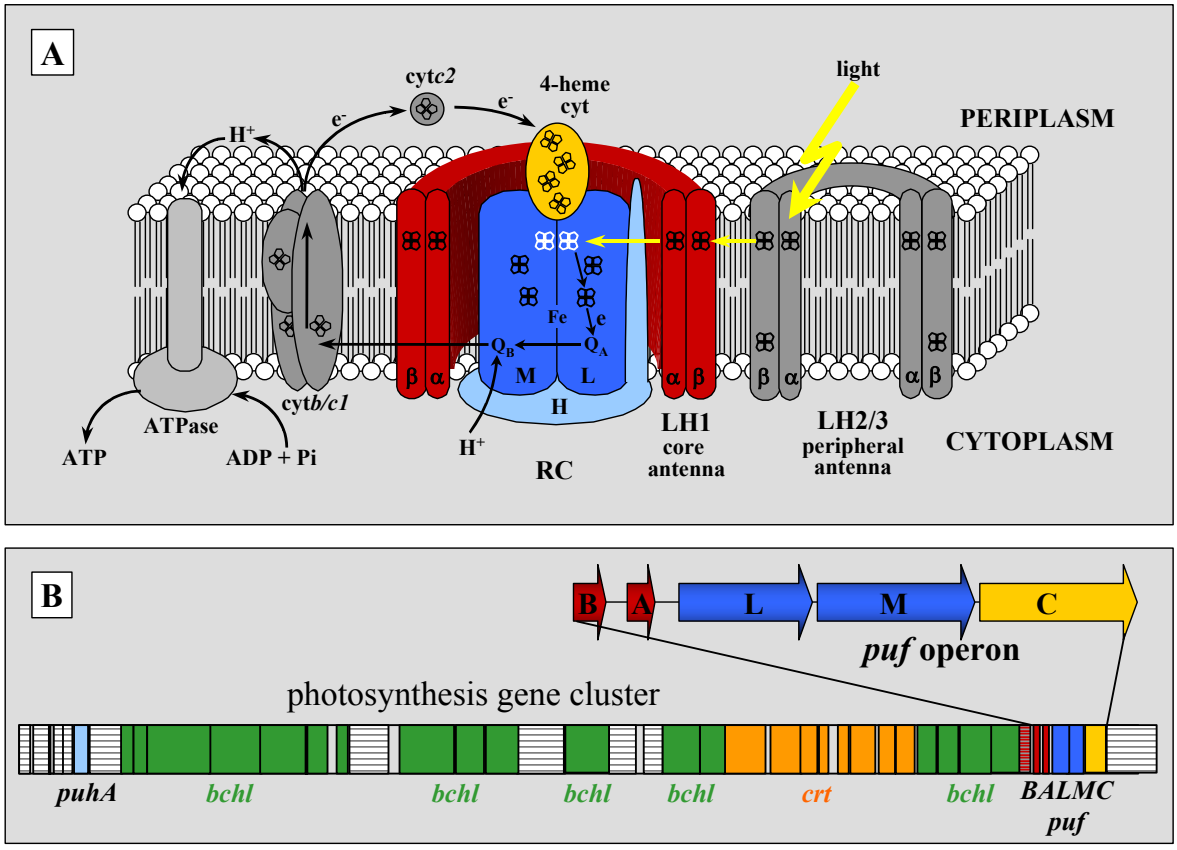

Figure 2: (A) Model of the photosynthetic apparatus of purple bacteria. Light, electron and proton pathways are indicated by arrows. Tetracycles represent porphyrin cofactors (Bchls, BPhe or cytochrome hemes) The special pair BChls are given in white. Q_A, Q_B = quinones; cyt = cytochrome. **(B)** Representation of a photosynthesis gene cluster of purple bacteria (adopted and modified from BEATTY, 1995). The structural components of the photosynthetic apparatus and their encoding genes are given in blue for the reaction center, red for the LH1 complex and yellow for the tetraheme cytochrome. In addition, bacteriochlorophyll (*bch*) and carotenoid (*crt*) biosynthesis genes within the photosynthesis gene cluster are indicated in green and orange.

flow (see above). The third subunit is a non-pigmented apoprotein, the H-subunit. It exhibits a cytoplasmic domain which shows evidence of facilitating proton uptake from the cytoplasm (ADELROTH *et al.*, 2001; ABRESCH *et al.*, 1998). In addition, a single transmembrane domain is present, which seems to be important for the stability of the whole complex (CHENG *et al.*, 2000).

Besides the reaction center the antenna complexes contain the majority of pigments, and confer the characteristic phenotypic coloured appearance to the cultures. Two types of antennae are known in purple bacteria (Fig. 2 and 3): (1) The reaction center (RC) associated core antenna, which is present in all purple bacteria and is also referred to as light harvesting complex (LH) 1 (Fig. 3). (2) Peripheral antennas, which are present in most, but not all purple bacteria. These antennas surround the RC-LH1 core unit (Fig. 3) and are also referred to as LH2 or LH3, depending on their spectral characteristics (MCLUSKEY *et al.*, 2001; ZUBER & COGDELL, 1995) (see further below). The amount of peripheral antennae per RC-LH1 unit can reach maximally eight (PAPIZ *et al.*, 1996) and might vary in dependence of environmental factors such as light (OVERMANN & GARCIA-PICHEL, 2000; GARDINER *et al.*, 1993). The smallest unit of the antennae is a heterodimer that consists of an α and a β polypeptide. Both peptides are integral membrane proteins with a single transmembrane domain, which is flanked by a cytoplasmic N-terminus and a periplasmic C-terminus. In LH1 about 16 αβ heterodimers are known to form a ring-like structure that encloses the reaction center (COGDELL *et al.*, 1999; PAPIZ *et al.*, 1996; KARRASCH *et al.*, 1995), in which the α polypeptides form the inner and the β polypeptides the outer ring (Fig. 3A). Whether the LH1 antenna is a completely closed ring or possesses a portal remains unclear (FRESE *et al.*, 2000; JUNGAS *et al.*, 1999). However, quinone molecules have to pass the core antenna to shuttle electrons from the reaction center to the cytochrome b/c_1 complex. The peripheral light-harvesting complexes are smaller. Eight or nine αβ heterodimers form a cycle which is built on the same modular principle as the core antenna (Fig. 3A) (COGDELL *et al.*, 1999; HU *et al.*, 1998; PAPIZ *et al.*, 1996). However, these apoproteins are chemically different from their LH1 analogues (see also chapter I.3).

The spectral properties of purple bacteria are mainly determined by the bacteriochlorophyll molecules bound to the antenna complexes. BChl *a* is the major pigment in most species, while a few purple sulfur bacteria employ BChl *b* in their antennas. In both light-harvesting complexes each polypeptide of the αβ heterodimer binds one BChl molecule. This pair of BChls is coordinately bound to the imidazole group of highly conserved histidine residues present in each antenna apoprotein (COGDELL *et al.*,

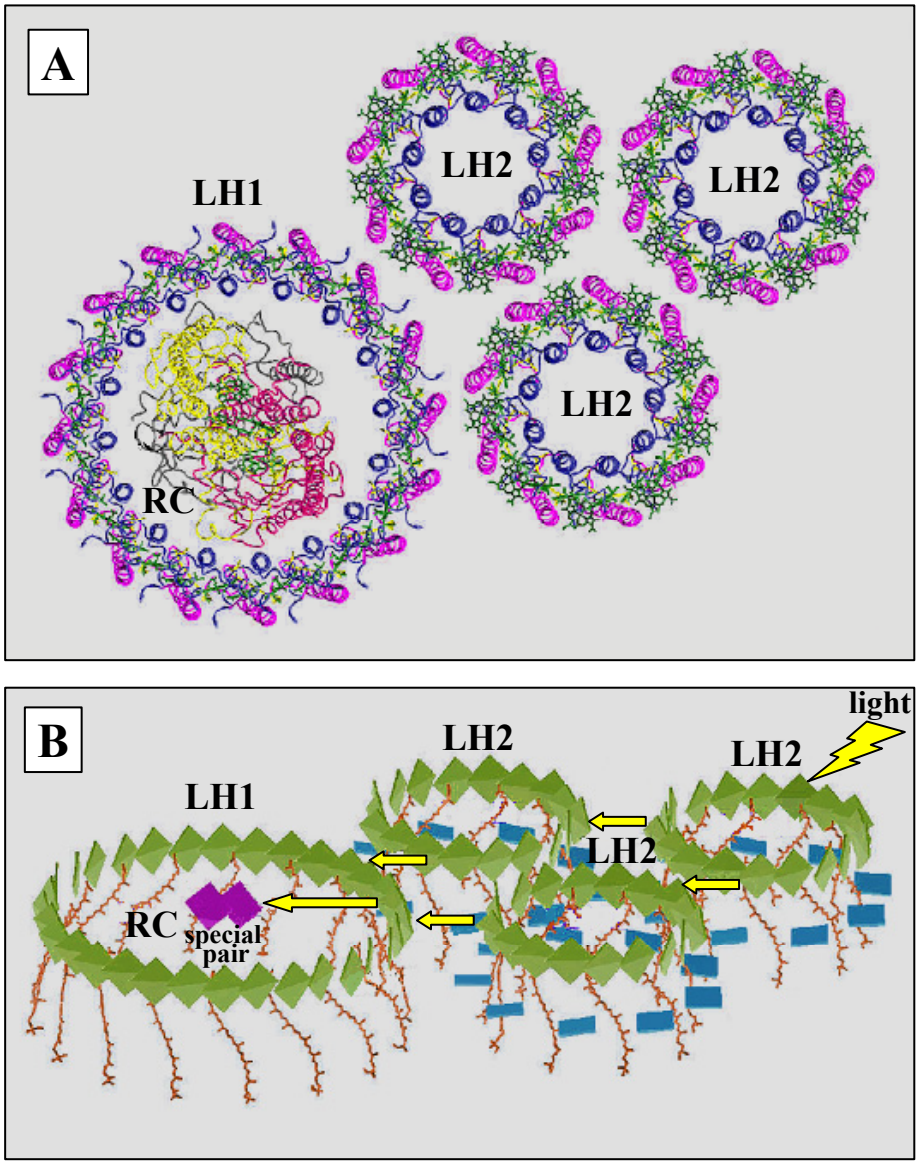

Figure 3: Schematic view of the bacterial photosynthetic unit modelled on structural information from *Rhodobacter sphaeroides* (adopted and modified from www.ks.uius.edu, HU *et al.*, 1998). Displayed are the LH1 core complex harbouring the reaction center (RC) and three peripheral light harvesting complexes (LH2). **(A)** View from top of the membrane. In both antenna complexes the α apoproteins from an inner ring and the β apoproteins an outer ring, shown in blue and magenta. The RC subunits L, M and H are displayed in yellow, red and gray, respectively. BChls and carotenoids are sandwiched between the α helices of the antenna proteins are given in green and yellow. **(B)** Side view. Representation of the pigment cofactors of the antenna complexes. Energy migration path towards the RC special pair BChls (represented as magenta squares) is indicated by arrows. The rings of excitonically coupled BChl dimers (green squares) are perpendicular to the membrane plane and in the same depth as the RC special pair. The monomeric BChls (blue squares) of the peripheral light harvesting complexes are arranged parallel to the membrane plane. Carotenoids (orange) are also shown.

1999; ZUBER & COGDELL, 1995; ZUBER & BRUNISHOLZ, 1991). However, a few other amino acids (argenine, asparagine or glutamine) might serve as ligands to BChls, too (OVERMANN & GARCIA-PICHEL, 2000; OLSEN *et al.*, 1997; ZUBER & BRUNISHOLZ, 1991). Due to their close spatial orientation these BChls are also referred to as dimeric BChls.

These BChls overlap with each other and with the BChls of the neighbouring αβ dimer and form altogether an excitonically coupled pigment ring within the antenna (Fig. 3B) (COGDELL *et al.*, 2002). In addition a single BChl is associated with each β polypeptide of the peripheral antenna, which is referred to as monomeric BChl.

On the basis of crystallographic data the detailed architecture of the reaction centers and light harvesting complexes are resolved in molecular detail (KOEPKE *et al.*, 1996; KARRASCH *et al.*, 1995; MCDERMOTT *et al.*, 1995; DEISENHOFER *et al.*, 1985). Embedded in the membrane both components are aligned in a way that the excitonically coupled BChl rings of the antennae complexes and the special pair of the reaction center are in the same plane within the membrane, close to the periplasmic side (Fig. 3B) (HU *et al.*, 1998; PAPIZ *et al.*, 1996). This geometry facilitates an ultrafast transfer of excitation energy on a picosecond time scale as revealed by laser-flash spectroscopy (HUNTER *et al.*, 1989). Within the BChl-rings of the antennae energy is delocalized until further transfer of photons to the reaction center, where the photochemical primary reaction is initiated.

Although many purple bacteria use the same type of BChl (*a*), different *in vivo* absorption characteristics are observed (ZUBER & BRUNISHOLZ, 1991) (see also Fig. 4). Embedded in the protein environment, BChl absorption is red-shifted in comparison to its free, monomeric form (BChl *a* absorbs at 770 nm in organic solvent). Shifts in absorption mainly depend on pigment-protein and pigment-pigment interaction (COGDELL *et al.*, 2002; ZUBER & COGDELL, 1995). BChl *a* associated LH1 antennae exhibit typically a single near infrared (NIR) absorption maximum between 870 and 890 nm (Q_y absorption) (ZUBER & BRUNISHOLZ, 1991). For some purple bacteria values up to 963 nm were reported (PERMENTIER *et al.*, 2001; GLAESER & OVERMANN, 1999; GARCIA *et al.*, 1986). BChl *b* associated antennae can reach NIR absorption maxima of about 1020 nm (ZUBER & BRUNISHOLZ, 1991).

The peripheral antennae typically exhibit two NIR absorption peaks. A maximum around 800 nm is usually related to the monomeric BChl *a*. A second peak at either 820 or 850 nm is present in dependence of the antenna type, LH2 or LH3 respectively. This absorption maximum is due to the dimeric BChls. Usually both peripheral antenna peaks are the most prominent in absorption spectra and often overlay the LH1 maximum.

Different spectral properties enable phototrophic bacteria to occupy different ecological niches. Modulation of spectral properties depends on pigment-protein and pigment-pigment interaction. However the fundamentals of these interactions and the molecular determinants of spectral properties are still not fully understood.

3.3 Photosynthesis genes of the purple bacteria

In purple bacteria most structural and functional photosynthesis genes are organized in a single photosynthesis gene cluster of about forty-thousand nucleotides (BEJA *et al.*, 2002; IGARASHI *et al.*, 2001; CHOUDHARY & KAPLAN, 2000; NAYLOR *et al.*, 1999; ALBERTI *et al.*, 1995). Within this cluster most genes are grouped into several operons. This "superoperonal" organization allows the coordinated expression and regulation of photosynthesis genes (BEATTY, 1995). The transcriptional units encompass especially the BChl biosynthesis genes (*bch*), the carotenoid biosynthesis genes (*crt*) and the structural genes for the photosynthetic core unit (*puf* and *puhA*) (Fig. 2B). The *puf* operon encompasses the genes *pufB* and *pufA* that encode for the light harvesting β and α polypeptides and the genes *pufL* and *pufM* that encode for the reaction center L- and M-subunit. The gene motive *pufBALM* is conserved in all purple bacteria. In most *puf* operons a *pufC* gene further encodes for a RC bound tetraheme cytochrome (Fig. 2B). However, presence or absence of the cytochrome gene 3' of *pufM* is species specific. Additional genes such as *pufQ* (5') or *pufX* (3') of the *pufBALM* core motive are restricted to a few species only (MASUDA *et al.*, 1999; HUNTER *et al.*, 1991; BAUER *et al.*, 1988). PufQ is thought to be involved in BChl synthesis (FIDAI *et al.*, 1995), whereas PufX seems to mediate electron transfer from and protein interactions within the RC-LH1 core unit (FRANCIA *et al.*, 2002; PARKES-LOACH *et al.*, 2001). Which genes accomplish these functions in the purple bacteria where *pufQ* and *pufX* are absent is currently unknown. Possible variations in the *puf* operon composition and extent became obvious only recently, when more sequence data became available for different purple bacteria (BEJA *et al.*, 2002; NAGASHIMA *et al.*, 2002; MASUDA *et al.*, 1999; GONG & KAPLAN, 1996). Also present within the photosynthesis gene cluster is *puhA*, the structural gene encoding the RC H-subunit. However, *puhA* is not part of the *puf* operon and located on the opposite side of the photosynthesis gene cluster (Fig. 2B).

In all investigated *puf* operons the affiliated genes were co-transcribed in a single transcript (BEATTY, 1995). The primary polycistronic messenger RNA is highly unstable and gets rapidly degraded into smaller, more stable segments. Degradation occurs via endonucleic and exonucleic events in which RNase E seems to play an important role (RAUHUT & KLUG, 1999; FRITSCH *et al.*, 1995; KLUG, 1995). The stability varies for the different degradation segments to meet the stoichiometric needs for a functional photosynthetic apparatus (the ratio between LH1 polypeptides PufBA and the RC proteins PufLM is about 16:1 per photosystem) (HECK *et al.*, 2000; KLUG, 1995). The resulting

transcripts encompass at least *pufBALM* and a more stable *pufBA* message. Secondary structures such as hairpins and loop structures act as stabilizing elements.

The peripheral antenna genes are also organized in a transcriptional unit. However, the so called *puc* operon is not part of the photosynthesis gene cluster. Located elsewhere on the chromosome it is less investigated. It consists at least of the structural genes *pucB* and *pucA* (SIMMONS *et al.*, 1999) and encode for the β and α subunits of the peripheral antennas, analogous to the *pufBA* genes. In addition *pucC*, *pucD* and *pucE* genes might be present, which are assumed to have regulatory and complex stabilizing functions (KLUG, 1995). Expression and regulation of the *puc* genes occur in close concert with the *puf* operon. Multiple copies of the structural *pucBA* genes were reported (SIMMONS *et al.*, 2000; GARDINER *et al.*, 1996; TADROS & WATERKAMP, 1989).

The current understanding about genetics and regulation of the photosynthetic apparatus is mainly derived from facultative phototrophic purple bacteria, such as *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*. The reason for this is (1) the good transformability of both species and (2) the ability to express their photosynthetic apparatus under low oxygen tension. Furthermore, cultivation of *Rhodobacter* species under low oxygen conditions still facilitates rapid growth rates, which makes handling convenient. Indeed oxygen is one of the major regulatory factors for photosynthesis gene expression in both species (GREGOR & KLUG, 1999). In contrast, the many purple (sulfur) bacteria that dominate in natural ecosystems are obligate phototrophs (VAN GEMERDEN & MAS, 1995) and express photosynthesis genes under anoxic conditions only. Genetic systems are difficult to establish for strictly anaerobes, thus knowledge about genetic and regulatory mechanisms for those species is poor.

4 Aims of this study

The main subjects explored in this thesis were (1) the variability of photosynthesis genes of purple bacteria and (2) molecular factors that determine spectral properties. Moreover, questions about transcription, phylogeny and evolution were addressed in terms of photosynthesis.

As outlined above, molecular aspects of photosynthesis are mainly derived from studies on purple non-sulfur bacteria. Especially members of the genera *Rhodobacter*, *Rhodospirillum* and *Rhodopseudomonas* received the major attention in this field of research (BLANKENSHIP *et al.*, 1995b).

In contrast little is known about the genetic background of photosynthesis genes and gene products in purple sulfur bacteria. For these bacteria complete *puf* operon sequences are available only for *Allochromatium vinosum* (NAGASHIMA *et al.*, 2002; CORSON *et al.*, 1999) and *Ectothiorhodospira shaposhnikovii* (Zhang & Gingras, 1997, Genbank record AF018955, unpublished data). This gap in knowledge is in sharp contrast to the important role purple sulfur bacteria can play in many natural environments (OVERMANN, 1997; VAN GEMERDEN & MAS, 1995). One aim of the present study was therefore to elucidate organization, structure, transcription and phylogenetic relations of photosynthesis genes of purple sulfur bacteria. To address these questions, the study focussed on the γ Proteobacterium *Amoebobacter* (*Amb.*) *purpureus* ML1, a purple sulfur bacterium from the family of the *Chromatiaceae*. It was isolated from the meromictic Mahoney Lake in North Western Canada, where it plays a crucial role in the ecology of the lake (OVERMANN, 1997). Cells of *Amb. purpureus* thrive in the chemocline as a dense purple layer of several centimeters in depth. Concentrations up to 27500 µg BChl *a* per liter were reported, the highest concentration ever found in a natural body of water (OVERMANN *et al.*, 1994). Gas vacuoles are characteristic and enable the spherical cells to move vertically by buoyancy regulation (OVERMANN & PFENNIG, 1992). *In vivo* NIR absorption peaks of *Amb. purpureus* indicated the presence of a LH3 type peripheral antenna complex with maxima at 800-825 nm and a LH1 core antenna complex that absorbs at 875 nm (Fig. 4) (OVERMANN *et al.*, 1991).

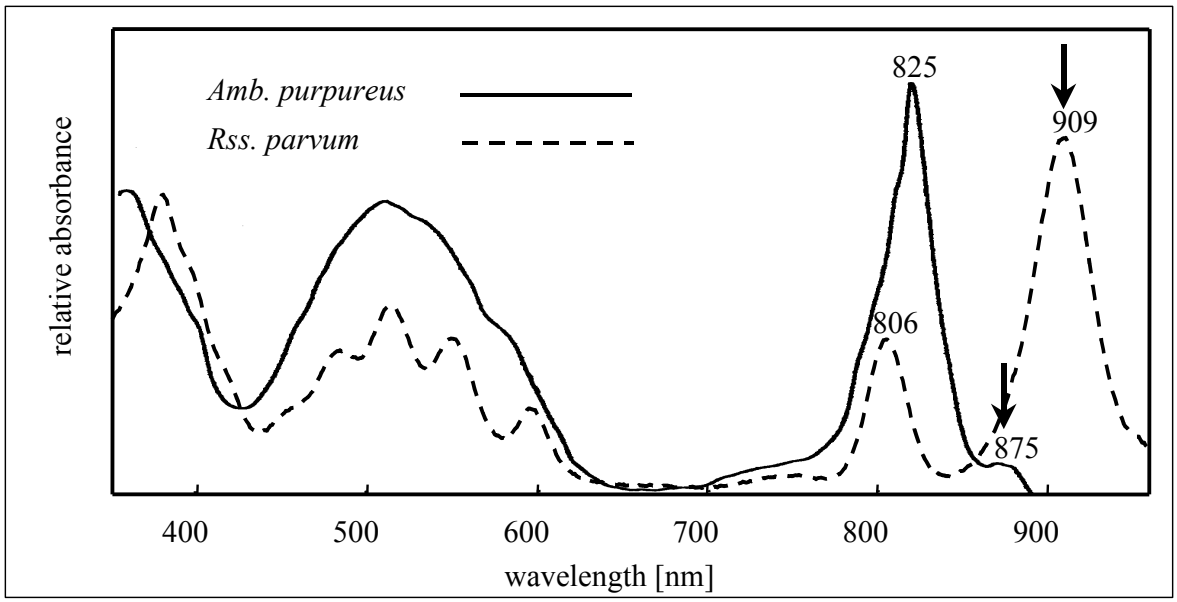

Figure 4: *In vivo* absorption spectra of *Amb. purpureus* and *Rss. parvum.* The near-infrared peak of the LH1 complex with 875 nm for *Amb. purpureus* and 909 nm for *Rss. parvum* are marked with arrows. Absorption peaks of the peripheral antenna complexes are also indicated (Spectra were adopted from OVERMANN *et al.*, 1991).

Only recently purple bacteria were described that exhibit red-shifted BChl *a* associated core antennae with absorption above 900 nm (PERMENTIER *et al.*, 2001; GLAESER & OVERMANN, 1999; FATHIR *et al.*, 1998; GARCIA *et al.*, 1986). This indicates that the variety of light harvesting complexes is greater than assumed so far. Pigment-pigment and pigment-protein interactions have the main impact on spectral variations (COGDELL *et al.*, 2002; COGDELL *et al.*, 1997; ZUBER & COGDELL, 1995). Although investigated by mutagenesis (OLSEN *et al.*, 1997; STURGIS *et al.*, 1997; HUNTER, 1995), the molecular details that govern those spectral variations are not fully understood. Purple bacteria that exhibit extraordinary spectral properties are model organisms for our understanding of spectral determinants. To explore these molecular determinants the thesis focussed on the purple non-sulfur bacterium *Roseospirillum* (*Rss.*) *parvum* 930I. It has been isolated from intertidal sediments of Great Sippewissett Salt Marsh, USA and was recently characterized and described as a new species (GLAESER & OVERMANN, 1999). Based on 16 rRNA phylogeny *Rss. parvum* is affiliated to the α-1 subclass of the Proteobacteria, where it forms a monophyletic branch. *Rss. parvum* is obligate anaerobic and cells are spirilloid shaped and highly motile. The strain exhibits an unusual LH1 long-wavelength absorption maximum at 909 nm (Fig. 4). No peripheral light harvesting complexes were described. Interestingly, a monomeric BChl *a* absorption peak at 805 nm is present that was attributed to the LH1 core antenna complex (PERMENTIER *et al.*, 2000).

5 This work

This thesis is presented in form of two manuscripts that have been submitted for publication.

Chapter II:

C. Tuschak, J.T. Beatty, J. Overmann (2003): The *puf* operon of the purple sulfur bacterium *Amoebobacter purpureus*: Structure, transcription and phylogenetic analyses. Archives of Microbiology, submitted.

Chapter III:

C. Tuschak, J.T. Beatty, J. Overmann (2003): Photosynthesis genes and pigment binding proteins of *Roseospirillum parvum* 930I, a purple non-sulfur bacterium with unusual spectral properties. Photosynthesis Research, submitted.

Parts have been published before in form of oral and poster presentations:

Oral presentations:

C. Tuschak, C. Höfler, J.T. Beatty, J. Overmann (2002): Modeling of pigment binding proteins from purple bacteria with unusual spectral properties: Structural and evolutionary implications. Oral presentation at the Annual Meeting of the Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM 2002), March 24-27, 2001, Göttingen, Germany.

C. Tuschak, J. Overmann, J.T. Beatty (2001): Structural basis for the variability of light-harvesting and reaction center proteins in purple bacteria. Oral presentation at the Annual Meeting of the Vereinigung für Allgemeine und Angewandte Mikrobiologie (VAAM 2001), March 25-28, 2001, Oldenburg, Germany.

Poster presentations:

C. Tuschak, J.T. Beatty, J. Overmann (2000): Variability of light-harvesting (LH1) and reaction center photosynthesis genes in purple bacteria. Poster presentation at the 10th International Symposium on Phototrophic Prokaryotes (ISPP), August 26-31, 2000, Barcelona, Spain.

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CHAPTER II

The *puf* operon of the Purple Sulfur Bacterium *Amoebobacter purpureus*: Structure, Transcription and Phylogenetic Analysis

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Keywords: *puf* genes · light-harvesting complexes · reaction center · purple sulfur bacteria · Chromatiaceae

Abbreviations: *aa* amino acids, *Alc*. *Allochromatium*, *BChl* bacteriochlorophyll, *Ect*. *Ectothiorhodospira*, *EDTA* ethylendiamine tetraacetic acid, *LH* light harvesting complex, *RC* reaction center, *Rbc. Rhodobacter*, *Rps*. *Rhodopseudomonas*, *Rsp. Rhodospirillum*, *MOPS* 3-(N-morpholino)propanesulfonic acid, *orf* open reading frame, *Thc*. *Thermochromatium*

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1 Abstract

The *puf* operon of the purple sulfur phototrophic bacterium *Amoebobacter* (*Amb.*) *purpureus* was cloned and sequenced, revealing an unusual operon consisting of the genes *pufB*$_1$*A*$_1$*LMCB*$_2$*A*$_2$*B*$_3$*A*$_3$. The presence of a *bchZ* gene 5' of *pufB*$_1$ indicates a linkage of *puf* and *bch* genes as in other species. This sequence represents the second complete *puf* operon available for *Chromatiaceae*. The presence of additional sets of light-harvesting (LH) genes, *pufB*$_2$*A*$_2$ and *pufB*$_3$*A*$_3$ at the 3' terminus of *pufC* so far has been described only for *Allochromatium* (*Alc.*) *vinosum*. Along with reports of multiple LH1 polypeptides found in some *Ectothiorhodospiraceae* by direct protein sequencing, our results indicate that multiple LH1 genes may occur frequently in phototrophic γ proteobacteria. Phylogenetic analyses suggested a coevolution of the core *puf* genes *pufB*$_1$*A*$_1$*LM*. Previously, the occurrence of multiple *pufBA* genes in *Amb. purpureus* and *Alc. vinosum* has been attributed to gene duplications in a common ancestor. Separate analysis of the LH1 α and β polypeptides indicated a high intraspecies relatedness for the secondary LH1β polypeptides, which is possibly caused by functional constraints. In contrast, LH1α subunits of *Amb. purpureus* and *Alc. vinosum* are closely related (85 % sequence identity) which could reflect horizontal gene transfer. RNA analyses suggested co-transcription of all *puf* genes in *Amb. purpureus* on an unusually long primary transcript of 5.8 kb. Although presumed mRNA degradation products of 2.7 and 1.5 kb and strong 0.8, 0.6 and 0.5 kb signals were detected, the primary transcript may be more stable than the *puf* operon primary transcripts of purple non-sulfur phototrophic bacteria. Regulation of expression of *puf* genes in *Amb. purpureus* under different light condition also differs from the pattern observed in purple non-sulfur bacteria.

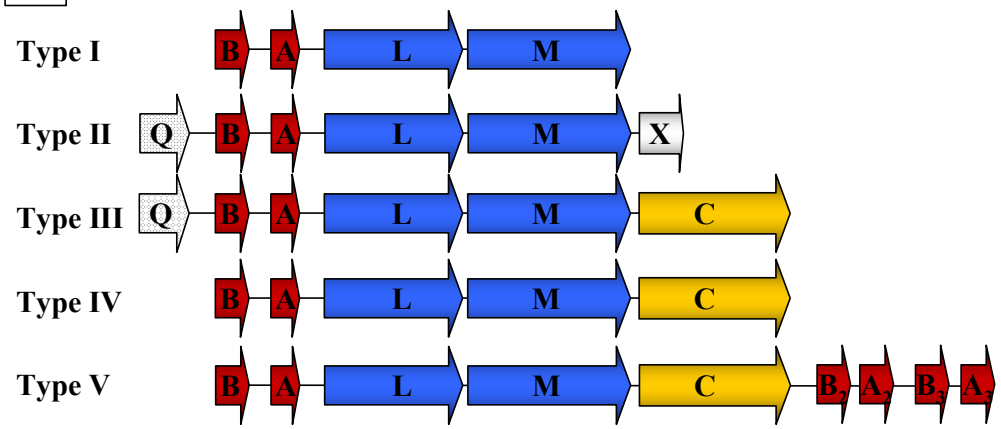
2 Introduction

Compared to oxygenic phototrophs, purple bacteria contain a less complex photosynthetic apparatus, consisting of one to three types of antenna complexes and the reaction center. All purple bacteria contain a core light-harvesting antenna complex (LH1). LH1 is thought to consist of about 16 heterodimeric protein subunits (α and β), which possibly form a ring around the reaction center (RC) (COGDELL *et al.*, 1999; PAPIZ *et al.*, 1996). In most purple bacteria, additional light-harvesting antenna complexes (LH2 or LH3) are present (MCLUSKEY *et al.*, 2001; ZUBER & COGDELL, 1995). The rings of these peripheral antenna complexes are formed by eight to nine heterodimeric subunits (α and β) (HU *et al.*, 1998; PAPIZ *et al.*, 1996).

The genes encoding the RC-LH1 core unit and the peripheral antenna complexes LH2 or LH3 are clustered in operons, called *puf* and *puc*, respectively. In all purple bacteria investigated so far, the *puf* operon contains the structural genes *pufBALM*, which code for the core antenna complex (β and α subunits of LH1) and the reaction center L and M apoproteins. With the exception of *Rhodospirillum* (*Rsp.*) *rubrum* (BELANGER & GINGRAS, 1988), *Rhodopseudomonas* (*Rps.*) *palustris* (http://genome.ornl.gov/microbial/rpal/1/rpal_1.html) and *Bradyrhizobium sp. ORS278* (GIRAUD *et al.*, 2000), additional *puf* genes are found in the other purple bacteria. Five different *puf* operon types can be distinguished (Fig. 1). A *pufQ* gene was reported to occur 5' of *pufB* in three species of purple non-sulfur bacteria (MASUDA *et al.*, 1999; BAUER *et al.*, 1988), and is thought to play a role in bacteriochlorophyll (BChl) synthesis (FIDAI *et al.*, 1995). In addition, a *pufK* gene was reported to exist in *Rhodobacter* (*Rbc.*) *sphaeroides*, and to be involved in translational control of *puf* operon expression (GONG & KAPLAN, 1996). A *pufX* gene was found 3' of *pufM* in *Rbc. capsulatus* and *Rbc. sphaeroides*; the PufX protein appears to mediate polypeptide interaction in the RC-LH1 core complex and to facilitate electron transfer from the reaction center to the bc_1-complex (FRANCIA *et al.*, 2002; PARKES-LOACH *et al.*, 2001). All other *puf* operons contain a terminal *pufC* gene 3' of *pufM*. The *pufC* gene encodes a reaction center-bound tetraheme cytochrome that serves as the electron donor for the oxidized reaction center after photon excitation (NITSCHKE & DRACHEVA, 1995). An orf 641 homologue is located 5´of *pufC* in several species.

With a few exceptions, the structure, function and evolution of genes and proteins involved in bacterial photosynthesis have only been investigated for purple non-sulfur

A



B

Operon	Species	Strain	Phylogenetic affiliation
Type I	*Rhodospirillum rubrum*	ATCC 11170^T	α-proteobacterium
	Rhodopseudomonas palustris	ATCC BAA-98	α-proteobacterium
	Bradyrhizobium sp.	ORS278	α-proteobacterium
Type II	*Rhodobacter capsulatus*	ATCC 11166^T	α-proteobacterium
	Rhodobacter sphaeroides	ATCC 17023^T	α-proteobacterium
Type III	*Rhodovulum sulfidophilum*	DSM 1374^T	α-proteobacterium
Type IV	*Blastochloris viridis*	DSM 133^T	α-proteobacterium
	Acidiphilium angustum [a]	ATCC 35903^T	α-proteobacterium
	Acidiphilium rubrum [a]	ATCC 35905^T	α-proteobacterium
	Phaeospirillum molischianum	ATCC 14031^T	α-proteobacterium
	Roseobacter denitrificans	ATCC 33940^T	α-proteobacterium
	Roseateles depolymerans	DSM 11813^T	β-proteobacterium
	Rubrivivax gelatinosus	IL144	β-proteobacterium
	Ectothiorhodospira shaposhnikovii [a]	DSM 243^T	γ-proteobacterium
	Thermochromatium tepidum [a]	ATCC 43061^T	γ-proteobacterium
Type V	*Allochromatium vinosum*	ATCC 17899^T	γ-proteobacterium
	Amoebobacter purpureus	ML1	γ-proteobacterium

[a] uncertain assignment due to insufficient sequence information downstream of *pufC*

Figure 1: (**A**) Different types of *puf* operons reported for purple non-sulfur bacteria and purple sulfur bacteria (*Chromatiaceae* and *Ectothiorhodospiraceae*). A common core motif in all *puf* operons reported is *pufBALM*. Variations are found 5' and 3' of this core motif. (**B**) Purple bacteria that can be assigned to the five defined operon types, of which type IV occurs most frequently. Species with only partial *puf* operon information available were excluded.

bacteria (NAGASHIMA *et al.*, 1997a; LANCASTER *et al.*, 1995; LOACH & PARKES-LOACH, 1995; ZUBER & COGDELL, 1995), and current models of the photosynthetic apparatus are largely based on studies of members of the genera *Rhodobacter*, *Rhodopseudomonas* and *Rhodospirillum* (HU *et al.*, 1998; PAPIZ *et al.*, 1996). Complete *puf* operon sequences are available from only two purple sulfur bacteria, *Allochromatium* (*Alc.*) *vinosum* (NAGASHIMA *et al.*, 2002; CORSON *et al.*, 1999) and *Ectothiorhodospira* (*Ect.*) *shaposhnikovii* (Zhang, S. and Gingras, G. 1997, unpublished, GenBank record AF018955). An incomplete *puf* operon sequence is available for *Thermochromatium* (*Thc.*) *tepidum* (FATHIR *et al.*, 1998; FATHIR *et al.*, 1997).

The *puf* operon of *Alc. vinosum* features an unusual organization that includes additional sets of *pufBA* genes 3' of *pufC* (CORSON *et al.*, 1999) (Fig. 1A). Furthermore, direct protein sequencing of antenna subunits of some *Ectothiorhodospira* spp. revealed that more than one type of LH1 α and β polypeptides exists in these purple sulfur bacteria (WAGNER-HUBER *et al.*, 1992; ZUBER & BRUNISHOLZ, 1991). The so far unique genetic organization in *Alc. vinosum* raises the question whether fundamental differences exist between purple sulfur and purple non-sulfur bacteria with respect to the structure and organization of the photosynthetic apparatus. In contrast to purple non-sulfur bacteria, *Chromatiaceae* form dense blooms in many aquatic environments. In the majority of cases, however, bacteria containing the carotenoid okenone dominate (VAN GEMERDEN & MAS, 1995). The purple sulfur bacterium *Amoebobacter* (*Amb.*) *purpureus* strain ML1, a dominant anoxygenic phototroph in a natural saltwater lake (OVERMANN, 1997; OVERMANN *et al.*, 1991), contains okenone, and absorption spectra indicate the presence of a LH3 type peripheral antenna complex (800-820 nm) and a LH1 core antenna complex (875 nm) (OVERMANN *et al.*, 1991). *Amb. purpureus* ML1 therefore differs considerably from *Alc. vinosum*. The structure, function and evolution of pigment-binding proteins in *Amb. purpureus* ML1 were investigated in the present study.

3 Materials and Methods

Cell cultivation

Amb. purpureus strain ML1 was grown anaerobically using the basal medium for *Chromatiaceae* (OVERMANN & PFENNIG, 1992). Sulfide served as an electron-donating substrate and CO_2 and acetate as carbon sources for mixotrophic growth (OVERMANN & PFENNIG, 1992). Cultures were routinely incubated at an incident light intensity of 100 µmol quanta · m^{-2} · s^{-1}, which was decreased to 50 µmol quanta · m^{-2} · s^{-1} in experiments on light regulation of mRNA transcripts. Light intensities were measured with a LI-185B light meter equipped with a LI-200SB pyranometer sensor (LI-COR, Lincoln, Neb., USA). Growth was monitored by epifluorescence microscopy of cells collected on polycarbonate filters (Millipore GmbH, Schwalbach, Germany), after staining with 0.2 mg · ml^{-1} of 4',6-diamidino-2-phenylindole.

For cloning and subcloning, *Escherichia coli* strain DH5α (GIBCO-BRL, Invitrogen Canada Inc., Burlington, Ontario, Canada) was employed. This strain was grown in Luria Bertani medium (SAMBROOK *et al.*, 1989), supplemented with ampicillin (200 µg · ml^{-1}) for plasmid maintenance.

PCR

Polymerase chain reactions (PCR) were performed in a GeneAmp PCR system 2400 using the GeneAmp PCR Reagent Kit (Applied Biosystems Canada, Streetsville, Ontario, Canada). A 1.5 kb-fragment from the L and M subunit of the reaction center was amplified with primers pufL-F and pufM-R (Table 1) (NAGASHIMA *et al.*, 1997a). For PCR, a touchdown protocol was employed, using 50 nanograms of genomic DNA. After a hot start at 95 °C for 5 min, 30 cycles followed, each consisting of a denaturation step at 94 °C for 30 s, an annealing step for 30 s and an extension step at 72 °C for 2 min. For touchdown, annealing started at 60 °C and the temperature was lowered by an increment of -0.4 °C per cycle for 25 cycles, after which 5 cycles at 50 °C followed. The PCR product was purified with the QIAquick PCR purification kit (Qiagen Inc., Mississauga, Ontario, Canada), directly sequenced for verification, and employed in the generation of probe II (Fig. 2B) for hybridizations.

Table 1: Oligonucleotides used for PCR and sequencing

Primer	Sequence	Source
Z-292-F	5´-agg cga tcc aca cca aga c	this study
Z-F	5´-tgt tcg a(t/c)g c**c**c tgt t	(NAGASHIMA *et al.*, 1997b) *
Z-R	5´-aac ag**g** gc(a/g) tcg aac a	(NAGASHIMA *et al.*, 1997b) *
Ap-B2-R	5´-ctg cgt gaa gat gcc gtg	this study
pufL-F	5´-ct(t/g) ttc gac ttc tgg gt(c/g) gg	(NAGASHIMA *et al.*, 1997a)
pufL-R	5´-cc(g/c) acc cag aag tcg aa(a/c) ag	(NAGASHIMA *et al.*, 1997a)
L250-F	5´-ct(**g/t**) tgg cag atc atc ac	(NAGASHIMA *et al.*, 1997b) *
L250-R	5´-gtg atg atc tgc ca(**a/c**) ag	(NAGASHIMA *et al.*, 1997b) *
L480-F	5´-tgg gtg tcg aac **gtc** gg**c** tac	(NAGASHIMA *et al.*, 1997b) *
M2-F	5´-ccg ttc gg(g/c) atc ttc c	this study
M2-R	5´-gga aga t(c/g)c cga acg g	this study
M3F	5´-ggc ggc gac cgt gag	this study
M3R	5´-ctc acg gtc gcc gcc	this study
pufM-R	5´-ccc at(c/g) gtc cag cgc cag aa	(NAGASHIMA *et al.*, 1997a)
C310-F	5´-gt**c** acc tgc t(**g**/a)(**g**/c) acc tgc ca	(NAGASHIMA *et al.*, 1997b) *
ApC310-R	5´-tgg cag gtg tag cag gt**g** ac	(NAGASHIMA *et al.*, 1997b) *
ApC2-F	5´-cag gga gcc tat aag ccg	this study
ApC2-R	5´-cgg ctt ata ggc tcc ctg	this study
ApCend-F	5´-ggc gga cga tgc acc ac	this study
Ap-puf386-R	5´-cag aat cgg cgg gcg tta tg	this study
Ap-pufB2A-F	5´-ccg ccg att ctg aag ttc t	this study
Ap-puf766-R	5´-gga agt tac ctc cag ttt gct c	this study
Ap-pufB3-F	5´-cga gca aac tgg agg taa ctt c	this study
Ap-puf934-R	5´-gga gaa acc ctg agg cgt ag	this study
Ap-puf1193-F	5´-atc cga ctc agc gta tgg	this study
Ap-puf1363-R	5´-cgt gag tcc gag atg ggg ctg	this study
Ap-puf1553-F	5´-ttc aga atc agc cat caa cg	this study
Ap-puf1951-F	5´-cca ggc ggt ggc gga tac	this study
Ap-Sac01-R	5´-gcc gaa gat cgc tgt cca a	this study
Ap-puf2256F	5´-gca gcc aaa cgc tcg cag ac	this study
Ap-pufC18-R	5´-gcg aga acc agc gat ttg	this study
Ap-puf2633-F	5´-cgg gag cga cca act act g	this study
Ap-Pae01-R	5´-ggc gat cag caa cac acc ct	this study
Ap-puf2994-F	5´-ggc gtg aag gca aag gac	this study
Ap-Sac02-R	5´-gcg ttg atg gct gat tct g	this study
Ap-pufA3-F	5´-ccg act tca act ggc tcg	this study
-21M13f	5' -tgt aaa acg acg gcc agt	Applied Biosystems
M13R	5´-cag gaa aca gct atg acc	Applied Biosystems

* Primers that were modified in comparison to the reference. Bold letters indicate modified nucleotides

Sequence information for the region 3' of *pufC* was obtained by an inverse-PCR (iPCR) approach (OCHMAN *et al.*, 1993). For iPCR, chromosomal DNA was digested with the restriction endonucleases *Bgl* II, *Pae* I or *Sac* I (MBI Fermentas GmbH, St. Leon-Rot, Germany). Restriction enzymes were chosen based on 5' *puf* sequences as determined in the present study. Digested DNA was purified with the QIAquick PCR purification kit (Qiagen). Three micrograms of DNA fragments were ligated for 16 hours at 15 °C in a volume of 1.5 ml using 30 U T4 DNA ligase (MBI Fermentas). Afterwards, samples were concentrated and washed once with water in Centricon 100 units (Millipore). Subsamples containing 100 - 250 ng of DNA were amplified with the primer ApCendF and ApC310R (Table 1) using a PCR protocol consisting of a hot start at 95 °C for 5 min, followed by 30 cycles with a denaturation step at 94 °C for 30 s, an annealing step at 62 °C for 1 min and an extension step at 72 °C for 4 min. For iPCR, AmpliTaq Gold DNA Polymerase (Applied Biosystems) was used. The addition of 5 % acetamide to the reaction mix turned out to be indispensable to obtain specific products. The iPCR products from all three different restriction approaches were sequenced in order to minimize sequencing errors.

Cloning and Sequencing

Standard recombinant DNA procedures were employed throughout (SAMBROOK *et al.*, 1989). For chromosomal DNA extraction, cells were harvested by centrifugation, resuspended in TE-buffer, and were lysed by addition of sodium dodecyl sulfate (SDS) to a final concentration of 1 % and incubation at 70 °C for 30 minutes. The crude extract was cooled, proteinase K (0.5 mg · ml^{-1}) added, and the extract incubated at 37 °C for 30 minutes. During all incubation steps the samples were vortexed repeatedly. Samples were extracted with phenol and genomic DNA purified with the Genomic-tip System 100 (Qiagen). One microgram of chromosomal DNA of *Amb. purpureus* was digested with restriction endonucleases, separated by gel-electrophoresis and transferred to a Biotrans nylon membrane (ICN, Irvine, Canada) (SAMBROOK *et al.*, 1989). Two *Pst* I fragments 2.2 and 2.4 kb in size were identified by hybridization with probe II (see below) to contain portions of the genes *pufL* and *pufM* (Fig. 2). Both fragments were cloned into pUC13 and the correct clones identified by hybridization of colonies blotted onto cellulose filter discs (Whatman 541; Whatman Inc., Clifton, New Jersy, USA). Plasmid DNA was purified with the QIAprep Spin Miniprep Kit (Qiagen), clones from individual positive colonies were tested by restriction analysis, and inserts of the expected size further subcloned for sequencing.

Cloned and subcloned fragments were sequenced with standard -21M13 and M13R primers (Table 1), and missing sequence stretches were obtained by primer walking (see Table 1 for primer sequences).

Generation of hybridization probes

Three probes were used in DNA and RNA blot analysis (as indicated in Fig. 2B). Based on restriction analysis of the sequences obtained from the cloned *Pst* I-fragments (Fig. 2A), a sub-fragment containing the *pufBA* genes (0.6 kb, *Sma* I to *Sac* I) and a sub-fragment containing almost the entire *pufC* gene (1.1 kb, *Ava* I to *Pst* I) were released from corresponding plasmids by digestion. After gel electrophoresis, these sub-fragments were purified and used as templates for the generation of probes I and III. The 1.5 kb PCR product complementary to *pufLM* was employed as the template for probe II (see above). Probes were random prime labeled with radioactive [^{32}P]dCTP using the Rediprime DNA labeling system (Amersham Pharmacia Biotech Inc., Baie d'Urfé, Québec, Canada) according to the manufacturer´s instructions.

Extraction of RNA, gel electrophoresis, blotting and hybridization

Cells of *Amb. purpureus* were grown to the late exponential phase (~1.6 - 1.8 · 10^8 cells · ml^{-1}) and total RNA was extracted using the Qiagen RNeasy Midi kit (Qiagen) according to the manufacturer´s protocol with the following modifications. 14 ml liquid culture (~2.2 · 10^9 cells) were centrifuged at 17,000 × *g* for 3 min at 4 °C. Cells were resuspended in TE and, since *Amb. purpureus* forms a strong capsule, treated with an elevated lysozyme concentration (5 mg · ml^{-1}) for 8 min at room temperature. Subsequent addition of the Qiagen kit lysis buffer (RLT-buffer, containing guanidine isothiocyanate) halted nucleolytic degradation. Samples were frozen at -80 °C until further processing. RNA concentrations were determined spectrometrically and 10 µg of RNA were ethanol precipitated and dissolved in 5 µl water. Ten microliters of denaturing mix consisting of 14 % 1 × MOPS buffer (20 mM 3-(N-morpholino)propanesulfonic acid, 1 mM EDTA and 5 mM sodium acetate; pH 6.5), 17 % formaldehyde and 69 % formamide were added to the samples. Samples were then denatured at 68 °C for 10 min (ROSEN *et al.*, 1990). RNA was cooled down on ice for 10 min and 5 µl loading buffer (50 % glycerol with ethidium bromide 1 mg · ml^{-1}) were added to samples prior to loading. Electrophoresis was performed in agarose-formaldehyde gels (1 % agarose, in 1 × MOPS-buffer, supplemented with 2 M formaldehyde) overnight at 30 V, with constant recirculation of the buffer. RNA

blotting and hybridization were performed essentially as described previously (AKLUJKAR *et al.*, 2000). After gel equilibration in 5 × and 0.5 × TBE, RNA was transferred onto a Biotrans-(+) nylon membrane (ICN) by electroblotting in 0.5 × TBE for 2 hrs at 80 V with a plate electrode Trans-Blot transfer cell (Bio-Rad). The membrane was baked at 80 °C for two hours under vacuum, and prehybridized at 42 °C in 10 ml of hybridization buffer (50 % formamide, 10 % dextran sulfate, 1 % SDS, 0.2 % bovine serum albumin (BSA), 0.2 % Ficoll, 0.2 % polyvinylpyrrolidone, 0.1 % sodium pyrophosphate, 50 mM Tris-HCl pH 7.5 and 0.1 mg · ml^{-1} sheared salmon sperm DNA). After 2 - 4 hours of prehybridization, a radiolabeled probe (see above) was added and hybridized for 16 - 20 hours. All incubation steps were carried out in a Tek*Star hybridization oven (Bio/Can Scientific Inc., Mississauga, Ontario, Canada) under constant agitation. Subsequently, the membrane was washed twice with 2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate) for 10 min at room temperature, rinsed once with 2 × SSC (containing 1 % SDS) for 20 min at 60 °C, and once with 0.1 × SSC for 10 min at room temperature. Finally, membranes were exposed to X-ray film (Kodak Canada Inc., Burnaby, British Columbia, Canada). For reprobing, blots were stripped in boiling SDS (0.1 %) according to the manufacturer´s instruction (ICN).

Sequence analyses

Sequences were compared to databases using the BlastX program (ALTSCHUL *et al.*, 1997) and were processed with DNA-Strider version 1.3 (DOUGLAS, 1995), GeneDoc 2.6.001 (NICHOLAS & NICHOLAS, 1997) or GeneWorks 2.5 (Oxford Molecular Group) (BROVEAK, 1996). Stem loop structures were predicted with the software DNA Strider. Free energy for stabilities of secondary structures was calculated using the program mfold version 3.1 by Zuker and Turner (ZUKER *et al.*, 1999). Related sequences of photosynthesis genes and proteins were retrieved from the GenBank database (BENSON *et al.*, 2002) for comparison. Preliminary sequence data for *Rhodopseudomonas palustris* were obtained from the DOE Joint Genome Institute (JGI) at http://www.jgi.doe.gov/JGI_microbial/html/index.html. Raw sequence data were aligned with Clustal X version 1.8 (THOMPSON *et al.*, 1997).

Both ends of the amino acid alignments showed a high variability in length of individual sequences. Therefore, the terminal regions were omitted from further alignment analysis if more than 50 % of the sequences showed gaps. Some PufC sequences were incomplete at the C-terminus, and so information C-terminal of the fourth heme motif of all species was omitted from analysis. These procedures yielded amino acid (aa) data sets

of 51 aa for the LH1 β-polypeptide, 61 aa for the LH1 α-polypeptide, 293 aa for the RC L-subunit, 252 aa for the RC M-subunit and 346 aa for the tetraheme cytochrome. Phylogenetic analysis was performed with individual and concatenated (β, α, L and M polypeptide) alignments, using the programs in the Phylogeny Inference Package, PHYLIP, Version 3.6(alpha3) (FELSENSTEIN, 2002). Since not all purple bacteria possess a tetraheme cytochrome, the PufC data set was omitted from the concatenated analysis and analyzed separately. The LH antennae genes and proteins of γ-proteobacteria were also subjected to phylogenetic analyses in a data set of 51 aa created from a combined alignment of α and β polypeptides from LH1 and LH2. These amino acid sequences were either deduced from gene sequences or derived from polypeptide sequences determined by direct protein sequencing.

Distance matrices were calculated with PROTDIST f the PHYLIP package, employing the PAM 001 matrix (DAYHOFF *et al.*, 1979) and trees generated with FITCH. For comparison, additional trees were calculated with the algorithm for maximum likelihood (PRO TML) and maximum parsimony (PROTPARS). Trees were constructed using the global rearrangement option with a randomized species input order (jumble: random number seed 133, 10 times). Bootstrap analysis was performed with 100 data sets (jumble: random number seed 133, 3 times) employing all three algorithms. A table of pairwise % sequence identity was created with PROTDIST.

Nucleotide sequence accession number

Nucleotide and deduced protein sequence information of the *puf* operon of *Amb. purpureus* has been deposited in the GenBank database under accession number AY177752.

4 Results

Structure and organization of *puf* genes in *Amb. purpureus*

Employing the homologous probe II (Fig. 2) two *Pst* I fragments (2.2 and 2.4 kb) were detected which contained the *puf* operon of *Amb. purpureus*. Both fragments were cloned and subsequently sequenced with a two- to four-fold coverage. The nucleotide sequence obtained encompasses five open reading frames, each with a high homology to known structural *puf* genes, $pufB_1$ and $pufA_1$ (encoding the LH1 β and α subunits), *pufL* and *pufM* (encoding the RC L and M subunits) and *pufC* (encoding the RC-bound tetraheme cytochrome) (Fig. 2). Because the *puf* operon of the purple sulfur bacterium *Alc. vinosum* contains additional LH1 genes 3' of *pufC* (Fig. 1) (CORSON *et al.*, 1999), the nucleotide sequence of *Amb. purpureus* 3' of the cloned *Pst* I fragments was analyzed by an inverse PCR approach. Within this region sequence information was verified by three-fold coverage up to nucleotide position 6000. Sequence analysis revealed the presence of two additional sets of LH1 genes ($pufB_2A_2$ and $pufB_3A_3$, Fig. 2). The region 3' of nucleotide 6000 was covered one-fold by sequencing and showed the highest sequence similarity to cytochrome P450 homologues of, among others, *Rhodopseudomonas palustris*, *Chloroflexus aurantiacus*, *Agrobacterium tumefaciens* and *Arabidopsis thaliana*. The 3' end of a *bchZ* gene homologue, encoding a chlorophyllide reductase subunit (ALBERTI *et al.*, 1995), is located 5' of $pufB_1$. In the intergenic region between *bchZ* and $pufB_1$ a putative orf is located that starts within the *bchZ* stop codon and encodes 69 amino acids with no significant similarity to known proteins. Overall, a total of 7444 bases were sequenced, revealing a *puf* operon structure of $pufB_1A_1LMCB_2A_2B_3A_3$.

As in other purple bacteria, putative mRNA secondary structure determinants could be identified within intercistronic regions (Fig. 2; Fig 3, I - VII). One putative hairpin is predicted 5' of *pufB* (I: ΔG = -16.6 kcal · mol^{-1}), and two possible hairpin structures 3' of *pufA* (II: ΔG = -20.8 kcal · mol^{-1} and III: ΔG = -14.4 kcal · mol^{-1}). Additional stem-loops were predicted for the intercistronic region between *pufC* and $pufB_2$ (IV: ΔG = -14.4 kcal · mol^{-1}), between the second and third set of LH1 genes (V, ΔG = -15.0 kcal · mol^{-1}) and at the end of the *puf* operon 5' of $pufA_3$ (VI, ΔG = -20.4 kcal · mol^{-1} and VII: ΔG = -26.4 kcal · mol^{-1}). A sequence motif was found within the *bchZ* gene approximately 600 nucleotides 5' of the $pufB_1$ start codon (Fig. 2 and 3) that is similar to *puf* and related promoters of *Rhodobacter* species (SWEM *et al.*, 2001).

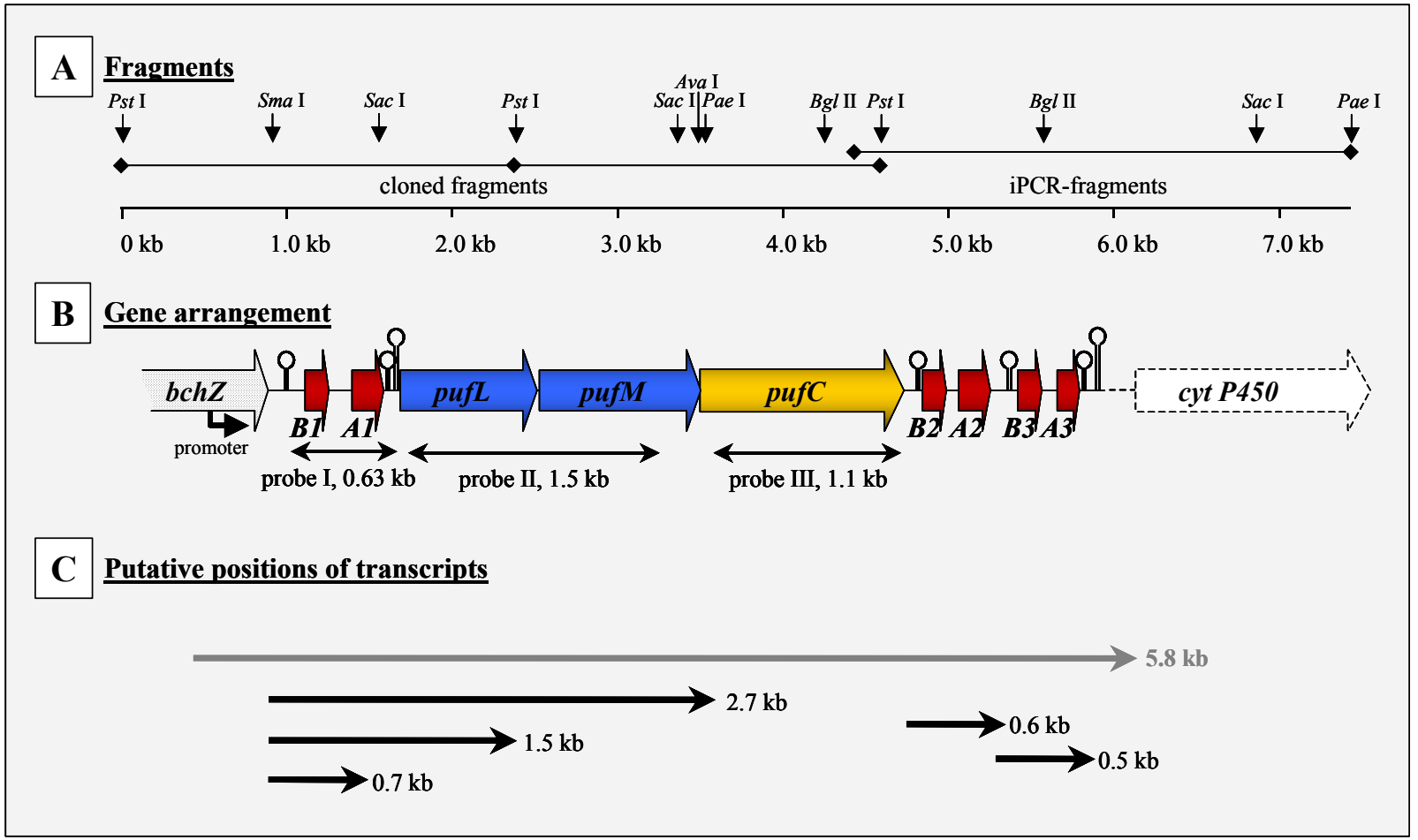


Figure 2: The *puf* operon of *Amb. purpureus*. (**A**) Cloned and iPCR-amplified fragments. *Pst* I restriction sites used for cloning, *Bgl* II, *Pae* I and *Sac* I sites used for iPCR and restriction sites relevant for probe construction are also shown. (**B**) Large filled horizontal arrows indicate the arrangement of putative genes identified on a 7444 bp long section of the chromosome. Predicted hairpin structures are indicated as loops. Double-headed arrows mark probes used for hybridization. A putative promoter was localized within the 3'-end of the *bchZ* gene and is shown. (**C**) Transcripts deduced from RNA blot hybridizations (compare Fig. 3) are indicated by bold arrows. The primary 5.8 kb message is assumed to be initiated at the putative promoter site, within the 3' end of the *bchlZ* gene. The smaller, post-transcriptionally modified messages are predicted to start just 5' of the various *pufB* genes.

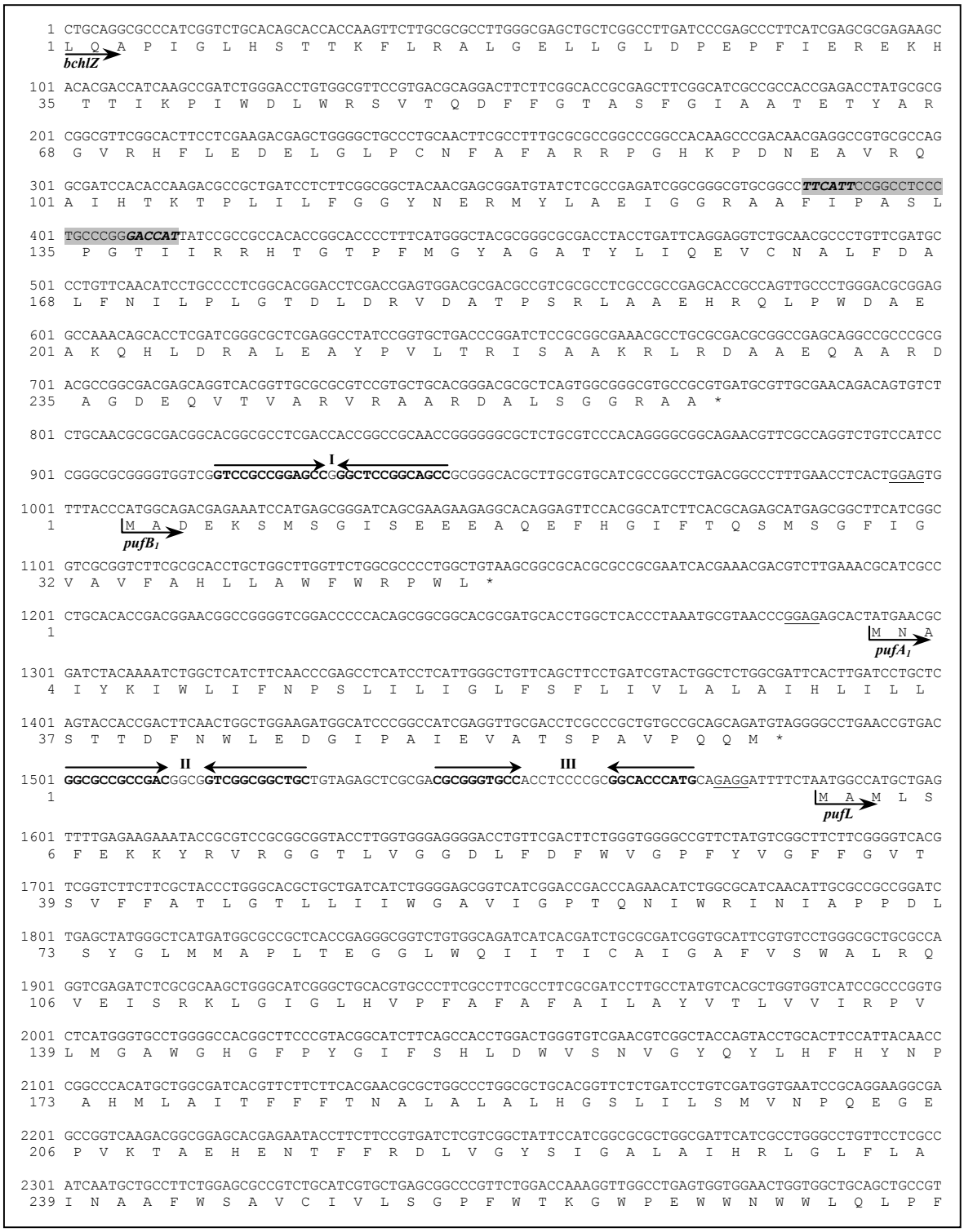


Figure 3...

Figure 3: continued...

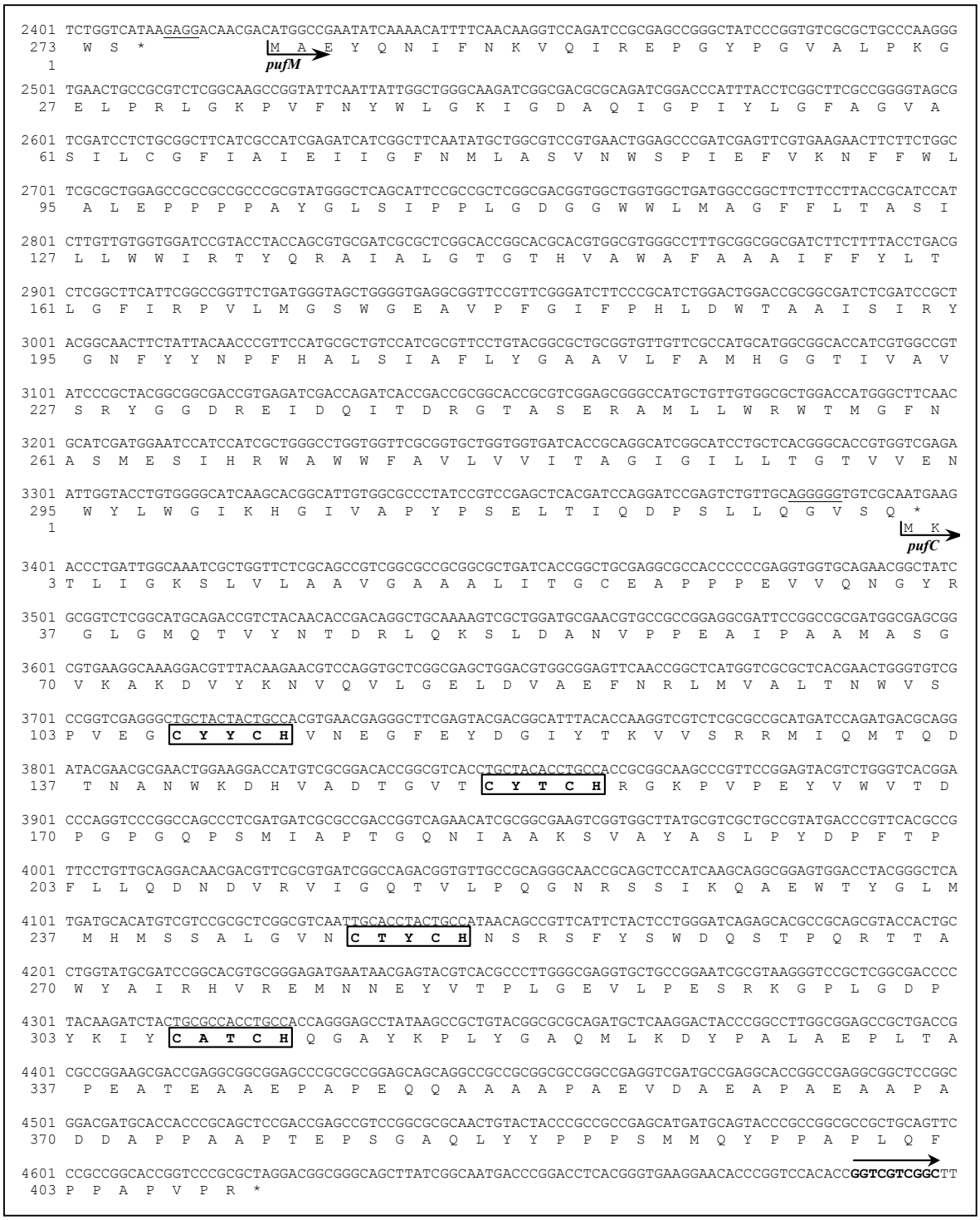


Figure 3...

Figure 3: continued

Figure 3: Nucleotide sequence and the deduced amino acid sequences of predicted *puf* genes of *Amb. purpureus*. The start codon for each gene is marked with an angled arrow below the amino acid sequence. Predicted hairpin-loop structures are indicated by head to head arrows above the DNA sequence and numbered I to VII. Putative Shine-Dalgarno sequences upstream the 5'-ends of the predicted genes are underlined. Possible -10 and -35 *puf* promoter motifs in the *bchZ* gene are shaded gray. Four heme-binding motifs (C-x-x-C-H) in the translated *pufC* sequence are framed in boxes.

Analysis of *puf* transcripts in *Amb. purpureus*

Initiation of transcription within the *bchZ* gene coding sequence has been demonstrated for *Rbc. capsulatus*, *Rbc. sphaeroides* and other purple bacteria (WIESSNER *et al.*, 1990; BELANGER & GINGRAS, 1988). In *Amb. purpureus*, transcription initiation within the 3' portion of the *bchl Z* gene (Fig. 3) would result in a primary transcript with a minimum size of ~5.4 kb if all genes are co-transcribed. Accordingly, the length of *puf* transcripts was tested by RNA blot analysis (Fig. 4). When probes I, II and III (which are derived

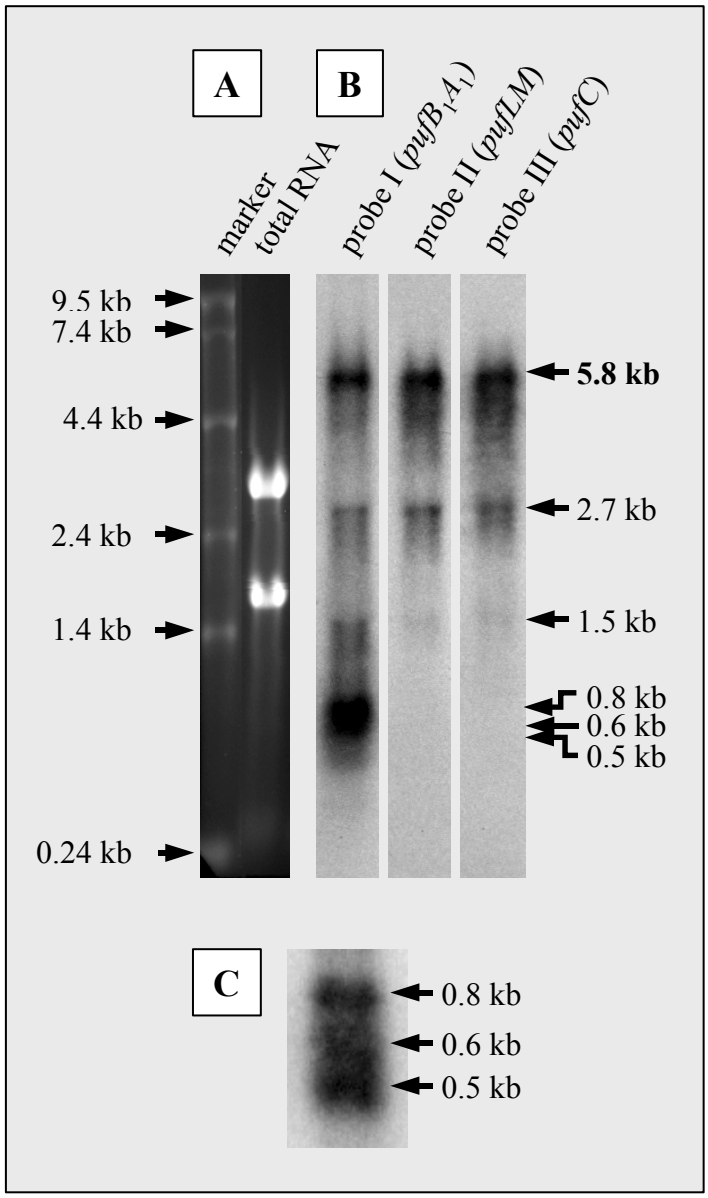


Figure 4: RNA blot analysis of *Amb. purpureus puf* transcripts. (**A**) Total RNA after separation by gel electrophoresis and ethidium bromide staining. The two signals visible on the gel represent ribosomal 16S and 23S rRNA. A size marker is shown for reference. (**B**) RNA hybridized with radiolabeled probe I (complementary to $pufB_1A_1$), probe II (complementary to *pufLM*) and probe III (complementary to *pufC*) (compare Fig. 2). (**C**) The low molecular weight transcripts detected with probe I only (panel B), could be separated at a higher resolution into three distinct mRNA species.

from three different portions of the *puf* operon) were used, a prominent message of 5.8 kb was detected with all three probes (Fig. 4). It is therefore feasible that the primary transcript encodes at least the *pufB*$_1$*A*$_1$*LMC* genes. If transcription starts at the putative promoter site, *pufB*$_2$*A*$_2$ and *pufB*$_3$*A*$_3$ would also be part of this transcript. In fact, co-transcription of these additional *puf* genes within a 4.4 kb polycistronic transcript has been demonstrated for *Alc. vinosum* (NAGASHIMA *et al.*, 2002).

All probes yielded a hybridization signal of 2.7 kb. Two additional transcripts were detected with probe I only, and consisted of a weak signal at 1.5 kb and a strong signal at around 0.6 kb. The latter could be separated into three distinct bands (0.8 kb, 0.6 kb and 0.5 kb) (Fig. 4C), which possibly encode the *puf(BA)*$_1$, *puf(BA)*$_2$ and *puf(BA)*$_3$ transcript species. This is in accordance with the slightly different sizes of all three *pufBA* elements which, if the flanking stem-loop structures marked the ends of degradation products derived from a larger molecule, would yield segments of 654 bp, 589 bp and 538 bp in length, respectively. The detection of a 2.7 kb transcript with all three probes indicates that this transcript either encompasses the genes *pufB*$_1$*A*$_1$*LM* and the 5' region of *pufC*, or alternatively consists of the 3' region of *pufM* and *pufCB*$_2$*A*$_2$*B*$_3$*A*$_3$. We attribute the faint bands in between the major bands to intermediates in the degradation of longer precursors to smaller, more stable products.

The expression of *puf* genes was assessed under different light conditions (Fig. 5). Doubling times (33 hrs ± 1.9) were significantly lower for cells incubated at an incident light intensity of 50 µmol quanta · m^{-2} · s^{-1} than for cells incubated at 100 µmol quanta · m^{-2} · s^{-1} (22 hrs ± 3.1 and 25 hrs ± 2.5) (Fig. 5A). After growth at 50 µmol quanta · m^{-2} · s^{-1}, cellular RNA levels (2.62 µg RNA per 10^9 cells) were lower by a factor of 5.2 compared to cells incubated at 100 µmol quanta · m^{-2} · s^{-1} (13.6 µg RNA per 10^9 cells). One of the cultures incubated at an incident light intensity of 100 µmol quanta · m^{-2} · s^{-1} was sulfide depleted at the point of harvest, and cellular RNA content was lower than in the sulfide replete cells (by a factor of 2.6, equivalent to 5.14 µg RNA per 10^9 cells). These values are reflected in the intensity of rRNA bands stained with ethidium bromide after gel electrophoresis (Fig. 5B, left panel). Northern hybridization with probe I showed that under light limitation or sulfide depletion the levels of *puf* messages per *Amb. purpureus* cell were greatly reduced (Fig. 5B, right panel). The amount of transcripts relative to RNA in low light or sulfide depleted cells, however, was only slightly reduced in comparison to cells grown under high light without sulfide limitation (Fig. 5C, compare right and left panels).

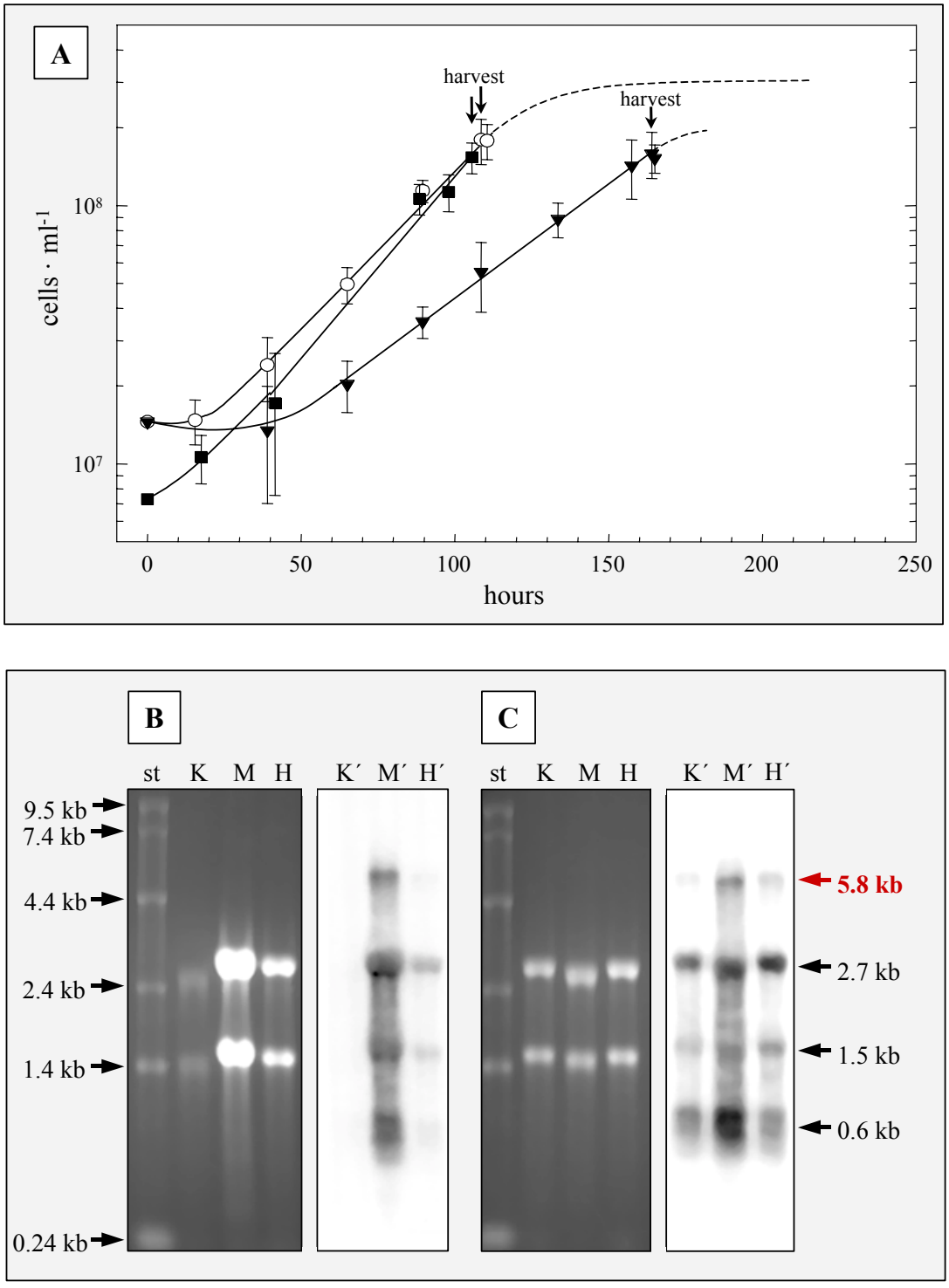


Figure 5: RNA blot analysis of *Amb. purpureus* cells grown under different light conditions and sulfide availability. (**A**) Growth curves of *Amb. purpureus* under 50 µmol quanta · m^{-2} · s^{-1} (culture K = ▼) and 100 µmol quanta · m^{-2} · s^{-1} (culture M = ■ and H = ○). Culture H was sulfide depleted at the time point of harvest. The dashed lines indicate the time course of growth of parallel cultures. The times when cells were harvested for RNA extraction are shown by arrows. (B and C) RNA was separated by electrophoresis on an agarose-formaldehyde gel (left side of panels B and C). The distinct signals at 1.5 and 2.7 kb represent ribosomal bulk RNA. For size comparison an RNA standard is given in lane 1. After Northern-blot transfer the RNA was hybridized with probe I, which is complementary to *pufBA* (right side of panels B and C). (**B**) *puf* transcripts normalized to cell biomass. Each well was loaded with 30 % of the total RNA extracts from ~2.2 · 10^9 cells, with 2 µg for culture K (lane 2), 11 µg for culture M (lane 3) and 4 µg for culture H (lane 4). (**C**) *puf* transcripts normalized to ribosomal RNA. 3 µg of RNA were applied to each well.

Analysis of amino acid sequences

Amino acid (aa) sequences were deduced from the DNA sequences (Fig. 3). The LH1 *pufBA* gene products consist of 47 aa for all three β polypeptides (β_1, β_2 and β_3), 63 aa for two of the α subunits (α_1 and α_2), and 44 aa for α_3. In comparison to the β polypeptides, the α polypeptides show more variability not only in size but also in their amino acid sequences (Fig. 6). Variable positions were detected in those portions of the polypeptides close to the membrane surface, in the *Amb. purpureus* LH1α_1 N-terminal domain (residues -17, -18 and -20) and in the LH1α_2 C-terminal domain (residues +9, +10 and +12). In other purple bacteria, these residues show a high degree of conservation. In contrast, the LH1α_3 sequence contains most of the consensus motifs characteristic of other purple bacteria. Differences among the LH1β polypeptides of both species are less pronounced (Fig. 6B). As expected from their close 16S phylogenetic affiliation, the antenna polypeptides of *Amb. purpureus* and *Alc. vinosum* share some amino acid conservation in addition to the commonly conserved residues (Fig. 6, arrows). The deduced size of the L and M subunits of the reaction center are 274 aa and 322 aa, respectively, whereas the tetraheme cytochrome (PufC) is predicted to be 409 aa long. Four heme-binding motifs (C-x-x-C-H) were identified within the sequence of PufC (Fig. 3).

Phylogenetic relationships of LH1 genes

Figure 7A shows a phylogenetic tree of concatenated sequences, in which all four polypeptides that were derived from the *puf* operon core unit (*pufBALM*) were analyzed as one continuous sequence. Tree topology was supported by all three methods employed, and the branching pattern was congruent to that of the individual phylogenetic trees for each of the four structural polypeptides (data not shown). Individual trees for the small LH1α and LH1β polypeptides had lower bootstrap support, however.

The topology of the concatenated tree in Fig. 7A is largely in agreement with that of the 16S rRNA phylogeny. In all trees analyzed (concatenated and individual trees), four clusters corresponding to the established 16S rRNA clusters of the α-3, the α-4, the *Acidiphilium* and the β subgroup of the proteobacteria were found. A fifth major cluster is dominated by the γ-proteobacteria. Within this major cluster, the three purple sulfur bacteria *Amb. purpureus*, *Alc. vinosum* and *Thc. tepidum* form one distinct linage with high statistical support. Other members of this cluster are *Ectothiorhodospira shaposhnikovii* (a member of the *Ectothiorhodospiraceae*) and the α proteobacteria *Blastochloris viridis* and

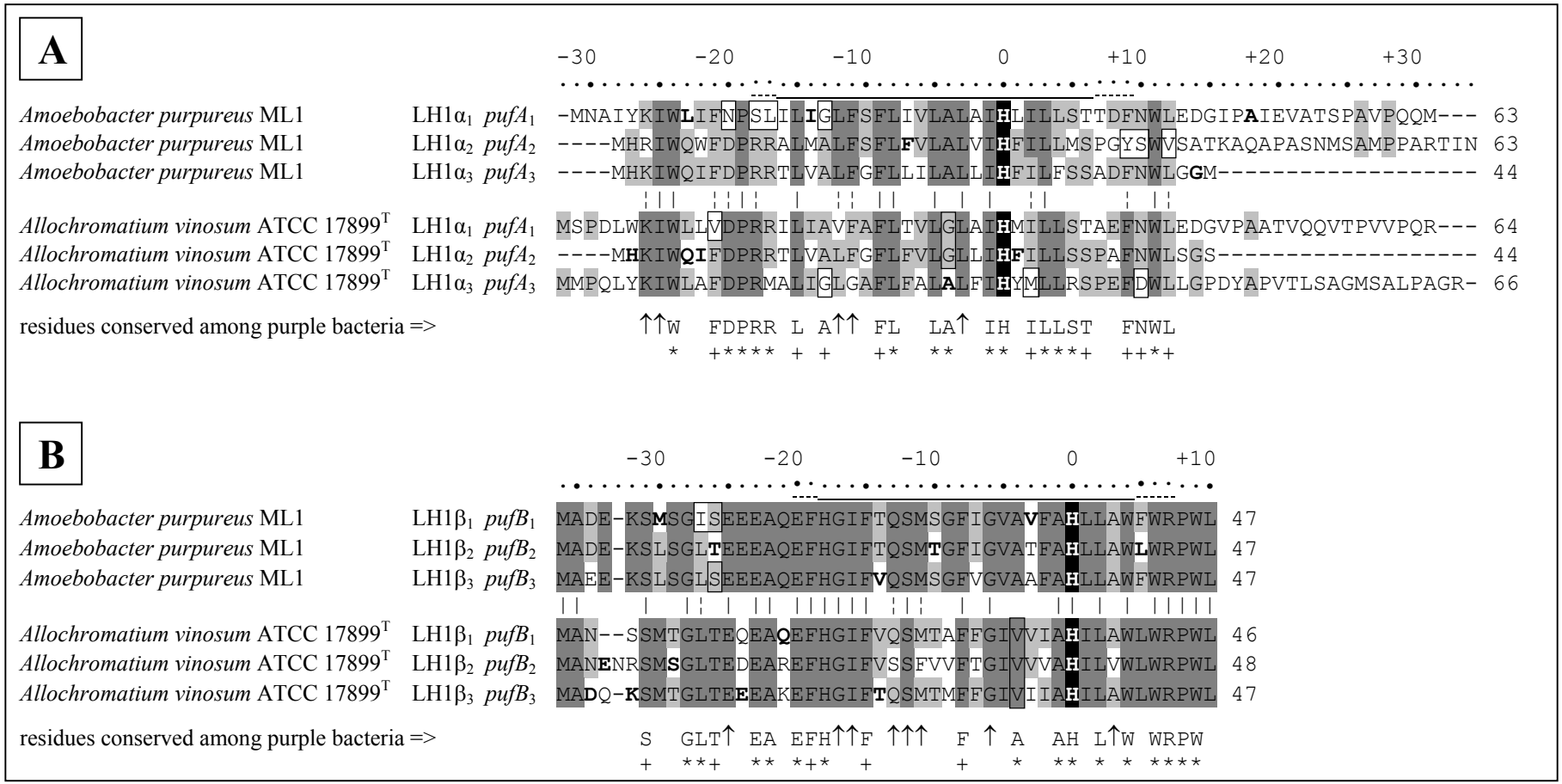


Figure 6: Alignment of the amino acid sequences encoded by multiple *pufA* (**A**) and *pufB* (**B**) loci in the *puf* operon of *Amb. purpureus* and *Alc. vinosum*. Amino acid residues conserved in Puf A_1, A_2, and A_3 of the same species shaded in *dark gray*; residues conserved in two of the three sequences shaded in *light gray*. Amino acids that differ from motifs which are conserved among purple nonsulfur bacteria are *framed*. Residues with ≥ 80 % conservation among all sequences included in this study are marked by an asterisk; ≥ 70 % conservation is indicated by a plus sign. Vertical arrows indicate sites conserved in both species but not conserved among other purple bacteria. Residue numbering on top is given with reference to the BChl *a* binding histidine. The *horizontal line on top* indicates the membrane spanning domain.

Phaeospirillum molischianum. The position of these α proteobacteria within this cluster is inconsistent with their affiliation in 16S rRNA phylogeny. PufC was not included in the concatenated tree, because not all purple bacteria possess a tetraheme cyochrome. However, it is remarkable that only species which contain a *pufC* gene in their operon (type IV and V *puf* operons, Fig. 1) are found in the cluster dominated by the γ-proteobacteria. When only the PufC sequences were analyzed phylogenetically, the clusters detected were similar to those of the PufBALM tree and most were well supported statistically (Fig. 7B).

So far, multiple sets of LH1α and β genes or polypeptides have been found only within the γ proteobacteria. Therefore the corresponding polypeptide sequences were analyzed separately (Fig. 7C). Amino acid sequences deduced from the multiple *pufBA* genes of *Amb. purpureus* and *Alc. vinosum* were included, as well as the various multiple LH1 and LH2 polypeptide sequences which have been detected in members of the *Ectothiorhodospiraceae* and of *Alc. vinosum* by direct protein sequencing (WAGNER-HUBER *et al.*, 1992; BISSIG *et al.*, 1990). Our phylogenetic analysis clearly separated the

Table 2: Identity table of amino acid sequence from α- and β-polypeptides of LH1 derived from members of the *Chromatiaceae*. Values in the upper right triangle display LH1α and those in the lower left triangle display identity values for LH1β polypeptides. Values ≥80% are shaded in dark gray. Set 1: α_1- and β_1-polypeptides, set 2: α_2- and β_2-polypeptides and set 3: α_3- and β_3-polypeptides.

	pairwise % sequence identity						
	***Alc. vinosum* LH1 set 1**	***Alc. vinosum* LH1 set 2**	***Alc. vinosum* LH1 set 3**	***Thc. tepidum* LH1**	***Amb. purpureus* LH1 set 1**	***Amb. purpureus* LH1 set 2**	***Amb. purpureus* LH1 set 3**
***Alc. vinosum* LH1 set 1**		63	44	52	63	43	55
***Alc. vinosum* LH1 set 2**	80		58	61	61	74	85
***Alc. vinosum* LH1 set 3**	86	70		38	44	39	50
***Thc. tepidum* LH1**	72	58	71		56	36	53
***Amb. purpureus* LH1 set 1**	73	63	74	60		38	58
***Amb. purpureus* LH1 set 2**	74	63	77	64	90		64
***Amb. purpureus* LH1 set 3**	73	63	69	64	86	88	

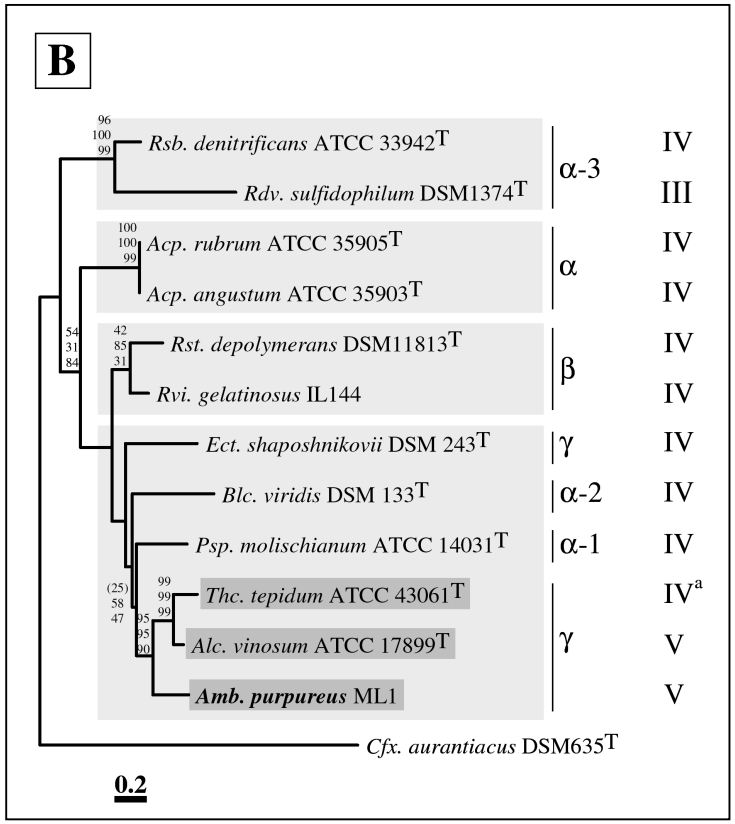
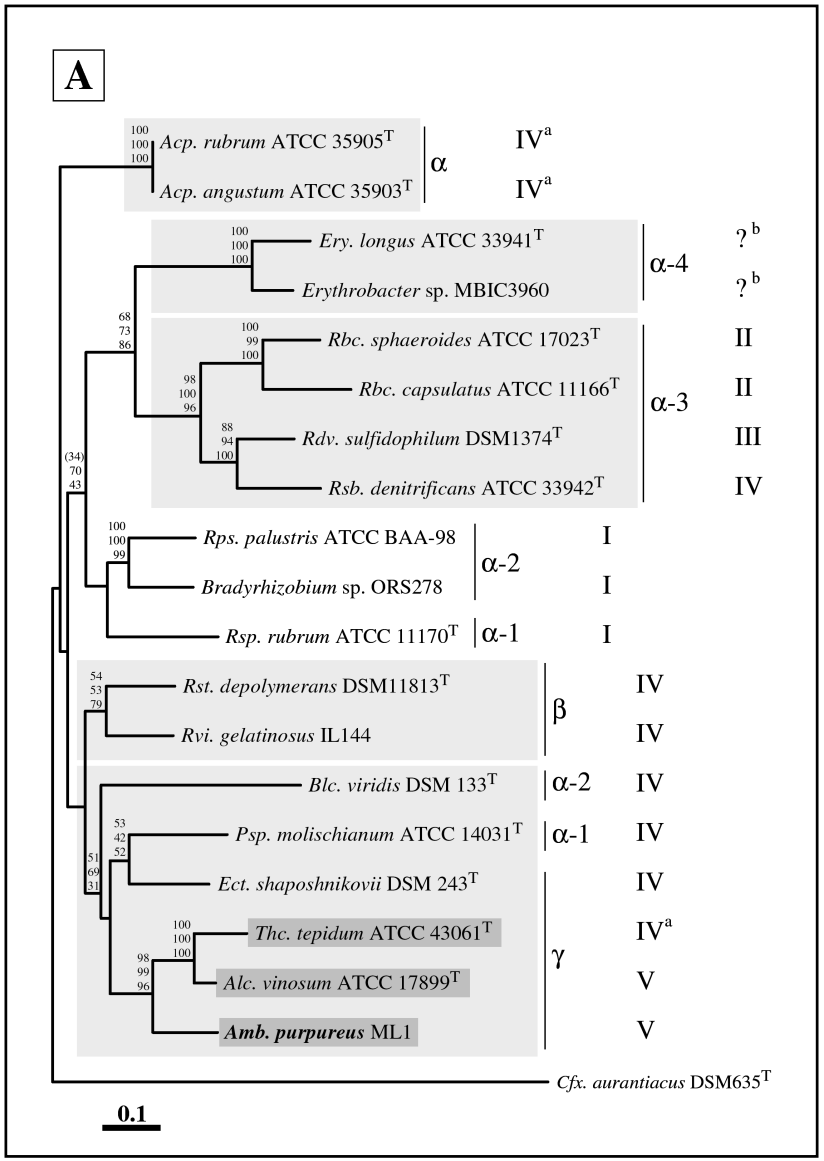

B
Rsb. denitrificans ATCC 33942T
Rdv. sulfidophilum DSM1374T
α-3
IV
III
Acp. rubrum ATCC 35905T
Acp. angustum ATCC 35903T
α
IV
IV
Rst. depolymerans DSM11813T
Rvi. gelatinosus IL144
β
IV
IV
Ect. shaposhnikovii DSM 243T
Blc. viridis DSM 133T
Psp. molischianum ATCC 14031T
γ
α-2
α-1
IV
IV
IV
Thc. tepidum ATCC 43061T
Alc. vinosum ATCC 17899T
Amb. purpureus ML1
γ
IVa
V
V
Cfx. aurantiacus DSM635T
0.2

Figure 7A + B


A
Acp. rubrum ATCC 35905T
Acp. angustum ATCC 35903T
α
IVa
IVa
Ery. longus ATCC 33941T
Erythrobacter sp. MBIC3960
α-4
?b
?b
Rbc. sphaeroides ATCC 17023T
Rbc. capsulatus ATCC 11166T
Rdv. sulfidophilum DSM1374T
Rsb. denitrificans ATCC 33942T
α-3
II
II
III
IV
Rps. palustris ATCC BAA-98
Bradyrhizobium sp. ORS278
Rsp. rubrum ATCC 11170T
α-2
α-1
I
I
I
Rst. depolymerans DSM11813T
Rvi. gelatinosus IL144
β
IV
IV
Blc. viridis DSM 133T
Psp. molischianum ATCC 14031T
Ect. shaposhnikovii DSM 243T
Thc. tepidum ATCC 43061T
Alc. vinosum ATCC 17899T
Amb. purpureus ML1
α-2
α-1
γ
IV
IV
IV
IVa
V
V
Cfx. aurantiacus DSM635T
0.1

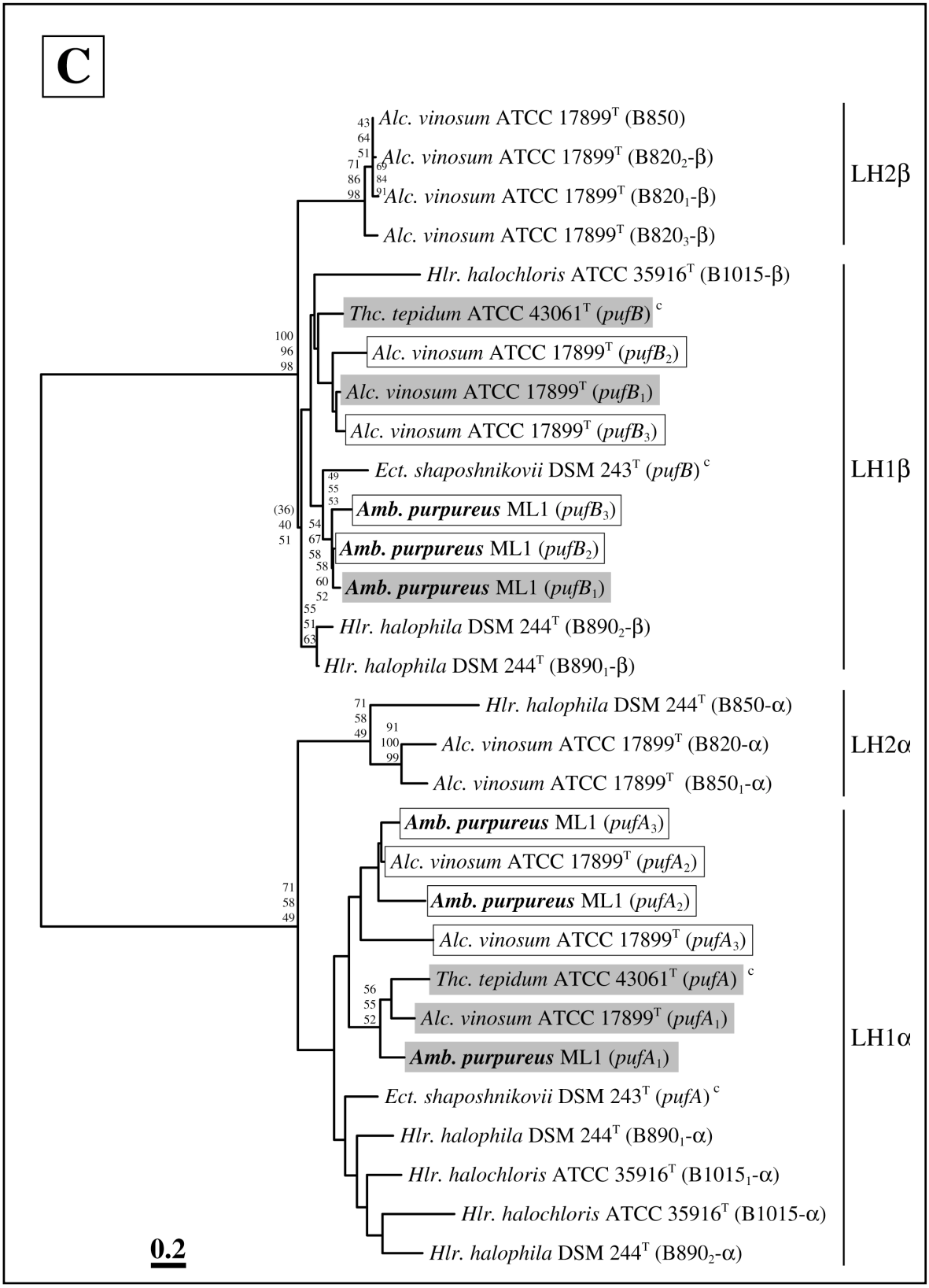

C
Alc. vinosum ATCC 17899T (B850)
Alc. vinosum ATCC 17899T (B820₂-β)
Alc. vinosum ATCC 17899T (B820₁-β)
Alc. vinosum ATCC 17899T (B820₃-β)
LH2β
Hlr. halochloris ATCC 35916T (B1015-β)
Thc. tepidum ATCC 43061T (pufB) c
Alc. vinosum ATCC 17899T (pufB₂)
Alc. vinosum ATCC 17899T (pufB₁)
Alc. vinosum ATCC 17899T (pufB₃)
Ect. shaposhnikovii DSM 243T (pufB) c
Amb. purpureus ML1 (pufB₃)
Amb. purpureus ML1 (pufB₂)
Amb. purpureus ML1 (pufB₁)
Hlr. halophila DSM 244T (B890₂-β)
Hlr. halophila DSM 244T (B890₁-β)
LH1β
Hlr. halophila DSM 244T (B850-α)
Alc. vinosum ATCC 17899T (B820-α)
Alc. vinosum ATCC 17899T (B850₁-α)
LH2α
Amb. purpureus ML1 (pufA₃)
Alc. vinosum ATCC 17899T (pufA₂)
Amb. purpureus ML1 (pufA₂)
Alc. vinosum ATCC 17899T (pufA₃)
Thc. tepidum ATCC 43061T (pufA) c
Alc. vinosum ATCC 17899T (pufA₁)
Amb. purpureus ML1 (pufA₁)
Ect. shaposhnikovii DSM 243T (pufA) c
Hlr. halophila DSM 244T (B890₁-α)
Hlr. halochloris ATCC 35916T (B1015₁-α)
Hlr. halochloris ATCC 35916T (B1015-α)
Hlr. halophila DSM 244T (B890₂-α)
LH1α
0.2

Figure 7C

Figure 7: Phylogenetic trees based on structural polypeptides of the photosynthetic apparatus of purple bacteria. (**A**) Concatenated tree calculated from the combined amino acid sequences deduced from the *pufBALM* core unit. (B) Phylogenetic tree based on *pufC* polypeptide sequences. Major clusters underlain in gray. Members of the *Chromatiaceae* are shaded in dark gray. 16S phylogenetic affiliation is indicated in Greek letters and corresponding *puf* operon types are given in Roman numerals. The green non-sulfur bacterium *Chloroflexus* (*Cfx.*) *aurantiacus* was used as outgroup. (**C**) Phylogenetic tree based on α and β polypeptides from LH1 and LH2 complexes of purple sulfur bacteria. Besides sequences deduced from *pufBA* genes (*Amb. purpureus*, *Alc. vinosum*, *Thc. tepidum* and *Ect. shaposhnikovii*) sequences determined by direct protein sequencing (WAGNER-HUBER, *et al.*, 1992; BISSIG, *et al.*, 1990) were included (indicated in parenthesis by their specific long wavelength absorption maxima as B820, B850, B890 or B1015). LH1 antenna polypeptides of the *Chromatiaceae* deduced from the core *puf* unit ($pufB_1$ and $pufA_1$) are shaded in dark gray, whereas sequences deduced from additional copies ($pufB_2$, $pufB_3$ and $pufA_2$, $pufA_3$) are framed in boxes. All trees displayed were determined by maximum likelihood analysis. Bootstrap values > 40 % are given beside the nodes with maximum likelihood (top), matrix based FITCH (middle) and parsimony (bottom). Bars represent 0.1 substitutions per site. (Abbreviations: *Acp*. *Acidiphilium*, *Alc*. *Allochromatium*, *Amb*. *Amoebobacter*, *Blc*. *Blastochloris*, *Cfx*. *Chloroflexus*, *Ect*. *Ectothiorhodospira*, *Ery*. *Erythrobacter*, *Hlr*. *Halorhodospira*, *Rbc*. *Rhodobacter*, *Rps*. *Rhodopseudomonas*, *Rsp*. *Rhodospirillum*, *Rdv*. *Rhodovulum*, *Rst*. *Roseateles*, *Rsb*. *Roseobacter*, *Rvi*. *Rubrivivax*, *Psp*. *Phaeospirillum*, *Thc*. *Thermochromatium*.)

[a] strains which were assigned to the *puf* operon type IV due to insufficient or lack of sequence information 3' of *pufC*

[b] lack of sequence information 3' of *pufM*

[c] strains for which no multiple *pufBA* genes or multiple LH1 antenna polypeptides were reported

antenna α and β polypeptide chains. In the LH1β subcluster, the multiple peptides of both *Amb. purpureus* and *Alc. vinosum* show a high degree of intraspecies relatedness (Fig. 6B). Correspondingly, *Amb. purpureus* LH1β_1, β_2, β_3 sequences and *Alc. vinosum* LH1β_1, β_2, β_3 sequences form separate clusters. Identity values were 70 to 86 % for *Alc. vinosum* and 86 to 90 % for *Amb. purpureus* (Table 2, lower left triangle). However, the identity of β-polypeptides of different species was generally low (≤ 74 %), with the possible exception of *Alc. vinosum* LH1β_3 and *Amb. purpureus* LH1β_2 (77 % identity).

In contrast to the sequences of the β polypeptides, the α_1 polypeptides of *Amb. purpureus* and *Alc. vinosum* were closely related and formed a robust separate cluster (Fig. 7C). This is in accordance with the topology found in the concatenated *pufBALM* tree (Fig. 7A) and the 16S rRNA tree. However, the additional LH1 α polypeptides of *Amb. purpureus* ML1 (derived from $pufA_2$ and $pufA_3$) do not cluster with the α_1 polypeptide, but show a high degree of relatedness with α-polypeptides of *Alc. vinosum* (see also Fig. 6A). Identity was generally lower than for the β polypeptide (36 to 85 % for the α polypeptides; Table 2, upper right triangle). Remarkably, the highest identity was calculated for *Alc. vinosum* LH1α_2 and *Amb. purpureus* LH1α_2 and LH1α_3 with levels of 74 % and 85 %, respectively.

5 Discussion

Structure and organization of the *puf* operon

The sequence obtained in the present study covers 7444 bp and comprises the structural photosynthesis genes $pufB_1A_1LMCB_2A_2B_3A_3$. Previously, this type of organization of *puf* genes had been described only for *Alc. vinosum*ATCC 17899^T (NAGASHIMA *et al.*, 2002; CORSON *et al.*, 1999). The presence of a 3'-portion of the *bchZ* gene found 5' of the *Amb. purpureus puf* operon indicates that the genes detected in *Amb. purpureus* form part of a photosynthesis gene cluster as in other purple bacteria (BEJA *et al.*, 2002; IGARASHI *et al.*, 2001; NAYLOR *et al.*, 1999; ALBERTI *et al.*, 1995). In most purple bacteria, the 3´ terminal gene flanking the *pufBALM* core motif of the *puf* operon is *pufC*. *PufC* is completely absent in only few of the purple bacteria so far investigated, or a *pufX* gene was reported instead in the case of *Rhodobacter* species. *Alc. vinosum* and *Amb. purpureus* are the only species so far known to contain secondary sets of *pufBA* genes. However, LH1 antenna complexes consisting of various types of α and β polypeptides were reported for the purple sulfur bacteria *Halorhodospira halochloris* and *Halorhodospira halophila* (ZUBER & COGDELL, 1995; WAGNER-HUBER *et al.*, 1992; ZUBER & BRUNISHOLZ, 1991; BISSIG *et al.*, 1990). These data were derived from direct protein sequencing, however, and no corresponding *puf* operon sequence information is available for the latter two species. The *Ectothiorhodospira shaposhnikovii* "complete" *puf* operon sequence consisting of the genes *pufBALMC* can be retrieved from the databases (ZHANG & GINGRAS, 1997, unpublished GenBank record AF018955). This sequence includes 180 nucleotides 3' of *pufC* with no further homology to known *puf* genes. In *Alc. vinosum* the intercistronic region between *pufC* and $pufB_2$ covers 175 nucleotides (CORSON *et al.*, 1999). Therefore, additional *pufBA* sets may be present in *Ectothiorhodospira shaposhnikovii*. Some *puf* sequences reported do not provide sufficient information beyond the terminal *puf* gene (especially type IV operons) to rule out the presence of additional antenna genes. Therefore it is possible that additional sets of *pufBA* genes are also present in other *Chromatiaceae* or *Ectothiorhodospiraceae* in contrast to the purple non-sulfur bacteria.

Phylogenetic and functional aspects of the multiplicity of LH1 antenna genes

In general, our concatenated phylogenetic tree is in good agreement with previous analyses of L and M gene sequences (BEJA *et al.*, 2002; NAGASHIMA *et al.*, 1997a), and amino acid

sequences of LH1 polypeptides (NAGASHIMA *et al.*, 1996). The congruence in tree topology of PufB, A, L and M and, if present, also of PufC indicate a coevolution of these structural components of the photosynthetic apparatus. The high values for amino acid sequence identity between *Amb. purpureus* and *Alc. vinosum* suggest that the evolution of light harvesting structures is limited by functional constraints.

In general, the tree topology of $PufB_1A_1LM$ and of PufC is also in good agreement with the 16S rRNA tree topology (data not shown). However the $PufB_1A_1LM$ polypeptides of *Phaeospirillum molischianum* (α-1 subgroup of proteobacteria) and *Blastochloris viridis* (α-2 subgroup) clearly had a different phylogenetic affiliation than their 16S rRNA genes. This is also supported by the PufC tree. Horizontal transfer of *puf* genes has been invoked previously to explain such incongruent phylogenetic patterns (NAGASHIMA *et al.*, 1997a; NAGASHIMA *et al.*, 1993). Our data indicate that horizontal transfer of *pufBALM* genes may have occurred in 2 out of 19 species analyzed and therefore appears to be rather rare. Similar to recent results (RAYMOND *et al.*, 2002) the deep branching pattern and the considerable bootstrap values suggest that the gene exchange has occurred early in the evolution of photosynthetic proteobacteria.

Currently, additional sets of *pufBA* genes have been detected only in *Alc. vinosum* (NAGASHIMA *et al.*, 2002; CORSON *et al.*, 1999). Because different genuine LH1 α and β polypeptides have been reported for *Halorhodospira* species (WAGNER-HUBER *et al.*, 1992) and *Alc. vinosum* (WAGNER-HUBER *et al.*, 1992; BISSIG *et al.*, 1990), the existence of multiple *pufBA* genes might be a common trait of purple sulfur bacteria.

The multiplicity of antenna genes raises the question of their evolutionary advantage and origin. In some purple bacteria, a multiplicity of LH2 peripheral antenna polypeptides has been found (BRUNISHOLZ & ZUBER, 1992; ZUBER & BRUNISHOLZ, 1991; BISSIG *et al.*, 1990), where spectral properties were modulated (B800-820, B800-850) by employing chemically different LH2 polypeptides in response to changing environmental conditions, such as light intensity or temperature (ZUBER & BRUNISHOLZ, 1991). However, to our knowledge no corresponding spectral shifts have been reported for native core antenna complexes. It could be argued that duplicate antenna genes serve as a protective mechanism against genetic perturbation. However, all three $PufA_1$, A_2 and A_3 polypeptides are present in functional membrane bound antenna complexes of *Alc. vinosum* (NAGASHIMA *et al.*, 2002; WAGNER-HUBER *et al.*, 1992; BISSIG *et al.*, 1990). It is still unclear whether these different antenna polypeptides fulfill different functions and whether they are of selective advantage.

In *Amb. purpureus* and *Alc. vinosum*, the phylogeny of the *pufB*$_1$*A*$_1$ genes is congruent with that of the reaction center genes *pufLM* and *pufC*. The additional β_2 and β_3 subunits show the highest relatedness to the corresponding β_1 polypeptide of the same species. In contrast, some additional α polypeptides of *Amb. purpureus* and *Alc. vinosum* are closely related. The close affiliation of the *Alc. vinosum* LH1α_2, and *Amb. purpureus* LH1α_2 and LH1α_3 might reflect a past event of horizontal gene exchange between the two *Chromatiaceae* species. From our phylogenetic analysis it can be concluded that considerable functional constraints differ for the α and β polypeptides and may result in the more conservative character of the multiple β LH polypeptides. Functional constraints may be explained by experimental evidence indicating that the LH1 β polypeptide inserts first into the cytoplasmic membrane and is a prerequisite for the stable assembly of a functional antenna complex (PUGH *et al.*, 1998; DREWS & GOLECKI, 1995). Other data indicate that α and β subunits interact before insertion into the membrane (DREWS, 1996; MERYANDINI & DREWS, 1996).

Transcription and regulation

The discovery of multiple core antenna genes in the purple sulfur bacterium *Amb. purpureus* besides *Alc. vinosum* raises the question of whether they are transcribed and transcriptionally regulated independently. The *puf* genes from various purple bacteria are known to be transcribed as a polycistronic unit (BEATTY, 1995). The primary transcript is segmentally degraded, resulting in RNA species with different degrees of stability (HECK *et al.*, 2000; KLUG, 1995). This results in a differential expression of the genes to meet the stoichiometric needs of the different components in the photosynthetic apparatus. Polycistronic transcripts of 2.5 kb to 4.0 kb were found in four species of purple non-sulfur bacteria by end-mapping experiments (BEATTY, 1995), which correspond to the approximate size of the *puf* operons in these species. In contrast, a significantly larger 5.8 kb transcript is present in high abundance in *Amb. purpureus*. At least the genes *pufBALMC* are part of this polycistronic unit as shown by hybridization with different probes. Since co-transcription of all *puf* genes within a major transcript of 4.4 kb was described recently for *Alc. vinosum* (NAGASHIMA *et al.*, 2002), the *Amb. purpureus puf* genes may similarly co-transcribed. However, since the transcript species of 2.7 and 1.5 kb as described in our study are absent in *Alc.vinosum* (NAGASHIMA *et al.*, 2002), post-transcriptional RNA processing might differ in *Amb. purpureus*.

In *Rbc. capsulatus*, the half-life of the *puf* operon primary transcript was reported to be < 0.5 min, such that only minor amounts can be detected by RNA blot (KLUG, 1993; ADAMS *et al.*, 1989). The more stable mRNA products are 2.7 and 0.5 kb long and have half-lifes on the order of 5 to 8 and 20 to 33 min, respectively (CHEN *et al.*, 1988). In contrast to the *Rbc. capsulatus* primary transcript, the 5.8 kb *puf* message in *Amb. purpureus* was present as a prominent band in the RNA blots, indicating a high stability. The pattern and intensity of smaller bands is consistent with a degradation pathway through quasi-stable intermediates to yield *pufBA* segments of the greatest stability. The very end of the *puf* operon 3' of $pufA_3$ contains sequences that could encode thermodynamically stable RNA hairpin structures, which would be resistant to 3'-exonucleolytic degradation.

Changes in RNA levels in response to environmental changes is a common mode of genetic regulation (RAUHUT & KLUG, 1999). In *Amb. purpureus* the RNA levels (*puf* and rRNA) at the late exponential phase were reduced in cells that were either light limited or sulfide depleted. Therefore availability of substrates seems to play an important role in regulating photosynthesis gene expression as well as the translational machinery. A positive correlation between total RNA content and light intensity was reported for *Rbc. capsulatus* (ZUCCONI & BEATTY, 1988), and a correlation exists between growth rate and rRNA content of bacteria in general (MADIGAN *et al.*, 2000). In *Amb. purpureus*, the drop of RNA levels was paralleled by a decreased BChl *a* content (data not shown). These findings are in contrast to the pattern of light intensity regulation of photosynthesis genes observed in *Rhodobacter* species, in which decreased light intensities increase photosynthetic protein and BChl *a* contents (BAUER & BIRD, 1996).

Concluding remarks

Our studies revealed the presence of an unusual type of *puf* operon in *Amb. purpureus*. It contains sets of *pufBA* genes at both ends of the operon to yield the gene order $pufB_1A_1LMCB_2A_2B_3A_3$. This type of organization was previously described only for *Alc. vinosum* (NAGASHIMA *et al.*, 2002; CORSON *et al.*, 1999). Although no additional data for purple sulfur bacteria exist, it is possible that a multiplicity of LH1 genes might be common in purple sulfur bacteria. Our phylogenetic studies suggest that LH1 genes 3' of *pufC* might be characteristic for members of a distinct cluster of purple bacteria that encompasses β, γ and some members of the α-1 and α-2 proteobacteria. Interestingly, all members of this cluster contain a reaction center bound tetra-heme cytochrome.

Furthermore, exchange of *puf* genes via horizontal transfer was hypothesized for the same cluster (NAGASHIMA *et al.*, 1997a) and could contribute to the multiplicity of LH1 genes within this group.

Our analysis showed that, besides unusual structural features of the *puf* operon of purple sulfur bacteria, transcription and gene regulation also differs from that of the well-characterized purple non-sulfur bacteria. Future studies are needed to reveal the details of the molecular mechanisms that govern the photosynthetic machinery in purple sulfur bacteria, a group that contains members like *Amb. purpureus* which play a dominant role in natural environments.

6 Acknowledgements

This investigation was supported by NSERC grants to J. T. Beatty, and scholarships of the German academic exchange service (DAAD) and the Heinz Neumüller Stiftung to Christian Tuschak. For *Rhodopseudomonas palustris* preliminary sequence data were obtained from the DOE Joint Genome Institute (JGI) at http://www.jgi.doe.gov/JGI_microbial/html/index.html.

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CHAPTER III

Photosynthesis genes and LH1 proteins of *Roseospirillum parvum* 930I, a purple non-sulfur bacterium with unusual spectral properties

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Running head: Photosynthesis genes of *Roseospirillum parvum*

Key words: purple sulfur bacteria, purple non-sulfur bacteria, light-harvesting complex, *puf* genes, anoxygenic photosynthesis, 3D modeling, pigment binding, evolution

1 Abstract

The purple bacterium *Roseospirillum (Rss.) parvum* 930I, like most other purple bacteria, contains bacteriochlorophyll (BChl) *a* as a LH chromophore, but exhibits an extremely red-shifted Q_y absorption maximum centered at 909 nm. The *puf* operon encoding the LH1 and other pigment binding proteins was cloned and sequenced, revealing the gene structure *pufBALMC*. Comparative analysis of predicted amino acid sequences of the α and β core LH polypeptides (PufA and PufB) revealed five amino acid substitutions at positions that are highly conserved in other purple bacteria. In the primary structure, these residues are in close proximity to the BChl *a* ligating histidine residue (α^{+3}, α^{+5}, α^{+6} and β^{-4}, β^{+9}, with respect to the central histidine, His^0). The nature of the enormous red-shifts to the near-infrared region of the light were attributed to those residues, with special emphasis to cysteine residues rarely present in other purple bacterial antenna polypeptides. Three dimensional structural models of the *Rss. parvum* LH1 α and β polypeptides suggest that the thiol groups of αCys^{+3} and βCys^{-4} are oriented toward the BChl *a* macrocycle in the native antenna complex. Because of their predicted close proximity to BChl, these thiol groups are likely to modulate the spectral properties of the LH1 complex. Phylogenetic comparisons of LH1 polypeptides indicate that *Rss. parvum*-like amino acid substitutions in proteobacterial LH1 complexes arose independently during evolution.

Abbreviations: *Acp. Acidiphilium, Alc. Allochromatium, Amb. Amoebobacter,*BChl bacteriochlorophyll*, Blc. Blastochloris, Blm, Blastomonas, Cfx. Chloroflexus, Ect. Ectothiorhodospira, Ery. Erythrobacter, Hlr. Halorhodospira,* LH light harvesting, *Psp. Phaeospirillum, Rbc. Rhodobacter, Rbi. Rhodobium, Rbl. Rhodoblastus,*RC reaction center, *Rcy. Rhodocyclus, Rdv. Rhodovulum, Rps. Rhodopseudomonas, Rsb. Roseobacter, Rsp. Rhodospirillum, Rss. Roseospirillum, Rst. Roseateles, Rvi. Rubrivivax, Thc. Thermochromatium.*

2 Introduction

Purple bacteria are anoxygenic phototrophic proteobacteria which typically contain bacteriochlorophyll *a* (Bchl *a*) as the essential photosynthetic pigment. Although containing the same BChl *a*, different species exhibit distinct long-wavelength absorption spectra *in vivo* (PERMENTIER, 2001; PERMENTIER *et al.*, 2001; GLAESER & OVERMANN, 1999; ZUBER & BRUNISHOLZ, 1991). Such variations in their spectral properties enable phototrophic bacteria to exploit different wavelengths of light and hence to occupy different ecological niches (GLAESER & OVERMANN, 1999).

In purple bacteria, BChl and carotenoid pigments are non-covalently bound to the reaction center (RC) PufL and M proteins, and the α and β subunits of light-harvesting (LH) complexes. Each LH1 complex contains BChl dimers sandwiched between ~16 αβ proteins assembled around the RC in a ring-like or crescent structure (LOACH, 2000; COGDELL *et al.*, 1999; PAPIZ *et al.*, 1996). Besides this core LH1 complex, peripheral antenna complexes (LH2 and LH3) are present in many purple bacteria. Each peripheral complex contains one monomeric BChl molecule in addition to the BChl dimer. LH2 complexes form a ring-like structure which is composed of eight to nine transmembrane αβ heterodimers (MCLUSKEY *et al.*, 2001; KOEPKE *et al.*, 1996; MCDERMOTT *et al.*, 1995).

While monomeric BChl *a* exhibits an absorption maximum at 770 nm in organic solvents, the long-wavelength (Q_y) absorption bands of dimeric BChl *a* in the LH1, LH2 and LH3 complexes are found at 870-890, 850 or 820 nm, respectively (MCLUSKEY *et al.*, 2001; ZUBER & BRUNISHOLZ, 1991). The additional monomeric BChl *a* bound in LH2 or LH3 complexes exhibits a Q_y absorption peak at about 800 nm (COGDELL *et al.*, 1999; ZUBER & COGDELL, 1995; ZUBER & BRUNISHOLZ, 1991). These red-shifts in the absorption maxima of intact LH complexes are caused by pigment-pigment interactions (COGDELL *et al.*, 2002; COGDELL *et al.*, 1997) and by interactions of BChl *a* with the pigment binding polypeptides (ZUBER & COGDELL, 1995). Within the α and β LH polypeptides, BChl *a* dimers are coordinately bound by highly conserved histidine residues (COGDELL *et al.*, 1999; ZUBER & COGDELL, 1995; ZUBER & BRUNISHOLZ, 1991). Excitonic coupling of BChl *a* molecules in dimers has the largest impact on the red-shift of the Q_y band (COGDELL *et al.*, 2002; COGDELL *et al.*, 1997). Further fine tuning of the Q_y absorption appears to be mediated by polar residues and point charges in the local pigment environment (ECCLES & HONIG, 1983), by deformation of the BChl macrocycle (COGDELL

et al., 2002), and by hydrogen bonding to the BChl macrocycle (GUDOWSKA-NOWAK *et al.*, 1990; HANSON *et al.*, 1987). In addition, the *in vivo* absorption of the LH2 monomeric BChl *a* is influenced by interaction with a formyl adduct of the N-terminal methionine residue of the α protein (MCLUSKEY *et al.*, 2001; COGDELL *et al.*, 1999; PRINCE *et al.*, 1997).

So far, the molecular basis of LH absorption properties has been studied mainly by site-directed mutagenesis. Spectral bandshifts were generated by, and thus linked to, substitutions of specific amino acid residues, in particular the LH1 αTrp^{+11} (Trp43) and βTrp^{+9} (Trp44) of *Rhodobacter capsulatus*, and the LH2α Tyr^{+13} (Tyr44) and LH2α Tyr^{+14} (Tyr45) in *Rhodobacter sphaeroides* (STURGIS *et al.*, 1997; HUNTER, 1995). (Note: a plain number following an amino acid designation indicates the position relative to the N-terminus of the protein, whereas a superscript number indicates the position relative to the His ligand of BChl; + indicates residues in the C-terminal direction from His whereas - indicates the N-terminal direction.) Crystallographic data support an interaction of these and other amino acid residues with BChl *a* (MCLUSKEY *et al.*, 2001; PRINCE *et al.*, 1997; KOEPKE *et al.*, 1996; MCDERMOTT *et al.*, 1995).

A second approach towards the identification of functional groups which influence the absorption properties of photosynthetic complexes is the analysis of native complexes with unusual absorption properties. Recently, three purple bacteria have been described which contain BChl *a* but display extremely red-shifted Q_y LH1 absorption bands at wavelengths >900 nm. The purple sulfur bacterium *Thermochromatium* (*Thc.*) *tepidum* strain ATCC 43061^T has a Q_y absorption maximum at 915 nm (FATHIR *et al.*, 1998; GARCIA *et al.*, 1986). Secondly, the Q_y band of an isolate from a marine microbial mat (strain 970) was detected at 963 nm (PERMENTIER *et al.*, 2001). Thirdly, the purple nonsulfur α proteobacterium *Roseospirillum* (*Rss.*) *parvum* strain DSMZ 12498^T exhibits a long wavelength absorption at 909 nm and thus resembles *Thc. tepidum* (GLAESER & OVERMANN, 1999). The LH1 absorption maxima of *Rss. parvum* are positioned at 909 nm and 805 nm (GLAESER & OVERMANN, 1999). Energy transfer experiments confirmed that both peaks belong to the same LH complex (PERMENTIER *et al.*, 2000). It was therefore suggested that *Rss. parvum* harbors a single and novel type of LH1 complex, which contains both dimeric and monomeric BChl *a*, similar to LH2 complexes (PERMENTIER *et al.*, 2000).

In the present study, *Rss. parvum* was chosen as a model organism to evaluate the molecular determinants of LH spectral variation. The organization and sequence of the *puf*

operon genes of *Rss. parvum* were determined, and the deduced amino acid sequences revealed the presence of unusual LH1 amino acid residues. Together with the results of molecular modeling, our data suggest that particular amino acid residues are responsible for the extremely red-shifted *in vivo* absorption properties of LH1 BChl *a* in this bacterium.

3 Materials and Methods

Cultivation and DNA extraction

Rss. parvum strain 930I was grown under anaerobic conditions in modified RS medium for purple non-sulfur bacteria at a light intensity of 100 µmol quanta · m^{-2} · s^{-1} as described previously (GLAESER & OVERMANN, 1999). Sulfide served as an electron-donating substrate and CO_2 and acetate as carbon sources for mixotrophic growth.

For extraction of chromosomal DNA, cells were harvested by centrifugation at 17,000 × *g* for 3 min at 4 °C and resuspended in TE-buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Cells were lysed by addition of an equal volume of Tris-buffered phenol (pH 8.0) and an incubation at 55 °C for 30 minutes. Extracts were vortexed frequently during incubation and finally centrifuged at 10,000 × *g* for 15 minutes at 4 °C. The aqueous phase was extracted with phenol-chloroform-isoamylalcohol (25:24:1) and subsequently with chloroform. Genomic DNA was purified by ammonium acetate precipitation (SAMBROOK *et al.*, 1989).

For cloning and sub-cloning, *Escherichia coli* strain DH5α (GIBCO-BRL, Invitrogen Canada Inc., Burlington, Ontario, Canada) was used. This strain was grown in Luria Bertani (LB) medium (Sambrook et al., 1989) supplemented with ampicillin (200 µg · ml^{-1}) as the selective antibiotic. Plasmid DNA was extracted with the QIAprep Spin Miniprep Kit (Qiagen Inc., Mississauga, Ontario, Canada).

PCR amplification of photosynthesis genes

Polymerase chain reactions (PCR) were performed in a GeneAmp PCR system 2400 using the GeneAmp PCR Reagent Kit (Applied Biosystems Canada, Streetsville, Ontario, Canada). A 1.5 kb-fragment from the L and M subunit of the reaction center was amplified with the primers pufL-F and pufM-R (Table 1) (NAGASHIMA *et al.*, 1997a). A touchdown protocol was employed, using 50 ng of genomic DNA. After a hot start at 95 °C for 5 min, 30 cycles followed, each comprising a denaturation step at 94 °C for 30 s, an annealing step for 30 s and an extension step at 72 °C for 2 min. For touchdown, annealing started at 60 °C and the temperature was lowered by an increment of -0.4 °C per cycle for 25 cycles, after which 5 cycles at 50 °C followed. The PCR product was purified with the QIAquick PCR purification kit (Qiagen), directly sequenced for verification, and used to generate a probe for Southern hybridization (Fig. 1B).

Table 1 Oligonucleotides used for PCR and sequencing

Primer	Sequence	Source
Z-R	5´-aac ag**g** gc(a/g) tcg aac a	(NAGASHIMA *et al.*, 1997b) *
pufL-F	5´-ct(t/g) ttc gac ttc tgg gt(c/g) gg	(NAGASHIMA *et al.*, 1997a)
L250-F	5´-ct(**g/t**) tgg cag atc atc ac	(NAGASHIMA *et al.*, 1997b) *
L480-F	5´-tgg gtg tcg aac **gtc** gg**c** tac	(NAGASHIMA *et al.*, 1997b) *
930-M380-R	5´-cgg acc cac cac aac ag	this study
M2-F	5´-ccg ttc gg(g/c) atc ttc c	this study
M2-R	5´-gga aga t(c/g)c cga acg g	this study
930-M624-F	5´-gtt cct cta cgg ttc ggc	this study
M3-F	5´-ggc ggc gac cgt gag	this study
M3-R	5´-ctc acg gtc gcc gcc	this study
pufM-R	5´-ccc at(c/g) gtc cag cgc cag aa	(NAGASHIMA *et al.*, 1997a)
930-C406-F	5´-gac gac ctc acc gcc ga	this study
930-C406-R	5´-tcg gcg gtg agg tcg tc	this study
C310-F	5´-gt**c** acc tgc t(**g**/a)(**g**/c) acc tgc ca	(NAGASHIMA *et al.*, 1997b) *
C310-R	5´-tgg cag gt(**c**/g) (**c**/t)ag cag gt**g** ac	(NAGASHIMA *et al.*, 1997b) *
930-Cend-F	5´-tgt cac aac agc cag tcg t	this study
930-Xho01-R	5´-gca act cgc cga tca gcc	this study
-21M13F	5' -tgt aaa acg acg gcc agt	Applied Biosystems
M13R	5´-cag gaa aca gct atg acc	Applied Biosystems

* Primers that were modified in comparison to the reference. Bold letters indicate modified nucleotides

Sequence information for the 3' terminal region of *pufC* and beyond was obtained by an inverse-PCR (iPCR) approach (OCHMAN *et al.*, 1993; OCHMAN *et al.*, 1990). Based on sequence information obtained from a cloned fragment (see below), a *Xho* I restriction site suitable for iPCR strategy was identified 485 nucleotides 5' of the fragment end (Fig. 1A). Accordingly, chromosomal DNA was digested with the restriction endonuclease *Xho* I (MBI Fermentas GmbH, St. Leon-Rot, Germany) and the digested DNA purified with the QIAquick PCR purification kit (Qiagen). Three µg of DNA fragments were ligated in a volume of 1.5 ml using 30 U T4 DNA ligase (MBI Fermentas) for 16 hours at 15 °C. Afterwards, samples were concentrated and washed once with water in Centricon 100 units (Millipore GmbH, Schwalbach, Germany). Subsamples containing 200 ng of DNA were amplified with the primer C310R and 930CendF (Table 1), employing a step-down PCR protocol which consisted of a hot start at 95 °C for 5 min, followed by 30 cycles with denaturation at 94 °C for 30 s, annealing for 1 min and extension at 72 °C for 4 min.

Annealing was carried out 10 times at 62 °C and 25 times at 56 °C to increase the yield, and AmpliTaq Gold DNA Polymerase (Applied Biosystems) was used. The addition of 5 % acetamide to the reaction mix turned out to be indispensable to obtain specific products. Amplification products were separated by electrophoresis on 1 % agarose gels in 0.5 × TBE buffer (45 mM Tris-HCl [pH 8.0], 45 mM boric acid, 1 mM EDTA), stained with ethidium bromide (0.5 $\mu g \cdot ml^{-1}$) and visualized under UV light.

Generation of nucleotide probes

For screening of DNA blots, the 1.5 kb PCR product amplified from the *pufLM* region served as a template for probe construction. The DNA template was labeled with [^{32}P]dCTP using the Rediprime DNA labeling system (Amersham Pharmacia Biotech Inc., Baie d'Urfé, Québec, Canada) according to the instructions of the manufacturer.

Cloning and Sequencing

Standard recombinant DNA procedures were employed throughout (SAMBROOK *et al.*, 1989). Briefly, one µg of chromosomal DNA of *Rss. parvum* was digested with restriction endonucleases, separated by gel-electrophoresis and transferred by electroblotting to a Biotrans nylon membrane (ICN, Irvine, Canada). Hybridization with the 1.5 kb *pufLM* probe identified a *Pst* I fragment of 4.3 kb (Fig. 1).This fragment was ligated with *Pst* I-digested pUC13, and clones identified using colony hybridization. Plasmid DNA from positive colonies was tested by restriction analysis and inserts of the expected size were subcloned for sequencing. Cloned and subcloned fragments were sequenced with the standard primers M13-21F and M13R (Table 1). Missing sequence stretches were obtained by primer walking. Sequencing was performed with an ABI Prism 310 or 377 Genetic Analyzer and the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Primer oligonucleotides employed for sequencing are listed in Table 1 and were synthesized by the Nucleic Acids Protein Services (NAPS unit, University of British Columbia, Vancouver, Canada) and MWG Biotech (Ebersberg, Germany).

Sequence analyses

Sequences were analyzed by comparative database search using the BLAST X program version 2.2.3 (ALTSCHUL *et al.*, 1997) and processed with GeneDoc 2.6.001 (NICHOLAS & NICHOLAS, 1997), DNA-Strider version 1.3 (DOUGLAS, 1995; MARCK, 1988) or GeneWorks 2.5 (Oxford Molecular Group Inc.) (BROVEAK, 1996). Stem-loop structures

were predicted with the software DNA Strider. Free energy for stabilities of secondary structures was calculated using the program mfold version 3.1 by Zuker and Turner (ZUKER *et al.*, 1999).

Related sequences of photosynthesis genes and proteins were retrieved from the GenBank database (BENSON *et al.*, 2002) for comparison. These amino acid sequences were either deduced from gene sequences or derived from polypeptide data originally determined by direct protein sequencing. Raw sequence data were aligned with Clustal X version 1.8 (THOMPSON *et al.*, 1997). The terminal sections of individual α and β polypeptide sequences showed a high variability in size. In order to compensate for bias due to the different sequence lengths, our phylogenetic analysis was restricted to those amino acid sequence positions which were present in all polypeptides (Fig. 3, double-headed arrows). The resulting amino acid (aa) alignments were 42 aa long for the LH1 α polypeptide and 47 aa long for the LH1 β-polypeptide. Phylogenetic analyses were performed with LH1 polypeptides alone and in combination with LH2 amino acid sequences to unravel any possible relatedness of the *Rss. parvum* LH polypeptides to peripheral antennae. For phylogenetic analyses, the programs of the Phylogeny Inference Package (PHYLIP) Version 3.6(alpha3) (FELSENSTEIN, 2002) were employed. Phylogenetic trees were calculated with the algorithm for maximum likelihood (PROTML) and maximum parsimony (PROTPARS). For matrix based tree calculation (FITCH), distances were generated with the PROTDIST program, employing the PAM 001 matrix (DAYHOFF *et al.*, 1979). Trees were constructed using the global rearrangement option with a randomized species input order (jumble: random number seed 133, 10 times). Bootstrap analysis was performed with 100 data sets (jumble: random number seed 133, 3 times) employing all three algorithms. The table of pairwise % sequence identity values was created with PROTDIST.

16S rRNA relatedness was also determined, employing the same criteria, algorithms and parameters as described above. From sequences of 32 species, a 1335 nucleotide alignment was generated for tree construction with the programs DNAML, DNAPARS and DNADIST/FITCH. In DNADIST, the algorithm of JUKES & CANTOR (1969) was used to calculate a distance matrix. Phylogenetic trees were inferred based on the algorithm of FITCH & MARGOLIASH (1967) as implemented in the program FITCH of the PHYLIP package.

Modeling of three-dimensional structures

In order to identify polypeptide residues which potentially interact with BChl *a* in the antenna complex of *Rss. parvum*, three dimensional (3-D) models were constructed. Initially, a SWISS-Model BLAST search was performed to find appropriate modeling templates in the ExPDB database (http://swissmodel.expasy.org/). 3-D modeling was performed with the Deep View - Swiss-Pdb Viewer version 3.7b2 (GUEX *et al.*, 1999; GUEX & PEITSCH, 1997). Using this SWISS-MODEL interface, initial models were constructed by superimposing the *Rss. parvum* raw sequence with template structures via the "Magic fit" tool of the Deep View program. Model projects were then submitted via the "Optimise Mode" option to the SWISS-MODEL Expert Protein Analysis System (ExPASy) web server, which returned the final models.

To date, only a few entries of 3-D structures of purple bacterial antenna systems are available in the Protein Data Bank (PDB) for comparative model prediction. These are the peripheral antenna structures from *Rhodopseudomonas acidophila* and *Phaeospirillum molischianum* (PAPIZ *et al.*, 2003; SORGEN *et al.*, 2002; MCLUSKEY *et al.*, 2001; PRINCE *et al.*, 1997; KOEPKE *et al.*, 1996) and one core antenna chain of *Rhodobacter sphaeroides* (CONROY *et al.*, 2000). The LH2 subunit of *Phaeospirillum molischianum* (crystallographic X-ray data at 2.4 Å resolution; PDB record 1lgh) (KOEPKE *et al.*, 1996) showed the highest sequence similarity (42.5 % identity, BLAST E-value: 6×10^{-6}) to the *Rss. parvum* LH1β subunit, followed by the LH1 β subunit of *Rhodobacter sphaeroides* (34.8 % identity, BLAST E-value: 10^{-5}). The latter represents the only core antenna subunit which is available in the database (NMR data; PDB record 1dx7) (CONROY *et al.*, 2000); no core antenna structure is available for LH1α polypeptides. Because the *Rss. parvum* antenna resembles LH2 with respect to absorption characteristics (PERMENTIER *et al.*, 2000), the LH2α polypeptide of *Phaeospirillum molischianum* was used as a template for the *Rss. parvum* LH1α polypeptide (27 % identity). It has been shown that the amino acid sequences of the peripheral antenna proteins of *Phaeospirillum molischianum* are highly similar to purple bacterial core antenna proteins (NAGASHIMA *et al.*, 1996; GERMEROTH *et al.*, 1993). However, similarity between the *Phaeospirillum molischianum* peripheral antenna polypeptide sequences and purple bacterial antenna core sequences is more pronounced for the β polypeptide (identity range 27.5 - 53.8 %) than for the α polypeptide (identity range 18.2 - 34.8 %). The *Phaeospirillum molischianum* LH2 structural templates contained pigment cofactors that are included in our models.

Nucleotide sequence accession number

Gene and protein sequence information of the *puf* operon of *Rss. parvum* has been deposited in the GenBank database under accession number AY242845.

4 Results

Structure of the *puf* operon in *Roseospirillum parvum*

Figure 1B shows the genetic map of the *Rss. parvum puf* operon. A 4.3 kb *Pst* I fragment was obtained by cloning and a 0.9 kb *Xho* I fragment by iPCR (Fig. 1A). A total of 5118 nucleotides (nt) was sequenced (Fig. 2). A BLASTX search identified five open reading frames, which exhibited high similarity to *pufBALMC* genes of other species (Fig. 1B). These genes encode the β and α subunits of the LH1 complex (*pufBA*), the L and M subunits of the RC (*pufLM*) and a RC-bound tetraheme cytochrome (*pufC*). In addition, the 3'-portion of a *bchZ* gene, encoding a chlorin reductase subunit, was deduced from the nt sequence 5' of *pufB*. The organization of the *puf* genes in *Rss. parvum* therefore is very similar to that in other anoxygenic phototrophic proteobacteria (TUSCHAK *et al.*, 2003).

Individual *puf* genes were 207 nt (*pufB*), 204 nt (*pufA*), 828 nt (*pufL*), 972 nt (*pufM*) and 1089 nt (*pufC*) long (Fig. 1 and Fig. 2). An additional open reading frame (orf) was detected in the intergenic region between *bchZ* and *pufB*. This orf starts within the *bchZ* stop codon and encodes a putative peptide of 65 amino acids with no significant similarity to known proteins.

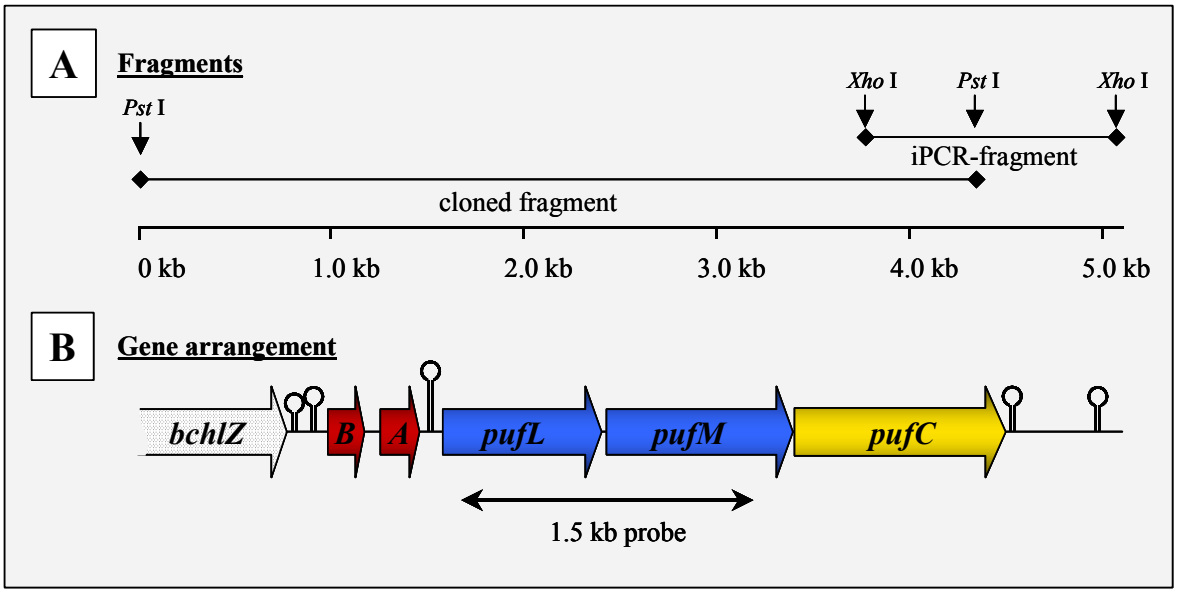
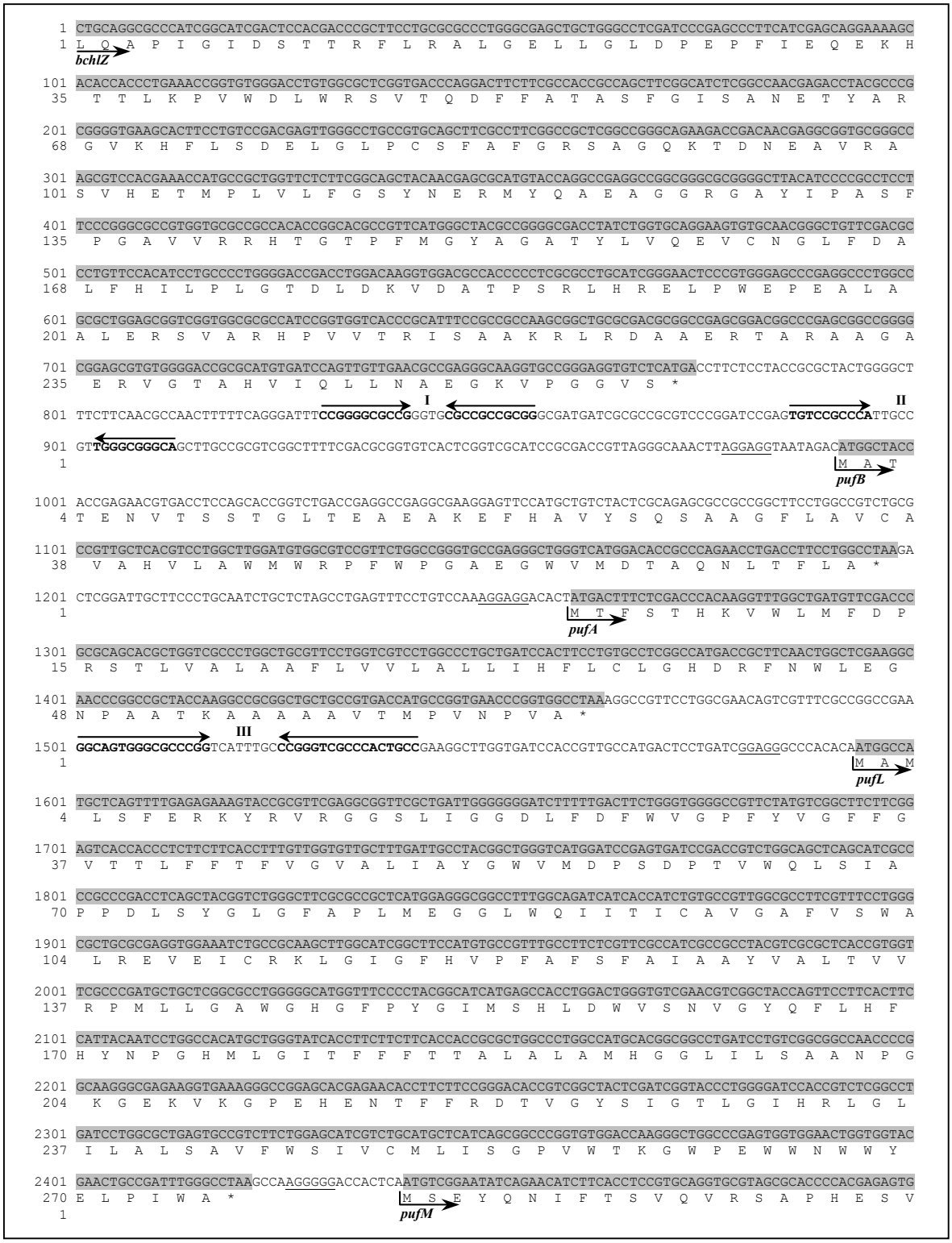


Figure 1: Genetic map of the *puf* operon of *Rss. parvum*. (**A**) Cloned and iPCR-amplified fragments. *Pst* I restriction sites used for cloning and *Xho* I sites used for iPCR are shown. (**B**) Gene arrangement. Large filled horizontal arrows indicate putative genes identified on a 5118 bp long section of the chromosome. Predicted hairpin structures are indicated as loops. The double-headed arrow marks the probe used for Southern hybridization.

As in other purple bacteria, putative mRNA secondary structures were identified within intercistronic regions (Fig. 1B; Fig. 2, I - V). Two predicted hairpin structures were localized between *bchZ* and *pufB* (I: ΔG = -14.3 kcal · mol^{-1}, II: ΔG = -19.1 kcal · mol^{-1}). The most stable predicted hairpin was found in the non-coding region between *pufA* and *pufL* (III: ΔG = -34.9 kcal · mol^{-1}). Additional hairpins were predicted 3' of *pufC* (IV: ΔG = -27.1 kcal · mol^{-1}, V: ΔG = -16.7 kcal · mol^{-1}), and may represent transcription terminators.



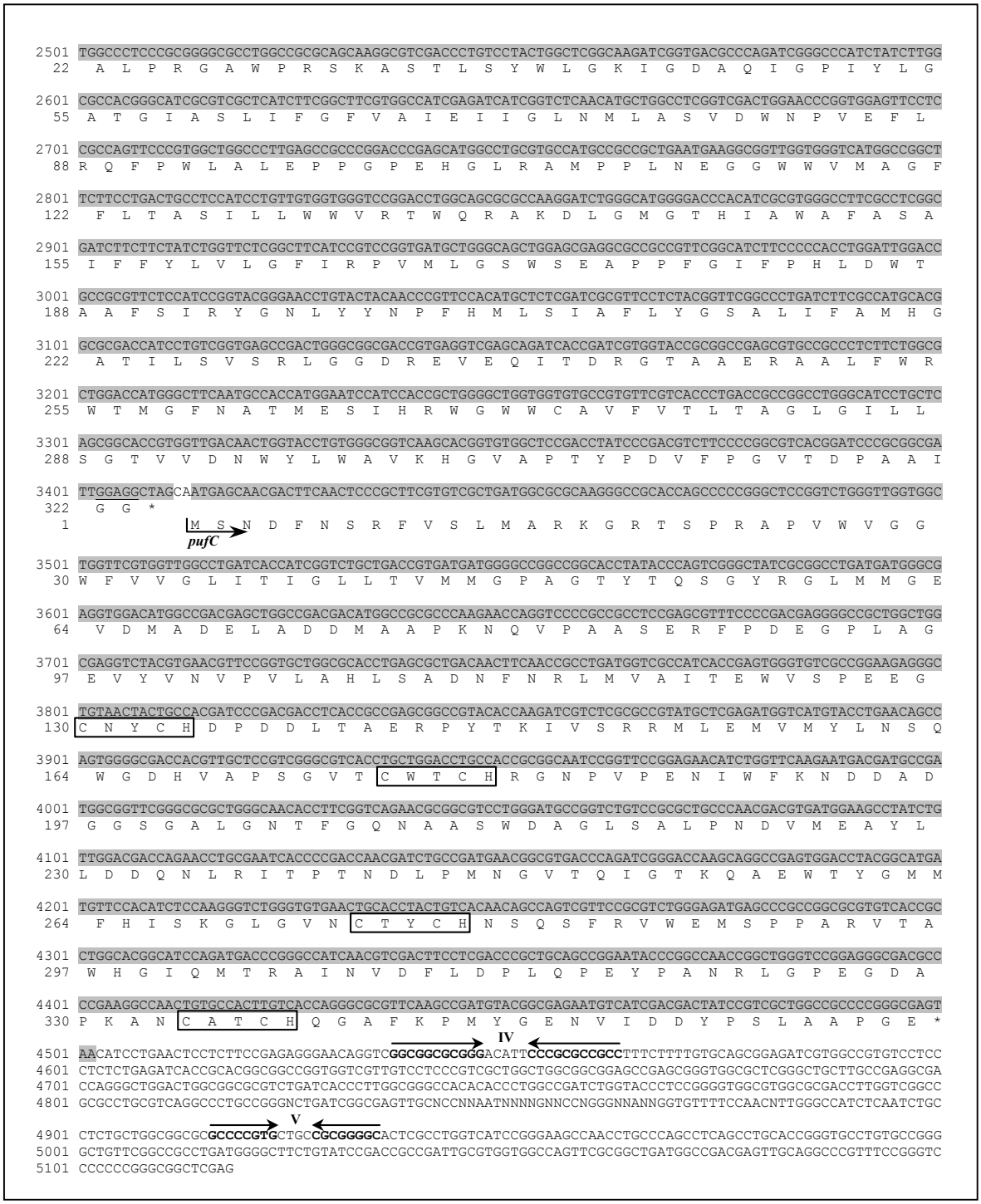


Figure 2: Nucleotide sequence and the deduced amino acid sequences of predicted *puf* genes of *Rss. parvum*. Coding regions are shaded in gray. The start codon for each gene is marked with an angled arrow below the amino acid sequence. Predicted hairpin-loop structures are indicated by head to head arrows above the DNA sequence and numbered I to V. Putative Shine-Dalgarno sequences upstream the 5'-ends of the predicted genes are underlined. Four heme-binding motifs (C-x-x-C-H) in the translated *pufC* sequence are framed in boxes.

Analysis of amino acid sequences

Polypeptide sizes of 68 aa for PufB, 67 aa for PufA, 275 aa for PufL, 323 aa for PufM and 362 aa for PufC were deduced from the nucleotide sequence (Fig. 2). Amino acid residues potentially involved in the Q_y absorption of LH1 BChl *a* were identified by comparison of the α polypeptide (PufA) and β polypeptide (PufB) amino acid sequences with those of other phototrophic proteobacteria. An amino acid sequence alignment (Fig. 3) revealed that both polypeptides of *Rss. pavum* clearly differ with respect to otherwise conserved amino acids. Almost all of these changes (highlighted in black; Fig. 3) were located close to the conserved histidine residues (His^0, underlain in black in Fig. 3), which serve as a fifth ligand to the central Mg^{2+} ion of the BChl macrocycle.

The LH1α polypeptide of *Rss. parvum* differs from most other homologues at four sites (Fig. 3A). The conserved arginine residue at position α^{-17} is replaced by serine in *Rss. parvum*. This residue is located where the transmembrane domain is predicted to leave the lipid bilayer (ZUBER & COGDELL, 1995). At this sequence position, uncharged amino acids are also present in α polypeptide chains of *Rhodospirillum rubrum*, *Rhodocyclus tenuis*, *Halorhodospira halochloris*, *Allochromatium vinosum* and *Amoebobacter purpureus* (Fig. 3). The latter three species contain multiple antenna polypeptides, which differ at position α^{-17} (Fig. 3A).

Most differences were found in close proximity of the conserved histidine residue (αHis^0). The positions αCys^{+3}, αGly^{+5} and αHis^{+6} in *Rss. parvum* differ from the consensus residues αLeu^{+3}, αSer^{+5} and αThr^{+6}, respectively (Fig. 3A, marked in black). Thus, this conserved amino acid sequence (LLST) differs by three residues in *Rss. parvum* (***C***L***GH***), suggesting that these amino acids may be involved in the red-shift of the Q_y absorption band of BChl *a* in the LH1 complex. The cysteine residue at position α^{+3} in the LH1α of *Rss. parvum* is of special interest, because it replaces the nonpolar, aliphatic residues found in almost all other purple bacteria. A cysteine at position α^{+3} is also found in the 2nd LH1 α protein of *Halorhodospira halochloris*, which however contains BChl *b* as the chromophore and thus cannot be directly compared to *Rss. parvum*. As in *Rss. parvum*, a glycine at residue α^{+5} is also present in *Rubrivivax gelatinosus* and *Rhodocyclus tenuis*, whereas a methionine, arginine or threonine is present at this position in the purple sulfur bacteria *Amoebobacter purpureus* (2nd α chain), *Allochromatium vinosum* (3rd α chain) and *Halorhodospira halochloris*, respectively. In contrast to the two other positions, the αHis^{+6} in *Rss. parvum* is unique among the known LH1 of phototrophic proteobacteria.

A

	BChl	*in vivo* absorption LH1	LH2 / LH3	(Ref.)
Acidiphilium acidophilum ATCC27807^T	Zn *a*	(~864) [b]	(792) [b]	(1)
*Acidiphilium angustum** ATCC 35903^T	Zn *a*	(~864) [b]	(792) [b]	(1)
*Acidiphilium rubrum** ATCC 35905^T	Zn *a*	(~864) [b]	(792) [b]	(1)
Blastomonas natatoria DSM 3183^T	*a*	(867) [b]	(absent) [b]	(1)
Blastomonas ursincola DSM 9006^T	*a*	867	absent	(1)
Bradyrhizobium sp. ORS278	*a*	875	absent	(2)
Amoebobacter purpureus ML1 *pufA*$_1$	*a*	878	798/826	(3)
Amoebobacter purpureus ML1 *pufA*$_2$	*a*	878	798/826	(3)
Amoebobacter purpureus ML1 *pufA*$_3$	*a*	878	798/826	(3)
Allochromatium vinosum ATCC 17899^T *pufA*$_1$	*a*	892	797/808/822/850	(3)
Allochromatium vinosum ATCC 17899^T *pufA*$_2$	*a*	892	797/808/822/850	(3)
Allochromatium vinosum ATCC 17899^T *pufA*$_3$	*a*	892	797/808/822/850	(3)
*Thermochromatium tepidum** ATCC 43061^T	*a*	917	800/805/855	(3)
*Ectothiorhodospira shaposhnikovii** DSM 243^T	*a*	890	795/855	(3)
Halorhodospira halochloris ATCC 35916^T (1st α)	*b*	1018	800/830	(3)
Halorhodospira halochloris ATCC 35916^T (2nd α)	*b*	1018	800/830	(3)
Halorhodospira halophila SL1 (1st α)	*a*	890	800/840	(3)
Halorhodospira halophila SL1 (2nd α)	*a*	890	800/840	(3)
Roseobacter denitrificans ATCC 33940^T	*a*	870	806	(1)
Rubrivivax gelatinosus IL144	*a*	880	803/859	(3)
Erythrobacter sp. MBIC3960	*a*	(~869) [b]	(absent) [b]	(1)
Erythrobacter longus ATCC 33941^T	*a*	870	absent	(1)
Rhodobacter capsulatus ATCC 11166^T	*a*	875	806/863	(3)
Rhodobacter sphaeroides ATCC 17023^T	*a*	890	801/852	(3)
Rhodocyclus tenuis DSM 109^T	*a*	893	n. a.	(3)
Roseateles depolymerans DSM 11813^T	*a*	870	absent	(5)
Rhodoblastus acidophilus ATCC 25092^T	*a*	890	800/820/855)	(3)
Rhodobium marinum DSM 2698^T	*a*	884	absent	(3)
Rhodopseudomonas palustris ATCC 17001^T	*a*	880-885	800/830/850	(3)
Blastochloris viridis DSM 133^T	*b*	1015	absent	(3)
Phaeospirillum molischianum ATCC 14031^T	*a*	885	801/825/845	(3)
Rhodospirillum rubrum ATCC 11170^T	*a*	883	absent	(3)
Rhodovulum sulfidophilum DSM 1374^T	*a*	890	803/855	(3)
Roseospirillum parvum* 930I DSM 12498^T *pufA	***a***	**805/911**	**absent**	(4)
Chloroflexus aurantiacus DSM635^T	*a*	806-866	absent	(6)

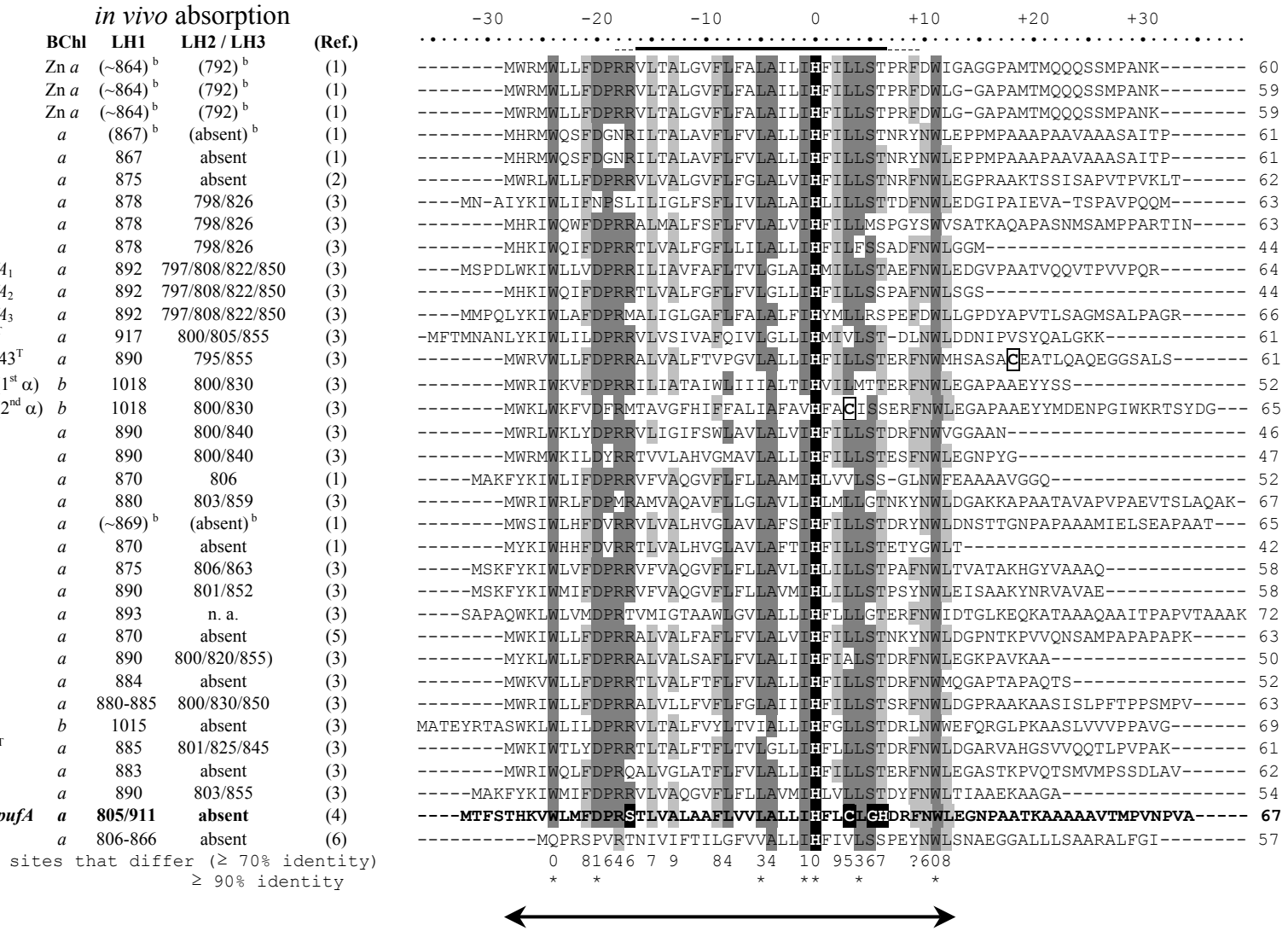


Figure 3A

B

	BChl	*in vivo* absorption LH1	LH2 / LH3	(Ref.)
*Acidiphilium angustum** ATCC 35903^T	Zn *a*	(~864) [b]	(792) [b]	(1)
*Acidiphilium rubrum** ATCC 35905^T	Zn *a*	(~864) [b]	(792) [b]	(1)
Bradyrhizobium sp. ORS278	*a*	875	absent	(2)
Amoebobacter purpureus ML1 *pufB*$_1$	*a*	878	798/826	(3)
Amoebobacter purpureus ML1 *pufB*$_2$	*a*	878	798/826	(3)
Amoebobacter purpureus ML1 *pufB*$_3$	*a*	878	798/826	(3)
Allochromatium vinosum ATCC 17899^T *pufB*$_1$	*a*	892	797/808/822/850	(3)
Allochromatium vinosum ATCC 17899^T *pufB*$_2$	*a*	892	797/808/822/850	(3)
Allochromatium vinosum ATCC 17899^T *pufB*$_3$	*a*	892	797/808/822/850	(3)
*Thermochromatium tepidum** ATCC 43061^T	*a*	917	800/805/855	(3)
*Ectothiorhodospira shaposhnikovii** DSM 243^T	*a*	890	795/855	(3)
Halorhodospira halochloris ATCC 35916^T (2nd β)	*b*	1018	800/830	(3)
[a]*Halorhodospira halochloris* ATCC 35916^T (1st β)	*b*	1018	800/830	(3)
Halorhodospira halophila SL1 (1st β)	*a*	890	800/840	(3)
Halorhodospira halophila SL1 (2nd β)	*a*	890	800/840	(3)
Roseobacter denitrificans ATCC 33940^T	*a*	870	806	(1)
Rubrivivax gelatinosus IL144	*a*	880	803/859	(3)
Erythrobacter sp. MBIC3960	*a*	(869) [b]	(absent) [b]	(1)
Erythrobacter longus ATCC 33941^T	*a*	870	absent Y	(1)
Rhodobacter capsulatus ATCC 11166^T	*a*	875	806/863	(3)
Rhodobacter sphaeroides ATCC 17023^T	*a*	890	801/852	(3)
Rhodocyclus tenuis DSM 109^T	*a*	893	n. a.	(3)
Roseateles depolymerans DSM 11813^T	*a*	870	absent	(5)
Rhodoblastus acidophilus ATCC 25092^T	*a*	890	800/820/855)	(3)
Rhodobium marinum DSM 2698^T	*a*	884	absent	(3)
Rhodopseudomonas palustris ATCC 17001^T	*a*	880-885	800/830/850	(3)
Blastochloris viridis DSM 133^T	*b*	1015	absent	(3)
Phaeospirillum molischianum ATCC 14031^T	*a*	885	801/825/845	(3)
Rhodospirillum rubrum ATCC 11170^T	*a*	883	absent	(3)
Rhodovulum sulfidophilum DSM 1374^T	*a*	890	803/855	(3)
Roseospirillum parvum* 930I DSM 12498^T*pufB	***a***	**805/911**	**absent**	(4)
Chloroflexus aurantiacus DSM635^T B806-866	*a*	806-866	absent	(6)

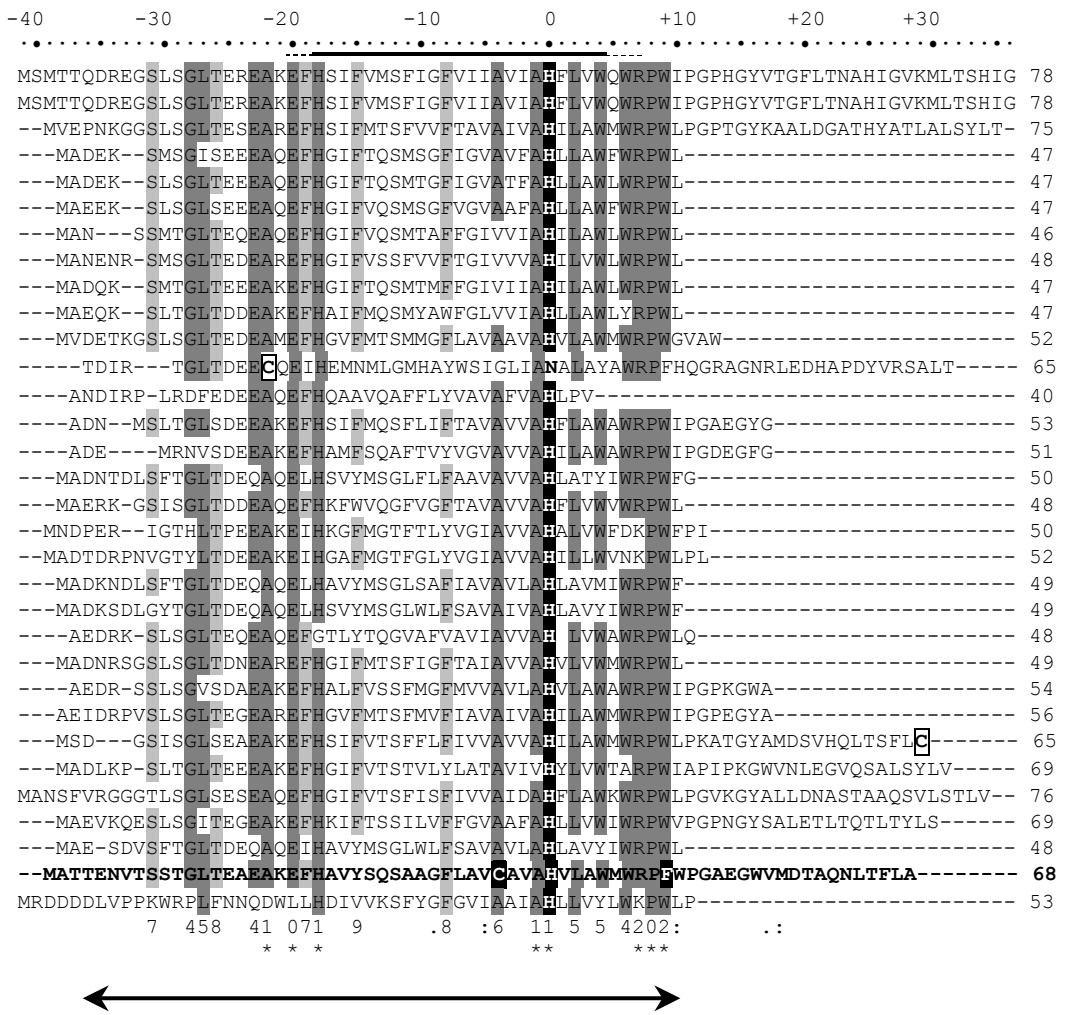


Figure 3B

Figure 3: Amino acid sequence alignments of LH1α (**A**) and LH1β (**B**) polypeptides. Amino acid numbering is relative to the highly conserved histidine residues (His^0, shaded black) and indicated on top. Negative numbers indicate positions towards the N-terminus and positive numbers indicate positions towards the C-terminus. Strongly conserved residues are shaded in light (≥ 70 %) and dark (≥ 80 %) gray. Numbers below the sequence indicate the amount of residues that differ from the consensus. The transmembrane domain of the polypeptides is indicated as a solid line on top of the sequences. The *Rss. parvum* sequences obtained in the present study are shown in boldface and residues that differ from the consensus motif are highlighted in black. Cysteine residues are indicated in framed boxes. The double-headed arrows indicate the section of amino acids used for phylogenetic analysis. [a], sequences excluded from phylogenetic analyses. [b], sequences data available from direct protein sequencing only. *n.a.* data not available. References: (1), (YURKOV & BEATTY, 1998); (2), (GIRAUD *et al.*, 2000); (3), (PERMENTIER, 2001); (4), (PERMENTIER *et al.*, 2000); (5), (SUYAMA *et al.*, 1999); (6), (ZUBER & COGDELL, 1995).

The β polypeptide of *Rss. parvum* differs from that of other species at two conserved positions (Fig. 3B). A cysteine at position β^{-4} replaces the conserved amino acid βAla^{-4} (or a valine or glycine residue present in polypeptides of three other species). In the *Rss. parvum* LH1β, a phenylalanine is present at position β^{+9}, replacing the βTrp^{+9} that is highly conserved in the other purple bacteria. A tryptophan residue at position β^{+10} is present next to βPhe^{+9} in *Rss. parvum* (Fig. 3B). The only other species in which a phenylalanine at residue β^{+9} is found is *Halorhodospira halochloris* (2^{nd} chain); here it is followed by a histidine, however.

In general, cysteine residues are rare in LH polypeptides . Besides in *Rss. parvum*, cysteine exists only in the α protein of *Ectothiorhodospira shaposhnikovii*, in the β protein of *Rhodopseudomonas palustris* and in the α_2 and β_2 proteins of *Halorhodospira halochloris* (Fig. 3A and B, three framed positions).

Structural models of the LH1 polypeptides

In *Rss. parvum* the unusual cysteine residues are positioned at a distance of three (αCys^{+3}) or four (βCys^{-4}) amino acids from the primary BChl ligand His^0 (Fig. 3). Since one turn of an alpha-helical protein structure corresponds to 3.6 amino acids, the cysteine residues in both polypeptides potentially have the correct orientation to interact with the BChl *a* molecules. The orientation of the unusual amino acids in the α and β polypeptides of *Rss. parvum* was therefore evaluated in three dimensional structural models (Fig. 4). Three structural models were constructed: One based on the *Rhodobacter sphaeroides* LH1 β apoprotein template (Fig. 4A,B), one on the *Phaeospirillum molischianum* LH2 β (Fig. 4C,D) and one on the LH2 α apoprotein template (Fig. 4E,F).

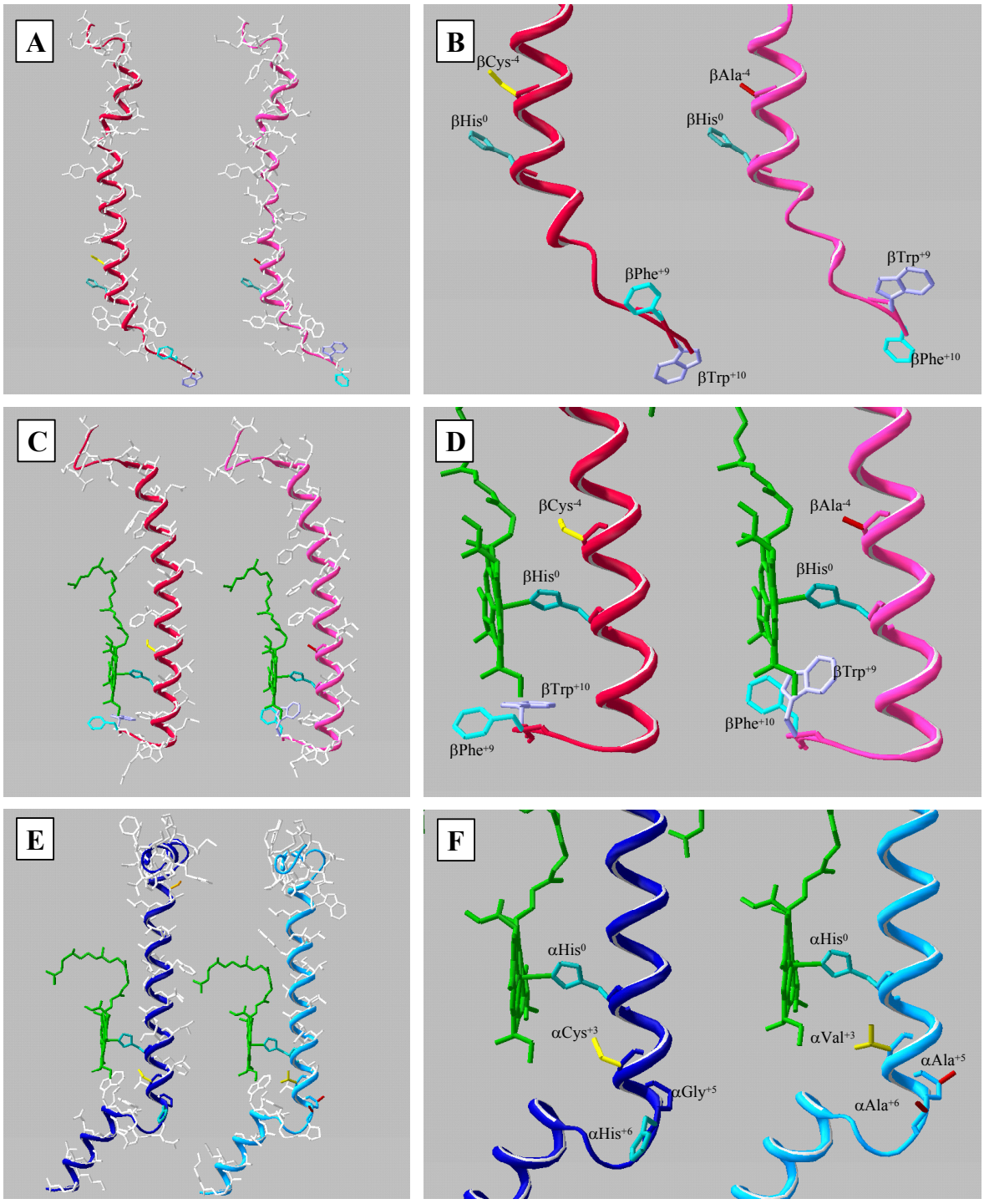


Figure 4: Comparative 3D models of the LH1 polypeptides of *Rss. parvum*. (**A,B**) Model based on structural data available for LH1 β core antenna polypeptide template of *Rhodobacter sphaeroides*. (**C-G**) Models based on the LH2 α and β peripheral antenna polypeptides templates of *Phaeospirillum molischianum*. In panels **A-F** the *Rss. parvum* polypeptide is depicted on the left side and its template model is displayed on the right side for reference. (**G**) Combination of the peripheral antenna based models into two building blocks. For clarity some of the polypeptide backbone structures were omitted. The helical structures represent the protein backbone. Color code: red, *Rss. parvum* β polypeptides; pink, template β polypetides; blue, *Rss. parvum* α polypeptide; light cyan, template α polypetide; green: BChl *a* molecules; light blue, tryptophan; turquoise, histidine; cyan, phenylalanine; yellow, cysteine; orange, serine; white, all other amino acid side chains.

Modeling based on the β polypeptide templates indicates that the *Rss. parvum* βCys^{-4} is oriented in the same direction as the BChl binding histidine (Fig. 4A-D). The spatial orientation of the thiol side chain, however, differs in these two models (compare Fig. 4B and D). Another difference is the orientation of the C-termini. In the model based on the core antenna of *Rbc. sphaeroides* LH1 β, the C-terminus projects in the opposite direction of the BChl coordinating βHis^{0} (Fig. 4B). In the model based on the the peripheral antenna, the C-terminus folds back in a loop towards the BChl (Fig 4D). As a consequence, the aromatic amino acid βPhe^{+9} present in *Rss. parvum* approaches the BChl *a* macrocycle from the opposite side compared to the βTrp^{+9}, which is highly conserved among other LH complexes and also present in the *Phaeospirillum molischianum* template (Fig 4D, right side). Similar to the βCys^{-4} in the β polypeptide of *Rss. parvum*, the αCys^{+3} in the α polypeptide also faces toward the BChl macrocycle (Fig. 4F,G). The αGly^{+5} and αHis^{+6} of the *Rss. parvum* α polypeptide point away from the BChl *a* molecule (Fig. 4E,F).

By varying the rotational angle of the thiol side chains, both the αCys^{+3} and the βCys^{-4} could reach van der Waals distance to the BChl *a* macrocycles. The βCys^{-4} approaches the α chain BChl as close as ~3.1 Å, and the phytol side chain of the β-chain BChl ~3.3 Å. Minimum distances for αCys^{+3} were determined to be ~3.6 Å to the β-chain BChl and ≥ 4.5 Å to the α chain BChl (Fig. 4G).

Phylogenetic relationships of LH1 sequences

The αCys^{+3}, αGly^{+5} and βPhe^{+9} of *Rss. parvum* are also found in LH1 sequences of some other species, namely *Halorhodospira halochloris* (αCys^{+3} and βPhe^{+9}), *Rubrivivax gelatinosus* (αGly^{+5}) and *Rhodocyclus tenuis* (αGly^{+5}) (Fig. 3). It thus appeared possible that LH polypeptides containing these unusual amino acids derived from a common ancestor and hence are phylogenetically closely related. Therefore, the overall relatedness of both LH1 proteins of *Rss. parvum* with those found in other purple bacteria was evaluated for a total of 35 α polypeptides and 31 β polypeptides. The N- and C-terminal sequences were omitted from the analyses because of high variability, and so the phylogenetic trees (Fig. 5) are based on a total of 42 and 47 informative amino acid positions of the LH1 α and β proteins, respectively. These sites were mainly located in the transmembrane domain (Fig. 3, double-headed arrow).

Table 2 Table of % idenity of selected LH1 polypetides that are most closley related to the *Rss parvum* 930I LH1 α and β subunits. Values > 65 % identity are underlain in gray. Multiplicity of LH1 polypeptides is indicated in parenthesis if present.

	pairwise % amino acid sequence identity to																
	Alc. vinosum (1st) ATCC 17899^T	*Alc. vinosum* (2nd) ATCC 17899^T	*Alc. vinosum* (3rd) ATCC 17899^T	*Amb. purpureus* (1st) ML1	*Amb. purpureus* (2nd) ML1	*Amb. purpureus* (3rd) ML1	*Blm. natatoria* DSM 3183^T	*Blm. ursincola* DSM 9006^T	*Bradyrhizobium* sp. ORS278	*Ect. shaposhnikovii* DSM 243^T	*Hlr. halophila* (1st) DSM 244^T	*Rbi. marinum* DSM 2698^T	*Rbl. acidophilus* 7050 DSM 137^T	*Psp. molischianum* ATCC 14031^T	*Rps. palustris* ATCC BAA-98	*Rsp. rubrum* ATCC 11170^T	*Rst. depolymerans* DSM11813^T	*Rvi. gelatinosus* IL144
Rss. parvum 930I ***pufB*** DSM 12498^T	51.3	50.0	51.2	52.6	55.0	57.9	n.a.	n.a.	55.0	**66.7**	56.4	54.8	55.3	46.2	59.5	46.3	51.2	51.2
Rss. parvum 930I ***pufA*** DSM 12498^T	55.0	**68.4**	52.5	55.3	54.1	64.1	61.5	61.5	62.5	60.0	48.7	**72.5**	**74.4**	65.0	56.4	65.0	62.5	46.3

n.a. not available

In general, phylogenetic trees generated for the LH1 α and LH1 β polypeptides are similar in topology and encompass several clusters, which are supported by bootstrap analysis and also correspond to clusters found in the 16S rRNA phylogeny (Fig 5, underlain in gray). More specifically, stable clusters were detected for (1) the *Acidiphilium* group of the α proteobacteria, (2) the α-3 proteobacteria and (3) the *Erythrobacter* species within the α-4 proteobacteria. In addition, clustering of *Blastomonas* species in the α polypeptide tree is supported by bootstrap analysis.

However, the affiliation of the *Rss. parvum* antenna polypeptides differs in the two trees. *Rhodoblastus acidophilus* shows the highest identity to the LH1α of *Rss. parvum* (>74 %, Table 2), closely followed by *Rhodobium marinum* (>72 %). This relationship is reflected in the clustering of the respective phylogenetic tree (Fig. 5A). In contrast, the β polypeptide clustered with *Ectothiorhodospira shaposhnikovii* (Fig. 5B). An identity value of 66.7 % was determined, which is by far the highest value for the *Rss. parvum* β chain (Table 2). Relatedness between purple bacterial LH1 α polypeptides was in general higher (on average 57.6 % identity) than between β polypeptides (on average 53.1 % identity). However, a specifically tight affiliation of LH1 polypeptides of species that share unusual spectral properties or unusual amino acid residues with the *Rss. parvum* LH1 was not observed.

Because the *Rss. parvum* LH1 antenna displays spectroscopic characteristics of LH2 complexes (PERMENTIER *et al.*, 2000), the phylogenetic relatedness to peripheral LH2 antenna complexes was determined separately. However, both polypeptides of *Rss. parvum* consistently clustered with other LH1 polypeptides, which was supported by high bootstrap values (phylogenetic tree not shown). Accordingly, the identity between the *Rss. parvum* LH1α and the LH2α polypeptide sequences of other species was significantly lower (18.4 - 35.9 %) than to other LH1α polypeptides (43.6 - 74.4 %). The range of identity values for the β polypeptides was 34.3 - 47.1 % between *Rss. parvum* LH1β and the other LH2β, whereas identity to *Phaeospirillum molischianum* was 51.4 %. In contrast, sequence identity to other LH1α polypeptides was in the range of 48.7 - 75.0 %.

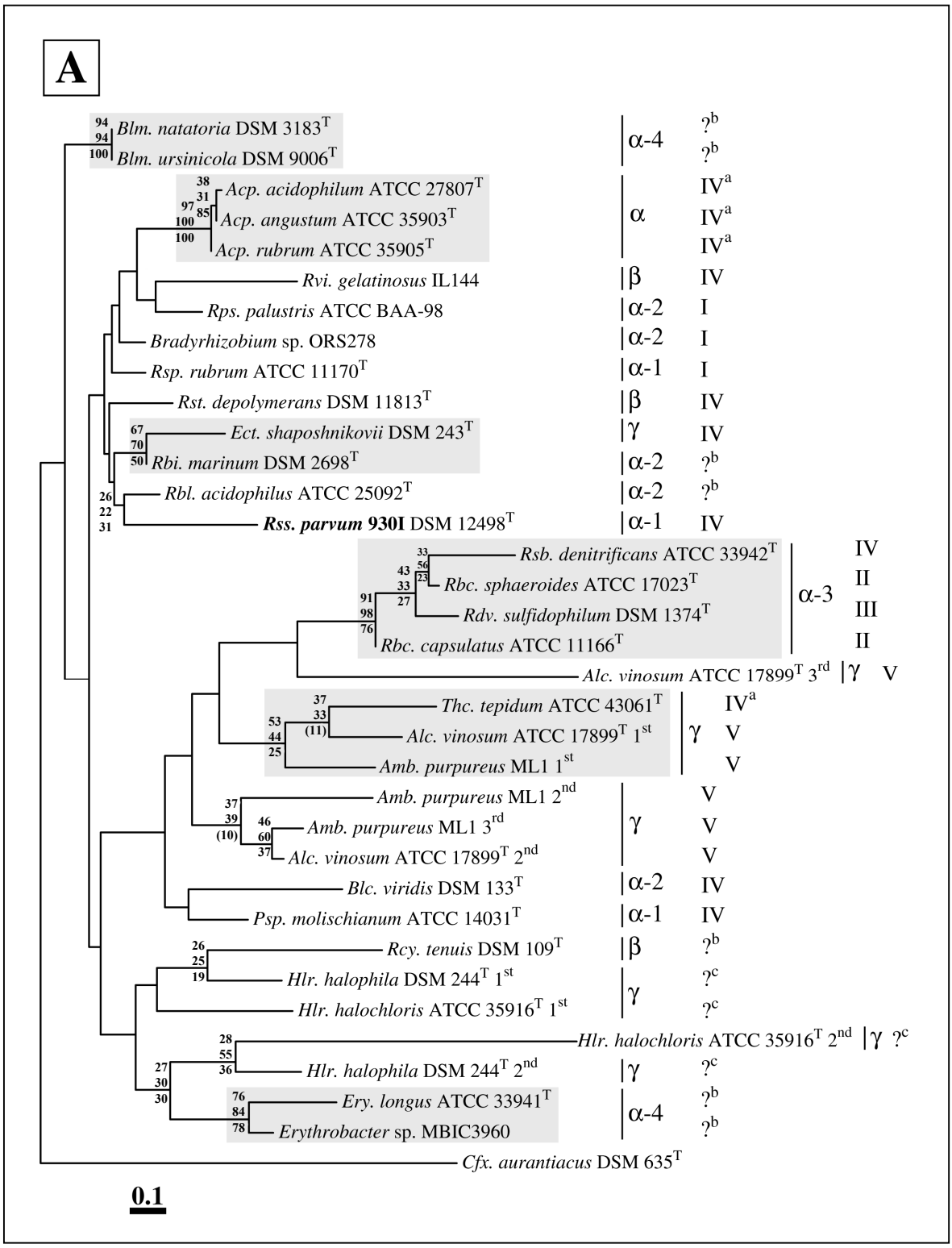

A
Blm. natatoria DSM 3183T
Blm. ursinicola DSM 9006T
α-4 ?b ?b
Acp. acidophilum ATCC 27807T
Acp. angustum ATCC 35903T
Acp. rubrum ATCC 35905T
α IVa IVa IVa
Rvi. gelatinosus IL144 β IV
Rps. palustris ATCC BAA-98 α-2 I
Bradyrhizobium sp. ORS278 α-2 I
Rsp. rubrum ATCC 11170T α-1 I
Rst. depolymerans DSM 11813T β IV
Ect. shaposhnikovii DSM 243T γ IV
Rbi. marinum DSM 2698T α-2 ?b
Rbl. acidophilus ATCC 25092T α-2 ?b
Rss. parvum 930I DSM 12498T α-1 IV
Rsb. denitrificans ATCC 33942T
Rbc. sphaeroides ATCC 17023T
Rdv. sulfidophilum DSM 1374T
Rbc. capsulatus ATCC 11166T
α-3 IV II III II
Alc. vinosum ATCC 17899T 3rd γ V
Thc. tepidum ATCC 43061T
Alc. vinosum ATCC 17899T 1st
Amb. purpureus ML1 1st
γ IVa V V
Amb. purpureus ML1 2nd
Amb. purpureus ML1 3rd
Alc. vinosum ATCC 17899T 2nd
γ V V V
Blc. viridis DSM 133T α-2 IV
Psp. molischianum ATCC 14031T α-1 IV
Rcy. tenuis DSM 109T β ?b
Hlr. halophila DSM 244T 1st
Hlr. halochloris ATCC 35916T 1st
γ ?c ?c
Hlr. halochloris ATCC 35916T 2nd γ ?c
Hlr. halophila DSM 244T 2nd γ ?c
Ery. longus ATCC 33941T
Erythrobacter sp. MBIC3960
α-4 ?b ?b
Cfx. aurantiacus DSM 635T
0.1

Figure 5A

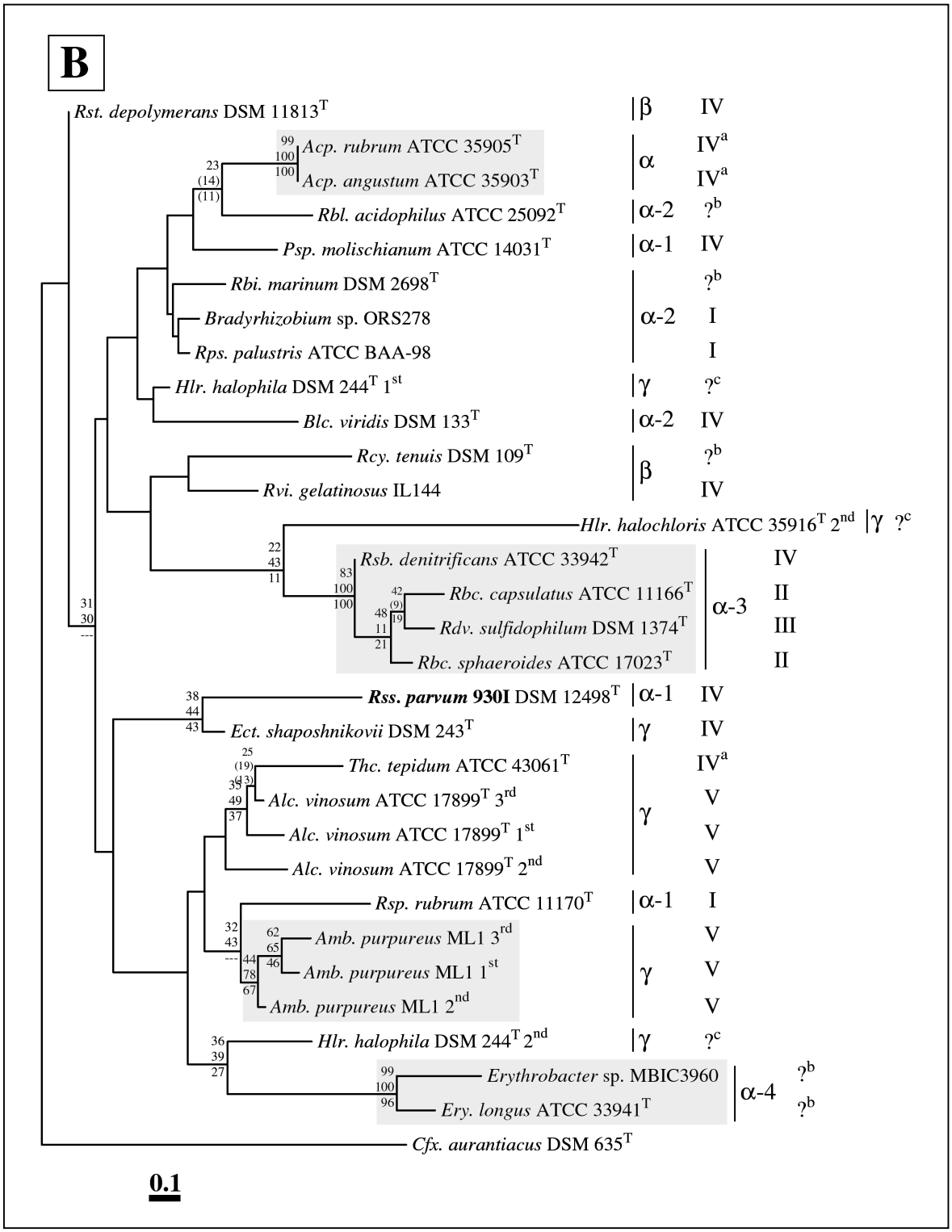

B
Rst. depolymerans DSM 11813T
Acp. rubrum ATCC 35905T
Acp. angustum ATCC 35903T
Rbl. acidophilus ATCC 25092T
Psp. molischianum ATCC 14031T
Rbi. marinum DSM 2698T
Bradyrhizobium sp. ORS278
Rps. palustris ATCC BAA-98
Hlr. halophila DSM 244T 1st
Blc. viridis DSM 133T
Rcy. tenuis DSM 109T
Rvi. gelatinosus IL144
Hlr. halochloris ATCC 35916T 2nd
Rsb. denitrificans ATCC 33942T
Rbc. capsulatus ATCC 11166T
Rdv. sulfidophilum DSM 1374T
Rbc. sphaeroides ATCC 17023T
Rss. parvum 930I DSM 12498T
Ect. shaposhnikovii DSM 243T
Thc. tepidum ATCC 43061T
Alc. vinosum ATCC 17899T 3rd
Alc. vinosum ATCC 17899T 1st
Alc. vinosum ATCC 17899T 2nd
Rsp. rubrum ATCC 11170T
Amb. purpureus ML1 3rd
Amb. purpureus ML1 1st
Amb. purpureus ML1 2nd
Hlr. halophila DSM 244T 2nd
Erythrobacter sp. MBIC3960
Ery. longus ATCC 33941T
Cfx. aurantiacus DSM 635T
0.1
β IV
α IVa
IVa
α-2 ?b
α-1 IV
?b
α-2 I
I
γ ?c
α-2 IV
?b
β IV
γ ?c
IV
α-3 II
III
II
α-1 IV
γ IV
IVa
γ V
V
V
α-1 I
γ V
V
V
γ ?c
?b
α-4 ?b

Figure 5B

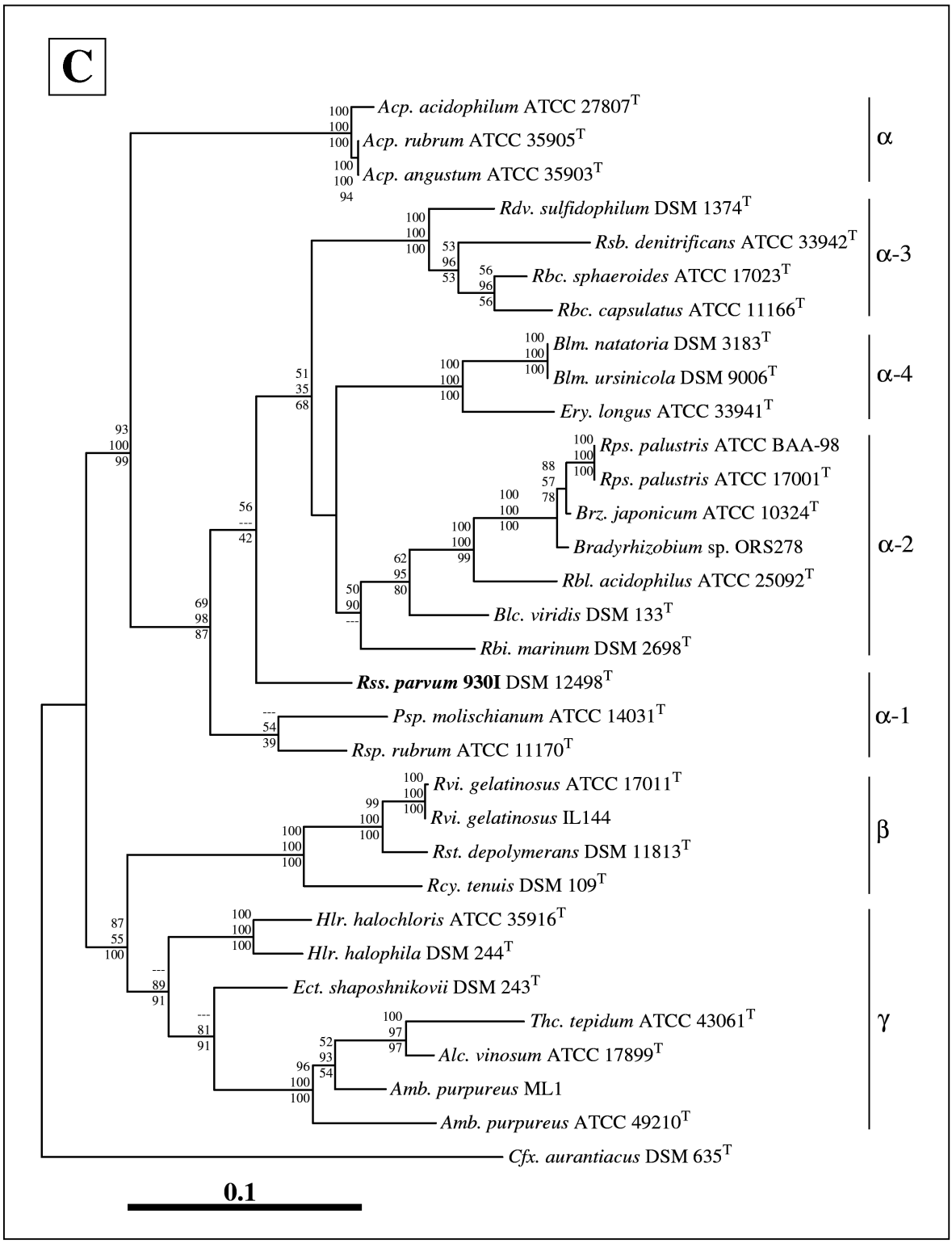

C
Acp. acidophilum ATCC 27807T
Acp. rubrum ATCC 35905T
Acp. angustum ATCC 35903T
α
Rdv. sulfidophilum DSM 1374T
Rsb. denitrificans ATCC 33942T
Rbc. sphaeroides ATCC 17023T
Rbc. capsulatus ATCC 11166T
α-3
Blm. natatoria DSM 3183T
Blm. ursinicola DSM 9006T
Ery. longus ATCC 33941T
α-4
Rps. palustris ATCC BAA-98
Rps. palustris ATCC 17001T
Brz. japonicum ATCC 10324T
Bradyrhizobium sp. ORS278
Rbl. acidophilus ATCC 25092T
Blc. viridis DSM 133T
Rbi. marinum DSM 2698T
α-2
Rss. parvum 930I DSM 12498T
Psp. molischianum ATCC 14031T
Rsp. rubrum ATCC 11170T
α-1
Rvi. gelatinosus ATCC 17011T
Rvi. gelatinosus IL144
Rst. depolymerans DSM 11813T
Rcy. tenuis DSM 109T
β
Hlr. halochloris ATCC 35916T
Hlr. halophila DSM 244T
Ect. shaposhnikovii DSM 243T
Thc. tepidum ATCC 43061T
Alc. vinosum ATCC 17899T
Amb. purpureus ML1
Amb. purpureus ATCC 49210T
γ
Cfx. aurantiacus DSM 635T
0.1

Figure 5C

Figure 5: Phylogenetic trees based on the LH1α (**A**) and LH1β (**B**) antenna polypeptides of purple bacteria. The data sets were based on the alignment in Figure 3. Major clusters underlain in gray. 16S phylogenetic affiliation is indicated in Greek letters and corresponding *puf* operon types as defined recently (TUSCHAK *et al.*, 2003) are given in Roman numerals. The green non-sulfur bacterium *Chloroflexus* (*Cfx.*) *aurantiacus* was used as an outgroup. (**C**) 16S rRNA phylogenetic tree of all species included in this study. For non-type strain species used in the antenna polypeptide analysis, the respective type strains or type species were additionally included, as for the genera *Amoebobacter*, *Bradyrhizobium*, *Rhodopseudomonas* and *Rubrivivax*, respectively. All trees displayed were determined by maximum likelihood analysis. Bootstrap values > 35 % are given beside the nodes, with maximum likelihood on top, matrix based FITCH in the middle and parsimony at the bottom. Bars represent 0.1 substitutions per site.

[a] strains which were assigned to the *puf* operon type IV due to insufficient or lack of sequence information 3' of *pufC*
[b] lack of sequence information 3' of *pufM*
[c] multiple *pufBA* genes or multiple LH1 antenna polypeptides were reported

5 Discussion

Structure of the *Rss. parvum puf* operon

The structure of the *Rss. parvum puf* operon with the gene organization *pufBALMC* represents the most common type of *puf* operon found in purple bacteria (TUSCHAK *et al.*, 2003; BEJA *et al.*, 2002). The presence of a 3' portion of the *bchZ* gene 5' of the *puf* operon indicates that the *puf* genes detected in *Rss. parvum* are part of a photosynthesis gene cluster similar to other purple bacteria (BEJA *et al.*, 2002; IGARASHI *et al.*, 2001; CHOUDHARY & KAPLAN, 2000; ALBERTI *et al.*, 1995). In all purple bacteria, the *pufBALM* genes form the core unit of the *puf* operon. Besides *pufBALMC*, several other types exist, which are characterized by the presence of additional genes 5' (*pufQ*) or 3' (*pufX*) of the core genes, or by the absence of *pufC*. No such additional genes were found in *Rss. parvum*. With the sequence of *Rss. parvum* extending more than 600 nt 3' of the *pufC* gene, the presence of additional *pufBA* genes as found in some *Chromatiaceae* (TUSCHAK *et al.*, 2003; CORSON *et al.*, 1999) can also be excluded.

Significance of amino acid substitutions in the *Rss. parvum* LH1 polypeptides

Both LH1 polypeptides of *Rss. parvum* differ considerably from LH polypeptides of other bacteria with respect to several usually highly conserved amino acids. Our results indicate that αCys^{+3}, βCys^{-4} and βPhe^{+9} are in close proximity to the BChl *a* molecules in *Rss. parvum*.

The local environment of the BChl *a* binding pocket contributes to shifts in the Q_y absorption of LH complexes (ECCLES & HONIG, 1983). Thus, hydrogen bonding to the BChl *a* macrocycle (GUDOWSKA-NOWAK *et al.*, 1990; HANSON *et al.*, 1987) may contribute to the red-shift observed in *Rss. parvum*. Amino acid residues at positions ±4 relative to the central His^0 are important secondary ligands of the BChl *a* molecules (ZUBER & COGDELL, 1995) and possible hydrogen bonds have been suggested between βTyr^{+4} and BChl *a* coordinated to the α polypeptide in *Rhodobacter sphaeroides* (LOACH & PARKES-LOACH, 1995). Especially in the vicinity of the His^0 all models exhibit high accuracy, due to a high degree of sequence similarity in this region as seen in the alignments (Fig. 6). In conclusion, the cysteine residues at position α^{+3} and β^{-4} are likely to change the local environment of the BChl *a* dimer in the LH1 antenna of *Rss. parvum*.

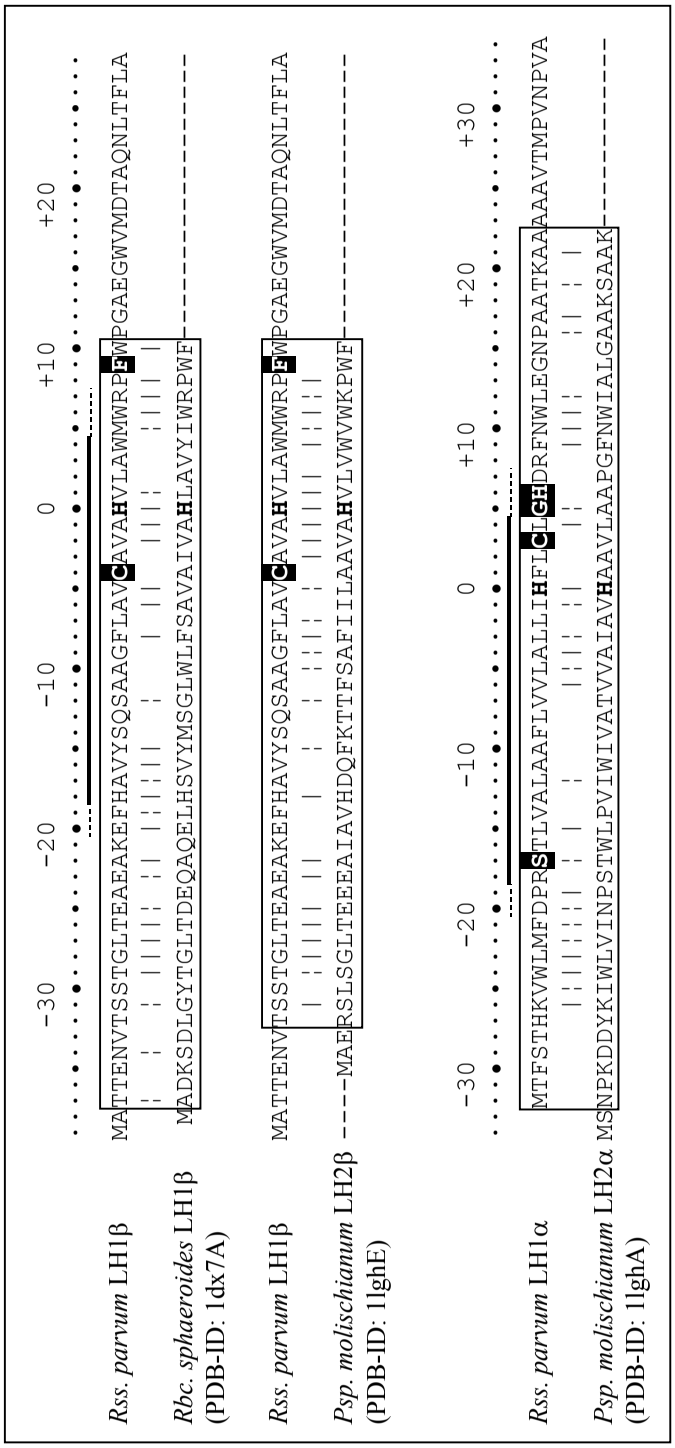


Figure 6: Amino acid sequence alignments of the *Rss. parvum* LH1 polypeptides and the templates used for 3D modeling.

In general, the presence of cysteine residues in LH1 polypeptides is particularly noticeable in the phylogenetic group of *Ectothiorhodospiraceae* (Fig. 3) (WAGNER-HUBER *et al.*, 1992). *Halorhodospira halochloris* contains a cysteine residue in at least one of the two known α polypeptides (2nd chain), similar to *Rss. parvum* at position α^{+3}. Although the tremendous red-shift in *Halorhodospira halochloris* is mainly attributed to BChl *b*, it is possible that αCys^{+3} plays a role in the red-shift of the Q_y of both, BChl *a* and BChl *b* associated LH complexes. Interestingly, the consensus motive LLST at position α^{+3} - α^{+6} is altered in *Rss. parvum* (***C***L***GH***) and in both α polypetides of *Halorhodospira halochloris* (L***MT***T, 1st α chain and ***CI***S***S***, 2nd α chain) (Fig. 3A).

A contribution of α^{+6} to possible cross-hydrogen bonding was discussed previously (LOACH & PARKES-LOACH, 1995). Our modeling predicts that the αHis^{+6} is not likely to make direct contact to the BChl *a*, because this His is oriented in the opposite direction of the BChl *a* macrocycle. This was also concluded from site-directed mutagenesis experiments in *Rhodobacter sphaeroides*, where the amino acids αSer^{+5} and αThr^{+6} did not participate in hydrogen bonding to the $C13^1$ keto group of the BChl macrocycle, contrary to previous assumptions (OLSEN *et al.*, 1997). Instead, it was proposed that in *Rhodobacter sphaeroides* the conserved Trp^{+11} of the C-terminus of the LH1 α polypeptide forms a hydrogen bond to the $C3^1$-acetyl carbonyl of one of the dimeric BChl *a* molecules (STURGIS *et al.*, 1997; OLSEN *et al.*, 1994). However, for a possible pigment interaction of residue α^{+11} a back-folding of the C-terminus is mandatory. Also, the αSer^{-17} at the N-terminus of the α polypeptide of the *Rss. parvum* might influence the absorption properties because the N-terminus was shown to participate in binding of the monomeric BChl in peripheral antenna complexes (MCLUSKEY *et al.*, 2001; COGDELL *et al.*, 1999; PRINCE *et al.*, 1997).

In antenna complexes of other species of purple bacteria, the β^{+9} residue is of relevance to the absorption properties. Mutagenesis experiments on *Rhodobacter sphaeroides* revealed significant wavelength shifts when the conserved βTrp^{+9} was changed to other aromatic amino acids (STURGIS *et al.*, 1997). However, Q_y absorption was blue-shifted when tryptophan was substituted for phenylalanine. In *Rss. parvum*, the highly conserved tryptophan βTrp^{+9} is substituted by phenylalanine. Contrary to the *Rbc. sphaeroides* mutants however, a red-shifted absorption maximum exists in *Rss. parvum* antenna complexes. This unexpected property may be related to the presence of the atypical adjacent βTrp^{+10}.

To our knowledge the sites α^{+3}, $\alpha^{+6}$and β^{-4} have not been subjected to mutagenesis studies to assess possible spectral contributions of these positions. Our data indicate that future studies on LH1 absorption properties could benefit from focussing on these residues.

Phylogenetic aspects

The phylogenetic analyses of LH1 antenna polypeptides were not entirely congruent with the 16S rDNA phylogeny of the respective purple bacteria. Specifically, the LH1 polypeptides of α-1 and α-2 proteobacteria do not form coherent groups. Horizontal gene transfer may have been significant in the evolution of phototrophic prokaryotes (RAYMOND *et al.*, 2002) and has been invoked to explain inconsistencies between phylogenies of *puf* and 16S rRNA gene sequences (NAGASHIMA *et al.*, 1997a; NAGASHIMA *et al.*, 1993). However, due to the small sizes of the antenna polypeptides, such inconsistencies might also reflect statistical fluctuations (NAGASHIMA *et al.*, 1996).

In any event, our phylogenetic analyses indicate that a monophyletic origin of LH polypeptides with rare amino acid substitutions as present in *Rss. parvum*, *Halorhodospira halochloris*, *Rubrivivax gelatinosus* and *Rhodocyclus tenuis* clearly can be excluded. Instead, the phylogenetic analysis suggests a closer relatedness of the LH polypeptides of *Rss. parvum* to the LH1α chain of *Rhodoblastus acidophilus* and to the LH1β chain of *Ectothiorhodospira shaposhnikovii*. Yet, neither of the latter two species exhibits extraordinary spectral properties, with LH Q_y bands at 890 nm in both cases (PERMENTIER, 2001). Thus, the structural similarities between LH polypeptides containing unusual amino acid sequences most likely represent independent adaptations to similar ecological niches (light harvesting above 900 nm), and consequently appear to be the result of convergent evolution.

Summary and outlook

Clearly, *Rss. parvum* contains unique and unusual amino acid sequences in LH1 proteins. The residues that differ from highly conserved sequences in other purple bacteria are likely to account for the unusual red-shift observed in *Rss. parvum*. In this context the occurrence of cysteine residues is especially notable. Possible cysteine-BChl interactions are supported by our 3-D models. Future experiments on LH1 residue-specific effects on the Q_y absorption band could exploit the cloned *Rss. parvum puf* operon and the genetic power of *Rhodobacter* species, for heterologous expression of *Rss. parvum* LH1 genes and site-directed mutations. Alternatively, expression of *Rhodobacter* species LH1 genes modified

by site-directed mutagenesis of the residues that differ in *Rss. parvum* may provide insights into the possible spectral role of these amino acids.

6 Acknowledgements

This investigation was supported by DFG grants to J. Overmann, NSERC grants to J.T. Beatty and scholarships of the German academic exchange service (DAAD) and the Heinz Neumüller Stiftung to C. Tuschak We thank J. Glaeser for growing *Rss. parvum* cultures. Preliminary sequence data for *Rhodopseudomonas palustris* were obtained from the DOE Joint Genome Institute (JGI) at http://www.jgi.doe.gov/tempweb/JGI_microbial/html/index.html.

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IV Summary

In the present thesis the structural genes of the photosynthetic apparatus from two purple bacteria, *Amoebobacter* (*Amb*.) *purpureus* ML1 and *Roseospirillum* (*Rss*.) *parvum* 930I, were investigated by molecular biological methods. Concomitant analyses of transcription, phylogeny, structure and evolutionary aspects were focused on their light harvesting (LH) 1 core antenna, which is encoded together with the pigment binding proteins of the reaction center within the *puf* operon. *Amb. purpureus* was chosen as a representative of the little investigated purple sulfur bacteria because of their importance in natural ecosystems. In contrast *Rss. parvum*, a purple non-sulfur bacterium, fascinates by its unusual spectral properties.

Chapter II describes the study of the *puf* operon of *Amb*. *purpureus*. The operon was cloned and sequenced, revealing an unusual operon consisting of the genes *pufB*$_1$*A*$_1$*LMCB*$_2$*A*$_2$*B*$_3$*A*$_3$. The presence of a *bchZ* gene 5' of *pufB*$_1$ indicates a linkage of *puf* and *bch* genes as in other species. The sequence data obtained represent the second complete *puf* operon available for *Chromatiaceae*. The unusual presence of additional sets of light-harvesting genes, *pufB*$_2$*A*$_2$ and *pufB*$_3$*A*$_3$ at the 3' terminus of *pufC* has been described so far only for *Allochromatium* (*Alc.*) *vinosum*. These discoveries, along with reports of multiple LH1 polypeptides found in some *Ectothiorhodospiraceae* by direct protein sequencing, indicate that multiple LH1 genes may occur frequently in phototrophic γ Proteobacteria. Phylogenetic analyses suggested a coevolution of the core *puf* genes *pufB*$_1$*A*$_1$*LM*. Separate analysis of the LH1 α and β polypeptides indicated a high intraspecies relatedness for the secondary LH1 β polypeptides, which is possibly caused by functional constraints. In contrast, LH1α subunits of *Amb. purpureus* and *Alc. vinosum* are closely related (85 % sequence identity) which could reflect horizontal gene transfer. RNA analyses suggested co-transcription of all *puf* genes in *Amb. purpureus* on an unusually long primary transcript of 5.8 kb. Although presumed mRNA degradation products of 2.7 and 1.5 kb and strong 0.8, 0.6 and 0.5 kb signals were detected, the primary transcript may be more stable than the *puf* operon primary transcripts of purple non-sulfur phototrophic bacteria. Regulation of expression of *puf* genes in *Amb. purpureus* under different light conditions also differs from the pattern observed in purple non-sulfur bacteria.

In chapter III the focus was set on *Rss. parvum*. *Rss*. *parvum*, like most other purple bacteria, contains bacteriochlorophyll (BChl) *a* as a LH chromophore, but exhibits an extremely red-shifted Q_y absorption maximum centered at 909 nm. To unravel molecular determinants of the unusual absorption properties of its light harvesting core antenna, the *puf* operon of *Rss. parvum* was cloned and sequenced, revealing the gene structure *pufBALMC*. Comparative analysis of predicted amino acid sequences of the α and β core LH polypeptides (PufA and PufB) revealed five amino acid substitutions at positions that are highly conserved in other purple bacteria. In the primary structure, these residues are in close proximity to the BChl *a* ligating histidine residue (α^{+3}, α^{+5}, α^{+6} and β^{-4}, β^{+9}, with respect to the central histidine, His^0). The nature of the enormous red-shifts to the near-infrared region of the light were attributed to those residues, with special emphasis on cysteine residues rarely present in other purple bacterial antenna polypeptides. Three dimensional structural models of the *Rss. parvum* LH1 α and β polypeptides suggest that the thiol groups of αCys^{+3} and βCys^{-4} are oriented toward the BChl *a* macrocycle in the native antenna complex. Because of their predicted close proximity to BChl, these thiol groups are likely to modulate the spectral properties of the LH1 complex. Phylogenetic comparisons of LH1 polypeptides indicate that *Rss. parvum*-like amino acid substitutions in proteobacterial LH1 complexes arose independently during evolution.

Both studies revealed variability in structures that were previously believed to be conserved. For *Amb. purpures* this variation was observed on the level of the *puf* operon organization and for *Rss. parvum* on the molecular level of the antenna polypeptide sequences. Mutagenesis, cloning and expression experiments could reveal the importance and function of multiple *puf* genes in *Amp. purpureus* and confirm the possible spectral role of the unusual amino acids in *Rss. parvum*. Overall, studies on less investigated phototrophic bacteria contribute to a more complete understanding of the principles of photosynthesis.

V Deutsche Zusammenfassung

In der vorliegenden Arbeit wurden die Strukturgene des Photosyntheseapparates zweier Purpurbakterien, *Amoebobacter* (*Amb*.) *purpureus* ML1 und *Roseospirillum* (*Rss*.) *parvum* 930I, mit Hilfe molekularbiologischer Methoden untersucht. Der Schwerpunkt der einhergehenden Analysen von Transkription, Phylogenie, Struktur und evolutionären Aspekten wurde auf ihre zentralen Lichtsammelkomplexe (LH1) gelegt, welche zusammen mit den pigmentbindenden Proteinen des Reaktionszentrums innerhalb des *puf*-Operons kodiert sind. *Amb. purpureus* wurde als Repräsentant der wenig untersuchten Schwefelpurpurbakterien und aufgrund ihrer Bedeutung in natürlichen Ökosystemen ausgewählt. Im Gegensatz dazu fasziniert *Rss. parvum*, ein schwefelfreies Purpurbakterium, durch seine ungewöhnlichen spektralen Eigenschaften.

Kapitel II beschreibt die Untersuchungen des *puf*-Operons von *Amb*. *purpureus*. Das Operon wurde kloniert und sequenziert. Dabei wurde eine ungewöhnliche Operonstruktur entdeckt, bestehend aus den Genen $pufB_1A_1LMCB_2A_2B_3A_3$. Das Vorhandensein eines *bchZ* Gens 5' von $pufB_1$ weist, wie bei anderen Arten auch, auf eine Verbindung zwischen *puf* und *bch* Genen hin. Die erhaltenen Sequenzdaten repräsentieren das zweite vollständige *puf*-Operon für *Chromatiaceaen*. Die ungewöhnlichen, zusätzlichen Sätze von Lichtsammelkomplex-Genen, $pufB_2A_2$ und $pufB_3A_3$ am 3'-Terminus von *pufC*, wurden bisher nur bei *Allochromatium* (*Alc.*) *vinosum* beschrieben. Diese Entdeckungen, zusammen mit Berichten über multiple LH1-Polypeptide, welche in einigen *Ectothiorhodospiraceaen* durch direkte Proteinsequenzierung gefunden wurden, weisen darauf hin, dass multiple LH1 Gene in phototrophen γ-Proteobakterien häufig auftreten könnten. Phylogenetische Analysen legten eine Koevolution der "Kern"-*puf*-Gene $pufB_1A_1LM$ nahe. Die separate Analyse der LH1 α- und β-Polypeptide zeigte eine hohe Verwandtschaft der sekundären LH1 β Polypeptide innerhalb der selben Art (intraspezifisch). Dies ist möglicherweise durch funktionelle Einschränkungen bedingt. Im Gegensatz dazu sind die LH1α-Untereinheiten von *Amb. purpureus* und *Alc. vinosum* eng verwandt (85 % Sequenzidentität), was einen horizontalen Gentransfer widerspiegeln könnte. RNA Analysen legten die Kotranskription aller *puf*-Gene in *Amb. purpureus* auf einem ungewöhnlich langen primären Transkript von 5.8 kb nahe. Obwohl vermutete mRNA-Abbauprodukte von 2.7 und 1.5 kb und starke 0.8, 0.6 und 0.5 kb Signale beobachtet wurden, scheint das primäre Transkript stabiler zu sein, als die primären Transkripte von *puf*-Operonen der

schwefelfreien phototrophen Bakterien. Die Regulation der Expression von *puf*-Genen in *Amb. purpureus* unter verschiedenen Lichtbedingungen unterscheidet sich ebenfalls von dem Muster, dass von schwefelfreien Purpurbakterien bekannt ist.

In Kapitel III wurde der Schwerpunkt auf *Rss. parvum* gelegt. Wie die meisten anderen Purpurbakterien enthält *Rss. parvum* Bacteriochlorophyll (BChl) *a* als Lichtsammel-Chromophor, weist aber ein extrem in den roten Bereich des Lichtes verschobenes Q_y Absorptionsmaximum um 909 nm auf. Um molekularen Faktoren (Pigment-Protein Wechselwirkungen) für diese Absorptionseigenschaften der zentralen Hauptantenne (LH1) zu untersuchen, wurde das *puf*-Operon von *Rss. parvum* kloniert und sequenziert. Es wurde eine Genstruktur von *pufBALMC* ermittelt. Die vergleichende Analyse der abgeleiteten Aminosäuresequenzen der LH1 α- und β-Polypeptide (PufA und PufB) zeigte Substitutionen von fünf Aminosäuren an Positionen, die in anderen Purpurbakterien hoch konserviert sind. In der Primärstruktur sind diese Reste in naher Nachbarschaft zum BChl *a*-bindenden Histidin (α^{+3}, α^{+5}, α^{+6} und β^{-4}, β^{+9}, in Verhältnis zum zenralen Histidin His^0). Die enorme Rotverschiebung zum nahen Infrarotbereich des Lichts wurde diesen Aminosäuren zugeschrieben. Cysteinresten wurde hierbei eine besondere Rolle zugeschrieben, da sie selten in den Antennenpolypeptiden anderer Purpurbakterien auftreten. Dreidimensionale Strukturmodelle der LH1 α- und β-Polypeptide von *Rss. parvum* legten nahe, dass die Thiolgruppen von αCys^{+3} und βCys^{-4} zur Ringstruktur des BChl *a* Moleküls im nativen Antennenkomplex orientiert sind. Ihre vorhergesagte große Nähe zum BChl macht einen Einfluß dieser Thiolgruppen auf die spektralen Eigenschaften des LH1 Komplexes wahrscheinlich. Der phylogenetische Vergleich von LH1 Polypeptiden legt nahe, dass *Rss. parvum*-ähnliche Aminosäuresubstitutionen in LH1 Komplexen von Proteobakterien unabhängig während der Evolution entstanden sind.

Beide Studien zeigten Variationen in Strukturen auf, von denen bisher angenommen wurde, dass sie konserviert sind. Für *Amb. purpureus* wurde diese Variabilität auf der Organisationsebene des *puf*-Operon und für *Rss. parvum* auf der molekularen Ebene der Antennenpolypeptidsequenzen beobachtet. Mutagenese-, Klonier- und Expressionsexperimente könnten die Bedeutung und Funktion multipler *puf* Gene in *Amp. purpureus* erhellen und die mögliche spektrale Rolle der ungewöhnlichen Aminosäuren in *Rss. parvum* bestätigen. Insgesamt tragen Studien an weniger untersuchten phototrophen Bakterien zu einem vollständigeren Verständnis der Prinzipien der Photosynthese bei.

Ich möchte mich bedanken, bei...

...Prof. Dr. Jörg Overmann, für die in mir geweckte Begeisterung für phototrophe Bakterien, für die Themenstellung und Betreuung, sowie für den stetigen fachlichen Rat in all den Jahren und für die Ermöglichung meiner Aufenthalte in Kanada durch seine Kontakte zu Tom Beatty.

...Prof. Dr. Tom Beatty für die freundliche Aufnahme in seinem Labor an der University of British Columbia in Vancouver, Kanada, für die lehrreiche Einführung in das "Molecular Cloning" und seinem stetigen Interesse am Fortgang meiner Arbeiten, sowie seiner immer währenden Unterstützung und Betreuung. Erwähnt seien die Zeiten vor der Tafel im Flur, an der so manches Problem skizziert, erörtert und gelöst wurde und die zahlreichen Diskussionen bei denen ich so enorm viel gelernt habe.

...Prof. Dr. Heribert Cypionka für das Interesse an der vorliegenden Arbeit und für die Bereitschaft als Gutachter zur Verfügung zu stehen. Gern denke ich an seinen Kurzbesuch in Vancouver zurück und von dem ich die Weisheit gelernt habe: "Kaum macht man's richtig, schon funktioniert's".

...dem Deutschen Akademischen Austausch Dienst (DAAD) und der Heinz Neumüller-Stiftung, deren Stipendien das "Abenteuer" Vancouver erst ermöglichten.

...den zahlreichen Leuten des Beatty-Labs in Vancouver, der Cypionka Gruppe in Oldenburg und der Overmann Crew in München, ohne die diese Arbeit nur halb soviel Spaß gemacht hätte; besonders für die vielen wertvollen Gespräche bei den vielen Litern an Kaffee und Tee, die zusammen getrunken wurden, sowie für die Offenheit und Hilfsbereitschaft, die mir stets begegnet ist.

...Ali, Micha und Stephan für die gemeinsamen Cookies beim Blue-Chip Coffey Shop.

...Andrew für die unvergessene Zeit im Beatty-Lab, für die endlosen Tipps und Tricks im Laboralltag und vor allem für die vielen Unternehmungen in der kanadischen "Wildnis" und seine Freundschaft.

...Willm, für die zahlreichen Diskussionen bei Tag und bei Nacht, mit seinem Humor und seiner stets ansteckend guten Laune.

...Frederic, für seine spanische, oder genauer gesagt, für seine katalonische Art.

..Ann und Martina für ihre erfrischende Art und den vielen netten Gesprächen in Büro 1.

...Jens für die gemeinsam durchlebte Zeit in München, mit den vielen Abenden im Labor aber auch für die zahlreichen Unternehmungen und Snowboardausflüge, aus denen sich über die Arbeit hinaus eine feste Freundschaft entwickelt hat.

...Elke, für Ihre stets offenen Ohren, für Ihren Rat in jeder Lebenslage, aber vor allem für die langjährige Freundschaft.

...meinen Eltern und meiner Schwester Bettina für den Rückhalt und Ihre Unterstützung über die Jahre hinweg.

Für Judit, die mich in den Jahren durch alle Höhen und Tiefen begleitete, mich wieder und wieder motivierte, deren Geduld niemals endete und ohne die diese Arbeit nie zu Ende geschrieben worden wäre, gibt es kaum Worte... Danke! Danke! Danke!

Lebenslauf

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation selbstständig verfasst und nur die angegebenen Quellen und Hilfsmittel verwendet habe.

Augsburg, den 12. September 2003