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# Genomic Analysis of the Evolution of Phototrophy among Haloalkaliphilic *Rhodobacterales*

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## Abstract

A characteristic feature of the order *Rhodobacterales* is the presence of a large number of photoautotrophic and photoheterotrophic species containing bacteriochlorophyll. Interestingly, these phototrophic species are phylogenetically mixed with chemotrophs. To better understand the origin of such variability, we sequenced the genomes of three closely related haloalkaliphilic species, differing in their phototrophic capacity and oxygen preference: the photoheterotrophic and facultatively anaerobic bacterium *Rhodobaca barguzinensis*, aerobic photoheterotroph *Roseinatronobacter thiooxidans*, and aerobic heterotrophic bacterium *Natronohydrobacter thiooxidans*. These three haloalkaliphilic species are phylogenetically related and share many common characteristics with the *Rhodobacter* species, forming together the *Rhodobacter-Rhodobaca* (RR) group. A comparative genomic analysis showed close homology of photosynthetic proteins and similarity in photosynthesis gene organization among the investigated phototrophic RR species. On the other hand, *Rhodobaca barguzinensis* and *Roseinatronobacter thiooxidans* lack an inorganic carbon fixation pathway and outer light-harvesting genes. This documents the reduction of their photosynthetic machinery towards a mostly photoheterotrophic lifestyle. Moreover, both phototrophic species contain 5-aminolevulinic synthase (encoded by the *hemA* gene) incorporated into their photosynthesis gene clusters, which seems to be a common feature of all aerobic anoxygenic phototrophic *Alphaproteobacteria*. Interestingly, the *chrR-rpoE* (sigma24) operon, which is part of singlet oxygen defense in phototrophic species, was found in the heterotrophic strain *Natronohydrobacter thiooxidans*. This suggests that this organism evolved from a photoheterotrophic ancestor through the loss of its photosynthesis genes. The overall evolution of phototrophy among the haloalkaliphilic members of the RR group is discussed.

**Key words:** Anoxygenic photosynthesis, bacteriochlorophyll, horizontal gene transfer, photosynthesis gene cluster, *Rhodobacteraceae*.

## Introduction

The order *Rhodobacterales* (*Alphaproteobacteria*) encompasses a highly diverse ensemble of species (Simon et al. 2017). They largely differ in their phenotype, metabolic traits, and ecological niches they inhabit. Its members conduct many fundamental metabolic processes such as aerobic respiration,

anaerobic fermentation, sulfur oxidation, autotrophic carbon fixation, nitrogen fixation, or hydrogen production in various combinations (Garrity et al. 2005; Androga et al. 2012). One of the most interesting features of *Rhodobacterales* is the presence of many species that perform anoxygenic photosynthesis. Members of the *Rhodobacter* (*Rba.*) and *Rhodovulum* (*Rdv.*)

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genera represent classical examples of purple nonsulfur bacteria—organisms capable of photoautotrophic growth under anaerobic conditions utilizing the Calvin cycle, or heterotrophic growth in the presence of oxygen (Androga et al. 2012). On the other hand, there exist many marine photoheterotrophic species belonging to the so called *Roseobacter* group, which grow and conduct photosynthesis in the presence of oxygen (Wagner-Döbler and Biebl 2006; Moran et al. 2007; Brinkhoff et al. 2008), do not contain RuBisCO and use light only as an additional energy source (Koblížek et al. 2013).

In the last 20 years, seven haloalkaliphilic photoheterotrophic species were isolated from several saline and soda lakes in Africa, America, Asia, and Antarctica. Remarkably, these organisms are phylogenetically closer to freshwater *Rhodobacter* species than to marine photoheterotrophs. *Rhodobaca (Rca.) bogoriensis* was isolated from soda lakes in the East African Rift Valley (Milford et al. 2000), and is capable of both aerobic and anaerobic growth on organic substrates, however it does not contain Calvin cycle enzymes and thus cannot grow photoautotrophically. A closely related organism with the same physiology, *Rca. barguzinensis*, was later isolated from a small soda lake in the Barguzin valley in south-eastern Siberia (Boldareva et al. 2008). In contrast, the closely related aerobic anoxygenic phototrophic (AAP) bacterium *Roseinatronobacter (Rna.) thiooxidans*, isolated from a soda lake in the Kunkurskaya steppe (Chita region, south-easter Siberia) grows solely under aerobic conditions (Sorokin et al. 2000b; Stadnichuk et al. 2009). Later, a very similar species, *Rna. monicus* was isolated from the hypersaline Soda Mono lake in California, USA (Boldareva et al. 2006). Closely related to the above mentioned species are two Antarctic AAP isolates *Roseibaca ekhonensis* and *Roseicitreum antarcticum*, which were collected from Lake Ekho (Labrenz et al. 2009), and sandy intertidal sediments (Yu et al. 2011), respectively. The last described AAP bacterium in this group is *Roseibacula alcaliphilum*, which was isolated from Lake Doroninskoe, East Transbaikal region, Russia (Boldareva and Gorlenko 2014). This photoheterotrophic group is complemented by some non-phototrophic species such as the haloalkaliphilic heterotrophic bacterium *Natronohydrobacter (Nhb.) thiooxidans* isolated from a soda lake in Kenya.

Based on the close phylogenetic proximity of photoautotrophic, photoheterotrophic, and lithoautotrophic species, Keppen et al. (2013) suggested that the photoheterotrophic species such as *Rhodobaca* and *Roseinatronobacter* represent the intermediate species on the regressive evolutionary pathway leading from photoautotrophic purple nonsulfur bacteria to heterotrophic species. A similar scenario has also been proposed for photoheterotrophic and heterotrophic species in the marine *Roseobacter* group (Koblížek et al. 2013). However, the existence of photoheterotrophic species can be explained using an alternative scenario where the originally heterotrophic species acquired their photosynthesis genes *via* horizontal gene transfer (HGT). A typical feature of purple

phototrophic bacteria is that most of their genes involved in the light-phase of photosynthesis are organized in photosynthesis gene clusters (PGCs) (Zsebo and Hearst 1984; Zheng et al. 2011). It has been speculated that clustering of photosynthesis genes in PGCs can facilitate HGT. In line with this hypothesis is the report that the whole PGC was likely transferred between *Proteobacteria* and *Gemmatimonadetes* (Zeng et al. 2014). *Rhodobacterales* species often carry various extra chromosomal elements (Petersen et al. 2013), transposable elements (Vollmers et al. 2013), or gene transfer agents (Luo and Moran 2014), which can facilitate gene transfer. Moreover, PGC-containing plasmids have been identified in some *Roseobacter*-group representatives (Petersen et al. 2013). HGT has also been proposed for *puf* genes encoding the protein subunits of the bacterial reaction centers within the *Roseobacter* group (Koblížek et al. 2015).

To elucidate the most likely evolutionary pathway, we decided to sequence genomes of three closely related (on average 98.7% 16S rRNA pairwise similarity) haloalkaliphilic organisms differing in their metabolic capacities and oxygen preference. Photoheterotrophic *Rca. barguzinensis* strain alga05 exhibits both aerobic and anaerobic growth. It lacks RuBisCO and nitrogenase, but it has the capacity to utilize thiosulfate and sulfide as electron donors (Boldareva et al. 2008). The second organism, *Rna. thiooxidans* strain ALG1, is a photoheterotrophic bacterium combining aerobic respiration and photophosphorylation, but it can also oxidize thiosulfate and sulfide during aerobic lithoheterotrophic growth (Sorokin et al. 2000b). The last organism, *Nhb. thiooxidans* strain AH01, is a strictly aerobic, nonphototrophic bacterium. It is capable of growing lithoheterotrophically with acetate and thiosulfate, sulfide, polysulfide, and elemental sulfur which are oxidized to sulfate, and in addition it can oxidize H<sub>2</sub> (Sorokin et al. 2000a). We mostly focused on the presence of photosynthesis genes, their organization and phylogeny in order to test the two proposed evolutionary scenarios.

## Materials and Methods

### Bacterial Strains and Cultivation Conditions

*Rna. thiooxidans* strain ALG1, *Rca. barguzinensis* strain alga05 and *Nhb. thiooxidans* strain AH01 were obtained from the UNIQEM Culture Collection of the Winogradsky Institute of Microbiology, Russian Academy of Sciences. The phototrophic strains were grown under aerobic conditions in the presence of light at 28 °C in 250 ml Erlenmeyer flasks with 100 ml of medium under shaking (150 rpm). The media used was as described in Sorokin et al. (2000b) and Boldareva et al. (2008). The chemotrophic strain AH01 was grown in a 5 l gas bottle with 1 l of acetate-containing medium and with a 20% H<sub>2</sub>/80% air gas phase (Sorokin et al. 2000a). The purity of the cultures was controlled by repeated streaking of the individual colonies formed on the medium solidified with 2% (wt/vol) agar and verified by microscopy.

## Genome Sequencing and Data Analyses

About 45 ml of dense culture was harvested by centrifugation at  $13,000 \times g$  for 5 min. The genomic DNA was extracted and purified using a commercial kit (Tiangen Biotech, Beijing, China). DNA quantity and quality were determined using a NanoDrop ND-1000. The identity of the cultures was verified by amplification and sequencing their 16S rRNA genes. The whole-genome shotgun sequencing was performed using the Illumina HiSeq 2000 platform and a Roche 454 GS FLX+ system at Macrogen (Seoul, South Korea). Procedures for DNA shearing, library preparation and quality control, sample loading, and sequencer operation were performed according to Macrogen's standard protocols (<https://dna.macrogen.com>).

Raw reads produced from both types of whole-genome shotgun sequencing were trimmed and de novo assembled into contigs using CLC Genomics Workbench 9.0 software (QIAGEN Aarhus, Denmark). To decrease the number of final contigs, they were further processed using the "Genome Finishing Module" of the CLC software.

Annotation of contigs was performed with the RAST server (Aziz et al. 2008). The obtained genomic data was compared with other *Rhodobacterales* species [*Rba. sphaeroides* 2.4.1, *Rba. capsulatus* SB 1003, *Rdv. sulfidophilum* DSM 2351, *Paracoccus (Pco.) denitrificans* PD 1222] obtained from the NCBI GenBank.

To detect clustered regularly interspaced short palindromic repeats (CRISPR) in studied genomes, we used the web-based tool CRISPRFinder (Grissa et al. 2007).

Orthologous gene cluster analysis was performed using the OrthoVenn web server (Wang et al. 2015). Pairwise sequence similarities between all input protein sequences were calculated with an e-value cut-off of  $1e-5$ . An inflation value ( $-l$ ) of 1.5 was used to define orthologous cluster structure.

## Phylogenetic Analyses

16S rRNA gene sequences obtained from GenBank™ were aligned using ClustalX version 2.1. Ambiguously aligned regions and gaps were excluded from further analyses using Gblocks (Talavera and Castresana 2007). Phylogenetic trees based on 16S rRNA gene sequences were constructed using PhyML/MEGA 6.0 software using a maximum likelihood (ML) algorithm with a HKY85 nucleotide substitution model and a bootstrap of  $1,000 \times$ . For the BchDHLNB concatenated tree, amino acid sequences were retrieved from GenBank™ and aligned using ClustalX version 2.1. The sites containing gaps and ambiguously aligned regions were stripped from the final alignment using Gblocks. Amino acid sequence alignments of each peptide were concatenated with Geneious version 8.1.2 (Biomatters Ltd.). The 5-aminolevulinic synthase (ALAS) phylogenetic tree involves amino acid sequences derived by BLASTing from ALAS sequences of *Dinoroseobacter (Drb.) shibae* (Dshi\_3546, Dshi\_1182, and Dshi\_3190).

Phylogenetic trees were inferred by MEGA 6.0 software using ML algorithm with LG model. 16S rRNA sequence pairwise similarities were calculated using GGDC tool under the recommended settings (Meier-Kolthoff et al. 2013).

## Sequence Accession Numbers

The *Rca. barguzinensis* strain alga05, *Rna. thiooxidans* strain ALG1 and *Nhb. thiooxidans* strain AH01 genome sequences have been deposited at NCBI GenBank under the accession numbers: GCA\_001870665.1, GCA\_001870675.1, and GCA\_001884735.1.

## Results

The sequencing and assembly of the genomes of the three studied organisms resulted in draft assemblies consisting of 5–52 contigs (table 1). The smallest genome ( $\sim 3.5$  Mb) was found in *Rna. thiooxidans* ALG1, while *Rca. barguzinensis* alga05 and *Nhb. thiooxidans* AH01 had somewhat larger genomes ( $\sim 3.9$  and 4.1 Mb). The three strains share 2,240 orthologs which account for  $\sim 80\%$  of their genes (supplementary fig. 1, Supplementary Material online).

The 16S rRNA sequences of the three sequenced strains were compared with other members of order *Rhodobacterales*. The obtained phylogenetic tree documents that the sequenced haloalkaliphilic strains are related not only to *Roseibaca*, *Roseicitreum*, and *Roseibacula* species but more distantly also to *Rhodobacter* species *Rba. sphaeroides*, *Rba. capsulatus*, *Rba. sp. SW2*, and *Rba. veldkampii* (fig. 1). We denote this group *Rhodobacter-Rhodobaca* group, or, in shorthand *RR* group. Freshwater photoautotrophic bacterium *Rdv. sulfidophilum*, and *Paracoccus* species, were closely related to the *RR*-group. The marine species belonging to the so-called *Roseobacter* group were phylogenetically more distant (fig. 1).

As expected, a complete PGC was found in alga05 and ALG1 genomes. Its organization was identical in both strains, and it was very similar to the organization of the PGC in *Rhodobacter* species (fig. 2). The only difference was the presence of the *hemA* gene in the haloalkaliphilic strains and the transposition of *tspO-crtBIA-bchIDO* genes. The PGC in *Drb. shibae* and *Rvu. sulfidophilum* has a clearly different organization. Moreover, in *Rvu. sulfidophilum*, it is split in two parts placed  $\sim 300$  kb apart. A typical feature for all phototrophic *RR*-species is the divergent orientation ( $\leftarrow \rightarrow$ ) of superoperons *bchFNBHLM-lhaA-puh* and *crt-bchCXYZ-puf*, while in other *Rhodobacterales* groups they have convergent or colinear orientation (Zheng et al. 2011).

The major difference between the investigated species and *Rhodobacter* species was the complete absence of genes for carbon and nitrogen fixation. We detected a RuBisCO-like protein in the genome of the AH01 strain, but due to low homology (36% amino-acid identity) and absence of the

**Table 1**

General Features of the Studied Genomes as Compared with the Published Genomes of the Closest Relatives

	<i>Rca. barguzinensis</i> alga05	<i>Rna. thiooxidans</i> ALG1	<i>Nhb. thiooxidans</i> AH01	<i>Rba. sphaeroides</i> 2.4.1	<i>Rdv. sulfidophilum</i> DSM 2351	<i>Rba. capsulatus</i> SB 1003	<i>Pco. denitrificans</i> PD 1222
Total bases	3,893,644	3,504,907	4,075,255	4,628,173	4,732,772	3,871,920	5,236,194
No. of contigs	5	52	39	Closed	Closed	Closed	Closed
GC content [%]	59	60	63	69	67	67	67
No. of RNAs	53	42	43	60	59	66	61
chromosomes	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	2	1	1	2
plasmids	<i>n.a.</i>	<i>n.a.</i>	<i>n.a.</i>	5	3	1	1
ORFs	3,805	3,385	3,885	4,347	4,398	3,685	5,121
proteins	2,760 (73%)	2,502 (74%)	2,834 (73%)	3,076 (71%)	2,837 (65%)	3,100 (84%)	4,032 (79%)
hyp.proteins	1,045 (27%)	883 (26%)	1,051 (27%)	1,271 (29%)	1,561 (35%)	610 (16%)	1,089 (21%)
PGC length [kbp]	45.4	45.1	–	44.7	42.1	44.3	–
No. of CRISPR spacers	4	38	29	0	11	59	9

other necessary enzymes it does not seem to be involved in carbon fixation. All three studied strains also lack the *pucBAC* operon encoding the outer light harvesting complexes (table 2), which is consistent with the lack of LH2 bands in their absorption spectra (Sorokin et al. 2000a).

#### Tetrapyrrole Biosynthesis Genes

Bacteriochlorophylls are the main light-harvesting pigments in anoxygenic phototrophs (Willows and Kriegel 2009). The genes responsible for their biosynthesis have been routinely used as suitable phylogenetic markers for the investigation of the origin of photosynthesis in bacteria (Raymond et al. 2002; Koblížek et al. 2013).

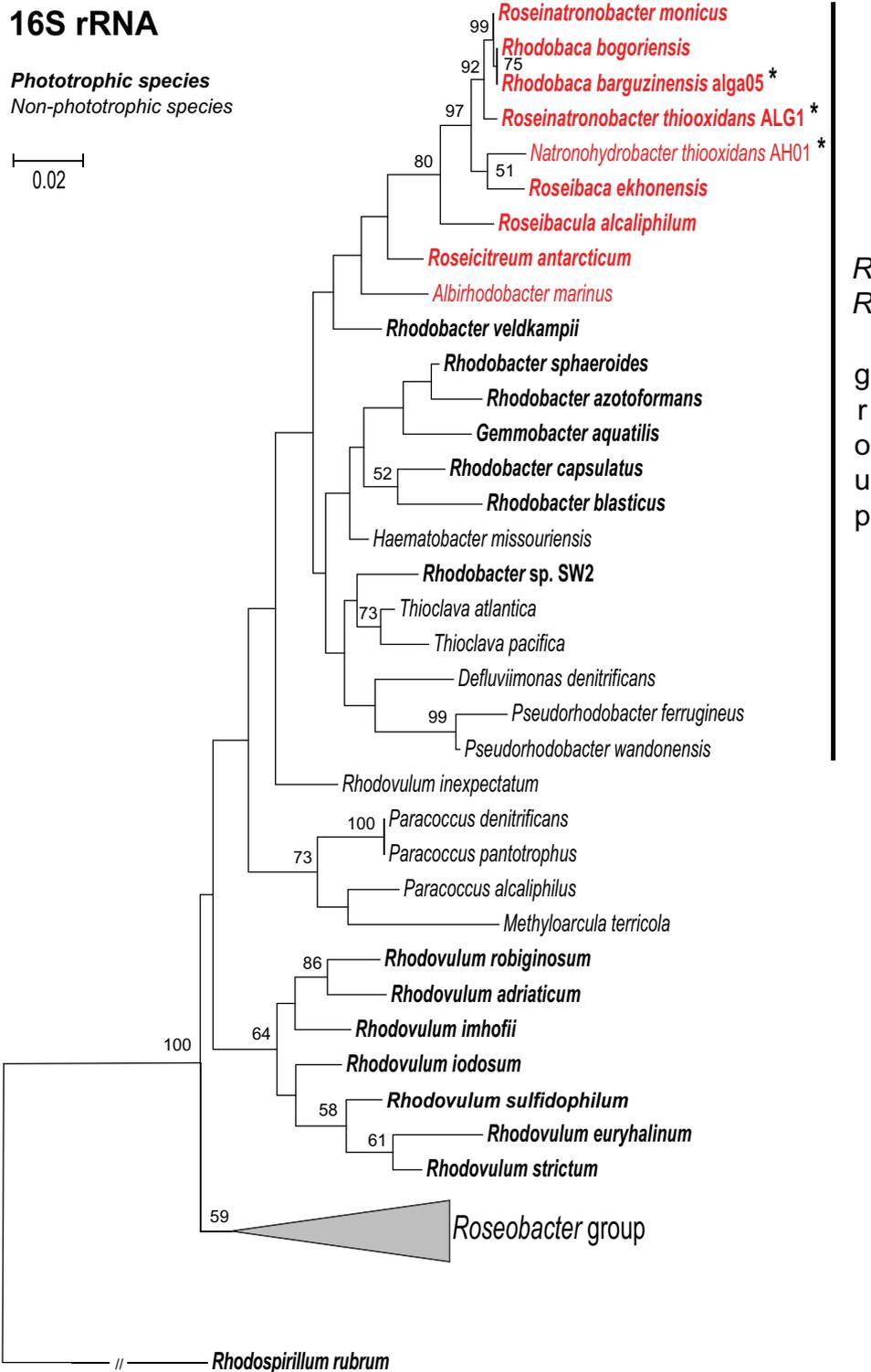
The biosynthesis of all tetrapyrrols, including BChl *a*, starts with the synthesis of 5-aminolevulinic acid (Willows and Kriegel 2009; Dailey et al. 2017). This compound is synthesized in all Proteobacteria via the Shemin pathway by 5-aminolevulinic acid synthase (ALAS). In *Rba. sphaeroides* 2.4.1., there exist two homologous isoenzymes coded by the *hemA* and *hemT* genes (Fanica-Gaignier and Clement-Metral 1973; Bolt et al. 1999). Similarly, there were both ALAS genes in the phototrophic strains alga05 and ALG1 (table 2). Only one gene was found (*hemA* form) in the heterotrophic strain AH01, in photoautotrophic bacteria *Rba. capsulatus*, *Rba. sp. SW2*, as well as in *Rvu. sulfidophilum*. To better understand the origin of the multiple ALAS forms, we constructed a maximum-likelihood tree with all ALAS genes found in various members of *Rhodobacterales*. The constructed tree clearly splits into two major groups (fig. 3). The first group encompasses *hemA*-like genes from both heterotrophic and phototrophic species, including the *hemA* gene from *Rba. sphaeroides*. It also contains *hemA* genes from *Rba. capsulatus*, *Rba. sp. SW2*, and *Rvu. sulfidophilum*. The second group contains *hemT* related genes, mostly from phototrophic *Rhodobacterales*. Both, alga05 and ALG1 contain one ALAS gene from the *hemA*

group, and one from the *hemT* group. Noteworthy, the latter is a part of the PGC (fig. 2), which seems to be a universal characteristic of all AAP *Rhodobacterales*. Interestingly, a homologue of this gene was also found in the PGC of AAP bacterium *Erythrobacter* sp. NAP1 (order *Sphingomonadales*), though, here the gene was likely acquired through the HGT.

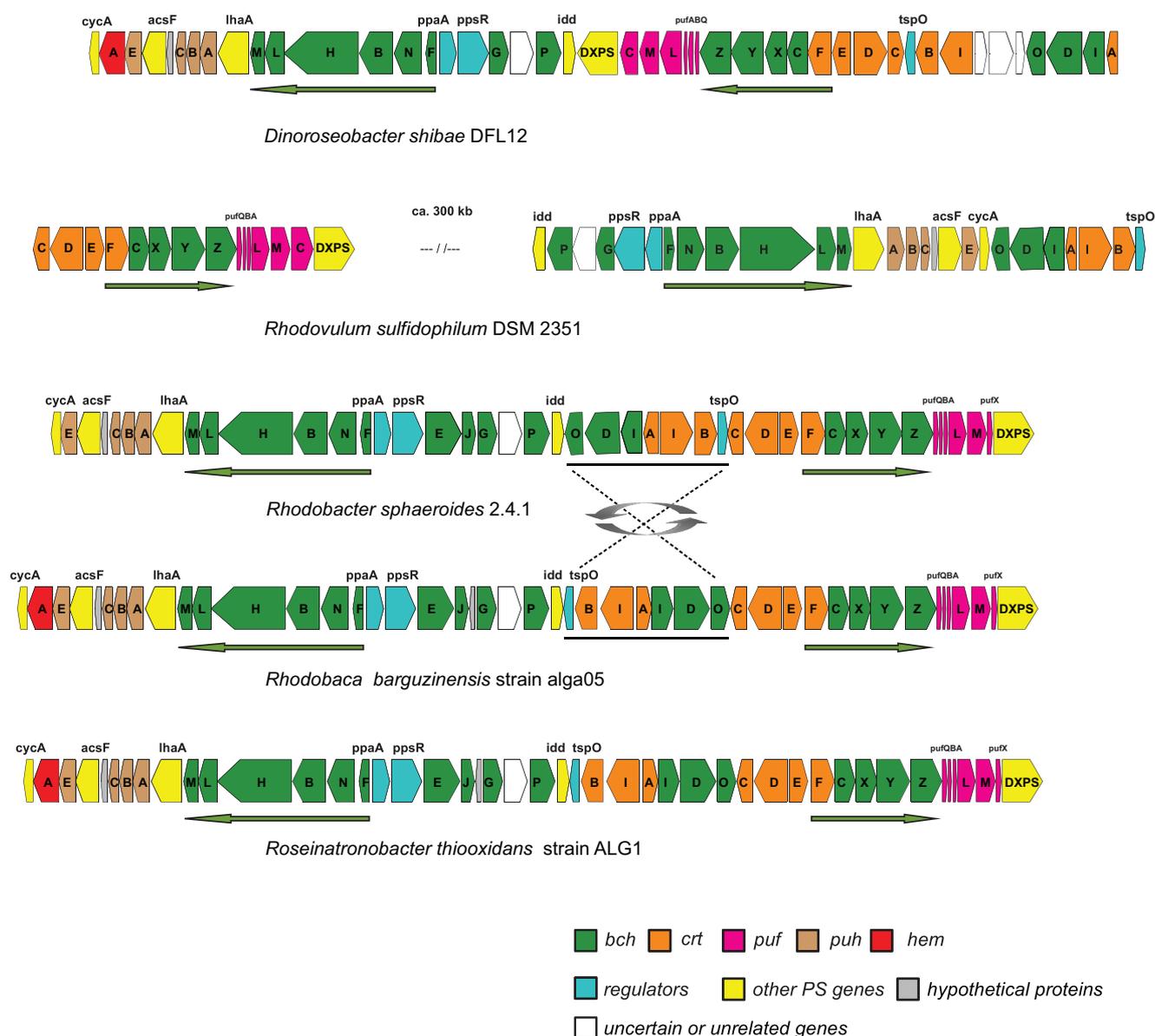
The tetrapyrrole pathway then proceeds from 5-aminolevulinic acid to protoporphyrin IX by action of six different enzymes. The last two are oxidases, which exist in oxygen-dependent and oxygen-independent forms. The coproporphyrinogen III oxidase is present in both forms (*hemF*, *hemN*) in all studied strains except *Rba. capsulatus*, which contains only the oxygen-independent form of this enzyme. In the case of protoporphyrinogen IX oxidase, all the investigated strains contained only the oxygen-dependent form encoded by the *hemY* gene (table 2).

#### Bacteriochlorophyll Biosynthesis

The BChl *a* pathway on its own starts with magnesium chelatase. This enzyme catalyzes the insertion of Mg into protoporphyrin IX. It is composed of three subunits encoded by the *bchl*, *bchD*, and *bchH* genes. Another enzyme of the BChl *a* biosynthesis pathway is light-independent protochlorophyllide reductase (subunits encoded by the *bchl*, *bchN*, and *bchB* genes). This protein is required for the light-independent reduction of protochlorophyllide to chlorophyllide *a* (Willows and Kriegel 2009). To investigate the phylogenetic relationship of the photosynthesis genes between studied and reference strains, we calculated the tree using the concatenated amino acid sequences of these two enzymes. The inferred phylogeny documents a very close relationship of the two photoheterotrophic strains, alga05 and ALG1, and all the *RR*-species (fig. 4).



**Fig. 1.**—16S rRNA phylogenetic tree showing the position of studied strains (marked with an asterisk) within the *Rhodobacterales* clade. Halophilic strains are highlighted in red. The phylogenetic tree was calculated using maximum likelihood algorithm with HKY85 nucleotide substitution model and bootstrap 1,000×. *Rhodospirillum rubrum* was used as an outgroup organism. Scale bar represents changes per position. Bootstrap values >50% are shown.



**Fig. 2.**—Comparison of gene organization in PGCs of the studied and reference genomes. Arrows show directions of superoperons *bchFNBHLM-lhaA-puh* and superoperons *crt-bchCXYZ-puf*. Note: *Rba. sp. SW2* has the same PGC organization as *Rba. sphaeroides*. PGC in *Rba. capsulatus* differs from *Rba. sphaeroides* only by the absence of the *acsF* gene.

Another important step of the BChl a biosynthetic pathway is the cyclization of its fifth ring by Mg-protoporphyrin IX monomethyl ester oxidative cyclase (Willows and Kriegel 2009). This enzyme has two forms: an oxygen-dependent and oxygen-independent form encoded by *acsF* and *bchE* gene, respectively. *Rba. sphaeroides*, *Rba. sp. SW2*, *Rvu. sulfidophilum*, *alga05* and *ALG1* contain both forms of this enzyme, while *Rba. capsulatus* contains only the oxygen independent form. Heterotrophic strains *AH01* and *Pco. denitrificans* obviously do not contain these genes. Interestingly these two forms of the cyclase have a very different phylogeny. The oxygen-dependent form of the cyclase (*acsF* gene product) was used previously as a convenient gene marker for

the detection of AAPs (Boldareva et al. 2013; Zeng et al. 2014). Indeed, all *RR*-species formed a distinct group, clearly separated from the marine species as well as *Rvu. sulfidophilum* (supplementary fig. 2, Supplementary Material online). The situation was very different for the oxygen-independent form (*bchE* gene product), where in *Rba. spp.*, *ALG1*, and *alga05*, the *bchE* gene is part of the PGC, while in *Rvu. sulfidophilum* and in many marine AAP species it is outside the PGC.

### Reaction Center Proteins

One part of the PGC comprises of *puf* (photosynthesis unit forming), an operon encoding the proteins for the inner

**Table 2**

The Presence of Genes Related to Photosynthesis in the Studied and Reference Genomes

	DSM 2351	2.4.1	SB 1003	alga05	ALG1	AH01	PD 1222
<b>Carbon fixation</b>							
RubisCO (large subunit)	○	○	○	–	–	–/?	○
<b>puf genes</b>							
<i>pufC</i>	●	–	–	–	–	–	–
<i>pufX</i>	–	●	●	●	●	–	–
<b>puh genes</b>							
<i>puhABC, puhE</i>	●	●	●	●	●	–	–
<b>puc genes</b>							
<i>pucBAC</i>	○	○	○	–	–	–	–
<b>Other PS genes</b>							
<i>cycA</i>	●	●	○	●	●	○	○
<i>lhaA</i>	●	●	●	●	●	–	–
<i>DXPS</i>	●○	●○	●○	●○	●○	○	○
<b>Porphyrin biosynthesis</b>							
<i>hemA</i>	○	○○	○	●○	●○	○	○
<i>hemF*</i>	○	○	–	○	○	○	○
<i>hemN</i>	○	○	○	○	○	○	○
<i>hemY*</i>	○	○	○	○	○	○	○
<i>hemG</i>	–	–	–	–	–	–	–
<i>acsF*</i>	●	●	–	●	●	–	–
<i>bchE</i>	○	●	●	●	●	–	–
<b>Regulatory proteins</b>							
<i>ppaA/aerR</i>	●	●	●	●	●	–	–
<i>appA</i>	–	○	–	–	–	–	–
<i>ppsR/crtJ</i>	●	●	●	●	●	–	–
<i>hvrB</i>	–	–	●	–	–	○	–
<i>tspO</i>	●	●	●	●	●	–	–
<i>pufQ</i>	●	●	●	●	●	–	–
<b>Transcription factors</b>							
Sigma factors							
RpoE, RpoH <sub>I</sub> , RpoH <sub>II</sub>	○	○	○	○	○	○	○
Anti-sigma factor ChrR							
	○	○	–	○	○	○	–

NOTE.—The PS genes present inside the PGC are marked “●”; the PS genes located outside the PGC are marked “○”; “●○” means two forms of gene are present; “–” means gene is absent in the genome. Genes coding for the oxygen-dependent form of the enzyme are marked with asterisk. *hemF* and *hemN* are aerobic and anaerobic forms of Coproporphyrinogen III oxidase, *hemY* and *hemG* are aerobic and anaerobic forms of Protoporphyrinogen IX oxidase. *acsF* and *bchE* are aerobic and anaerobic forms of Mg-protoporphyrin IX monomethylester oxidative cyclase. ‘/?’ = RubisCO-like protein identified in the genome of AH01.

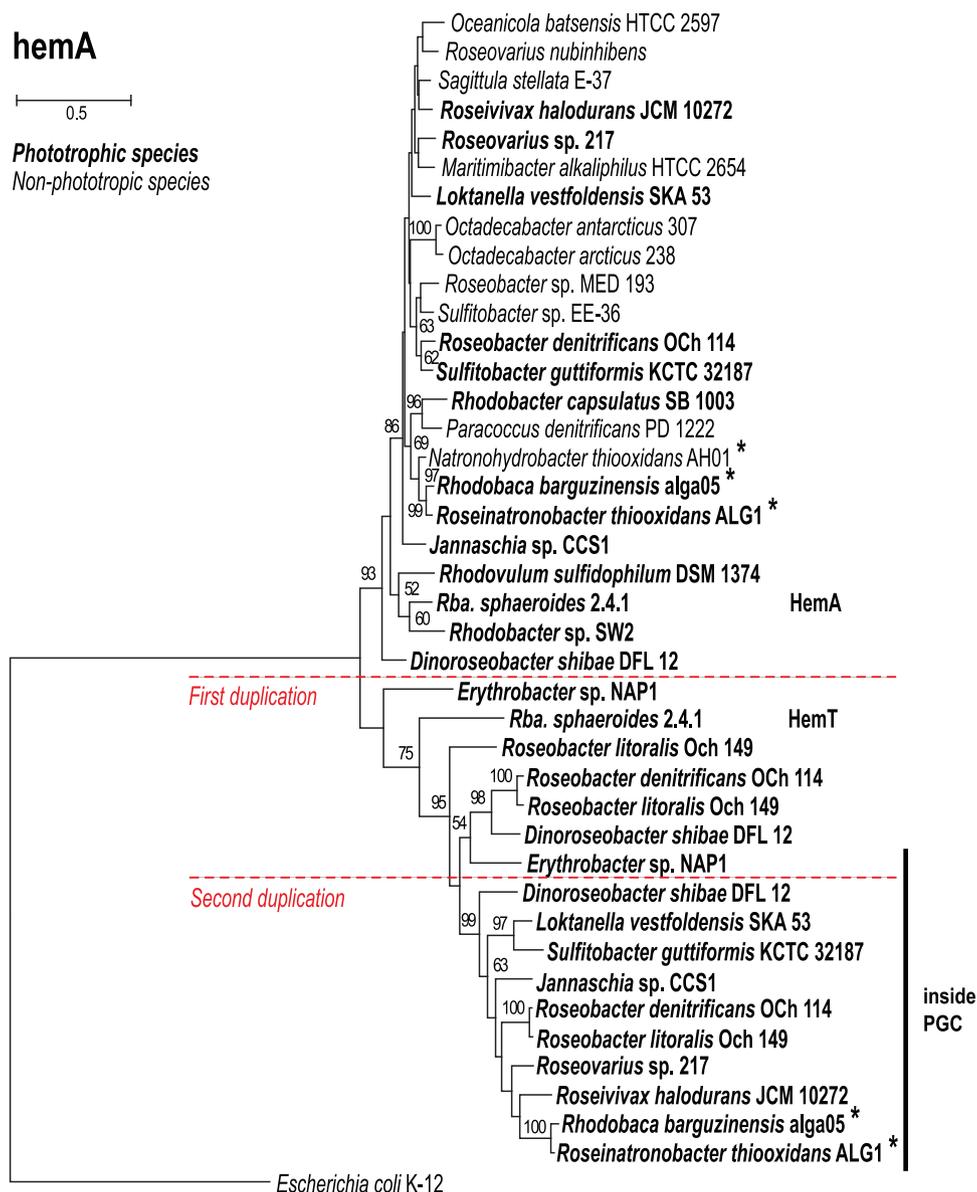
antenna (*pufBA*) and the photosynthesis reaction center subunits L and M (*pufLM*). In all *Rhodobacter* species (*Rba. sphaeroides*, *Rba. capsulatus*, and *Rba. sp. SW2*) these genes are accompanied by a *pufX* gene encoding the light-harvesting associated protein PufX, with the typical *pufQBALMX* operon organization. This gene was also present in the two phototrophic strains alga05 and ALG1. In contrast, *Rdv. sulfidophilum* contains the *pufC* gene encoding the cytochrome c subunit of the complex (table 2).

Since the *pufM* gene represents a convenient genetic marker of anoxygenic phototrophs with type 2 reaction centers, it is frequently used to infer phylogeny of new phototrophic organisms. The computed PufM phylogenetic tree of phototrophic *Rhodobacterales* shows a major split into two branches. The first encompasses *Rhodobacter* species, the phototrophic species alga05 and ALG1, and a few members

of the marine *Roseobacter* group. The second group contains *Rvu. sulfidophilum*, *Roseobacter (Rsb.) denitrificans*, *Drb. shibae* and most of the marine AAP species. This phylogeny further confirms the close relationship of the studied haloalkalophiles with *Rhodobacter* species, and their distinction from *Rhodovulum* and *Roseobacter* species (supplementary fig. 3, Supplementary Material online).

### Regulatory Proteins

All photosynthetic organisms control expression of their photosynthesis genes in response to changes in the environment. All seven studied strains contain these common regulators: AhcY, FnrL, HvrA, PrrA/RegA, PrrB/RegB, and PrrC/SenC. All studied phototrophic strains accommodate *ppaA/aerR*, *ppsR/crtJ*, *tspO*, and *pufQ* genes inside their PGCs. *Rba. sphaeroides*

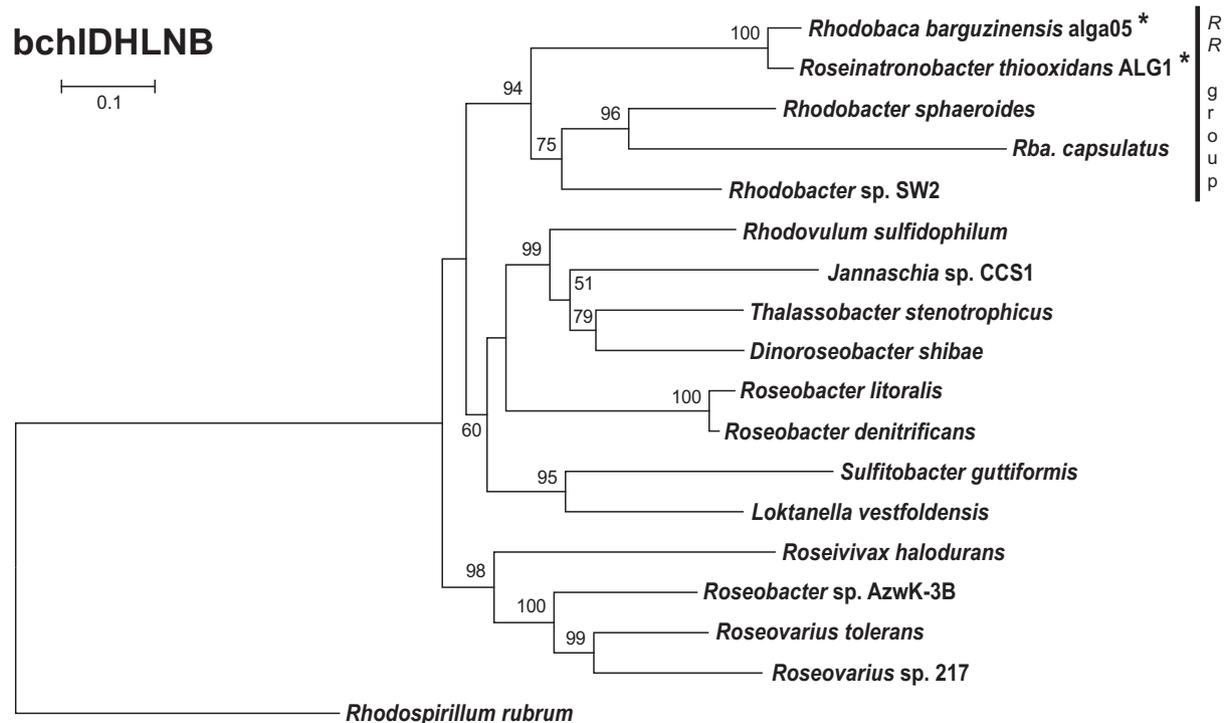


**Fig. 3.**—Phylogenetic analysis based on alignment of amino acid sequences of ALAS (HemA or HemT). Maximum likelihood (ML) tree with bootstrap 500× was constructed for studied strains and other representative species from the *Rhodobacterales* clade. *Escherichia coli* K-12 was used as an outgroup organisms. Scale bars represent changes per position. Bootstrap values >50% are shown. Horizontal bar marks sequences found inside the PGC. Studied strains are marked with an asterisk. Hypothetical gene duplication events are marked with horizontal dashed lines.

is the only strain with *appA* gene, which is located outside the PGC. Interestingly, *Rba. sp. SW2* also does not possess this gene in the genome (data not shown). Two investigated strains, that is, phototrophic *Rba. capsulatus* and chemotrophic strain AH01 possess the gene coding for the HvrB protein. The phototrophic strain contains this gene inside its PGC (table 2).

One of the most important physiological processes in phototrophic species is the protection of cells against reactive oxygen species. Singlet oxygen is generated under aerobic conditions by light-excited BChl *a* (Borland et al. 1989).

In *Rba. sphaeroides* three alternative sigma factors are involved in response to the singlet oxygen, the sigma-24 factor RpoE, and the sigma-32 factors RpoH<sub>I</sub> and RpoH<sub>II</sub>. While RpoH is part of a more general oxidative stress response, RpoE and ChrR have been shown to be triggered specifically by the oxygen singlet. In these organisms, RpoE and ChrR are encoded in an operon and together with a second operon transcribed from the same promoter in the opposite direction form a conserved gene cluster. This gene cluster has also been found in all investigated phototrophic species, but it was absent in most of the heterotrophic species. Surprisingly, the



**Fig. 4.**—Phylogenetic tree based on concatenated alignments of amino acid sequences of magnesium chelatase and light-independent protochlorophyllide reductase (BchIDHLNB; 2317 common amino acid positions). Maximum-likelihood (ML) tree with bootstrap 1,000× was constructed for studied strains and other representative species from the *Rhodobacterales* clade. *Rhodospirillum rubrum* was used as an outgroup organism. Scale bars represent changes per position. Bootstrap values >50% are shown. Studied strains are marked with an asterisk.

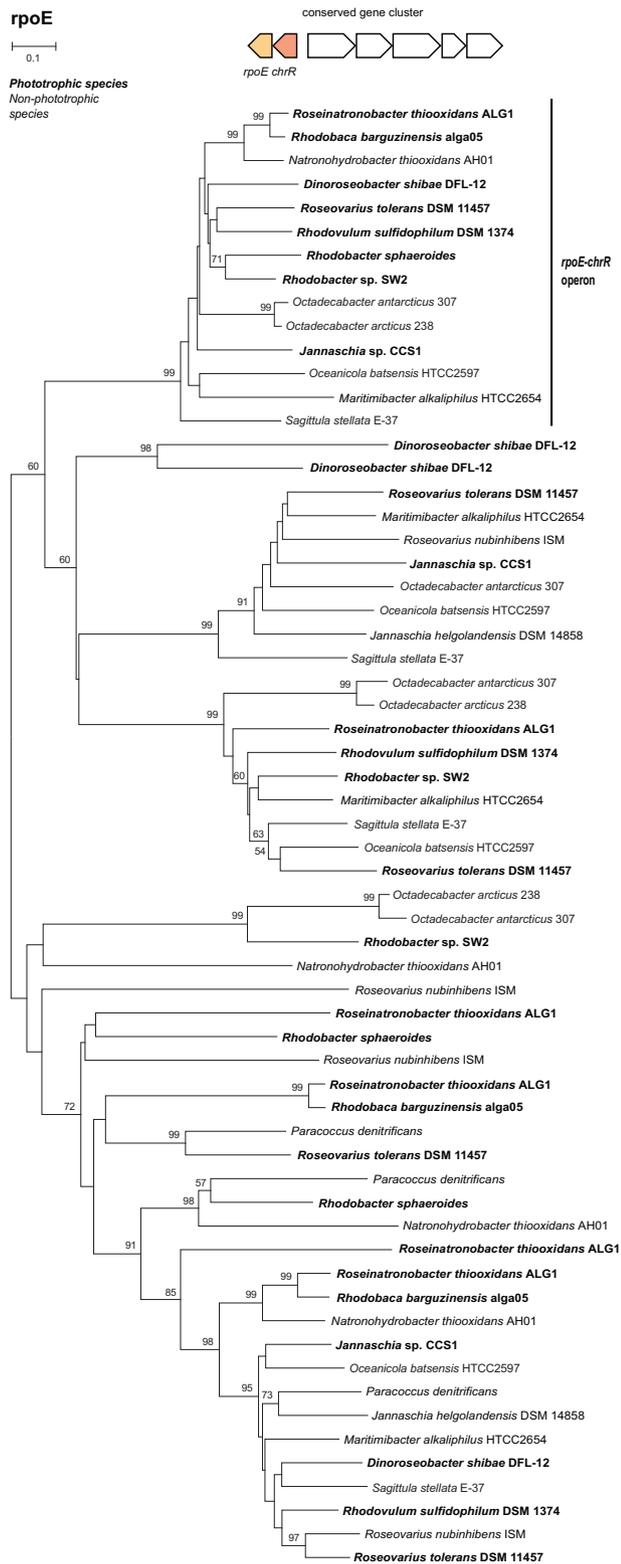
*rpoE-chrR* cluster was also present in the studied heterotrophic strain AH01, as well as in several marine heterotrophic bacteria *Maritimibacter alkaliphilus*, *Octadecabacter antarcticus*, *Octadecabacter arcticus*, *Oceanicola batsensis*, and *Sagittula stellata* which should not be prone to singlet oxygen formation (fig. 5).

## Discussion

Two evolutionary scenarios explaining the presence of phototrophic and heterotrophic strains among haloalkaliphilic species in the *RR*-group were considered in this study (1) the “regressive evolution” scenario, where the ancestors of the haloalkaliphilic *RR*-group were photoautotrophic organisms, which later lost part (or all) of their photosynthesis gene, or (2) the ancestors of the *RR*-group were heterotrophs, which adopted their photosynthesis genes *via* HGT of photosynthesis genes. Our data indicates that phototrophy is ancestral in the *RR*-group. There are two main lines of evidence. First, all the studied phototrophic organisms belonging to the *RR*-group shared an almost identical organization of their PGCs (fig. 2). A unique feature for all phototrophic *RR*-species is the divergent orientation of superoperons *bchFNBHLM-lhaA-puh* and *crt-bchCXYZ-puf*. This orientation has not been found in any other *Rhodobacterales* species (Zheng et al. 2011), which

represents the main evidence against the hypothesis that the *RR*-species adopted the PGC through HTG from other *Rhodobacterales* genera. Another distinctive feature present in all studied phototrophic members of the *RR*-group is the presence of the *pufX* gene, and the absence of the *pufC* gene (table 2). The gene *pufX* is also present in several marine AAP species, but they have likely received it *via* the HGT as discussed before (Koblížek et al. 2015). Second, the performed phylogenetic analyses on the photosynthesis genes *bchIDHLNB*, *acsF* and *pufM* (fig. 4, supplementary figs. 2 and 3, Supplementary Material online) document that the *RR*-group always cluster together, with an exception of the *pufM* tree where the *RR*-group is mixed with several marine AAP species (supplementary fig. 3, Supplementary Material online).

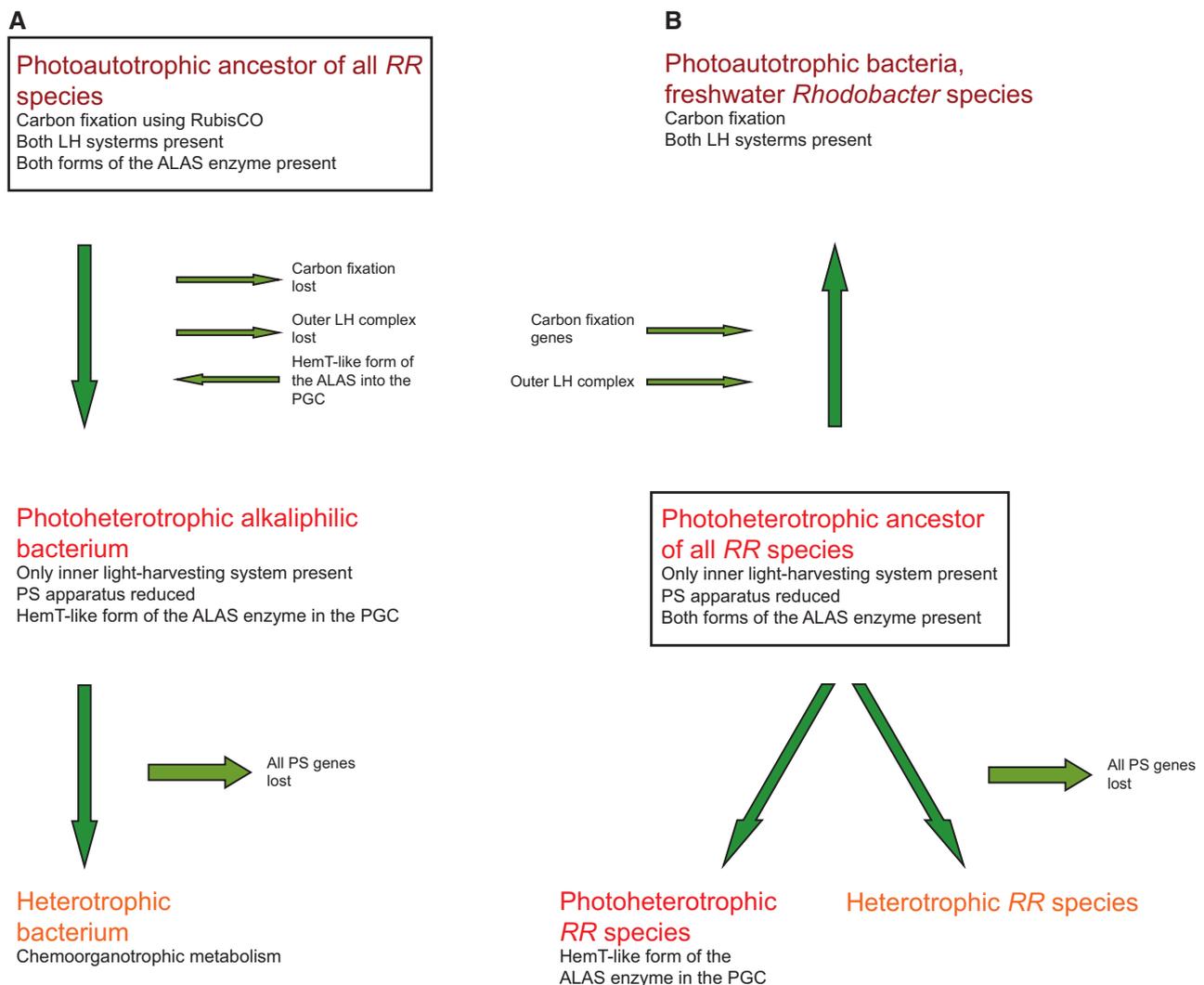
An important finding is the presence of genes connected with the singlet oxygen defense system in chemotrophic strain AH01. This strain contains the same singlet-oxygen-stress-response mechanism (encoded in *rpoE-chrR* gene operon) as *Rba. sphaeroides* (Anthony et al. 2004), as well as in ALG1 and alga05 strains. When bound to its anti-sigma factor ChrR, RpoE is inactive in the cell. The presence of singlet oxygen leads to a dissociation of the heterodimer, thus the activation of RpoE and subsequently its more than 180 target genes (Anthony et al. 2004). A similar response has been



**Fig. 5.**—Phylogenetic tree of sigma-24 factors in *Rhodobacterales*. Species with a photosynthesis gene cluster are in bold. Sigma-24 factors in an operon with *chrR* form a distinct phylogenetic group, marked by a vertical bar. The tree has been constructed using the Neighbour-joining (NJ) method with pairwise deletion of gaps and 500 bootstraps.

shown for the AAP species *Rsb. litoralis* (Berghoff et al. 2011) and *Drb. shibae* (Tomasch et al. 2011). As the main source of singlet oxygen in bacteria is BChl *a* (Borland et al. 1989), all the phototrophic *Rhodobacterales* contain the RpoE–ChrR system to protect their cellular machinery against damage. However, chemotrophic organisms (which lack BChl *a*) should not be prone to the formation of singlet oxygen, and the presence of RpoE–ChrR system is unnecessary. This suggests that the ancestor of AH01 was originally a phototrophic bacterium, which later lost its photosynthetic genes, but yet retained the RpoE–ChrR system.

From the presented pieces of evidence, we conclude that the ancestors of the haloalkaliphilic members of the *RR*-group were phototrophic organisms. A more difficult question to answer is whether these ancestral species were photoautotrophic and contained RubisCO. There are two possible scenarios (fig. 6). The first [compatible with Keppen et al. (2013)] assumes that the ancestors of the *RR*-group were photoautotrophs similar to most of the modern *Rhodobacter* species. Then, during evolution, some *RR*-lineages lost a part or all of their photosynthesis genes producing photoheterotrophic, lithoheterotrophic, or chemoheterotrophic species. In the second scenario, the ancestors of the *RR*-group were photoheterotrophs. Here, some lineages (e.g., *Rhodobacter* species) gained RubisCO genes and became photoautotrophs, whereas some lineages lost all their photosynthesis genes and became heterotrophs (fig. 6). These two scenarios are difficult to reconcile based on the available data, but based on the performed phylogenetic analyses we prefer the photoautotrophic ancestor scenario. Indeed, the photoautotrophic *Rhodobacter* species (especially *Rba. veldkampii*) are located closer to the root of the *RR*-group in our 16S rRNA tree, whereas the photoheterotrophic species branch off later (fig. 1). The weakness of this argument is the very low statistical support of the 16S phylogenetic tree. However, a similar conclusion can also be made based on the ALAS phylogeny. The ALAS (*hemA/hemT* gene) tree shows a major split, documenting the existence of two different forms of the gene in *Rhodobacterales* (*hemA*-like and *hemT*-like form). These two forms probably originate from a gene duplication event. Since the *hemA* and *hemT* genes of *Rba. sphaeroides* lay close to the split, it seems that the gene duplication occurred in the ancestors of *Rhodobacter*-like species and all the subsequent photoheterotrophic ancestors retained the two forms of the gene, which gradually diverged. Photoheterotrophic species always contain both forms, which indicate that they only evolved after *Rba. sphaeroides*. Later, the AAP species incorporated the *hemT*-like form of ALAS into the PGC. The presence of ALAS in the PGC seems to be a common characteristic of all the AAP species belonging to *Alphaproteobacteria* (Zheng et al. 2011). This arrangement is probably advantageous for convenient regulation of BChl *a* synthesis in AAP species, which need to tightly control the initial step of the tetrapyrrol pathway with the final part of the



**Fig. 6.**—Proposed schemes of the evolution of phototrophy among the members of the *RR*-group: (A) Regressive evolution model compatible with Keppen et al. (2013). (B) Mixed model assuming a photoheterotrophic ancestor of all modern *RR*-species—heterotrophs, photoheterotrophs, and photoautotrophs.

BChl *a* pathway. Indeed, this has been documented in *Drb. shibae*, where the inducible ALAS gene located in the PGC (*hemT*-like form) was under strong light regulation together with all *bch* genes (Tomasch et al. 2011). Interestingly, all these ALAS genes present in the PGC cluster together form a distinct subclade of the entire *hemT*-like group (fig. 3). This indicates that the *hemA* split occurred first in photoautotrophic *Rhodobacter*-like species, which probably needed to differentially regulate the tetrapyrrol biosynthesis pathway for heme (*hemA* form) and BChl *a* synthesis (*hemT* form). Later, after the evolution of the AAP species, the *hemT* form of the gene was moved under the common regulation of the PGC cluster. Moreover, in some species (*Drb. shibae* and *Roseobacter* species), the *hemT* form of ALAS underwent a second duplication, indicating the need for even more delicate regulation of the gene.

In conclusion, the presented data indicate that the ancestors of the haloalkaliphilic members of the *RR*-group were phototrophic species. The heterotrophic species have evolved through the regressive loss of their photosynthetic apparatus. In addition, we have demonstrated that the important step enabling the evolution of photoheterotrophic species was the duplication of the *hemA* gene, and its later incorporation into the PGC of the AAP species.

Many interesting questions were left to be answered such as if there is an alternative pathway of descent from photoautotrophic species through the facultatively autotrophic aerobic or facultatively anaerobic lithotrophs, such as *Paracoccus* (loss of the PGC), to aerobic chemolithoheterotrophs (loss of RubisCO) and finally organoheterotrophs? What was the role of the hydrogenase in this alternative scenario? Did *Rba. capsulatus* evolve regressively from a *Rba. sphaeroides*-like

species through the loss of genes (*acsF*, *hemF*, *hemT*, and anti-sigma factor), or does it represent a former (more ancient) photoautotrophic species? We believe, that the constantly expanding genomic data, will allow us to address these questions in the near future.

## Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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