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Phylogenetic relationship of phototrophic purple sulfur bacteria according to *pufL* and *pufM* genes

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Summary. The phylogenetic relationship of purple sulfur bacteria (PSB), of the order Chromatiales (class Gammaproteobacteria), was analyzed based on photosynthetic gene sequences of the *pufL* and *pufM* genes, and the results compared to phylogenetic trees and groupings of the 16S rRNA gene. Primers for *pufL* and *pufM* genes were constructed and successfully used to amplify the *pufLM* genes of members of 16 genera of Chromatiales. In total, *pufLM* and 16S rRNA gene sequences of 66 PSB strains were analyzed, including 29 type strains and 28 new isolates. The inferred phylogenetic trees of the *pufLM* and 16S rRNA genes reflected a largely similar phylogenetic development suggesting coevolution of these essential genes within the PSB. It is concluded that horizontal gene transfer of *pufLM* genes within the PSB is highly unlikely, in contrast to the situation in other groups of anoxygenic phototrophic bacteria belonging to Alpha- and Betaproteobacteria. The phylogeny of *pufLM* is therefore in good agreement with the current taxonomic classification of PSB. A phylogenetic classification of PSB to the genus level is possible based on their *pufL* or *pufM* sequences, and in many cases even to the species level. In addition, our data support a correlation between Puf protein structure and the type of internal photosynthetic membranes (vesicular, lamellar, or tubular). [**Int Microbiol** 2009; 12(3):175-185]

Keywords: *Chromatiaceae* · *Ectothiorhodospiraceae* · purple sulfur bacteria (PSB) · genes *pufLM* · phylogeny

Introduction

Purple sulfur bacteria (PSB) represent a physiological group of unicellular gram-negative bacteria phylogenetically classified in the class Gammaproteobacteria within the order Chromatiales [20]. They are ubiquitously distributed but restricted to aquatic habitats characterized by adequate light conditions, low oxygen tension, and moderate sulfide concentrations. Under optimal conditions in nature, PSB form visibly colored blooms. The colors are due to large amounts of carotenoids and bacteriochlorophylls (Bchl *a* or Bchl *b*), which are located in internal membranes. The most characteristic feature of all PSB is their ability to perform photosynthesis under anoxic conditions, mainly using reduced sulfur compounds such as H_2S or thiosulfate as electron donors. Anoxygenic phototrophy via bacteriochlorophylls is not restricted to the PSB but is also found in purple nonsulfur bacteria (PNSB, e.g., *Rhodovulum* spp. within the Alphaproteobacteria and *Rubrivivax* spp. within the Betaproteobacteria), aerobic anoxygenic photosynthetic bacteria (AAPB, e.g., *Erythrobacter* spp. within the Alphaproteobacteria and *Congregibacter litoralis* within the Gammaproteobacteria), green nonsulfur bacteria (*Chloroflexaceae*), green sulfur bacteria (GSB, i.e., *Chlorobiaceae*), and *Heliobacteriaceae,* as well as in some cyanobacteria under certain conditions. PSB represent a phylogenetic group that is clearly separated from all other anoxygenic photosynthetic bacteria, including the known gammaproteobacterial AAPB *Congregibacter litoralis* KT71 and strain HTCC2080 [8].

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PSB constitute the order Chromatiales, and comprise the *Chromatiaceae* and *Ectothiorhodospiraceae* families, which can be easily distinguished by their ability to store elemental sulfur inside or outside the cells as an intermediate during photosynthesis.

Functional gene approaches can support phylogenetic considerations concluded from 16S rRNA gene analysis. This has already been demonstrated for the Fenna-Maththews-Olson (FMO) protein in GSB [2]. In the case of anoxygenic phototrophic bacteria, genes of the photosynthetic apparatus, which are located on a photosynthetic gene cluster, have been used as targets in environmental studies [5]. Part of this photosynthetic gene cluster is the *puf* (photosynthetic unit forming) operon, containing genes coding for the photosynthetic reaction center type II structural proteins (Puf). Reaction center proteins encoded by *puf* are found in anoxygenic photosynthetic bacteria of the Alpha-, Beta-, and Gammaproteobacteria and in the *Chloroflexaceae.* Currently, five different *puf* operon types, differing in number and in the arrangement of the *puf* genes, are known [39]. Since the positions of the *pufL* and *pufM* genes do not differ in the different operon types of phototrophic purple bacteria (and *Chloroflexus*), they were the preferred target genes in our study. *pufL* and *pufM* code for the light and medium polypeptide chains of the photosynthetic reaction center, respectively [6,12]. Several previously conducted studies on the *pufL* and/or *pufM* genes of anoxygenic phototrophic bacteria [1,25,34,40] focused on PNSB (Alpha- and Betaproteobacteria), especially on AAPB [4,5,8,36]. However, genetic information on *puf* genes belonging to photosynthetic members of the PSB, the Chromatiales, is still sparse.

As *pufLM* genes are essential in photosynthesis by PSB, they may represent important conservative markers for phylogenetic analyses. Based on the limited number of available sequence data, we analyzed the *pufLM* genes in a large number of strains available in our strain collection (including 29 type strains) as well as in several new isolates. Sixty-six PSB strains of 16 PSB genera from all over the world, adapted to various environmental conditions, such as hypersaline, marine, brackish, or freshwater habitats, were analyzed. The primary structure of the PufLM proteins and their encoding nucleotide sequences showed distinct sequence signatures down to the species-specific level.

Materials and methods

Recommended abbreviations of purple bacteria genera names. *Alc.* – Allochromatium; *Blc.* – Blastochloris; *Chr.* – Chromatium; *Hch.* – Halochromatium; *Mch.* – Marichromatium; *Rba.* – Rhodobacter; *Rsp.* – Rhodospirillum; *Tca.* – Thiocapsa; *Tch.* – Thermochromatium; *Tco.* – Thiococcus; *Tcs.* – Thiocystis; *Tfc.* – Thioflavicoccus; *Thc.* – Thiohalocapsa; *Trc.* – Thiorhodococcus.

Cultivation of bacteria. In general, PSB were cultivated following previously described methods [19]. Bacterial strains from our culture collection and environmental isolates were grown in liquid culture (50- or 100-ml screw-cap bottles) using freshly prepared Pfennig's medium amended with 0.5 or 1 ml of acetate solution (NH$_4$-acetate/Mg-acetate; 2.5 g each/100 ml). The final acetate concentration of the medium was 0.05%. Depending on the species, NaCl was added to the medium to a final concentration of 0–10% (medium for marine isolates contained 2% NaCl). After inoculation, the bottles were stored in the dark for several hours. They were then exposed to light from a tungsten lamp (500–700 lux) and incubated at 25°C until turbidity was visible. To keep the concentration of toxic sulfide in the cultures low (up to 2 mM final concentration) but to assure a continued supply of sulfide, it was necessary to feed the cultures with sulfide solution after depletion of this substrate. Feeding of the cultures with sulfide and preparation of the feeding solution were done as previously described [19]. The exhaustion of sulfide in the cultures was monitored using Pb(II) acetate paper (Merck). Well-grown cultures were directly used for analyses and afterwards either stored at 4°C or frozen in liquid nitrogen.

Isolation of new environmental strains. To obtain pure cultures of environmental isolates, agar shake dilution series were prepared, as described previously [19]. The first agar tube was inoculated with 100 µl of an environmental sample, and the subsequently inoculated test tubes were treated as described for liquid cultures (see cultivation of bacteria). Dilution series were repeated until purity was achieved (at least 4 times). Pure isolates were transferred into liquid medium. The purity of the isolates was checked microscopically and acknowledged by the high sequence quality of the 16S rRNA and *pufLM* genes.

DNA extraction, primers, PCR, and sequencing. DNA was extracted by mechanical disruption of the cells using a bead mill (type MM200, Retsch, Germany). One ml of a freshly grown culture was centrifuged in a 1.5-ml tube (10 min, 8000 ×*g*). The supernatant was discarded and the cell pellet resuspended in 200 µl of DNA-free water. Zirconia/silica beads 0.1 mm in diameter (0.8 g) were filled into the tubes followed by bead mill treatment for 6 min with a frequency of 30 Hz/s. Finally, the suspensions were centrifuged for 10 min at 10,000 ×*g*. The extracted DNA was directly used for PCR or stored at –20°C until analysis.

The *pufL* and *pufM* genes were PCR-amplified using a modified primer set based on the primers previously published by Nagashima et al. [34]. The forward primer sequence (pufL67F) was 5′-TTCGACTTYTGGRTNG-GNCC-3′, and the reverse primer sequence (pufM781R) 5′-CCAKS-GTCCAGCGCCAGAANA-3′. The *pufLM* fragment length was ca. 1.5 kb. 16S rRNA genes were amplified using the primers 27F [7] and 1492R [37].

The *puf* and 16S rRNA genes were PCR-amplified using puReTaq Ready-To-Go PCR beads (GE Healthcare, USA) in a final volume of 25 µl. Conditions for 16S rRNA gene amplification that differed from those of *pufLM* amplification are indicated in parentheses: 3–5 µl (1–2 µl) of template and 1 µl of each primer, at a concentration of 10 µM, were used. PCR was carried out using the following conditions: initial denaturation step at 94°C for 2 min, primer annealing at 55°C (50°C) for 40 s, elongation step at 72°C for 1.5 min (1 min), and denaturation at 94°C for 40 s. Overall, 35 (30) cycles were performed. Before the PCR was cooled to 8°C, final annealing at 50°C (42°C) for 40 s and a final elongation step at 72°C for 5 min were conducted.

Sequencing was done using the BigDye Terminator v1.1 sequencing kit (Applied Biosystems) in a 3730 DNA analyzer (Applied Biosystems) as specified by the manufacturer. The *pufLM* gene fragment was sequenced using the PCR primers (pufL67F and pulM781R). 16S rRNA gene amplificates were sequenced with the primers 342f [31], 790f (5′-GATACCCTG-GTAGTCC-3′), and 534r [33]. Sequences obtained during this study were deposited in the EMBL database and were assigned the accession numbers given in Table S1. Sequences were edited using the Seq Man II program (DNASTAR, USA).

Phylogenetic analysis. With regard to the reliability and comparability of the phylogenetic trees, only the 16S rRNA gene and *pufLM* sequences derived from the same strain were taken into account. Only DNA fragments

of the 16S rRNA and *pufLM* genes with a minimum length of ~1400 bp were used in the analysis. To achieve comparability between the *pufLM* nucleotide and amino acid trees, the non-coding region between the *pufL* and *pufM* genes was not included in the phylogenetic analyses. In cases in which several sequences of identical strains were available in the databases, these were carefully compared and the best sequence was used for the analyses.

The nucleotide sequences of the *pufLM* genes were converted into amino acid sequences using BioEdit version 7.0.1 [38] and manually refined. The *pufLM* nucleotide sequence alignment was obtained by reconverting the amino acid sequence alignment. The corresponding 16S rRNA gene sequences were aligned using the integrated aligner function implemented in the ARB program package [32] and manually refined with respect to secondary structure information. The evolutionary models GTR+I+G and WAG+I+G, used for phylogenetic analyses of nucleotide and amino acid sequences, were determined using the program ModelGenerator, version 0.85 [Keane TM, et al. (2004) ModelGenerator: amino acid and nucleotide substitution model selection. National University of Ireland, Maynooth, Ireland]. Phylogenetic trees were calculated using the PhyML Online program [15]. *Chloroflexus aggregans* DSM 9485[T] and *Chloroflexus aurantiacus* DSM635[T] served as the outgroup species.

Nucleotide and amino acid sequence similarities were obtained using the DNAdist and Protdist program of the Phylip package [13], respectively, including the "similarity table" option with default parameters. The hydropathy plot was created using the Kyte and Doolittle algorithm [30] (scan window size 20) implemented in the BioEdit program package.

Results

Primer system. Due to bacterial whole-genome and metagenomic sequencing projects, the number of *puf* gene sequences available in the database that cover the entire *pufLM* gene has increased significantly over the past few years. Based on the available *pufLM* gene sequences, the previously published primer set by Nagashima et al. [34] were found to contain several mismatches with the target sequences. Therefore, the primer sequences were suitably modified to take into account all available sequences.

The modified primer system (pufL67F/pufM781R) reproducibly amplified *pufLM* genes of all PSB (Table S1) used in this study. The optimal annealing temperature for amplifying a PSB *pufLM* gene fragment was 55°C. It should be emphasized that the primers used in this study also amplified *pufLM* genes of PNSB, AAPB, and *Chloroflexus aurantiacus* (data not shown), and thus represent a universal primer system for these genes.

Identification of new strains. New strains were obtained from sampling locations in India and Germany and were assigned to several PSB groups (Table S1). Marine isolates from Indian east coast habitats were identified as *Halochromatium* spp. and *Marichromatium* spp. Freshwater isolates from the Indian east coast as well as one isolate from a polluted freshwater pond in the city of Hyderabad, India, belonged to the genus *Thiocapsa*. A second isolate (strain MTPPIF163) obtained from this pond was distinct from all

available sequences and most closely related to *Thiolamprovum pedioforme* DSM 3802[T] (16S rRNA gene similarity 96%). Marine isolates from the Indian west coast were most closely related or identical to *Marichromatium gracile* DSM 203[T] whereas two strains were affiliated with the genus *Allochromatium*, with *Alc. vinosum* DSM 180[T] as the next relative type strain (97%). Strains received from marine and freshwater samples from Germany belonged to the *Marichromatium* and the *Thiocapsa* groups. Additionally, strain MTWDM004, obtained from the Wadden Sea, was most closely related to *Thiorhodococcus kakinadensis* JA130[T] and *Marichromatium gracile* DSM 203[T], with 16S rRNA gene sequence similarities to both strains of 96%. Based on the 16S rRNA gene sequence, the freshwater pond isolate MTRDDF079 was determined to be 99% similar to the freshwater bacterium *Thiobaca trueperi* DSM 13587[T].

Genetic analysis of the PSB strains. In the present study, the phylogenetic relationship of 66 PSB strains was analyzed according to the *pufLM* gene sequences and compared with the phylogeny based on 16S rRNA genes. 16S rRNA gene sequences of previously analyzed strains were repeated and refined whenever necessary and the corresponding EMBL database entries were updated. Our datasets covered 16 out of 25 described genera of the PSB and included 29 validly described PSB type strains (Table S1). The majority of the 16S rRNA and *pufLM* gene sequence data were derived from strains of our culture collection.

***puf* gene sequences.** Nucleotide sequences of *pufL* and *pufM* as well as the deduced amino acid sequences obtained during this study formed a coherent cluster together with available database sequences. Compared to the 16S rRNA gene sequences, the *pufL* and *pufM* genes showed a wider range of variation. The similarity of nucleotide sequences of *pufLM* of the PSB varied within a wide range, from 63.8 to 100%. *pufLM* sequence similarities between PSB and AAPB varied from 61.5 to 73.8% (Table 1). Groups of *Chromatiaceae* species established on the basis of *pufLM* nucleotide sequence similarities of 86% corresponded to groups defined on the basis of 16S rRNA gene sequence similarities of >95.0. Members of the two *Ectothiorhodospiraceae* groups shared *pufLM* gene sequence similarities of 80.8–100% and 98.8%, which corresponded to 16S rRNA gene sequence similarities of 94.7–100% and 98.5%, respectively.

The deduced amino acid sequences of the *pufL* and *pufM* genes of PSB indicated highly conserved structures, and hydropathy plots of all analyzed *pufL* and *pufM* proteins revealed the characteristic presence of five α-helices, as was first reported for these proteins in *Blastochloris viridis*,

Table 1. Sequence similarities among groups of the purple sulfur bacteria (PSB)

	All PSB	Chromatiaceae	Chromatiaceae Bchl a	Chromatiaceae Bchl b	Ectothiorhodospiraceae	AAPB[*]
All PSB	63.8–100 (62.4–100)					
Chromatiaceae		63.8–100 (64.3–100)				
Chromatiaceae Bchl a			71.5–100 (77.9–100)			
Chromatiaceae Bchl b			63.8–71.3 (64.3–68.9)	89.2–100 (96.2–100)		
Ectothiorhodospiraceae		66.5–77.4 (71.3–77.5)	66.5–77.4 (71.3–77.5)	66.7–70.3 (62.4–64.8)	80.8–100 (87.5–100)	
AAPB[*]	61.5–73.8 (62.2–79.0)	61.5–73.8 (62.2–79.0)	67.3–73.8 (70.1–79.0)	61.5–65.8 (62.3–63.6)	67.1–73.0 (71.4–76.3)	70.5% (78.8%)

Values are nucleotide similarities (amino acid similarities are given in brackets), in percentage.
*AAPB = aerobic anoxygenic phototrophic Gammaproteobacteria.

Rhodobacter sphaeroides, and *Thermochromatium tepidum* [3,10,12]. Examined more closely, the PufLM proteins showed a number of distinctive features. Several insertions of amino acids were present in certain PSB between the helices A and B of the PufL protein. *Tch. tepidum* has an eight amino acid insertion (Gly-Pro-Thr-Ser-Asp-Leu-Gln-Thr) [12] between positions 59 and 60 (*Blc. viridis* numbering). At the same position, an insertion was identified in the phylogenetically defined groups (Table S1) representing species and strains of *Thiocapsa* (group V), *Allochromatium* (group VII), *Thiorhodococcus* A (group IX), *Tcs. violacea* DSM 207ᵀ (group X), *Thiorhodococcus* B (group XI), isolate MTWDM004 (group XII), *Thiocystis* (group XIII), *Chr. weissei* DSM 5161ᵀ (group XIV), and *Marichromatium* (group XVI). The insertion consisted of five amino acids of which four were identical and one differed between groups and/or strains (Ser/Ala/Val/Pro-Glu-Leu-Gln-Thr) and was similar to the insertion of *Tch. tepidum*. A different insertion of five amino acids (Glu-Val-Gly-Pro-Ala) at the same position was found for the Bchl-*b*-containing *Chromatiaceae* (group I). This insertion was identical in all Bchl-*b*-containing strains but consisted of amino acids completely different than those of the insertions of other *Chromatiaceae*. In addition, Bchl-*b*-containing *Chromatiaceae* had an additional leucine after PufL position 75. An extra amino acid (Ser) was also located at the end of PufL in all strains of groups I, II (except *Thc. marina*), and III (Table S1). All *Ectothiorodospira* species had an alanine at that position, while both *Halorhodospira halophila* strains had an elongation of 3 amino acids (Trp-Gly-Gly).

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

In addition to these insertions, there were several amino acid substitutions. All PSB (except species of *Thiorhodovibrio,* group IV) had a serine and a tryptophan at PufL positions 72 and 73, respectively. In *Thiorhodovibrio* species, aspartic acid and valine occurred at these positions. Another example of a signature amino acid was the cysteine at PufM position 15, found only in members of the *Marichromatium* group.

Despite the foregoing differences, which are suitable for differentiation, crucial parts of the sequences were highly conserved. Noticeably, none of the mentioned insertions were located in membrane-spanning regions of the proteins. In the membrane-spanning α-helices of both proteins, all important proline residues acting as helix turn motifs were located at the same position in all PSB. Histidine residues serving as ligands for the special pair of bacteriochlorophyll and the accessory bacteriochlorophylls were also conserved in both genes of all analyzed strains, including those containing Bchl *b*. In addition, the tryptophan residue at PufM position 130, acting as ligand for the bacteriophaeophytin, was conserved in all strains.

The *pufLM* sequence and deduced amino acid alignments showing the named signatures are available from the authors upon request.

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

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

Most remarkably, *pufLM*-based phylogenetic analyses led to phylogenetic clades within the *Chromatiaceae* containing the same strains as obtained with the 16S rRNA-gene-based phylogeny (groups I–XVIII, Table S1). This grouping was strongly supported by high bootstrap values (Figs. 1–3). The overall topology and the arrangement of the PSB groups with each other revealed several differences between the tree based on the *pufLM* gene and that of the 16S rRNA gene, e.g., the positions of the groups of *Halorhodospira* and *Ectothiorhodospira*. However, within these two groups, the tree topology of *pufLM* corresponded exactly to that of the 16S rRNA gene.

Phylogenetic analyses of the *pufLM* genes of *Chromatiaceae* revealed several analogies to the 16S rRNA gene data. Certain groups formed clusters based on the gene sequences of both the 16S rRNA gene and *pufLM*, such as the salt-dependent species of the genera *Halochromatium* and *Thiohalocapsa* (groups III + II). Moreover, within the *Halochromatium* clade the branch topology exactly corresponded to that of the 16S rRNA phylogenetic tree (group III, Figs. 2 and 3). A second cluster occurring in all phylogenetic analyses harbored *Thiocapsa* and *Thiolamprovum* species and relatives (groups V + VI). The *Thiocapsa* group comprised 12 sequences. While the results of the *pufLM* trees (especially the amino acid tree) indicated a division of the *Thiocapsa* group into two subclades (subclades one and two), in the 16S rRNA tree this was less well-defined. Subclade one harbored validly described species of *Tca. marina* and *Tca. pendens*. Subclade two was represented by the species *Tca. rosea* and *Tca. roseopersicina*. According to the phylogenetic results based on 16S rRNA gene analyses and supported by data on *pufLM* genes, *Tch. tepidum* and *Chromatium weissei* were most closely related to the *Allochromatium* and the *Thiocystis* group,

respectively. However, at the amino acid level *Tch. tepidum* was more closely related to the new *Thiobaca*-like isolate MTRDDF079 than to *Allochromatium*. Members belonging to the *Allochromatium* group (group XII) showed the same topology in the inferred *pufLM* trees but differed slightly compared with the topology in the 16S rRNA gene phylogenetic tree. *pufLM* trees showed that the isolates MTCH3IM013 and MTCH3IM086 were in close phylogenetic proximity to *Alc. vinosum* while in the tree inferred from the 16S rRNA they were located between *Alc. vinosum* and *Alc. warmingii*. The *Thiocystis* group, consisting of *Tcs. minor* and *Tcs. gelatinosa* (group XIII, Fig. 2), formed a distinguishable group with a quite similar topology in all inferred phylogenetic trees. *Thiocystis violacea* DSM 207^T formed a distinct and separate branch, with a close relationship to members of the *Thiorhodococcus* clade A and which did not cluster with the other *Thiocystis* species. The separation of members belonging to the genus *Thiorhodococcus* into two groups (clade A and B) as determined by *pufLM* phylogenetic analyses was also recognized by 16S rRNA gene sequences (Figs. 1–3). The *Thiorhodococcus* clade A (group IX) harbored *Trc. minor*, *Trc. mannitoliphagus*, and *Tcs. violacea* DSM 208 whereas *Trc. drewsii* and *Trc. kakinadensis* were located in the *Thiorhodococcus* clade B (group XI). Group XVI represented the coherent and robust clade of *Marichromatium* species and was highly supported by both 16S rRNA gene and *pufLM* phylogeny. On the 16S rRNA gene sequence level, *Mch. purpuratum* and *Mch. gracile* were closely related (99% similar to each other and 98% to *Mch. bheemlicum*) as reflected also by the tree topology. Based on *pufLM* sequences (amino acids and nucleotides), however, the okenone-containing *Mch. purpuratum* separated from the carotenoids-containing species of the spirilloxanthin series, *Mch. gracile* and *Mch. bheemlicum*, by sequence similarity and tree topology (Figs. 1 and 2). A quite distinct and outstanding group of the *Chromatiaceae* harbored the genera of the Bchl-*b*-containing *Chromatiaceae*, namely, *Tco. pfennigii* and *Tfc. mobilis* (group I), well-separated by long branches and well-established by high bootstrap values. This separate position of Bchl-*b*-containing *Chromatiaceae* was also seen in trees based on 16S rRNA gene sequence (Fig. 3).

Phylogenetic and 16S rRNA gene sequence analyses of PSB. As demonstrated earlier [21,23,24], species characterized as members of the *Chromatiaceae* and *Ectothiorhodospiraceae* formed distinct groups based on their 16S rRNA gene sequences. The *Ectothiorhodospiraceae* were divided into two well-separated groups containing *Ectothiorhodospira* species (group XVII) and *Halorhodospira* species (group XVIII). Within the *Chromatiaceae*, 16 groups were recognized (Fig. 3), which in general represent the different gen-
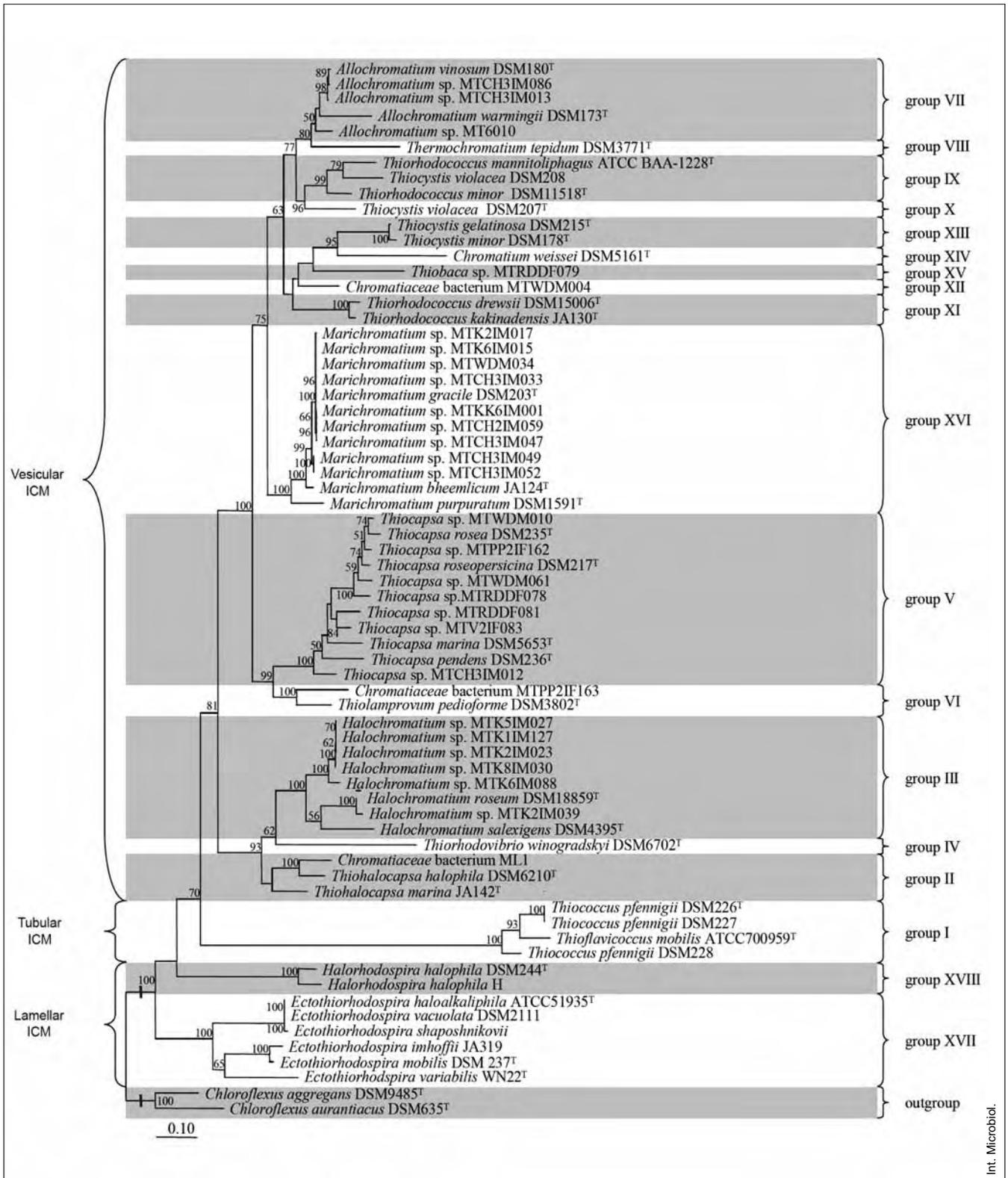
Fig. 1. Bootstrapped maximum-likelihood-based phylogenetic *pufLM* tree of nucleotide sequences of PSB. Bootstrap values of 50 or higher are given at the nodes. Parentheses on the left define the groups of vesicular, tubular, and lamellar intracytoplasmic membrane (ICM). Parentheses on the right define groups within the *Chromatiaceae* (I–XVI) whose members share nucleotide similarities ≥86% (≥81% for the *Ectothiorhodospiraceae* (XVII–XVIII), which coincides with ≥95% similarity for their corresponding 16S rRNA gene and thus concurrently represents the genus level.
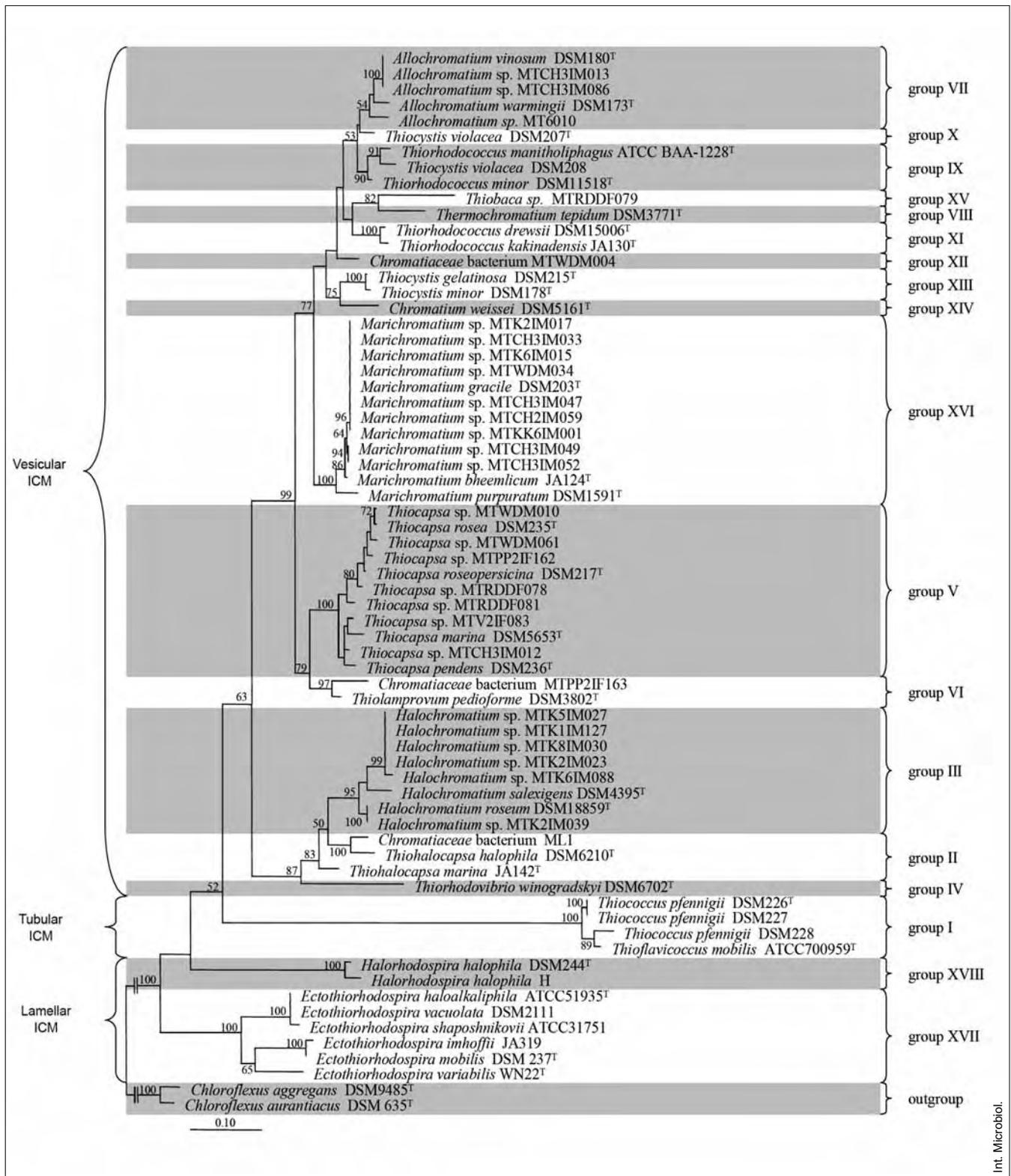
Fig. 2. Bootstrapped maximum-likelihood-based phylogenetic PufLM tree of deduced amino acids sequences of PSB. Bootstrap values of 50 or higher are given at the nodes. Parentheses on the left define the groups of vesicular, tubular, and lamellar intracytoplasmic membrane (ICM). Parentheses on the right define groups within the *Chromatiaceae* (I–XVI) whose members share nucleotide similarities ≥86% (≥81% for the *Ectothiorhodospiraceae* (XVII–XVIII), which coincides with ≥95% similarity for their corresponding 16S rRNA gene and thus concurrently represents the genus level.
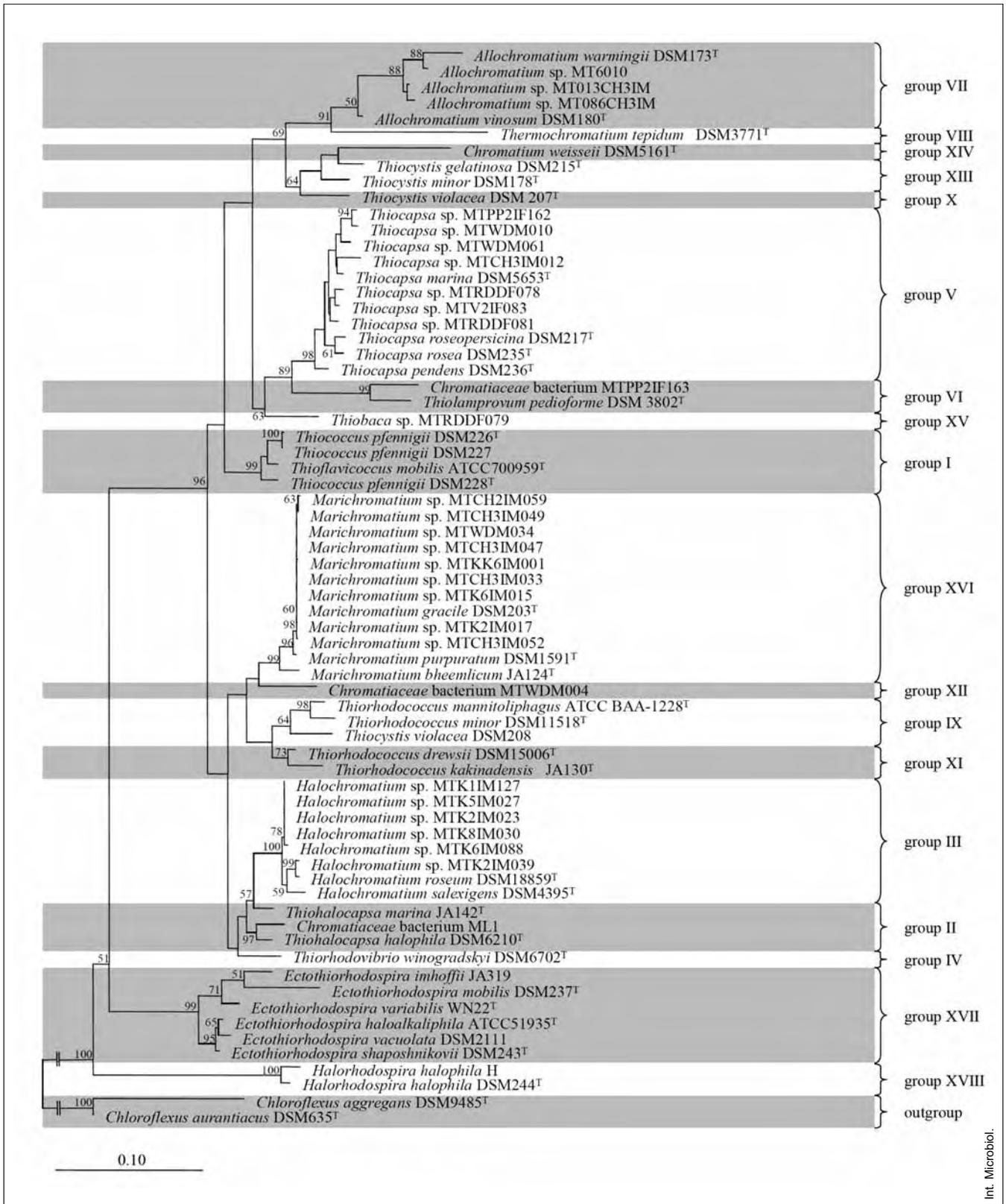
Fig. 3. Bootstrapped maximum-likelihood-based phylogenetic 16S rRNA gene tree of PSB. Bootstrap values of 50 or higher are given at the nodes. Parentheses on the right define groups within the *Chromatiaceae* (I-XVI) whose members share *pufLM* gene sequence nucleotide similarities ≥86% (≥81% for the *Ectothiorhodospiraceae* (XVII-XVIII) which coincides with ≥95% similarity for their corresponding 16S rRNA gene and thus concurrently represents the genus level.

era of *Chromatiaceae*, and were supported by high bootstrap values. There were a few exceptions, e.g., members of the genus *Thiorhodococcus* formed two distinct groups (groups IX and XI), as also seen in the *pufLM*-based phylogeny. Furthermore, the genus *Thiocystis* was divided into two groups (groups X and XIII). Indeed the type strain, *Thiocystis violacea* DSM 207[T], although closely related to other representatives of this genus, forms a distinct branch. This finding was also strongly supported by the *pufLM* gene tree and sequence analyses (see above and Fig. 2). Interestingly, *Thiohalocapsa* species (group II) and *Halochromatium* species (group III) showed a tendency to form a halophilic branch within the *Chromatiaceae*. *Thiolamprovum* (group VI) and *Thiocapsa* (group V) were robust sister groups consisting of non-motile cells and classified as mainly freshwater species [16,21].

Discussion

In general, both the amino acid sequences and the corresponding nucleotide sequences of *pufLM* were highly conserved. Hydropathy plots revealed five α-helices and the corresponding interhelical structures with highly conserved amino acid motifs. Regarding the resulting structure of the membrane-spanning protein, the conserved motifs may represent structural elements crucial for maintaining the functionality of the proteins. Correspondingly, *pufL* and *pufM* gene sequences were most highly conserved at the binding sites of the cofactors involved in the photochemical reaction. Additionally, the highly conserved motifs within the regions of the five α-helices indicate a functional relevance of these structural elements while the interhelical conserved regions are dominated by the amino acid proline, which is responsible for the helix turns. The conserved structure of the entire PufL and PufM proteins, within the α-helices, in interhelical regions, and at cofactor binding sites points to the importance of the structure of PufLM proteins as important factors in the functionality of the PSB photosynthetic reaction center.

In spite of the overall conserved feature of the proteins, differences in amino acid/nucleotide sequence similarities were as high as ~40%. Within the defined groups belonging to the *Chromatiaceae* (Table S1), nucleotide similarities of the *pufLM* genes always equaled or exceeded 86% (81% in *Ectothiorhodospiraceae*). The results obtained from the environmental isolates collected as part of this study specifically supported these findings. Isolates sharing *pufLM* gene sequence similarities 86% with any of the described species also revealed high 16S rRNA gene sequence similarities (95%) and could therefore be reliably assigned to one of the defined groups (Table S1). Thus, an assignment to genera of

the *Chromatiaceae* can be made according to *pufLM* nucleotide sequence similarities of 86.0%. By contrast, isolates with *pufLM* gene sequence similarities of <86% (e.g., strains MTWDM004 and MTRDDF079) could not be identified as belonging to any of the known groups and may thus represent new species or even genera. In this sense, *pufLM* sequences give confidence that an isolate originally assigned to "*Lamprocystis purpurea*," strain ML1 [35] has been misclassified. Strain ML1 contained *pufLM* nucleotide sequences most similar to *Thc. marina* JA142[T] and *Thc. halophila* DSM6210[T], strains with *pufLM* sequence similarities of 87% and 90% while those for the 16S rRNA gene were 97% and 98%, which suggests an affiliation with the genus *Thiohalocapsa*. A 16S rRNA gene sequence similarity between strain ML1 and *Lcs. purpurea* DSM4197[T] of only 94% further supports the conclusion that the classification is false.

Despite the overall nucleotide similarity values, the amino acid sequences had recurrent insertions and/or substitutions at distinct positions. Signatures found in both *pufL* and *pufM* are useful in differentiation of the PSB groups. For example, all non-motile *Halochromatium* species share an insertion of three amino acids at *pufM* position 33; *Ectothiorhodospiraceae* share a glycine at *pufM* position 230 while all *Chromatiaceae* contain a serine at that position. The amino acid insertions after *pufL* position 59 allow a member of the PSB to be distinguished from all other *pufL*-possessing bacteria, because these insertions only occur in gammaproteobacterial PSB. Furthermore, Glu-Val-Gly-Pro-Ala insertions at this position define the Bch-*b*-harboring genera *Thiococcus* and *Thioflavicoccus*. Our findings demonstrate that nucleotide sequence similarities together with specific signature amino acids are suitable properties in polyphasic taxonomy of PSB, as suggested in another study [22]. Overall, phylogenetic analyses based on *pufL* and *pufM* gene sequence revealed stable groups (Table S1, Fig. 1) that are in good agreement with the currently recognized genera. The two exceptions were in the genera *Thiocystis* and *Thiorhodococcus*, each of which was separated into two distinct groups. Both of these exceptions were also found in phylogenetic trees of the 16S rRNA gene and therefore support a possible reclassification of these bacteria.

A distinct positioning of *Thiococcus* and *Thioflavicoccus* within the *Chromatiaceae* was found in all trees and corresponds to the fact that the photosynthetic reaction centers expressed in these genera contain Bchl *b* instead of the Bchl *a* present in other *Chromatiaceae*. In addition, *Thiococcus* and *Thioflavicoccus* have a unique internal membrane system of tubular type, in contrast to the vesicular system in all other *Chromatiaceae* [20]. A third type of internal membrane system occurs in all *Ectothiorhodospiraceae* species having

lamellar internal membranes. These species also form a distinct major group according to their *pufLM* sequences. This further demonstrates coherence between PufLM protein structure and the type of internal membrane system. A correlation between protein structure and membrane type has been already proposed earlier [11]. For example, a mutant of *Rba. sphaeroides* lacking LH2 has been shown to form tubular membranes whereas the wild-type has vesicular internal membranes [18]. Furthermore, functional *puf* gene products have been shown to be necessary for the formation of intracytoplasmic membranes in *Rsp. rubrum* [17].

A separate grouping of salt-dependent genera (*Thiococcus, Thioflavicoccus*, *Thiorhodovibrio*, *Thiohalocapsa*, *Halochromatium*, *Marichromatium*, and *Thiorhodococcus*), as demonstrated earlier using 16S rRNA gene sequence-based trees [21], was confirmed in the *pufLM* trees. The *pufLM* phylogeny evidenced a close relationship between *Thiorhodovibrio*, *Thiohalocapsa*, and *Halochromatium* as well as the formation of distinct branches for the group around *Thiococcus* and *Thioflavicoccu*s and another branch for *Marichromatium* species (Figs. 1–3). This study confirmed an overall high consistency of tree topologies of the two *puf* genes and the 16S rRNA gene and supports a largely coherent evolution of these genes. This is in contrast to the situation in phototrophic Alpha- and Betaproteobacteria, in which horizontal gene transfer of the photosynthetic gene clusters discussed in another study [34]. Identical species clusters formed according to 16S rRNA gene and *pufLM* sequences as well as important signatures within the *pufLM* sequences do not lend support for horizontal gene transfer of these genes within the PSB.

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Supplementary Table S1. PSB strains analyzed in this study, their group affiliation and accession numbers of the corresponding *pufLM* and 16S rRNA genes

Group	Species	Strain no.	16S rRNA accession no.	*pufLM* accession no.
I	*Thiococcus pfennigii*	DSM 226[T]	Y12373[§]	FN257141[P]
I	*Thiococcus pfennigii*	DSM 227	FN293056[P]	FN257142[P]
I	*Thiococcus pfennigii*	DSM 228	FN293055[P]	FN257143[P]
I	*Thioflavicoccus mobilis*	ATCC 700959[T]	AJ010126[§]	FN257144[P]
	"*Lamprocystis purpurea*"			
II	= *Chromatiaceae* bacterium	ML1	AJ006212[a]	AY177752[k]
II	*Thiohalocapsa halophila*	DSM 6210[T]	AJ002796	FN257151[P]
II	*Thiohalocapsa marina*	JA142[T]	AM491592[b]	FN257154[P]
III	*Halochromatium roseum*	DSM 18859[T]	AM283535[c]	FN257161[P]
III	*Halochromatium salexigens*	DSM 4395[T]	X98597[§]	FN257159[P]
III	*Halochromatium* sp.[1]	MTK6IM088	FN293083[P]	FN257173[P]
III	*Halochromatium* sp.[1]	MTK2IM023	FN293080[P]	FN257169[P]
III	*Halochromatium* sp.[1]	MTK8IM030	FN293082[P]	FN257174[P]
III	*Halochromatium* sp.[1]	MTK5IM027	FN293081[P]	FN257171[P]
III	*Halochromatium* sp.[1]	MTK1IM127	FN293079[P]	FN257175[P]
III	*Halochromatium* sp.[1]	MTK2IM039	FN293068[P]	FN257170[P]

IV	*Thiorhodovibrio winogradskyi*	DSM 6702[T]	Y12368[§]	FN257136[p]
V	*Thiocapsa marina*	DSM 5653[T]	FM178270[p]	FN257140[p]
V	*Thiocapsa roseopersicina*	DSM 217[T]	Y12364[§]	FN257146[p]
V	*Thiocapsa rosea*	DSM 235[T]	FM178269[p]	FN257147[p]
V	*Thiocapsa pendens*	DSM 236[T]	AJ002797[§]	FN257145[p]
V	*Thiocapsa* sp.[2]	MTRDDF081	FN293073[p]	FN257163[p]
V	*Thiocapsa* sp.[2]	MTRDDF078	FN293074[p]	FN257164[p]
V	*Thiocapsa* sp.[3]	MTPP2IF162	FN293070[p]	FN257166[p]
V	*Thiocapsa* sp.[4]	MTWDM061	FN293078[p]	FN257178[p]
V	*Thiocapsa* sp.[4]	MTWDM010	FN293076[p]	FN257176[p]
V	*Thiocapsa* sp.[5]	MTCH3IM012	FN293065[p]	FN257182[p]
V	*Thiocapsa* sp.[6]	MTV2IF083	FN293075[p]	FN257180[p]
VI	*Thiolamprovum pedioforme*	DSM 3802[T]	FM178271[p]	FN257152[p]
VI	*Chromatiaceae* bacterium[3]	MTPP2IF163	FN293071[p]	FN257165[p]
VII	*Allochromatium warmingii*	DSM 173[T]	Y12365[§]	FN257132[p]
VII	*Allochromatium vinosum*	DSM 180[T]	FM178268[p]	FN257131[p]
VII	*Allochromatium* sp.[7]	MT6010	FN293054[p]	FN257155[p]
VII	*Allochromatium* sp.[5]	MTCH3IM013	FN293062[p]	FN257181[p]
VII	*Allochromatium* sp.[5]	MTCH3IM086	FN293063[p]	FN257186[p]
VIII	*Thermochromatium tepidum*	DSM 3771[T]	M59150[d]	D85518[1]
IX	*Thiorhodococcus mannitoliphagus*	ATCC BAA-1228[T]	FM178272[p]	FN257139[p]
IX	*Thiorhodococcus minor*	DSM 11518[T]	FN293057[p]	FN257138[p]
IX	*Thiocystis violacea*	DSM 208	FN293060[p]	FN257149[p]

X	*Thiocystis violacea*	DSM 207[T]	FN293059[p]	FN257148[p]
XI	*Thiorhodococcus drewsii*	DSM15006[T]	FM178273[p]	FN257137[p]
XI	*Thiorhodococcus kakinadensis*	DSM 18858[T]	AM282561[q]	AM944094[r]
XII	*Chromatiaceae* bacterium[4]	MTWDM004	FN293061[p]	FN257179[p]
XIII	*Thiocystis gelatinosa*	DSM 215[T]	FN293058[p]	FN257189[p]
XIII	*Thiocystis minor*	DSM 178[T]	Y12372[§]	FN257150[p]
XIV	*Chromatium weissei*	DSM 5161[T]	FN293053[p]	FN257133[p]
XV	*Thiobaca* sp.[2]	MTRDDF079	FN293072[p]	FN257162[p]
XVI	*Marichromatium gracile*	DSM 203[T]	X93473[§]	FN257134[p]
XVI	*Marichromatium purpuratum*	DSM 1591[T]	AJ224439[§]	FN257135[p]
XVI	*Marichromatium bheemlicum*	ATCC BAA-1316[T]	AM180952[e]	AM944099[m]
XVI	*Marichromatium* sp.[8]	MTKK6IM001	FN293069[p]	FN257167[p]
XVI	*Marichromatium* sp.[9]	MTCH2IM059	FN293064[p]	FN257188[p]
XVI	*Marichromatium* sp.[5]	MTCH3IM047	FN293085[p]	FN257184[p]
XVI	*Marichromatium* sp.[1]	MTK6IM015	FN293087[p]	FN257172[p]
XVI	*Marichromatium* sp.[4]	MTWDM034	FN293077[p]	FN257177[p]
XVI	*Marichromatium* sp.[1]	MTK2IM017	FN293086[p]	FN257168[p]
XVI	*Marichromatium* sp.[5]	MTCH3IM033	FN293084[p]	FN257183[p]
XVI	*Marichromatium* sp.[5]	MTCH3IM049	FN293066[p]	FN257185[p]
XVI	*Marichromatium* sp.[5]	MTCH3IM052	FN293067[p]	FN257186[p]
XVII	*Ectothiorhodospira vacuolata*	DSM 2111	X93478[g]	FN257157[p]
XVII	*Ectothiorhodospira variabilis*	WN22[T]	AM943121[s]	FN257153[p]
XVII	*Ectothiorhodospira shaposhnikovii*[†]	DSM243[T] /ATCC31751	M59151[f]	AF018955[n]

XVII	*Ectothiorhodospira haloalkaliphila*	ATCC51935	FN293052[p]	FN257156[p]
XVII	*Ectothiorhodospira imhoffii*	JA319	AM902494	AM944100[o]
XVII	*Ectothiorhodospira mobilis*	DSM237	X93481[g]	FN257158[p]
XVIII	*Halorhodospira halophila*	DSM 244[T]	CP000544[h]	CP000544[h]
XVIII	*Halorhodospira halophila*	H	FN293051[p]	FN257160[p]
Outgroup	*Chloroflexus aggregans*	DSM9485[T]	AAUI01000026[i]	AAUI01000013[i]
Outgroup	*Chloroflexus aurantiacus*	DSM635[T]	CP000909[j]	CP000909[j]

References for the sequences

[a][9]; [b][29]; [c][28]; [d](Woese, direct submission); [e][26]; [f](Woese, direct submission); [g][24]; [h](Copland et al., direct submission WGS (whole genome sequence));
[i](Copland et al., direct submission WGS); [j](Copland et al. direct submission WGS); [k][40]; [l][12]; [m](Kumar, direct submission); [n](Gingras, direct submission);
[o](Kumar, direct submission); [p](this study); [q][27]; [r](Kumar, direct submission);
[s][14]; [§]updated 16S rRNA sequences during this study; numerals denote origin of the new environmental isolates: 1, saltern near Kakinada, Indian east coast;
2, pond near Kiel, Germany; 3, pond in Hyderabad, India; 4, Wadden Sea sediment near Büsum, Germany; 5, sandy rock pool near Trivendrum, Indian
southwest coast; 6 Crocodile lake near Visakhapatnam, India; 7, old culture bottle, Kiel, Germany; 8, sandy rockpool near Kanyakumari, southern India; 9,
green colored rock pool near Trivendrum, Indian southwest coast;
[†]16S rRNA gene sequence