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Distribution and Origin of Oxygen-Dependent and Oxygen-Independent Forms of Mg-Protoporphyrin Monomethylester Cyclase among Phototrophic *Proteobacteria*

Ekaterina N. Boldareva-Nuianzina,[a] Zuzana Bláhová,[a,b] Roman Sobotka,[a,b] Michal Koblížek[a,b]

Institute of Microbiology CAS, Department of Phototrophic Microorganisms, Algatech, Opatovický mlýn, Třeboň, Czech Republic[a]; Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic[b]

Magnesium-protoporphyrin IX monomethylester cyclase is one of the key enzymes of the bacteriochlorophyll biosynthesis pathway. There exist two fundamentally different forms of this enzyme. The oxygen-dependent form, encoded by the gene *acsF*, catalyzes the formation of the bacteriochlorophyll fifth ring using oxygen, whereas the oxygen-independent form encoded by the gene *bchE* utilizes an oxygen atom extracted from water. The presence of *acsF* and *bchE* genes was surveyed in various phototrophic *Proteobacteria* using the available genomic data and newly designed degenerated primers. It was found that while the majority of purple nonsulfur bacteria contained both forms of the cyclase, the purple sulfur bacteria contained only the oxygen-independent form. All tested species of aerobic anoxygenic phototrophs contained *acsF* genes, but some of them also retained the *bchE* gene. In contrast to *bchE* phylogeny, the *acsF* phylogeny was in good agreement with 16S inferred phylogeny. Moreover, the survey of the genome data documented that the *acsF* gene occupies a conserved position inside the photosynthesis gene cluster, whereas the *bchE* location in the genome varied largely between the species. This suggests that the oxygen-dependent cyclase was recruited by purple phototrophic bacteria very early during their evolution. The primary sequence and immunochemical similarity with its cyanobacterial counterparts suggests that *acsF* may have been acquired by *Proteobacteria* via horizontal gene transfer from cyanobacteria. The acquisition of the gene allowed purple nonsulfur phototrophic bacteria to proliferate in the mildly oxygenated conditions of the Proterozoic era.

The anoxygenic phototrophic bacteria are among the oldest organisms on Earth. They evolved more than 3 billion years (3 Gyr) ago under the anoxic conditions of the Archean Ocean (1, 2). Hence, many species of anoxygenic phototrophs remained strictly anaerobic organisms inhabiting anoxic environments. More evolutionary advanced oxygenic cyanobacteria evolved somewhat later (approximately 2.7 Gyr ago), and their rapid spreading resulted in a gradual oxygenation of the Proterozoic Ocean (3, 4). The changing conditions forced many groups of anoxygenic phototrophs (such as purple sulfur bacteria [PSB]) to retreat to marginal anaerobic niches, whereas some adapted to new oxic conditions and became facultative aerobes (i.e., purple nonsulfur bacteria [PNB]). Later, some phototrophic groups embarked on a completely aerobic lifestyle; they lost the ability to fix inorganic carbon and use light energy only as a supplement of their predominantly heterotrophic metabolism (5, 6). These organisms, called aerobic anoxygenic phototrophs (AAPs), were found in marine environments (7–10), but recently their presence was also demonstrated in river estuaries (11) as well as freshwater and saline lakes (12, 13).

The main light-harvesting pigments in the anoxygenic phototrophs are porphyrin molecules called bacteriochlorophylls, whereas oxygenic organisms, cyanobacteria, algae, and plants utilize structurally very similar pigments called chlorophylls (14, 15). A universal structural feature of all these molecules is an isocyclic ring, the formation of which is an essential step of (bacterio)chlorophyll biosynthesis (16). This complex reaction is catalyzed by Mg-protoporphyrin IX monomethylester (oxidative) cyclase and results in the conversion of Mg-protoporphyrin IX monomethyl ester into divinyl protochlorophyllide (see Fig. 1). The anaerobic phototrophs possess an oxygen-independent form of this enzyme containing 4Fe-4S and cobalamin prosthetic groups. The enzyme catalyzes a complex multistep reaction in which the oxygen atom necessary for the reaction is extracted from water (14, 17). An essential component or perhaps the whole oxygen-independent cyclase enzyme is encoded by the *bchE* gene. A completely different form of this enzyme exists in cyanobacteria, algae, and plants as well as in many PNB and AAP species. Unlike the oxygen-independent form, this enzyme catalyzes the formation of the isocyclic ring using oxygen as a substrate (18). Oxygen-dependent cyclase is expected to be active as a multisubunit complex (19); however, the only known subunit was identified by mutational analysis of the photosynthetic bacterium *Rubrivivax gelatinosus* (20) and denoted as AcsF (abbreviated from aerobic cyclase). A subsequent genetic study revealed that *Rubrivivax* contained both forms of the cyclase, which allows the cells to synthesize bacteriochlorophyll under different oxygen concentrations (21). AcsF homologs have since been identified in many cyanobacteria and photosynthetic eukaryotes, including *Synechocystis* sp. strain PCC 6803 (22), *Chlamydomonas reinhardtii* (23), and *Arabidopsis thaliana* (24). More recently, the *acsF* gene was also identified in the green nonsulfur bacterium *Chloroflexus aurantiacus* (25).

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Address correspondence to Michal Koblížek, koblizek@alga.cz.
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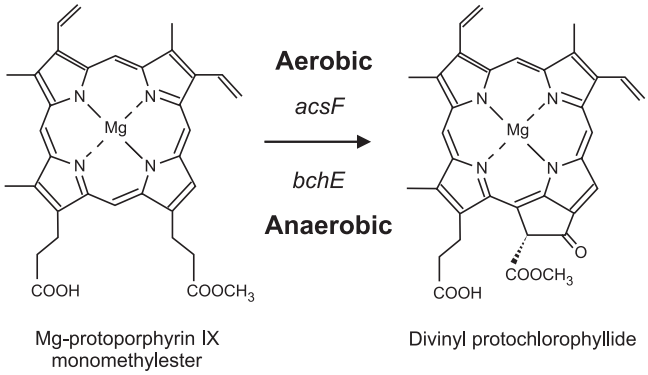

FIG 1 A simplified scheme of the conversion of Mg-protoporphyrin IX monomethylester into divinyl protochlorophyllide. The reaction is catalyzed by two fundamentally different forms of Mg-protoporphyrin monomethylester cyclase. The oxygen-dependent form of the enzyme encoded by the *acsF* gene operates under aerobic (oxic) conditions, whereas the oxygen-independent form encoded by the *bchE* gene operates under anaerobic (anoxic) conditions.

The acquisition of the oxygen-dependent form of the cyclase was proposed to be one of the key adaptations done by anoxygenic phototrophs during the evolutionary transition from anaerobic to aerobic conditions (26). In their recent review, Yurkov and Csotonyi (5) speculated that the presence of the oxygen-dependent cyclase and the absence of the oxygen-independent cyclase could serve as a convenient marker of predominantly aerobic phototrophic species. To test these hypotheses, we surveyed the presence and organization of *acsF* and *bchE* genes among various anoxygenic phototrophic *Proteobacteria*. The obtained molecular information was used to infer the evolutionary origin of the two forms of the cyclase and their current role in anoxygenic phototrophic *Proteobacteria*.

MATERIALS AND METHODS

Strains. Anoxygenic phototrophs belonging to alpha, beta, and gamma classes of *Proteobacteria* were used for the study. *Rhodobaca bogoriensis* (strain LBB1), *Rhodobaca barguzinensis* (strain alga-05), *Roseinatronobacter* strains Dor-vul, Dor-3.5, Da, and Khil-ros, and *Rhodobacterales* strains Zun-kh and Chep-kr have been isolated from soda lakes as described earlier (27, 28). Marine isolates SO3, SYOP2, BS110, COL2P, B09, and B11, belonging to the *Roseobacter* clade, *Roseovarius* sp. strain SL25, *Erythrobacter* sp. strain NAP1, and *Citromicrobium* sp. strain CV44, were described previously (29–32). Freshwater betaproteobacterium strain VA01 was isolated from Velka Amerika lake, Czech Republic, by the dilution-to-extinction technique (M. Mašín, unpublished data). *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* were cultured photoheterotrophically in organic medium as described previously (32). Cultures of *Roseibaca ekhonensis* (strain EL50), *Roseisalinus antarcticus* (strain EL88), and *Staleya guttiformis* (strain EL 38) were kindly provided by Matthias Labrenz. *Sandarakinorhabdus limnophila* (strain so42) was provided by Frederic Gich. *Congregibacter litoralis* strain KT71 (DSM 17192), *Roseateles depolymerans* (DSM 11813), *Roseobacter litoralis* (DSM 6996), and *Rhodovulum sulfidophilum* (DSM 1374) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. *Synechocystis* sp. strain PCC6803 was grown photoautotrophically in the BG11 mineral medium. The microbial cultures were grown at room temperature under a light/dark cycle with an irradiance of 150 μmol of photons m^{-2} s^{-1} in rotating Erlenmeyer flasks. If not stated otherwise, we used media recommended by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DMSZ).

PCR amplification. Bacterial DNA was extracted using the RTP bacteria DNA minikit (Invitek GmbH, Germany). Partial *acsF* gene sequences were PCR amplified using forward primer acsF-F and reverse primer acsF-R1, providing a 350-bp gene fragment (see Table 1). The program consisted of 35 cycles with 1 min of denaturation at 94°C, 45 s of annealing at 45°C, and 1 min of extension at 72°C. The final extension was conducted for 10 min at 72°C. The *bchE* gene was amplified with the forward primer bchE-f (see Results) and reverse primer bchE-r2. The PCR program was 30 cycles with 1 min of denaturation at 94°C, 45 s of annealing at 52°C, and 1 min of extension at 72°C. The size of the obtained product was 640 bp. In some cases, *bchE* gene presence was amplified using a bchE-f and bchE-r4 primer pair (*Roseococcus* sp. strain Da). The amplification conditions remained the same, while annealing was conducted at 48°C. The size of the amplified fragments was 405 bp. The obtained PCR products were purified using a GenElute PCR clean-up kit (Sigma-Aldrich Co. LLC) and directly sequenced using the appropriate forward primers. The sequencing results were analyzed and manually corrected using BioEdit (Sequence Alignment Editor) version 5.0.9, Chromas version 1.5.

GenBank accession numbers. GenBank accession numbers of partial *acsF* sequences are as follows: *Roseobacter* sp. strain Zun-kholvo, JF917109; betaproteobacterium strain VA01, JF917110; *Roseobacter* sp. strain SY0P2, JF917111; *Roseobacter* sp. strain SO3, JF917112; *Roseovarius* sp. strain SL25, JF917113; *Sandarakinorhabdus limnophila* so42, JF917114; *Rhizobiales* sp. strain RM11-8-1, JF917115; *Roseinatronobacter* sp. strain Khil, JF917116; *Staleya guttiformis* EL38, JF917117; *Roseinatronobacter* sp. strain Dor-vul, JF917118; *Roseinatronobacter* sp. strain Dor 3.5, JF917119; *Citromicrobium* sp. strain CV44, JF917120; *Roseobacter* sp. strain COL2P, JF917121; *Rhodobacterales* strain Chep-kr, JF917122; *Roseobacter* sp. strain B11, JF917123; *Roseobacter* sp. strain B09, JF917124; *Roseococcus* sp. strain Da, JF917126; and *Rhodobaca barguzinensis* alga05, JF917125.

Partial *bchE* sequences are as follows: *Roseobacter* sp. strain B09, JF917127; *Roseobacter* sp. strain B11, JF917128; *Citromicrobium* sp. strain CV44, JF917129; *Roseococcus* sp. strain Da, JF917126; *Rhodobacterales* strain Chep-kr, JF917131; *Roseinatronobacter* sp. strain Dor 3.5, JF917132; *Rhizobiales* strain RM11-8-1, JN018414; *Roseinatronobacter* sp. strain Khil, JN018416; *Roseinatronobacter* sp. strain Dor-vul, JN018415; *Roseobacter* sp. strain Zun_kholvo, JN018417; *Roseinatronobacter monicus* ROS35, JN018418; and *Rhodobaca bogoriensis* LBB1, JN018419.

Phylogenetic analyses. The obtained nucleotide sequences were translated into amino acids and aligned using the ClustalX2 program version 2.0.10. The alignments were manually checked; ambiguously

TABLE 1 Primers designed for PCR amplification of *bchE* and *acsF* genes

Primer	Direction	Target positions in the corresponding gene	Primer sequence
acsF-F	Forward	382–404	5′-ARTTYTCNGGCTGYGTNCTBTA-3′
acsF-R1	Reverse	731–754	5′-TGSSDRAAYTCRTCRTTGCACCA-3′
bchE-f	Forward	640–662	5′-AATGGAAATTCTGGCGCGACTA-3′
bchE-r2	Reverse	1320–1340	5′-GGRTARTGRAANAGCGCCTT-3′
bchE-r4	Reverse	1045–1063	5′-ACGATGAACTGNGCYTCG-3′

TABLE 2 Presence of the *acsF* and the *bchE* genes among full-genome-sequenced phototrophic *Proteobacteria*

Organism	GenBank no.	Group	Presence/absence[a] of:	
			acsF	*bchE*
Acidiphilium multivorum AIU301	AP012035	α1, AAP	●	○
Phaeospirillum molischianum DSM 120	ZP_09875400	α1, PNB	–	○
Rhodospirillum centenum SW[b]	CP000613	α1, PNB	●	○
Rhodospirillum rubrum ATCC 11170	CP000230	α1, PNB	–	○
Rhodospirillum photometricum DSM 122	YP_005416037	α1, PNB	–	○
Ahrensia sp. strain R2A130	NZ_AEEB01000017	α2, AAP	●	–
Agrobacterium albertimagni AOL15	ALJF00000000	α2, AAP	●	○
Hoeflea phototrophica DFL43	NZ_ABIA02000022	α2, AAP	●	–
Labrenzia alexandrii DFL11	NZ_EQ973123.1	α2, AAP	●	○
Methylobacterium sp. strain 4-46	CP000943	α2, AAP	●	○
Methylobacterium radiotolerans	CP001001	α2, AAP	●	–
Methylobacterium populi BJ001	YP_001927978	α2, AAP	●	–
Methylobacterium extorquens AM1	YP_002966142	α2, AAP	●	–
Methylocella silvestris BL2	CP001280	α2, AAP	●	–
Bradyrhizobium sp. strain BTAi1	CP000494	α2, PNB	●	●
Bradyrhizobium sp. strain ORS278	CU234118	α2, PNB	●	●
Rhodomicrobium vannielii ATCC 17100	NC_014664	α2, PNB	–	○
Rhodopseudomonas palustris	Multiple[d]	α2, PNB	●	○
Dinoroseobacter shibae DFL12	CP000830	α3, AAP	●	○
Jannaschia sp. strain CCS1	CP000264	α3, AAP	●	–
Loktanella vestfoldensis SKA53	AAMS01000000	α3, AAP	●	–
Roseobacter denitrificans Och 114	CP000362	α3, AAP	●	○
Roseobacter litoralis Och 149[c]	ABIG00000000	α3, AAP	●	○
Roseobacter sp. strain AzwK-3b	ABCR00000000	α3, AAP	●	●
Roseobacter sp. strain CCS2	AAYB00000000	α3, AAP	●	–
Roseovarius sp. strain TM1035	ABCL00000000	α3, AAP	●	○
Roseovarius sp. strain 217	AAMV00000000	α3, AAP	●	○
Rhodobacter capsulatus SB 1003	NC_014034	α3, PNB	–	●
Rhodobacter sphaeroides	Multiple[e]	α3, PNB	●	●
Rhodobacter sp. strain SW2	ZP_05842911	α3, PNB	●	●
Erythrobacter sp. strain NAP1	AAMW00000000	α4, AAP	●	–
Citromicrobium bathyomarinum JL354	ZP_06861151	α4, AAP	●	○
Sphingomonas spp.	Multiple[f]	α4, AAP	●	–
Brevundimonas subvibrioides ATCC 15264	CP002102	α4, AAP	●	–
Rubrivivax gelatinosus IL-114	NC_017075	β, PNB	●	○
Rubrivivax benzoatilyticus JA2	NZ_AEWG01000000	β, PNB	●	○
Methyloversatilis universalis FAM5	ZP_08506871	β, PNB	●	–
Limnohabitans sp. strain Rim28	ALKN00000000	β, PNB	●	–
Limnohabitans sp. strain Rim47	ALKO00000000	β, PNB	●	–
Allochromatium vinosum DSM 180	NC_013851	γ, PSB	–	○
Ectothiorhodospira sp. strain PHS-1	NZ_AGBG01000002	γ, PSB	–	○
Halorhodospira halophila SL1	CP000544	γ, PSB	–	●
Marichromatium purpuratum 984	NZ_AFWU01000001	γ, PSB	–	○
Thiocapsa marina 5811	NZ_AFWV01000003	γ, PSB	–	○●
Thiocystis violascens DSM198	AGFC00000000	γ, PSB	–	○
Thioflavicoccus mobilis 8321	NC_019940	γ, PSB	–	○
Thiorhodococcus drewsii AZ1	NZ_AFWT01000007	γ, PSB	–	○
Thiorhodospira sibirica ATCC 700588	AGFD01000016	γ, PSB		○
Congregibacter litoralis KT71	AAOA01000014	γ, AAP	●	●
Gammaproteobacterium strain NOR5-3	ZP_05125815	γ, AAP	●	●
Gammaproteobacterium strain NOR51-B	NZ_DS999411	γ, AAP	●	
Gammaproteobacterium strain HTCC2080	NZ_DS999405	γ, AAP	●	
Gammaproteobacterium strain HIMB55	ZP_09691978	γ, AAP	●	

[a] The genome data were obtained from GenBank. ●, gene present in the PGC; ○, gene present outside PGC; –, gene absent.
[b] Alternative name, *Rhodocista centenaria*.
[c] In *Roseobacter litoralis*, PGC is located on a plasmid whereas the *bchE* gene is located on the chromosome.
[d] Represents the following strains of *Rhodopseudomonas palustris*: TIE-1 (CP001096), BisA53 (CP000463), BisB18 (CP000301), BisB5 (CP000283), CGA009 (BX571963), HaA2 (NC_007778), and DX-1 (NC_014834).
[e] Represents the following strains of *R. sphaeroides*: 2.4.1. (CP000143), KD131 (NC_011963), WS8N (AFER01000001), ATCC 17029 (NC_009049), and ATCC 17025 (NC_009428).
[f] Represents the following *Sphingomonas* isolates: *S. echinoides* ATCC 14820 (AHIR00000000) and *Sphingomonas* sp. strains PAMC 26605 (AHIS00000000), PAMC 26617 (AHHA00000000), and PAMC 26621 (AIDW00000000).

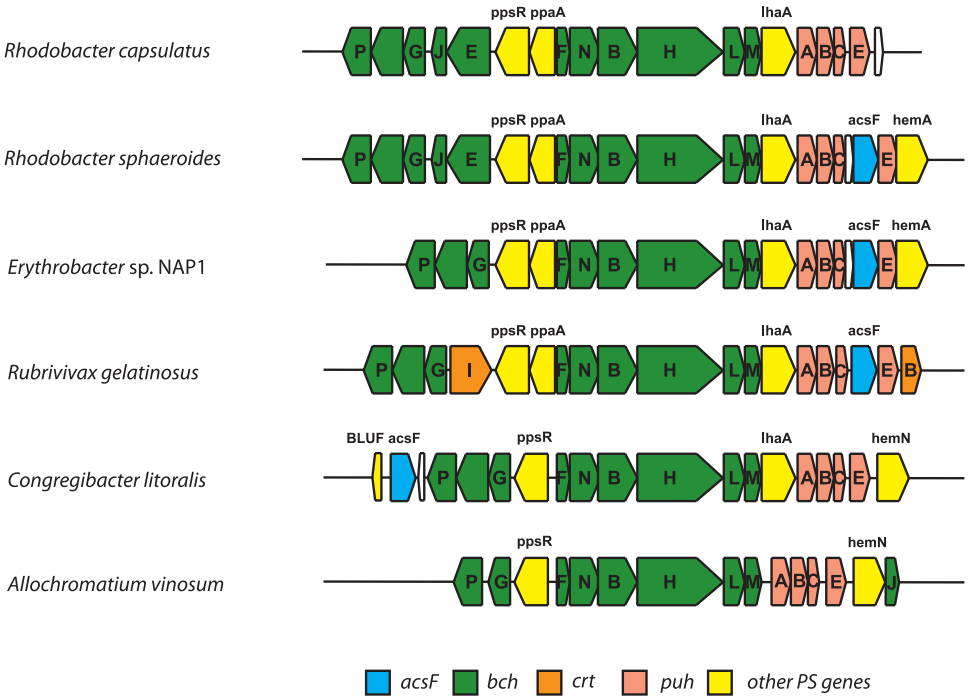

FIG 2 Positions of *acsF* genes in photosynthetic gene clusters (partial) in various phototrophic species. *Rhodobacter capsulatus* and *Allochromatium vinosum* are shown for comparison as an example of organisms devoid of the *acsF* gene.

aligned regions and gaps were excluded from further analyses. The redundant sequences representing different strains of the same species (i.e., *Rhodopseudomonas palustris*, *Rhodobacter sphaeroides*) were removed. The sequence identity matrix was calculated from the corrected alignment using the ClustalX2 program. The phylogenetic trees were computed from partial amino acid sequences using a neighbor-joining algorithm with 1,000 bootstraps. In addition, the inferred phylogeny was confirmed by the maximum likelihood algorithm (PhyML software) using the LG model chosen by the ProtTest 2.4 algorithm.

Isolation of cell membranes and immunodetection of AcsF homologs. The bacterial cells were pelleted by centrifugation and resuspended in a breakage buffer containing 25 mM MES buffer (pH 6.5), 10 mM $MgCl_2$, 10 mM $CaCl_2$, and 25% glycerol. The cells were disrupted in a beadbeater (BioSpec Products Inc.) using zirconia/silica beads 0.1 mm in diameter. The disruption was performed in four 1-min cycles interrupted by 5 min of cooling on ice to prevent potential protein degradation. Cell membranes were pelleted by centrifugation at 40,000 × *g* at 4°C for 20 min, washed in the breakage buffer, and finally resuspended in 100 μl of the same buffer.

The obtained membranes were solubilized by 2% dodecyl-β-maltoside, spun down to remove cell debris, and separated by 10% SDS polyacrylamide gel electrophoresis. The proteins were then transferred onto the Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences, Sweden), which was incubated with polyclonal antibody raised against the AcsF protein (CHL27) from *Arabidopsis thaliana* (Agrisera AB, Sweden) to detect the presence of AcsF homologs in the photosynthetic bacteria studied.

RESULTS

Genome survey. In the first stage of this work, we computationally surveyed the genome sequences of 53 phototrophic *Proteobacteria* available in GenBank. Twenty-one species contained both the *acsF* and *bchE* genes (see Table 2). All PSB species and five PNB species (*Rhodospirillum rubrum*, *Rhodospirillum photometricum*, *Phaeospirillum molischianum*, *Rhodomicrobium vannielii*, and *Rhodobacter capsulatus*) contained only the *bchE* gene, which was consistent with their anaerobic character. On the other hand, 18 predominantly aerobic species contained only the *acsF* gene (see Table 2). In our survey, we noticed that, if present, the *acsF* gene was always located inside the so-called photosynthesis gene cluster (PGC) together with other photosynthesis genes (see also reference 33). In all studied *Alpha-* and *Betaproteobacteria*, the *acsF* gene was placed inside the *puh* operon (between the *puhABC* and *puhE* genes), whereas in aerobic members of the NOR5 cluster (*Gammaproteobacteria*) it was located independently at the periphery of the PGC (see Fig. 2). In contrast, *bchE* was found inside (i.e., *Rhodobacter sphaeroides*), at the border of (i.e., *Congregibacter litoralis*), or outside (i.e., *Dinoroseobacter shibae*, *Roseobacter denitrificans*) the PGC. The presence of a *bchE* gene either inside or outside PGC is shown in Table 2. Frequently, the *bchE* gene was located together with the *bchJ* gene (i.e., *Acidiphilium multivorum*, *Rhodopseudomonas palustris*, *Rhodobacter capsulatus*); however, in some cases, *bchE* and *bchJ* were situated separately (i.e., *Roseobacter litoralis*, gammaproteobacterium strain HTCC2080).

Design of universal PCR primers. Apart from analyzing the GenBank data, we designed universal primers for both aerobic and anaerobic forms of oxidative cyclase to survey the presence of these genes in other species of phototrophic *Proteobacteria* (see Fig. S1 and S2 in the supplemental material). The new primers were used to survey for the presence of *acsF* and *bchE* genes in 22 strains of AAP bacteria belonging to various subgroups of *Alpha-* and *Betaproteobacteria*. In addition, we included three strains of PNB belonging to *Rhodobacterales* which exhibit both aerobic and anaerobic growth (Table 3). The *acsF* gene fragments were successfully amplified in all tested strains (Fig. 3A). However, while

TABLE 3 Presence of *acsF* and *bchE* genes in the tested strains of phototrophic *Proteobacteria*

Strain	Group	Environment	Presence/absence[a] of:	
			acsF	*bchE*
Roseococcus strain Da	α1, AAP	Mud volcano	+	+
Rhizobiales strain RM11-8-1	α2, AAP	Marine	+	+
Roseobacter strain SO3	α3, AAP	Marine	+	−
Roseobacter strain SYOP2	α3, AAP	Marine	+	−
Roseobacter strain BS110	α3, AAP	Marine	+	−
Roseobacter strain COL2P	α3, AAP	Marine	+	−
Roseobacter strain B09	α3, AAP	Marine	+	+
Roseobacter strain B11	α3, AAP	Marine	+	+
Roseisalinus antarcticus EL-88	α3, AAP	Hypersaline lake	+	−
Staleya guttiformis EL-38	α3, AAP	Hypersaline lake	+	−
Roseibaca ekhonensis EL-50	α3, AAP	Hypersaline lake	+	−
Roseovarius strain SL25	α3, AAP	Hypersaline lake	+	−
Roseinatronobacter monicus ROS35	α3, AAP	Soda lake	+	+
Roseinatronobacter strain Dor-vul	α3, AAP	Mud volcano	+	+
Roseinatronobacter strain Dor-3.5	α3, AAP	Soda lake	+	+
Roseinatronobacter strain Khil-ros	α3, AAP	Soda lake	+	+
Roseobacter strain Zun-kh	α3, AAP	Soda lake	+	+
Rhodobacterales strain Chep-kr	α3, AAP	Soda lake	+	+
Rhodobaca barguzinensis alga-05	α3, PNB	Soda lake	+	+
Rhodobaca bogoriensis LBB1	α3, PNB	Soda lake	+	+
Rhodovulum sulfidophilum DSM 1374	α3, PNB	Marine sediments	+	+
Citromicrobium strain CV44	α4, AAP	Marine	+	+
Sandarakinorhabdus limnophila so42	α4, AAP	Fresh water	+	−
Betaproteobacterium strain VA01	β, AAP	Fresh water	+	−
Roseateles depolymerans DSM11813	β, AAP	Fresh water	+	−

[a] +, gene present; −, gene absent or not amplified.

the *bchE* gene was detected in all PNB species, it was detected only in 10 AAP strains (Fig. 3B, Table 3). All the obtained PCR products were purified and sequenced.

Phylogeny of *acsF* and *bchE* genes. To analyze the phylogenetic relationship and potential evolutionary origin of genes coding for oxygen-dependent cyclase, we constructed phylogenetic trees using sequences obtained from both GenBank and *de novo* sequencing. We have also included the *acsF* sequences from various oxygenic organisms encompassing unicellular cyanobacteria, algae, and higher plants (here, *acsF* was encoded on chloroplast chromosomes). The phylogenetic tree was calculated from partial amino acid sequences using the neighbor-joining algorithm (the analysis was also performed using the maximum likelihood algorithm, but the maximum likelihood tree suffered from overall low bootstrap values). The constructed tree displayed relatively well-separated individual subgroups (α-1, α-2, α-3, α-4) of *Alphaproteobacteria*, as well as *Beta-* and *Gammaproteobacteria*, which signalized a good agreement with 16S rRNA-derived phylogeny (see Fig. S3 in the supplemental material). The proteobacterial sequences were clearly separated from those of various oxygenic organisms (cyanobacteria, algae, and plants) and green nonsulfur bacteria *Chloroflexus* and *Roseiflexus* (Fig. 4). The amino acid sequences of the selected oxygenic organisms had a 42% ± 3% (mean ± standard deviation) identity to the proteobacterial sequences, while the average sequence identity among tested *Proteobacteria* was 61% ± 7% (calculated for 34 organisms).

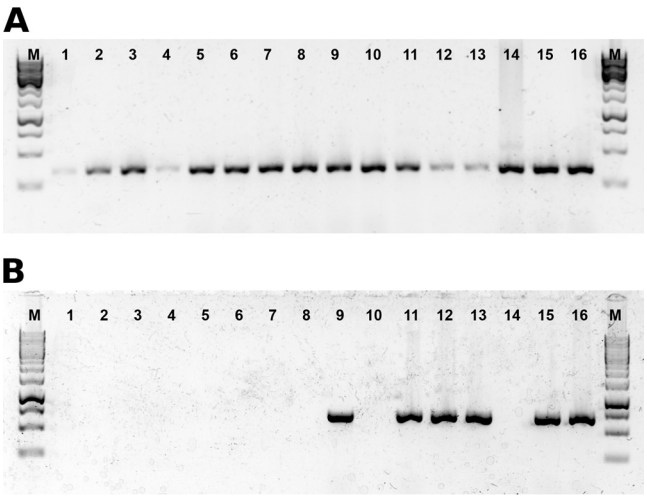

FIG 3 Electrophoretic analysis of PCR products for the *acsF* gene (A) and the *bchE* gene (B). Description of the lines: M, marker; 1, *Roseococcus* sp. Da; 2, *Hoeflea phototrophica*; 3, *Labrenzia alexandrii*; 4, *Rhodobacterales* strain SO3; 5, *Rhodobacterales* strain SYOP2; 6, *Roseobacter* sp. COL2P; 7, *Roseibaca ekhonensis*; 8, *Staleya guttiformis*; 9, *Roseinatronobacter* sp. Khil-ros; 10, *Sandarakinorhabdus limnophila* so42; 11, *Roseobacter* sp. Chep-kr; 12, *Rhodobacterales* sp. Dor-vul; 13, *Citromicrobium* sp. CV44; 14, Betaproteobacterium VA01; 15, *Rhodobaca barguzinensis*; 16, *Rhodobacter sphaeroides* (used as the positive control).

A different situation was encountered for the phylogeny of the oxygen-independent cyclase. Here, the constructed phylogenetic tree (Fig. 5) differed significantly from the 16S rRNA phylogeny (see Fig. S3 in the supplemental material). The *bchE* tree clearly split (bootstrap = 1,000/1,000) into two main clades, suggesting that two forms of *bchE* genes exist (Fig. 5). The first clade (cluster I) is formed by the majority of *Proteobacteria*, whereas the second clade (cluster II) was formed by other phototrophic phyla, such as

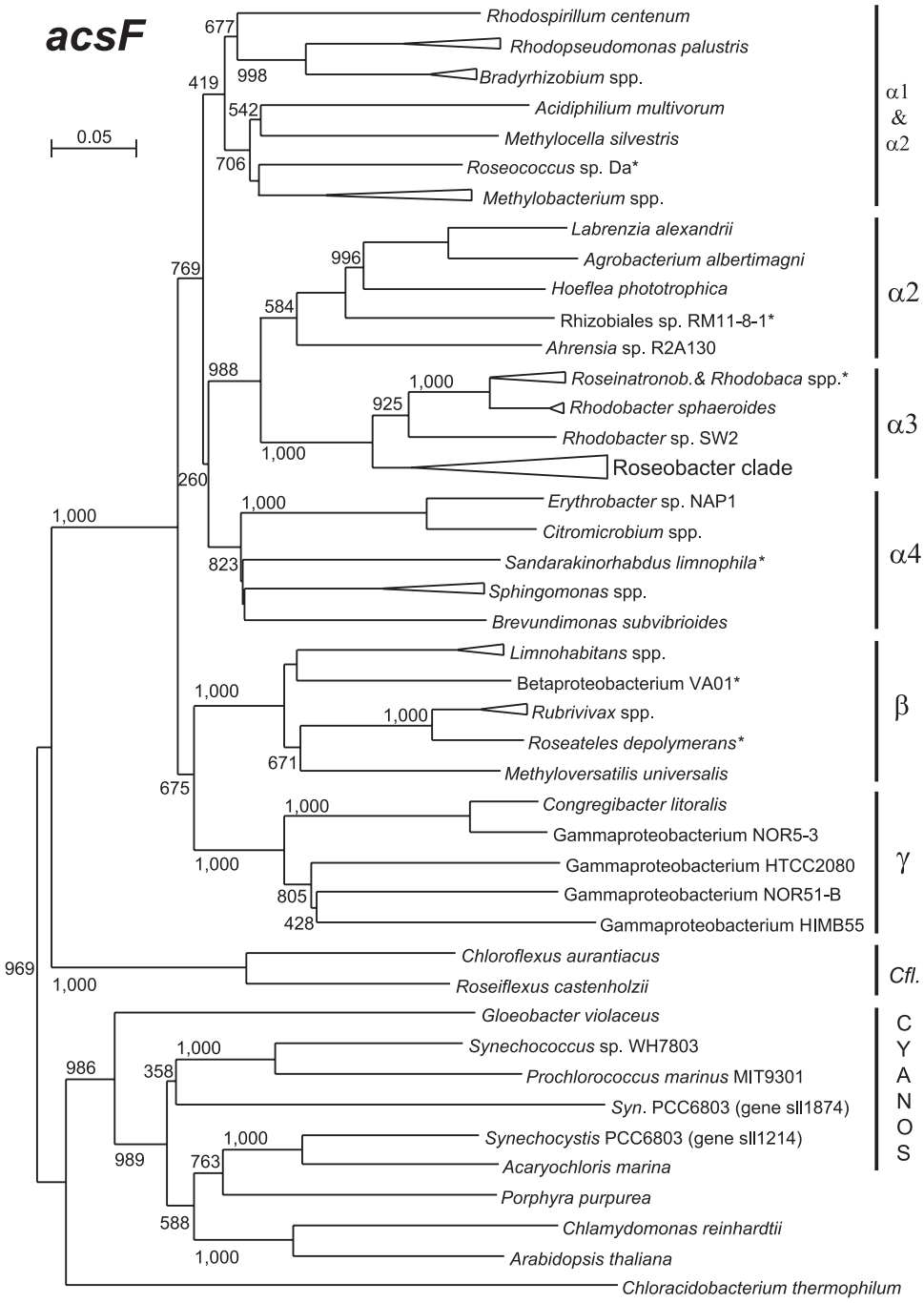

FIG 4 Phylogenetic tree as inferred from partial *acsF* amino acid sequences (312 amino acids). The tree was computed using a neighbor-joining algorithm. Species with shorter sequences obtained by PCR (marked with an asterisk) were added later to the tree. Numbers above protein trees indicate bootstrap values (1,000 replicates). *Roseinatronob.*, *Roseinatronobacter*; *Syn.*, *Synechocystis* sp.

Chlorobi, *Chloroflexi*, and *Chloracidobacterium tepidum*. Cluster I could be further divided into subcluster Ia, which contained mostly *Alphaproteobacteria*, and mixed subcluster Ib. The complicated phylogeny of the *bchE* gene can be best documented on the example of *Gammaproteobacteria*. While *Halorhodospira halophila* (*Ectothiorhodospiraceae*) and *Congregibacter litoralis* (NOR5 clade) clustered by *Alphaproteobacteria* forming cluster Ia, some PSB from the *Chromatiaceae* family (*Allochromatium vinosum* and *Marichromatium purpuratum*) belonged to the mixed cluster Ib. In addition, two other PSB species, *Thiocystis violascens* (*Chromatiaceae*) and *Thiorhodospira sibirica* (*Ectothiorhodospiraceae*), belonged to cluster II. Moreover, *Thiocapsa marina* contained two forms of the *bchE* gene, one belonging to cluster Ia and the other to cluster II (Fig. 5). Even within *Alphaproteobacteria*, the situation

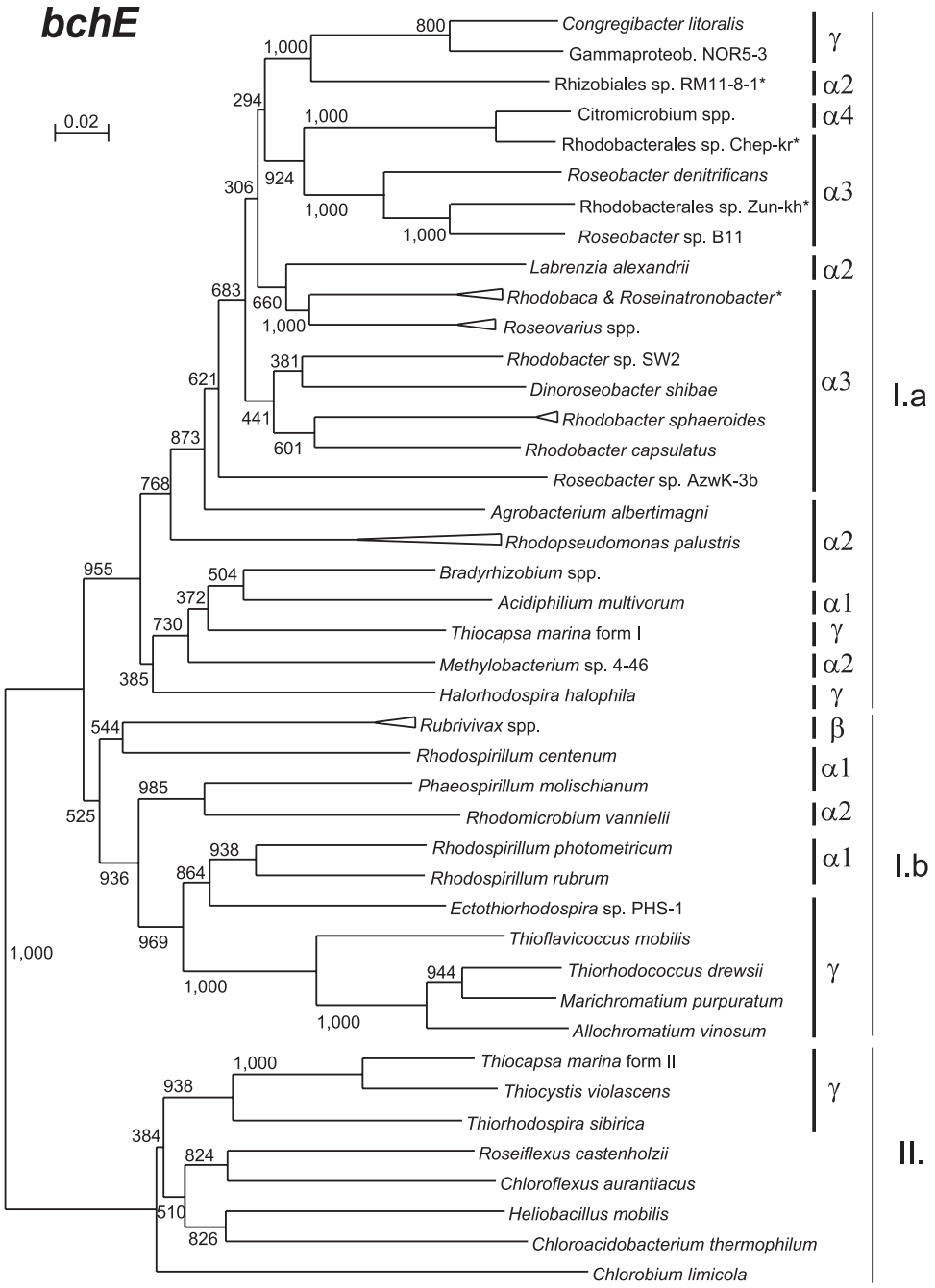

FIG 5 Phylogenetic tree as inferred from partial *bchE* amino acid sequences (202 amino acids). The tree was computed using the neighbor-joining algorithm. Numbers above protein trees indicate bootstrap values (1,000 replicates). Sequences obtained by the PCR are marked by an asterisk. Gammaproteob., Gammaproteobacteria.

was complex. The *bchE* sequences from α-1 and α-2 species were largely scattered across both clusters Ia and Ib. Such topology suggests relatively common horizontal gene transfers of this gene, perhaps driven by a strong environmental selection.

Immunodetection of AcsF homologs in photosynthetic bacteria. To confirm the presence of the AcsF protein and also to test its structural similarity in different groups of photosynthetic bacteria, we detected this protein using an anti-AcsF antibody raised against the AcsF homolog from *Arabidopsis thaliana* (24). As the AcsF protein is known to be tightly associated with membranes (24, 34), we first isolated membrane fractions from *Roseobacter litoralis* (*Alphaproteobacteria*), *Rubrivivax gelatinosus* (*Betaproteobacteria*), *Congregibacter litoralis* (*Gammaproteobacteria*), and *Chloroflexus aurantiacus* (green nonsulfur bacterium). The membrane proteins were then separated by SDS polyacrylamide gel electrophoresis (Fig. 6A), blotted, and probed with the anti-AcsF antibody (Fig. 6B). Membrane proteins from the cyanobacterium *Synechocystis* sp. strain PCC 6803, *Arabidopsis thaliana*, and *Rho-*

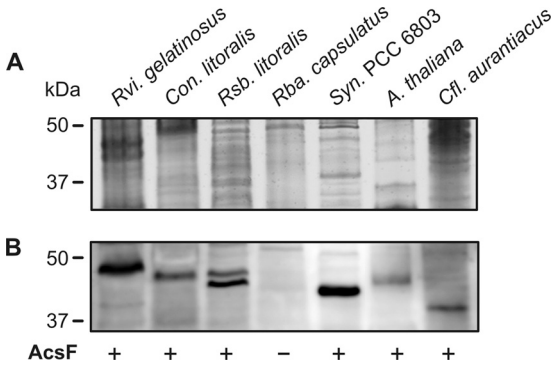

FIG 6 Isolation and separation of membrane proteins from photosynthetic organisms and immunodetection of AcsF protein. (A) Approximately 10 μg of solubilized membrane proteins was separated by SDS electrophoresis and stained with Coomassie blue. (B) The separated proteins were transferred onto a polyvinylidene difluoride membrane and probed by polyclonal anti-AcsF antibody generated using the AcsF homolog of *Arabidopsis thaliana* (see Materials and Methods). Membrane fractions of *Arabidopsis thaliana* and the cyanobacterium *Synechocystis* PCC 6803 were included as positive controls, and anaerobic bacterium *Rhodobacter capsulatus* was included as a negative control. *Rvi. gelatinosus*, *Rubrivivax gelatinosus*; *Rsb. litoralis*, *Roseobacter litoralis*; *Rba. capsulatus*, *Rhodobacter capsulatus*; *Cfl. aurantiacus*, *Chloroflexus aurantiacus*. *Con. Litoralis*, *Congregibacter litoralis*; *Syn.* PCC 6803, *Synechocystis* sp. PCC 6803; *A. thaliana*, *Arabidopsis thaliana*.

dobacter capsulatus were used as positive and negative controls, respectively. The antibody reacted with all four samples, producing bands of the expected sizes (39 to 42 kDa). It is noteworthy that AcsFs from *Roseobacter litoralis* appears to form a double band, indicating a specific protein modification or fast degradation not observed in cyanobacteria. Nonetheless, given that the used antibody was raised against the plant AcsF homolog, our results suggest that the oxygen-dependent cyclase is a conserved enzyme despite being employed by very evolutionarily distant organisms such as anoxygenic phototrophic bacteria and oxygenic cyanobacteria or plants.

DISCUSSION

The performed survey revealed that different functional groups of phototrophic *Proteobacteria* employ different forms of the cyclase. All investigated PSB contained the *bchE* gene only, which is consistent with their strictly anaerobic lifestyle. The majority of the tested PNB species contained both genes. As could be expected from their aerobic character, all AAP species contained the *acsF* gene; however, in contrast to Yurkov and Csotonyi's original assumption (5), about half of the AAP strains also contained the *bchE* gene.

The origin of the oxygen-independent form of the cyclase among phototrophic proteobacteria is not completely clear. Presumably, it represents an ancient form of the cyclase, which is consistent with its presence in all main phyla containing anoxygenic phototrophs. On the other hand, the *bchE* phylogenetic tree indicates a very complex evolutionary history (see Fig. 4). Moreover, in contrast to the conserved position of *acsF* inside the PGC, *bchE* occurs in different places in the genome, both inside or outside the PGC. All this suggests that the *bchE* gene was probably transferred several times during the evolution. This is especially evident among *Gammaproteobacteria*, where individual species possess completely unrelated forms of the cyclase. As the phylogeny partially displays ecological subclusters such as halophilic species (marine and soda and salt lake strains), freshwater, or strictly anaerobic purple sulfur bacteria, this may signalize a strong environmental selection pressure on this gene.

The function of the *bchE* gene in AAP species is not clear. One explanation is that AAPs may use this gene to supplement the activity of oxygen-dependent cyclase in situations when they are temporarily facing anaerobic conditions. On the other hand, a *bchE* gene homologue was also found in the heterotrophic organism *Acidiphilium cryptum* strain JF-5 (GenBank accession no. CP000697). Also, three *bchE* gene homologues were identified in the cyanobacterium *Synechocystis* sp. PCC 6803, which, however, did not seem to have any cyclase activity (22). All this indicates that the *bchE* genes in AAP species may encode proteins with another enzymatic function.

A very different situation was encountered in the case of the *acsF* gene. Unlike the *bchE* gene, the *acsF* gene phylogeny corresponds relatively well with 16S rRNA-based phylogeny (Fig. 4; see also Fig. S3 in the supplemental material). Moreover, in all investigated *Alpha-* and *Betaproteobacteria*, the *acsF* genes occupy the same conserved position inside the PGC (Fig. 2; see also reference 33). All this evidence suggests that the adoption of the oxygen-dependent cyclase by proteobacteria occurred in a single event. The origin of the *acsF* gene cannot be clearly reconciled based on available genetic data. However, based on both the similarity between proteobacterial and cyanobacterial *acsF* sequences (42% ± 3%) and the immunoreactivity of bacterial AcsF proteins with plant anti-AcsF antibody (Fig. 6), we speculate that the *acsF* gene may have been transferred between PNB and cyanobacteria via horizontal gene transfer. The direction of the transfer cannot be unambiguously determined using the current data. The fact that oxygenic cyanobacteria were naturally exposed to oxygen, whereas anoxygenic species were originally anaerobes, however, suggests that the gene first evolved in cyanobacteria and only later was it transferred to *Proteobacteria*.

The timing of the *acsF* adoption by *Proteobacteria* can be estimated using the available genomic data. The fact that *acsF* genes were found in all major proteobacterial lineages (*Alpha-*, *Beta-*, and *Gammaproteobacteria*) indicates that its acquisition probably occurred only shortly after the radiation of *Proteobacteria*. Using the molecular clock calculation, Battistuzzi et al. (35) estimated that the *Proteobacteria* evolved together with the onset of oxygenic photosynthesis approximately 2.6 to 2.3 Gyr ago. This suggests that the *acsF* gene may have been acquired during the early Proterozoic (Paleoproterozoic) period. Such a scenario is consistent with the current biogeochemical models. During the Archean period, the Earth's atmosphere was largely anoxic. At the beginning of the Proterozioc era, oxygen produced by cyanobacteria started to gradually oxygenate the Earth's atmosphere. Based on the geological record, it was estimated that the so-called Great Oxidation event occurred approximately 2.45 to 2.0 Gyr ago (4). After that, the concentration of oxygen stayed relatively constant (0.02 to 0.04 atm) through most of the Proterozoic era (till approximately 0.85 Gyr ago). During this period, the ocean was probably mildly oxygenated at its surface, whereas the deep waters remained anoxic, providing an environment supporting both anoxygenic and oxygenic phototrophs (36), facilitating the horizontal gene transfer event. Since the enzymatic activity of the oxygen-independent cyclase is strongly inhibited by free oxygen (21, 37), the adoption of the oxygen-dependent cyclase was a necessary step, allowing the early purple phototrophic bacteria to adapt to new conditions (26). Therefore, we suggest that the acquisition of the oxygen-

dependent cyclase by *Proteobacteria* was an important innovation which allowed the evolutionary transition from anaerobic to aerobic conditions and facilitated the radiation of purple nonsulfur species during the Proterozoic era. We speculate that the fully aerobic AAP species probably evolved only later, at the end of the Proterozoic era, when oxygen concentrations reached approximate present levels.

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