Three classes of oxygen-dependent cyclase involved in chlorophyll and bacteriochlorophyll biosynthesis

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The biosynthesis of (bacterio)chlorophyll pigments is among the most productive biological pathways on Earth. Photosynthesis relies on these modified tetrapyrroles for the capture of solar radiation and its conversion to chemical energy. (Bacterio)chlorophylls have an isocyclic fifth ring, the formation of which has remained enigmatic for more than 60 y. This reaction is catalyzed by two unrelated cyclase enzymes using different chemistries. The majority of anoxygenic phototrophic bacteria use BchE, an O_2-sensitive [4Fe-4S] cluster protein, whereas plants, cyanobacteria, and some phototrophic bacteria possess an O_2-dependent enzyme, the major catalytic component of which is a diiron protein, AcsF. Plant and cyanobacterial mutants in *ycf54* display impaired function of the O_2-dependent enzyme, accumulating the reaction substrate. Swapping cyclases between cyanobacteria and purple phototrophic bacteria reveals three classes of the O_2-dependent enzyme. AcsF from the purple betaproteobacterium *Rubrivivax* (*Rvi.*) *gelatinosus* rescues the loss not only of its cyanobacterial ortholog, *cycI*, in *Synechocystis* sp. PCC 6803, but also of *ycf54*; conversely, coexpression of cyanobacterial *cycI* and *ycf54* is required to complement the loss of *acsF* in *Rvi. gelatinosus*. These results indicate that Ycf54 is a cyclase subunit in oxygenic phototrophs, and that different classes of the enzyme exist based on their requirement for an additional subunit. AcsF is the cyclase in *Rvi. gelatinosus*, whereas alphaproteobacterial cyclases require a newly discovered protein that we term BciE, encoded by a gene conserved in these organisms. These data delineate three classes of O_2-dependent cyclase in chlorophototrophic organisms from higher plants to bacteria, and their evolution is discussed herein.

photosynthesis | chlorophyll | bacteriochlorophyll | cyclase

The (bacterio)chlorophylls, or (B)Chls, are ubiquitous pigments used by chlorophototrophic organisms for light harvesting and photochemistry, so elucidation of their biosynthetic pathways is of great importance. The least well-characterized step in the common pathway for all (B)Chls is formation of the isocyclic E ring, occurring via oxidation and cyclization of the C13 propionate group of magnesium protoporphyrin IX monomethyl ester (MgPME), producing 8-vinyl protochlorophyllide (8V Pchlide) (Fig. 1). The reaction is catalyzed by two distinct enzymes using different chemistries: an O_2-sensitive protein containing [4Fe-4S] and cobalamin prosthetic groups (1) that derives oxygen from water (2), and an oxidative diiron enzyme that requires molecular oxygen (3). The O_2-independent MgPME cyclase [EC:1.21.98.3] is believed to be encoded by a single gene, *bchE* (4), that is essential for BChl biosynthesis in bacterial phototrophs inhabiting anoxic environments. The O_2-dependent MgPME cyclase [EC:1.14.13.81] catalyzes this reaction in plants and cyanobacteria (5, 6), and is believed to be composed of multiple subunits (7).

The first subunit assigned to the O_2-dependent reaction was identified in the purple betaproteobacterium *Rubrivivax* (*Rvi.*) *gelatinosus* and was named AcsF (aerobic cyclization system Fe-containing subunit) (8). Subsequently, it was demonstrated that *Rvi. gelatinosus* contains both BchE and AcsF cyclases, conferring the ability to synthesize BChl under varying O_2 regimes (9). Orthologs of *acsF* are widely distributed in phototrophs and have been studied in higher plants (10, 11), algae (12) and cyanobacteria (13), the green nonsulfur bacterium *Chloroflexus aurantiacus* (14), and the purple alphaproteobacterium *Rhodobacter* (*Rba.*) *sphaeroides* (15) (Fig. S1).

Two isoforms of AcsF in the unicellular alga *Chlamydomonas reinhardtii*, CRD1 and CTH1, catalyze E ring formation under copper-deficient and -replete conditions, respectively (12, 16). These proteins are localized to both the thylakoid membrane and chloroplast envelope (17), a pattern shared with the single AcsF in *Arabidopsis thaliana*, CHL27 (10, 18). The cyanobacterium *Synechocystis* sp. PCC 6803 (hereinafter *Synechocystis*) also contains two isoforms of AcsF, designated CycI and CycII (19). Constitutively expressed *cycI* encodes the sole AcsF protein responsible for cyclase activity under oxic conditions, with *cycII* expressed only under microoxic conditions (13); overexpression of *cycII* cannot rescue cyclase function under a range of O_2 tensions when CycI levels are depleted (19).

The O_2-dependent enzyme has been resolved into membrane-bound and soluble fractions from cucumber chloroplasts and *Synechocystis*, suggesting that more than one subunit is required for a functional enzyme (7, 20). Later in vivo pulldown experiments in *Synechocystis* with tagged CycI and CycII identified Ycf54 as an interaction partner of both AcsF isoforms; tagged Ycf54 produced in the same manner was shown to interact with CycI (21). A mutant depleted in Ycf54 had a significantly reduced Chl content, accumulated MgPME, was only able to tolerate low light intensities and was unable to grow under photoautotrophic conditions (21, 22). Antisense mutants of the ortholog of *ycf54* in tobacco, *LCAA*, also demonstrated impaired cyclase activity, and the dimeric protein was shown to interact with CHL27 in the chloroplast (23). The foregoing findings suggest that Ycf54/LCAA, conserved among oxygenic phototrophs, is the "missing" soluble

Significance

(Bacterio)chlorophylls harvest and convert the solar energy that powers the biosphere. The absorption properties of these pigments are determined in part by formation of the isocyclic ring, which confers their characteristic green color. This last remaining uncharacterized biosynthetic step has remained enigmatic for more than 60 y, and only a single subunit of the O_2-dependent cyclase, AcsF, has been identified. Here we demonstrate that Ycf54 is a bona fide subunit of this enzyme in oxygenic phototrophs; identify a new cyclase subunit, BciE, in Alphaproteobacteria; and confirm that the AcsF found in other classes of bacterial phototrophs is the principal form of the cyclase, requiring neither Ycf54 nor BciE for activity.

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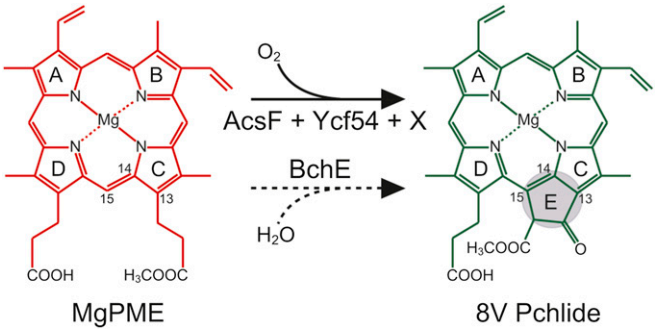

Fig. 1. Cyclization reactions involved in (B)Chl biosynthesis. Shown is isocyclic ring formation via O_2-dependent and -independent routes, catalyzed by AcsF and Ycf54 (solid arrow) and BchE (dashed arrow), respectively. Here x denotes the as-yet unassigned subunit required for the O_2-dependent reaction. International Union of Pure and Applied Chemistry numbering of the relevant macrocycle carbons is indicated, and formation of the ring E is highlighted. The oxygen sources for the O_2-dependent and -independent enzymes are molecular oxygen and water, respectively.

component of the O_2-dependent cyclase. The subunit requirement for this enzyme across phototrophic organisms has not been resolved, however.

In the present study, we demonstrate that *acsF* from *Rvi. gelatinosus* corrects the loss of both *cycI* and *ycf54* in *Synechocystis*, suggesting that this AcsF protein does not require a Ycf54 component. Reciprocally, CycI substitutes for AcsF in *Rvi. gelatinosus* only in the presence of Ycf54, providing validation of this protein as a subunit of the O_2-dependent cyclase. Furthermore, we identify BciE as a cyclase subunit conserved among AcsF-containing Alphaproteobacteria. This work delineates three distinct classes of O_2-dependent cyclase; phylogenetic analysis identifies defined clades, the evolution of which is discussed.

Results

***Rvi. gelatinosus acsF* Complements the Loss of *cycI* in *Synechocystis*, Regardless of the Presence of *ycf54*.** The apparent absence of *ycf54* orthologs in phototrophic bacteria containing orthologs of *acsF* suggests that the Ycf54 component of the O_2-dependent cyclase either is not required for function of the bacterial enzyme or that an unrelated protein performs the same function in its place. To determine which of these possibilities is the case, we integrated *acsF* from *Rvi. gelatinosus* into the genome of the *ycf54*-containing model cyanobacterium *Synechocystis* in place of the nonessential, light-responsive *psbAII* as described previously (24) (Fig. 2*A*). Deletion of the native CycI-encoding gene (sll1214) was attempted in this $acsF^{Rg+}$ background; a previous attempt to delete *cycI* in the wild type (WT) under oxic conditions proved unsuccessful (19). Full segregation of Δ*cycI* in $acsF^{Rg+}$ was achieved (Fig. 2*B*), indicating that *acsF* complements the loss of *cycI* in *Synechocystis*. Subsequently, deletion of *ycf54* (slr1780) in $acsF^{Rg+}$ Δ*cycI* was achieved by replacement of the native gene with a zeocin resistance cassette as described previously (22), yielding $acsF^{Rg+}$ Δ*cycI*Δ*ycf54* (Fig. 2*C*).

We performed phenotypic analyses of the $acsF^{Rg+}$ strains lacking *cycI* and both *cycI* and *ycf54*, along with WT and Δ*ycf54* controls. Liquid cultures were grown photomixotrophically under low light to an OD_{750} of ∼0.4. Absorption spectra of these suspensions indicate that deletion of *ycf54* almost abolishes the assembly of Chl-containing photosystems, as judged by the near absence of a peak at ∼680 nm (Fig. 2*D*). The restoration of a 680-nm absorption band by the introduction of $acsF^{Rg}$ into strains lacking *cycI*, irrespective of the presence of *ycf54*, shows that $acsF^{Rg}$ is necessary and sufficient for Chl *a* biosynthesis in *Synechocystis*. This conclusion is further reinforced by the Chl content of these strains grown under moderate light, calculated when all apart from Δ*ycf54* were grown without glucose ($mg \cdot L^{-1} \cdot OD_{750}^{-1}$, % relative to WT): WT, 3.22 ± 0.05, 100%; Δ*ycf54*, 0.24 ± 0.01, 7.5%; $acsF^{Rg+}$Δ*cycI*, 3.08 ± 0.07, 96%; $acsF^{Rg+}$ Δ*cycI*Δ*ycf54*, 3.08 ± 0.01, 96%. These data indicate that the O_2-dependent cyclase of *Rvi. gelatinosus* integrates into a

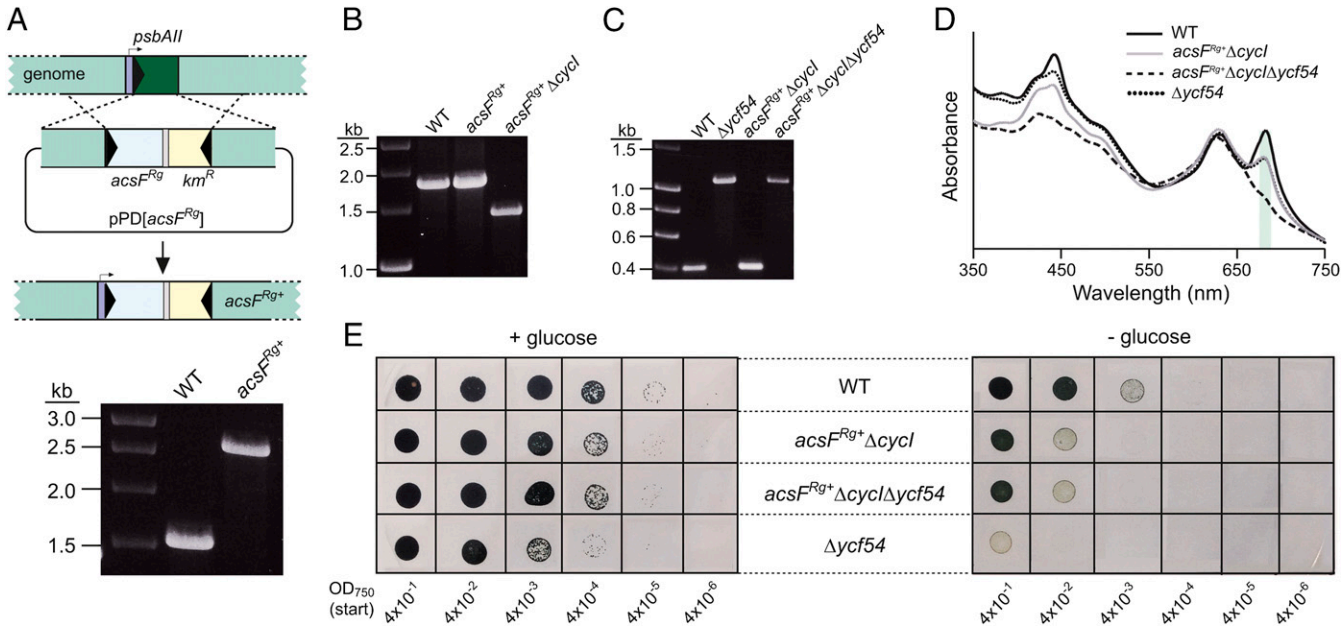

Fig. 2. Construction and phenotypic analyses of *Synechocystis* cyclase mutants. (*A*) Diagram depicting replacement of the *psbAII* gene with $acsF^{Rg}$ via pPD[$acsF^{Rg}$] (*Upper*), and construction of the fully segregated strain confirmed by colony PCR (*Lower*). (*B* and *C*) Inactivation of *cycI* (*B*) and *ycf54* (*C*) genes via replacement with chloramphenicol and zeocin resistance cassettes, respectively, confirmed by colony PCR. (*D*) Whole-cell absorption spectra of strains grown mixotrophically under low light conditions. The peaks for Chl-containing complexes are marked with a green shadow. (*E*) Drop growth assays of strains on solid agar, supplemented with or lacking glucose. Photographs were taken after incubation for 12 d.

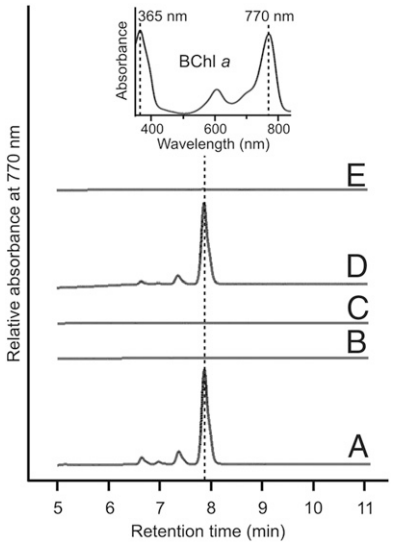

Fig. 3. HPLC analysis of pigments extracted from *Rvi. gelatinosus* strains. Pigments were extracted from the same number of cells of each strain except for the Δ*bchE* strain, which had a much greater BChl *a* content than the other strains. (*A*) Δ*bchE*. (*B*) Δ*bchE*Δ*acsF*. (*C*) Δ*bchE*Δ*acsF*::*cycI*. (*D*) Δ*bchE*Δ*acsF*::*cycI-ycf54*. (*E*) Δ*bchE*Δ*acsF*::$acsF^{Rs}$. (*Inset*) Retention times and Soret/Q_y maxima of peaks were used to identify BChl *a*.

cyanobacterial Chl pathway and dispenses with the requirement for Ycf54 normally exhibited by its native partner, CycI.

In addition, we performed drop growth assays on solid agar with and without 5 mM glucose (Fig. 2*E*). As expected, supplementation with glucose resulted in improved growth at identical dilutions for each strain. Strains containing $acsF^{Rg}$ showed the same pattern as seen in WT and grew under photoautotrophic conditions; they also were able to grow at higher dilutions than the Δ*ycf54* mutant under photomixotrophic conditions. These data suggest that $AcsF^{Rg}$ restores Chl biosynthesis and photoautotrophic growth to *Synechocystis* in the absence of Ycf54.

Ycf54 Is a Catalytic Component of the O_2-Dependent Cyclase Enzyme in Oxygenic Phototrophs. Previous work has shown that *Synechocystis* Δ*ycf54* makes a small amount of Chl, ∼13% of WT levels (21, 22); thus, Ycf54 appears to be important, but not essential, for cyclase activity. To assess the contribution of Ycf54 more precisely, we developed a reciprocal system for the heterologous expression of *Synechocystis* genes in *Rvi. gelatinosus*, which synthesizes BChl *a* under conditions ranging from oxic to anoxic using O_2-dependent and -independent cyclase enzymes, respectively (8, 9). Genes encoding the known components of these enzymes were removed using an in-frame, markerless deletion method to avoid the polar effects often encountered with resistance cassette-mediated gene disruption. The O_2-independent cyclase was inactivated by deletion of *bchE* (Fig. S2*A*), and BChl biosynthesis was completely inactivated by the subsequent deletion of *acsF* (Fig. S2*B*). This Δ*bchE*Δ*acsF* strain, which accumulates the cyclase substrate MgPME, provides a background for testing components of the O_2-dependent enzyme. The *cycI* gene from *Synechocystis* was integrated at the original *acsF* locus both alone and in combination with *ycf54* from the same organism encoded downstream of *cycI* (Fig. S2*B*). A third complemented strain used *acsF* from *Rba. sphaeroides*, which was recently shown to be essential for O_2-dependent cyclase activity in this model anoxygenic phototroph (15) (Fig. S2*B*).

The three resulting strains, Δ*bchE*Δ*acsF*::*cycI*, Δ*bchE*Δ*acsF*::*cycI-ycf54*, and Δ*bchE*Δ*acsF*::$acsF^{Rs}$, along with positive and negative control strains Δ*bchE* and Δ*bchE*Δ*acsF*, respectively, were cultured under oxic conditions in the dark in liquid medium, standardized by OD_{680}, and pelleted. The pigments accumulated by these strains were extracted, and BChl *a* content was analyzed by HPLC (Fig. 3). As expected, BChl *a* accumulated to a high level in Δ*bchE* (Fig. 3*A*), but Δ*bchE*Δ*acsF* was unable to synthesize BChl (Fig. 3*B*). The presence of *cycI* in this background did not restore BChl biosynthesis (Fig. 3*C*), whereas BChl was detected in the strain complemented with both *cycI* and *ycf54* (Fig. 3*D*). These data confirm that Ycf54 is essential for activity of the the O_2-dependent cyclase from oxygenic phototrophs. Surprisingly, although a cyanobacterial cyclase was functional, *acsF* from the more closely related *Rba. sphaeroides* was unable to restore BChl biosynthesis to this strain (Fig. 3*E*).

Bioinformatic Analysis of a Conserved ORF Upstream of *acsF* in Alphaproteobacteria Reveals a Cyclase Subunit. The unexpected lack of activity of $acsF^{Rs}$ in *Rvi. gelatinosus* led us to postulate that the functional enzyme from *Rba. sphaeroides* may require a "Ycf54-like" protein. The genome of *Rba. sphaeroides* does not contain an ortholog of *ycf54*, so there was no obvious candidate encoding a missing cyclase component. All known genes essential for BChl biosynthesis in this model organism are contained within a 40.7-kb photosynthesis gene cluster (PGC) (25). PGCs are conserved throughout bacterial phototrophs and are a likely location for a "purple bacterial *ycf54*." Analysis of the PGC of *Rba. sphaeroides* revealed a small ORF (rsp_6110) found directly upstream of, and overlapping with, *acsF*; orthologs of this gene are conserved upstream of *acsF* in all studied Alphaproteobacteria containing *acsF* but are absent in all other taxonomic groups (26), including the betaproteobacterium *Rvi. gelatinosus* (Table S1). To determine whether rsp_6110 encodes an essential component of the alphaproteobacterial O_2-dependent cyclase, the ORF was deleted from *Rba. sphaeroides* Δ*bchE*Δ*ccoP* (Fig. S3), in which native AcsF function has been detected via accumulation of BChl *a* chelated with either Mg or Zn (15). The resulting *Rba. sphaeroides* strain, Δ*bchE*Δ*ccoP*Δ6110, and its parent were cultured under oxic conditions in the dark, and pigments were extracted from pellets and analyzed as described previously (15).

Both Mg-BChl *a* and Zn-BChl *a* were detected in Δ*bchE*Δ*ccoP* (Fig. 4*A*), whereas these pigments were absent in Δ*bchE*Δ*ccoP*Δ6110 (Fig. 4*B*). To ensure that this result was due to deletion of rsp_6110, rather than a polar effect on *acsF*, rsp_6110 was expressed *in trans* in Δ*bchE*Δ*ccoP*Δ6110 from pBBRBB-$Ppuf_{843–1200}$ (27). Pigments from this strain were analyzed as above, and Mg-BChl *a* and Zn-BChl *a* were detected in this extract (Fig. 4*C*), confirming that the O_2-dependent cyclase from *Rba. sphaeroides* requires

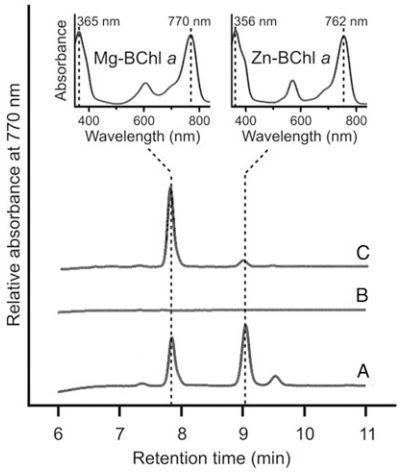

Fig. 4. HPLC analysis of pigments extracted from *Rba. sphaeroides* strains. Pigments were extracted from strains standardized by cell number. (*A*) Δ*bchE*Δ*ccoP*. (*B*) Δ*bchE*Δ*ccoP*Δ6110. (*C*) Δ*bchE*Δ*ccoP*Δ6110 + pBB[6110]. (*Insets*) Retention times and Soret/Q_y maxima of peaks were used to identify Mg- and Zn-chelated species of BChl *a*.

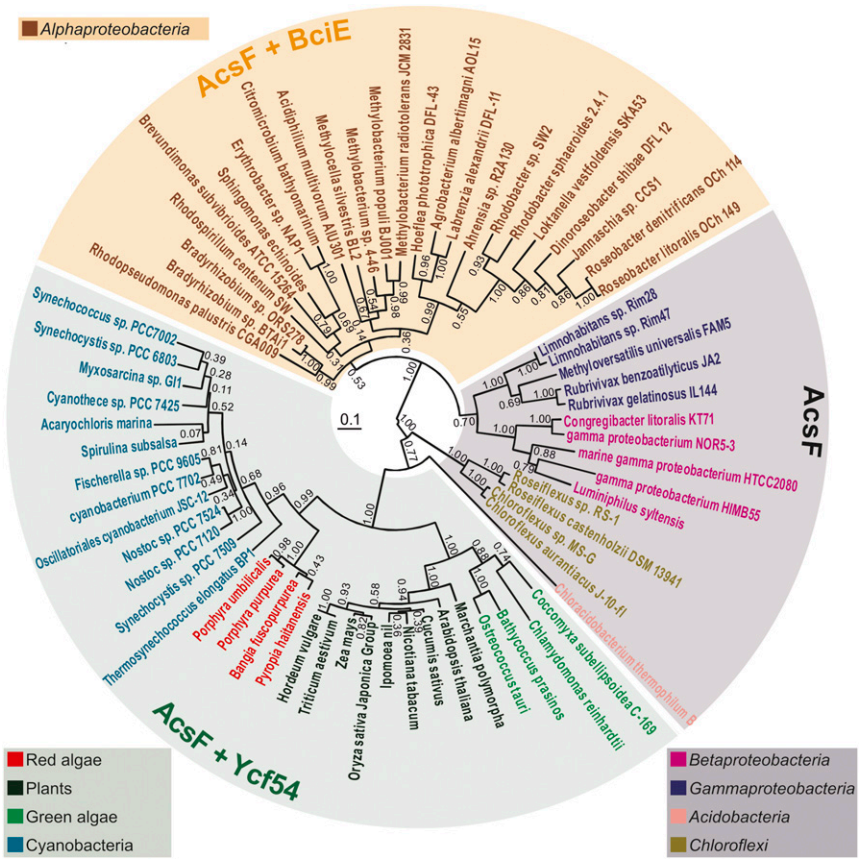

Fig. 5. Phylogenetic analysis of AcsF proteins. Evolutionary analysis via a phylogenetic tree was conducted in MEGA6 using the maximum likelihood method based on the JTT matrix-based model. The analysis involved 69 protein sequences. The tree with the highest log-likelihood (−17,513.1099) is shown. Numbers next to each node indicate bootstrap values (1,000 replicates) as percentages. Phyla are distinguished by color of species name. The length of each branch represents the number of amino acid substitutions per site in proportion to the scale bar at the center of the tree. The presence/absence of BciE/Ycf54 is indicated by shading over the species names: gray, no BciE or Ycf54; orange, BciE present; green, Ycf54 present. Note that orthologs of both *bciE* and *ycf54* are not found together in the genome of any organism sequenced to date.

Rsp_6110 to function. Therefore, we propose, according to the Demerec nomenclature, that rsp_6110 be renamed *bciE*.

To determine whether heterologous expression of *bciE* from *Rba. sphaeroides*, along with *acsF* from the same organism in *Rvi. gelatinosus* Δ*bchE*Δ*acsF*, is able to restore BChl biosynthesis, where $acsF^{Rs}$ alone is not, we amplified the overlapping *bciE* and *acsF* genes directly from the genome of *Rba. sphaeroides* and integrated at the *acsF* locus of *Rvi. gelatinosus*, as described earlier (Fig. S4*A*). This strain, Δ*bchE*Δ*acsF*::*bciE-*$acsF^{Rs}$, was grown, and its pigments were extracted and analyzed as described above. Coexpression of *bciE* and $acsF^{Rs}$ resulted in accumulation of BChl (Fig. S4*B*).

Phylogenetic Analysis of AcsF Proteins. To investigate the evolutionary history of AcsF orthologs, we conducted phylogenetic analysis with example protein sequences from plants, algae, and *acsF*-containing phyla of phototrophic (cyano)bacteria, as described in *Materials and Methods* (Fig. 5). AcsF proteins from species belonging to the same group cluster in the same clade and the topology of the tree correspond closely with the evolutionary relationships among the species being analyzed (28) (Fig. 5). In addition, we checked for the presence or absence of BciE and Ycf54 orthologs in the 69 studied species by performing DELTA-BLAST searches using either *Rba. sphaeroides* BciE (WP_002720458) or *Synechocystis* Ycf54 (P72777) as a query. Distribution patterns of BciE and Ycf54 orthologs are seemingly related to the phylogeny of AcsF proteins.

Discussion

The O_2-dependent cyclase is the "missing link" in (B)Chl biosynthesis, and it has remained enigmatic for more than 60 y. Before the present study, only AcsF had been identified as a bona fide cyclase subunit (8, 13). Ycf54 and its ortholog LCAA were subsequently discovered to be required for the normal activity of cyanobacterial and plant enzymes, respectively (21, 22), but the absence of genes encoding Ycf54 from *acsF*-containing anoxygenic bacterial phototrophs suggested that it might not be a catalytic subunit. Our demonstration that only the Ycf54-CycI combination replaces the native cyclase function of AcsF in *Rvi. gelatinosus* shows that, irrespective of the other roles of Ycf54 in *Synechocystis*, it can be considered a subunit of this enzyme. In the case of *Synechocystis* Δ*ycf54*, it may be that the small amount of Chl *a* produced by this strain is due to the presence of three orthologs of *bchE* in the genome of this organism. It once was believed that proteins encoded by these genes did not contribute to Chl biosynthesis (13); however, the recent finding that cyanobacterial *bchE* orthologs from two strains of *Cyanothece* restore BChl *a* biosynthesis in a *bchE* mutant of *Rba. capsulatus* demonstrates the activity of O_2-independent BchE orthologs from oxygenic phototrophs (29).

In the other half of the reciprocal experiment, expression of $acsF^{Rg}$ in *Synechocystis* was found to complement the loss of either the native *cycI* or *ycf54*. The step occurring after the formation of ring E in (B)Chl biosynthesis, conversion of Pchlide to chlorophyllide, can be catalyzed by two unrelated enzymes, light-activated Pchlide oxidoreductase (POR) and dark-operative POR (DPOR), both of which are present in *Synechocystis*. The light-activated POR is dominant under the conditions that we used for culturing the complemented strains of *Synechocystis* (30). This enzyme is absent from all anoxygenic bacterial phototrophs, so the recombinant bacterial AcsF is able to functionally integrate into the Chl pathway in an oxygenic phototroph; this tallies with observations that other recombinant (B)Chl enzymes expressed in cyanobacterial or purple phototrophic hosts can function in non-native pathways (31–33). These results suggest that there is much promise for combining pigment biosynthetic pathways with the aim of producing novel (B)Chls with unique spectral properties.

The existence of cyclase enzymes that require an extra subunit in plants and cyanobacteria is reminiscent of the role played by Gun4 in the activity of magnesium chelatase (MgCH), the

enzyme catalyzing the first committed step in (B)Chl biosynthesis. Plant and cyanobacterial mutants in *gun4* display reduced Chl content and impaired growth, and they accumulate the substrate for MgCH, whereas phototrophic bacteria lack orthologs of *gun4* (34). Gun4 was found to stimulate cyanobacterial MgCH activity in vitro (35) and to be involved in increasing flux into the Chl biosynthesis pathway in vivo (36). Similarly, it is conceivable that Ycf54 may play a role in substrate channeling; pull-down experiments identified protein–protein interactions between Ycf54 and the cyanobacterial AcsF, CycI (21), and CycI with other Chl biosynthesis enzymes (22). These interactions are abrogated in the absence of *ycf54*, and the level of CycI is reduced, suggesting that Ycf54 may stabilize the CycI protein (22).

We have identified and validated a subunit of the O_2-dependent cyclase in Alphaproteobacteria, which we have named BciE. The ORF encoding this protein is found directly upstream of *acsF* in this bacterial class. Deletion in *Rba. sphaeroides* led to the abolition of O_2-dependent cyclase activity, reinstatement of *bciE in trans* restored activity, and $AcsF^{Rs}$ was found to require BciE to function in the heterologous *Rvi. gelatinosus* system. No conserved domain can be identified in BciE based on the National Center for Biotechnology Information's Conserved Domain Database (37). Thus, BciE may represent a novel protein family; its precise role in the alphaproteobacterial enzyme is unclear, but it may play a role similar to that of Ycf54 or Gun4 in stabilization of the major subunit and/or stimulation of the forward reaction.

A cyanobacterial progenitor of algal and plant chloroplasts (38) is also the likely origin of the *acsF* and *ycf54* genes common to all oxygenic phototrophs. Our phylogenetic analysis is consistent with this theory, with Ycf54-requiring AcsF sequences clustering in a well-defined clade. In addition, it is believed that *acsF* was horizontally transferred from cyanobacteria to Proteobacteria before the divergence of Alphaproteobacterial, Betaproteobacterial, and Gammaproteobacterial lineages, because cyanobacteria were initially oxygenating the atmosphere, conferring an advantage to anoxygenic phototrophs previously reliant solely on O_2-sensitive BchE (26). A newly discovered bacterial phototroph belonging to the phylum Gemmatimonadetes is believed to have acquired an *acsF*-containing purple bacterial PGC via horizontal gene transfer (39), and thus is more recent than the acquisition of *acsF* by the Proteobacteria. The *acsF* gene is also found in Chloroflexi and the single phototrophic member of the phylum Acidobacteria discovered to date (28). Although Chloroflexi are more closely related to cyanobacteria than to other phyla mentioned here, their AcsF proteins more closely resemble the proteobacterial enzyme, whereas the acidobacterial AcsF is more similar to those from cyanobacteria, yet it does not require Ycf54. These observations imply that multiple horizontal transfers have occurred, possibly to a common ancestor of Acidobacteria and Proteobacteria, followed by transfer from a proteobacterium to the Chloroflexi. Because *acsF* and *ycf54* are not clustered in cyanobacteria, it follows that they would not be readily cotransferred to another organism. Mutations in the transferred *acsF* might have relieved the requirement for Ycf54, explaining the absence of *ycf54* in anoxygenic bacteria. The *bciE* gene may have appeared after the divergence of Alphaproteobacteria from other subgroups, and the emergence of BciE may be necessary for the function of the enzyme under as-yet unidentified cellular conditions.

It has long been believed that the O_2-dependent cyclase reaction requires NAD(P)H as a reductant (5), with dependence observed in plant, algal, and cyanobacterial systems (20, 40). The barley *xantha-l* and *viridis-k* mutants that accumulate MgPME are deficient in the membrane components of the O_2-dependent enzyme, with all *xantha-l* mutations mapping to the single *acsF* ortholog that is intact in the *viridis-k* mutants (11). Subsequently, Bollivar et al. (41) concluded that *viridis-k* mutants do not lack Ycf54; thus, it is possible that the missing subunit of the enzyme, disrupted in *viridis-k* mutants, is a membrane-associated NAD(P)H-binding protein. Recently it was proposed that the O_2-dependent cyclase may derive electrons from reduced quinone, as in the case for other diiron enzymes, plastid terminal oxidase and the mitochondrial alternative oxidase (42). The authors suggested that NAD(P)H may maintain the redox state of the plastoquinone pool, via NAD(P)H dehydrogenase, which they proposed to be directly coupled to efficient cyclase activity. Those studies indicated that the last remaining hurdle in the study of this enzyme, and thus in the pathway common to all (B)Chl pigments, is to determine the electron donor to the diiron center of AcsF (Fig. S5).

Conclusion

We have used genetic techniques to test and identify components of the O_2-dependent cyclase in three unrelated model organisms. Bioinformatic analyses coupled with heterologous gene expression (*i*) show that Ycf54 is essential for cyclase function in oxygenic phototrophs; (*ii*) identify a previously unknown protein component of the alphaproteobacterial enzyme, BciE; and (*iii*) indicate that the AcsF from other taxonomic groups of anoxygenic phototrophs requires neither Ycf54 nor BciE for activity.

Materials and Methods

Bacterial Strains and Growth Conditions. *Rvi. gelatinosus* strains were grown in a rotary shaker in the dark at 30 °C in PYS medium (43). Microoxic and oxic growth regimes were achieved as described previously (15). When required, kanamycin was added at 50 $\mu g \cdot mL^{-1}$. *Synechocystis* strains were grown photoautotrophically in a rotary shaker at 30 °C under low light (5 μmol photons $m^{-2} \cdot s^{-1}$) or moderate light (30 μmol photons $m^{-2} \cdot s^{-1}$) as described previously (35). Photomixotrophic growth was achieved by the addition of glucose to a final concentration of 5 mM. *Rba. sphaeroides* strains were grown in the dark at 30 °C as described previously (15). *Escherichia coli* strains were grown in LB medium, supplemented with 30 $\mu g \cdot mL^{-1}$ kanamycin when required. The bacterial strains and plasmids used in this study are listed in Table S2.

Construction of Mutants. Heterologous expression $acsF^{Rg}$ in *Synechocystis* was achieved by cloning the ORF between *NdeI*/*Bgl*II sites in pPD-FLAG (21). The resulting pPD[$acsF^{Rg}$] was transformed into WT *Synechocystis*, and transformants were isolated as described previously (21). The regions upstream and downstream of *cycI* were amplified with 1214UpF and 1214UpR and with 1214DownF and 1214DownR primers, respectively. The chloramphenicol (Cm) resistance cassette from pACYC184 was amplified with 1214UpCmF and 1214DownCmR. The three PCR products were fused by overlap extension PCR. The resulting fragment was used to disrupt *cycI*, and segregation was achieved by sequentially doubling the concentration of Cm from 5 to 80 $\mu g \cdot mL^{-1}$. A *ycf54* disruption fragment used in this study was constructed as described previously (22). Markerless deletion mutants in *Rba. sphaeroides* and *Rvi. gelatinosus* were constructed as described previously (15). Gene replacements were achieved by cloning ORFs into the *NdeI* site in the relevant pK18Δ*gene* construct. The pBB[*gene*] plasmid was used to express foreign genes in *Rba. sphaeroides* and was constructed by cloning the relevant ORF between *BglII*/*NotI* sites in pBBRBB-$Ppuf_{843–1200}$ (27). *Rvi. gelatinosus* was transformed using a method described by Nagashima et al. (43). Sequences of primers used in this study are listed in Table S3.

Whole-Cell Absorption Spectroscopy and Determination of Chl Content. Absorption spectra were obtained as detailed previously (15). Pigments were extracted from 4 mL of *Synechocystis* culture (OD_{750} = 0.4) with methanol, and Chl concentrations were determined spectroscopically from extracts of biological triplicates using a published method (44).

Pigment Extraction and Analysis by HPLC. Pigment extraction and HPLC analysis were carried out as described previously (15).

Plate-Based Growth Assays. *Synechocystis* drop growth assays were conducted using liquid cultures of *Synechocystis* grown under low light, adjusted to OD_{750} = 0.4, and serially diluted. Dilutions were spotted on solid medium ±5 mM glucose and incubated at low light.

Phylogenetic Tree Construction. The phylogenetic tree was built with MEGA6 (45). AcsF sequences from 24 Alphaproteobacteria, 5 Betaproteobacteria, 5 Gammaproteobacteria, 13 Cyanobacteria, 1 *Acidobacterium* sp., 4 Chloroflexi, 9 algae, and 8 plants were retrieved via DELTA-BLAST using *Rvi. gelatinosus* AcsF (RGE_33550) as a query. The protein sequences thus obtained (Table S1) were aligned using the ClustalW algorithm provided in MEGA6. All positions containing gaps and missing data in the alignment were removed. Initial

trees for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the Jones–Taylor–Thornton (JTT) amino acid substitution model (46). Evolutionary history was inferred by using the maximum likelihood method with the JTT model and was tested by the bootstrap method with 1,000 replicates. The tree with the highest log-likelihood (−17,513.1099) was adopted and visualized using Interactive Tree of Life v2 (47).

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1. Gough SP, Petersen BO, Duus JØ (2000) Anaerobic chlorophyll isocyclic ring formation in *Rhodobacter capsulatus* requires a cobalamin cofactor. *Proc Natl Acad Sci USA* 97: 6908–6913.
2. Porra RJ, et al. (1996) Origin of the two carbonyl oxygens of bacteriochlorophyll *a*: Demonstration of two different pathways for the formation of ring E in *Rhodobacter sphaeroides* and *Roseobacter denitrificans*, and a common hydratase mechanism for 3-acetyl group formation. *Eur J Biochem* 239:85–92.
3. Walker CJ, Mansfield KE, Smith KM, Castelfranco PA (1989) Incorporation of atmospheric oxygen into the carbonyl functionality of the protochlorophyllide isocyclic ring. *Biochem J* 257:599–602.
4. Hunter CN, Coomber SA (1988) Cloning and oxygen-regulated expression of the bacteriochlorophyll biosynthesis genes *bch E*, *B*, *A*, and *C* of *Rhodobacter sphaeroides*. *J Gen Microbiol* 134:1491–1497.
5. Chereskin BM, Wong YS, Castelfranco PA (1982) In vitro synthesis of the chlorophyll isocyclic ring: Transformation of magnesium-protoporphyrin IX and magnesium-protoporphyrin IX monomethyl ester into magnesium-2,4-divinyl pheoporphyrin A(5). *Plant Physiol* 70:987–993.
6. Beale SI (1999) Enzymes of chlorophyll biosynthesis. *Photosynth Res* 60:43–73.
7. Wong YS, Castelfranco PA (1984) Resolution and reconstitution of mg-protoporphyrin-IX monomethyl ester (oxidative) cyclase, the enzyme system responsible for the formation of the chlorophyll isocyclic ring. *Plant Physiol* 75:658–661.
8. Pinta V, Picaud M, Reiss-Husson F, Astier C (2002) *Rubrivivax gelatinosus acsF* (previously *orf358*) codes for a conserved, putative binuclear iron cluster-containing protein involved in aerobic oxidative cyclization of Mg-protoporphyrin IX monomethylester. *J Bacteriol* 184:746–753.
9. Ouchane S, Steunou AS, Picaud M, Astier C (2004) Aerobic and anaerobic Mg-protoporphyrin monomethyl ester cyclases in purple bacteria: A strategy adopted to bypass the repressive oxygen control system. *J Biol Chem* 279:6385–6394.
10. Tottey S, et al. (2003) *Arabidopsis* CHL27, located in both envelope and thylakoid membranes, is required for the synthesis of protochlorophyllide. *Proc Natl Acad Sci USA* 100:16119–16124.
11. Rzeznicka K, et al. (2005) *Xantha-l* encodes a membrane subunit of the aerobic Mg-protoporphyrin IX monomethyl ester cyclase involved in chlorophyll biosynthesis. *Proc Natl Acad Sci USA* 102:5886–5891.
12. Moseley JL, et al. (2002) Reciprocal expression of two candidate di-iron enzymes affecting photosystem I and light-harvesting complex accumulation. *Plant Cell* 14: 673–688.
13. Minamizaki K, Mizoguchi T, Goto T, Tamiaki H, Fujita Y (2008) Identification of two homologous genes, $chlA_I$ and $chlA_{II}$, that are differentially involved in isocyclic ring formation of chlorophyll *a* in the cyanobacterium *Synechocystis* sp. PCC 6803. *J Biol Chem* 283:2684–2692.
14. Tang K-H, Wen J, Li X, Blankenship RE (2009) Role of the AcsF protein in *Chloroflexus aurantiacus*. *J Bacteriol* 191:3580–3587.
15. Chen GE, Canniffe DP, Martin EC, Hunter CN (2016) Absence of the cbb_3 terminal oxidase reveals an active oxygen-dependent cyclase involved in bacteriochlorophyll biosynthesis in *Rhodobacter sphaeroides*. *J Bacteriol* 198:2056–2063.
16. Moseley J, Quinn J, Eriksson M, Merchant S (2000) The Crd1 gene encodes a putative di-iron enzyme required for photosystem I accumulation in copper deficiency and hypoxia in *Chlamydomonas reinhardtii*. *EMBO J* 19:2139–2151.
17. Allen MD, Kropat J, Merchant SS (2008) Regulation and localization of isoforms of the aerobic oxidative cyclase in *Chlamydomonas reinhardtii*. *Photochem Photobiol* 84: 1336–1342.
18. Bang WY, et al. (2008) Role of *Arabidopsis* CHL27 protein for photosynthesis, chloroplast development, and gene expression profiling. *Plant Cell Physiol* 49:1350–1363.
19. Peter E, et al. (2009) Differential requirement of two homologous proteins encoded by sll1214 and sll1874 for the reaction of Mg protoporphyrin monomethylester oxidative cyclase under aerobic and micro-oxic growth conditions. *Biochim Biophys Acta* 1787:1458–1467.
20. Bollivar DW, Beale SI (1996) The chlorophyll biosynthetic enzyme Mg-protoporphyrin IX monomethyl ester (oxidative) cyclase: Characterization and partial purification from *Chlamydomonas reinhardtii* and *Synechocystis* sp PCC 6803. *Plant Physiol* 112:105–114.
21. Hollingshead S, et al. (2012) Conserved chloroplast open-reading frame *ycf54* is required for activity of the magnesium protoporphyrin monomethylester oxidative cyclase in *Synechocystis* PCC 6803. *J Biol Chem* 287:27823–27833.
22. Hollingshead S, et al. (2016) Synthesis of chlorophyll-binding proteins in a fully-segregated Δ*ycf54* strain of the cyanobacterium *Synechocystis* PCC 6803. *Front Plant Sci* 7:292.
23. Albus CA, et al. (2012) LCAA, a novel factor required for magnesium protoporphyrin monomethylester cyclase accumulation and feedback control of aminolevulinic acid biosynthesis in tobacco. *Plant Physiol* 160:1923–1939.
24. Canniffe DP, Jackson PJ, Hollingshead S, Dickman MJ, Hunter CN (2013) Identification of an 8-vinyl reductase involved in bacteriochlorophyll biosynthesis in *Rhodobacter sphaeroides* and evidence for the existence of a third distinct class of the enzyme. *Biochem J* 450:397–405.
25. Naylor GW, Addlesee HA, Gibson LCD, Hunter CN (1999) The photosynthesis gene cluster of *Rhodobacter sphaeroides*. *Photosynth Res* 62:121–139.
26. Boldareva-Nuianzina EN, Bláhová Z, Sobotka R, Koblízek M (2013) Distribution and origin of oxygen-dependent and oxygen-independent forms of Mg-protoporphyrin monomethylester cyclase among phototrophic proteobacteria. *Appl Environ Microbiol* 79:2596–2604.
27. Tikh IB, Held M, Schmidt-Dannert C (2014) BioBrick™-compatible vector system for protein expression in *Rhodobacter sphaeroides*. *Appl Microbiol Biotechnol* 98:3111–3119.
28. Bryant DA, et al. (2012) Comparative and functional genomics of anoxygenic green bacteria from the taxa Chlorobi, Chloroflexi, and Acidobacteria. *Functional Genomics and Evolution of Photosynthetic Systems*, eds Burnap R, Vermaas W (Springer, Amsterdam, The Netherlands), pp 47–102.
29. Yamanashi K, Minamizaki K, Fujita Y (2015) Identification of the *chlE* gene encoding oxygen-independent Mg-protoporphyrin IX monomethyl ester cyclase in cyanobacteria. *Biochem Biophys Res Commun* 463:1328–1333.
30. Kopečná J, Sobotka R, Komenda J (2013) Inhibition of chlorophyll biosynthesis at the protochlorophyllide reduction step results in the parallel depletion of Photosystem I and Photosystem II in the cyanobacterium *Synechocystis* PCC 6803. *Planta* 237:497–508.
31. Wilks HM, Timko MP (1995) A light-dependent complementation system for analysis of NADPH:protochlorophyllide oxidoreductase: Identification and mutagenesis of two conserved residues that are essential for enzyme activity. *Proc Natl Acad Sci USA* 92:724–728.
32. Canniffe DP, Chidgey JW, Hunter CN (2014) Elucidation of the preferred routes of C8-vinyl reduction in chlorophyll and bacteriochlorophyll biosynthesis. *Biochem J* 462: 433–440.
33. Hitchcock A, et al. (2016) Biosynthesis of chlorophyll a in a purple bacterial phototroph and assembly into a plant chlorophyll-protein complex. *ACS Synth Biol* 5:948–954.
34. Wilde A, Mikolajczyk S, Alawady A, Lokstein H, Grimm B (2004) The *gun4* gene is essential for cyanobacterial porphyrin metabolism. *FEBS Lett* 571:119–123.
35. Davison PA, et al. (2005) Structural and biochemical characterization of Gun4 suggests a mechanism for its role in chlorophyll biosynthesis. *Biochemistry* 44:7603–7612.
36. Kopečná J, et al. (2015) Porphyrin binding to Gun4 protein, facilitated by a flexible loop, controls metabolite flow through the chlorophyll biosynthetic pathway. *J Biol Chem* 290:28477–28488.
37. Marchler-Bauer A, et al. (2015) CDD: NCBI's conserved domain database. *Nucleic Acids Res* 43:D222–D226.
38. McFadden GI, van Dooren GG (2004) Evolution: Red algal genome affirms a common origin of all plastids. *Curr Biol* 14:R514–R516.
39. Zeng Y, Feng F, Medová H, Dean J, Koblížek M (2014) Functional type 2 photosynthetic reaction centers found in the rare bacterial phylum *Gemmatimonadetes*. *Proc Natl Acad Sci USA* 111:7795–7800.
40. Nasrulhaq-Boyce A, Griffiths WT, Jones OT (1987) The use of continuous assays to characterize the oxidative cyclase that synthesizes the chlorophyll isocyclic ring. *Biochem J* 243:23–29.
41. Bollivar D, Braumann I, Berendt K, Gough SP, Hansson M (2014) The Ycf54 protein is part of the membrane component of Mg-protoporphyrin IX monomethyl ester cyclase from barley (*Hordeum vulgare* L.). *FEBS J* 281:2377–2386.
42. Steccanella V, Hansson M, Jensen PE (2015) Linking chlorophyll biosynthesis to a dynamic plastoquinone pool. *Plant Physiol Biochem* 97:207–216.
43. Nagashima KV, Shimada K, Matsuura K (1996) Shortcut of the photosynthetic electron transfer in a mutant lacking the reaction center-bound cytochrome subunit by gene disruption in a purple bacterium, *Rubrivivax gelatinosus*. *FEBS Lett* 385:209–213.
44. Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: Verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochim Biophys Acta* 975:384–394.
45. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 30:2725–2729.
46. Jones DT, Taylor WR, Thornton JM (1992) The rapid generation of mutation data matrices from protein sequences. *Comput Appl Biosci* 8:275–282.
47. Letunic I, Bork P (2011) Interactive Tree of Life v2: Online annotation and display of phylogenetic trees made easy. *Nucleic Acids Res* 39:W475-8.
48. Simon R, Priefer U, Pühler A (1983) A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Nat Biotechnol* 1:784–791.

Supporting Information

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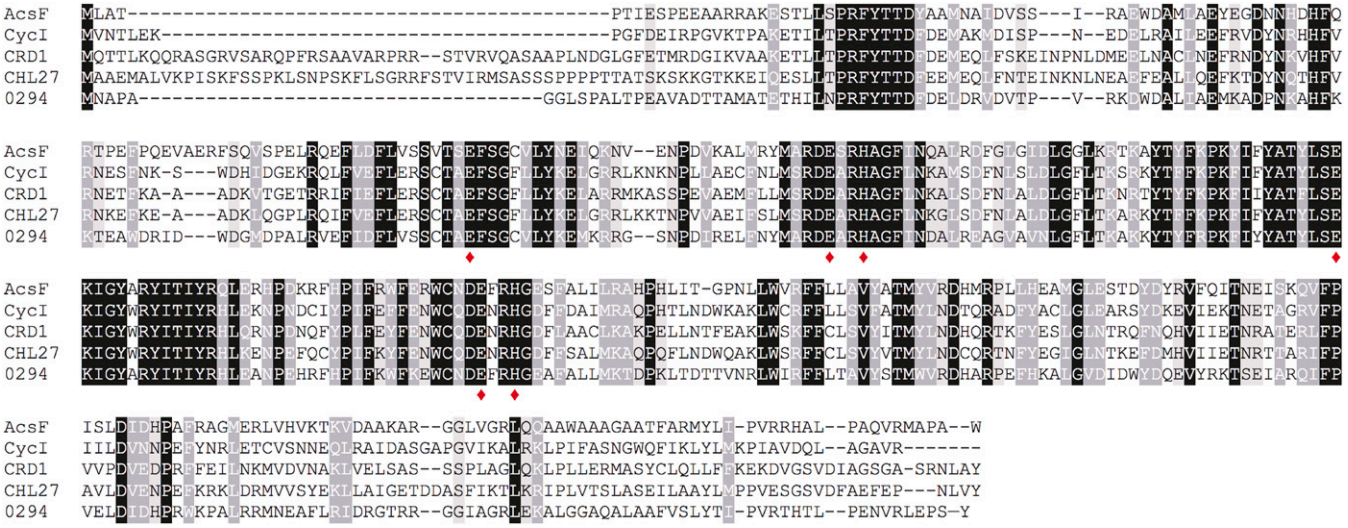

Fig. S1. Amino acid sequence alignments of known AcsF proteins. Sequences are those from *Rvi. gelatinosus* (AcsF), *Synechocystis* (CycI), *C. reinhardtii* (CRD1), *A. thaliana* (CHL27), and *Rba. sphaeroides* (Rsp_0294; abbreviated as 0294). Conserved, highly similar, and similar residues are highlighted in black, dark gray, and light gray, respectively. The putative diiron center ligands are marked by red diamonds.

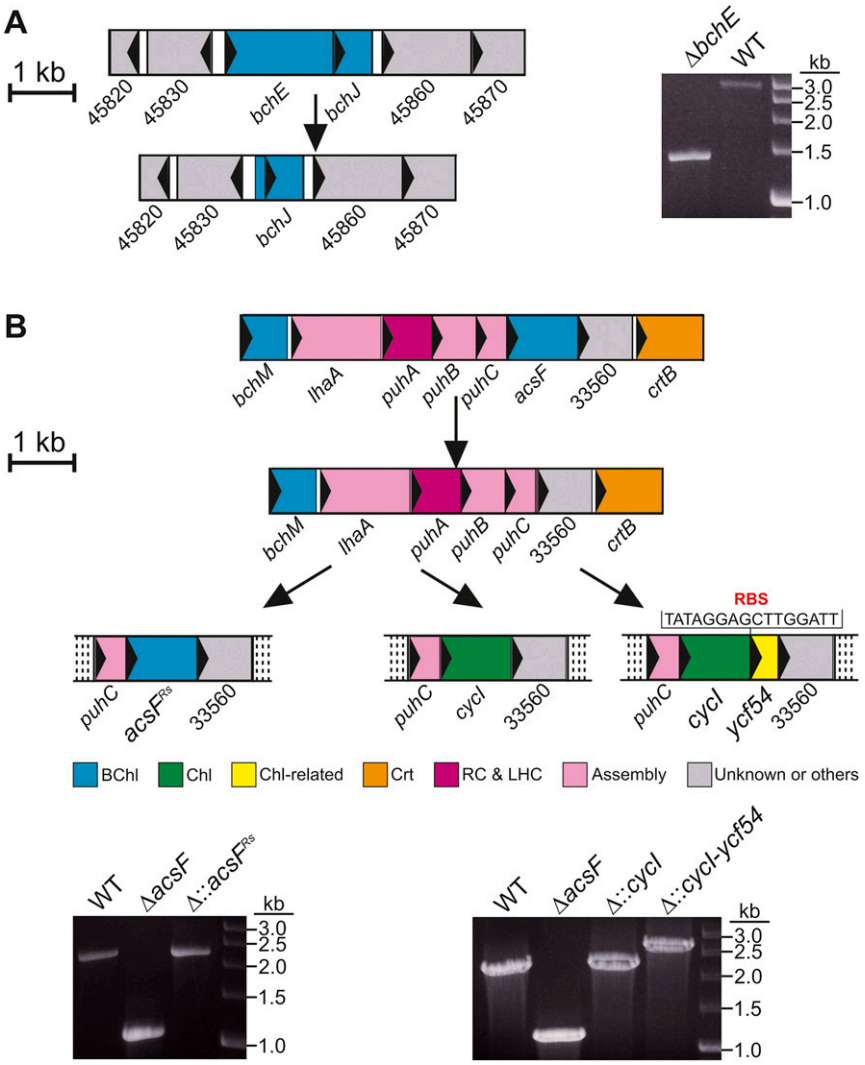

Fig. S2. Genetic knockouts and replacements in *Rvi. gelatinosus.* (*A*) Depiction of the deletion of *bchE* (*Left*), confirmed by colony PCR (*Right*). (*B*) Depiction of deletion of *acsF*, and subsequent integration of foreign genes at the *acsF* locus, under control of the native promoter (*Upper*), confirmed by colony PCR (*Lower*). The regions subjected to genetic manipulation are depicted in proportion to the scale bar. ORFs are represented as colored filled rectangles, within which the arrow indicates the direction of transcription. Crt, carotenoid biosynthesis; RC&LHC, reaction center and light-harvesting complexes.

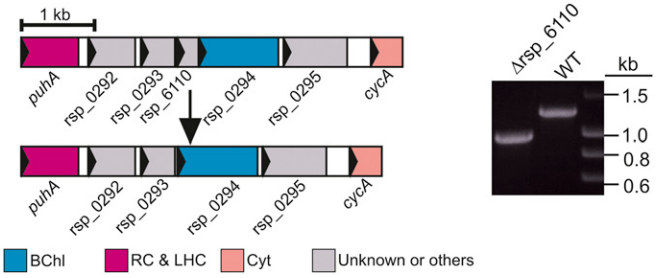

Fig. S3. Deletion of rsp_6110 in *Rba. sphaeroides.* Diagram depicting deletion of rsp_6110 (*Left*), and confirmation by colony PCR (*Right*).

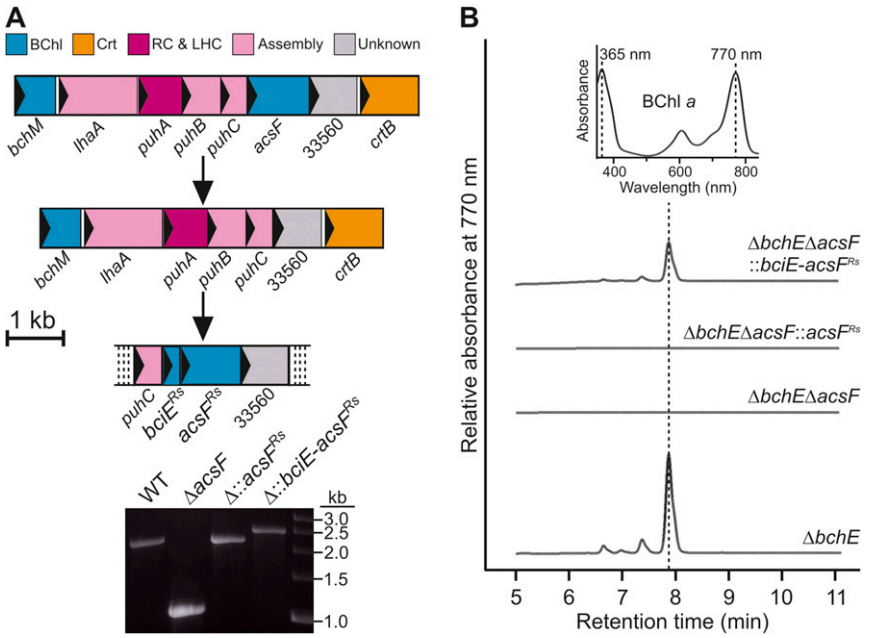

Fig. S4. Construction and phenotypic analysis of *Rvi. gelatinosus* mutant expressing *bciE* and *acsF* from *Rba. sphaeroides*. (*A*) Diagram depicting integration of *bciE* and *acsF* from *Rba. sphaeroides* in place of the native *acsF* in *Rvi. gelatinosus* (*Upper*), and confirmation by colony PCR (*Lower*). (*B*) HPLC analysis of pigments extracted from *Rvi. gelatinosus* strains, extracted from the same number of cells of each strain except for the Δ*bchE* strain, which had a much greater BChl *a* content compared with the other strains. (*Inset*) Retention times and Soret/Q_y maxima of peaks were used to identify BChl *a*.klj.

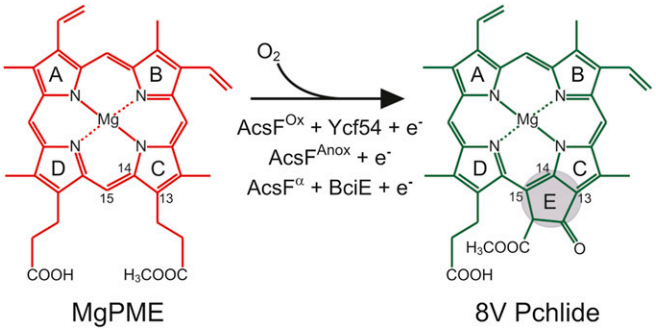

Fig. S5. Current status of known components of the oxygen-dependent cyclase. $AcsF^{\alpha}$, $AcsF^{Anox}$, and $AcsF^{Ox}$ represent AcsF proteins from Alphaproteobacteria, anoxygenic phototrophs other than the Alphaproteobacteria, and oxygenic phototrophs, respectively. e^- denotes the electron donor to the diiron center of AcsF.

Table S1. Distribution of *acsF* and *bchE* genes among sequenced phototrophic Proteobacteria, along with the presence of orthologs of rsp_6110

Organism	GenBank no.	Group	Presence/absence of: 6110	*acsF*	*bchE*
Acidiphilium multivorum AIU301	AP012035	α1, AAP	●	●	○
Phaeospirillum molischianum DSM 120	ZP_09875400	α1, PNB	X	X	○
Rhodospirillum centenum SW	CP000613	α1, PNB	●	●	○
Rhodospirillum rubrum ATCC 11170	CP000230	α1, PNB	X	X	○
Rhodospirillum photometricum DSM 122	YP_005416037	α1, PNB	X	X	○
Ahrensia sp. strain R2A130	NZ_AEEB01000017	α2, AAP	●	●	X
Agrobacterium albertimagni AOL15	ALJF00000000	α2, AAP	●	●	○
Hoeflea phototrophica DFL43	NZ_ABIA02000022	α2, AAP	●	●	X
Labrenzia alexandrii DFL11	NZ_EQ973123.1	α2, AAP	●	●	○
Methylobacterium sp. strain 4-46	CP000943	α2, AAP	●	●	○
Methylobacterium radiotolerans	CP001001	α2, AAP	●	●	X
Methylobacterium populi BJ001	YP_001927978	α2, AAP	●	●	X
Methylobacterium extorquens AM1	YP_002966142	α2, AAP	●	●	X
Methylocella silvestris BL2	CP001280	α2, AAP	●	●	X
Bradyrhizobium sp. strain BTAi1	CP000494	α2, PNB	●	●	●
Bradyrhizobium sp. strain ORS278	CU234118	α2, PNB	●	●	●
Rhodomicrobium vannielii ATCC 17100	NC_014664	α2, PNB	X	X	○
Rhodopseudomonas palustris	Multiple	α2, PNB	●	●	○
Dinoroseobacter shibae DFL12	CP000830	α3, AAP	●	●	○
Jannaschia sp. strain CCS1	CP000264	α3, AAP	●	●	X
Loktanella vestfoldensis SKA53	AAMS01000000	α3, AAP	●	●	X
Roseobacter denitrificans Och 114	CP000362	α3, AAP	●	●	○
Roseobacter litoralis Och 149	ABIG00000000	α3, AAP	●	●	○
Roseobacter sp. strain AzwK-3b	ABCR00000000	α3, AAP	●	●	●
Roseobacter sp. strain CCS2	AAYB00000000	α3, AAP	●	●	X
Roseovarius sp. strain TM1035	ABCL00000000	α3, AAP	●	●	○
Roseovarius sp. strain 217	AAMV00000000	α3, AAP	●	●	○
Rhodobacter capsulatus SB 1003	NC_014034	α3, PNB	X	X	●
Rhodobacter sphaeroides	Multiple	α3, PNB	●	●	●
Rhodobacter sp. strain SW2	ZP_05842911	α3, PNB	●	●	●
Erythrobacter sp. strain NAP1	AAMW00000000	α4, AAP	●	●	X
Citromicrobium bathyomarinum JL354	ZP_06861151	α4, AAP	●	●	○
Sphingomonas spp.	Multiple	α4, AAP	●	●	X
Brevundimonas subvibrioides ATCC 15264	CP002102	α4, AAP	●	●	X
Rubrivivax gelatinosus IL-114	NC_017075	β, PNB	X	●	○
Rubrivivax benzoatilyticus JA2	NZ_AEWG01000000	β, PNB	X	●	○
Methyloversatilis universalis FAM5	ZP_08506871	β, PNB	X	●	X
Limnohabitans sp. strain Rim28	ALKN00000000	β, PNB	X	●	X
Limnohabitans sp. strain Rim47	ALKO00000000	β, PNB	X	●	X
Allochromatium vinosum DSM 180	NC_013851	γ, PSB	X	X	○
Ectothiorhodospira sp. strain PHS-1	NZ_AGBG01000002	γ, PSB	X	X	○
Halorhodospira halophila SL1	CP000544	γ, PSB	X	X	●
Marichromatium purpuratum 984	NZ_AFWU01000001	γ, PSB	X	X	○
Thiocapsa marina 5811	NZ_AFWV01000003	γ, PSB	X	X	●○
Thiocystis violascens DSM198	AGFC00000000	γ, PSB	X	X	○
Thioflavicoccus mobilis 8321	NC_019940	γ, PSB	X	X	○
Thiorhodococcus drewsii AZ1	NZ_AFWT01000007	γ, PSB	X	X	○
Thiorhodospira sibirica ATCC 700588	AGFD01000016	γ, PSB	X	X	○
Congregibacter litoralis KT71	AAOA01000014	γ, AAP	X	●	●
gamma proteobacterium NOR5-3	ZP_05125815	γ, AAP	X	●	●
Luminiphilus syltensis NOR51-B	NZ_DS999411	γ, AAP	X	●	X
marine gamma proteobacterium HTCC2080	NZ_DS999405	γ, AAP	X	●	X
gamma proteobacterium HIMB55	ZP_09691978	γ, AAP	X	●	X

Modified from (26). ●, gene present in PGC; ○, gene present outside PGC; **X**, gene absent. The red box indicates an identical pattern of presence/absence of orthologs of rsp_6110 and *acsF* among Alphaproteobacteria. AAP, aerobic anoxygenic phototroph; PNB, purple nonsulfur bacterium; PSB, purple sulfur bacterium.

Table S2. Strains and plasmids described in this study

Strain/plasmid	Genotype/characteristics	Source
E. coli		
JM109	Cloning strain for plasmid constructs	Promega
S17-1	Conjugation strain for pK18*mobsacB* constructs	(48)
Rvi. gelatinosus		
WT	IL144	S. Nagashima*
Δ*bchE*	Unmarked deletion mutant of *bchE* in WT	This study
Δ*bchE*Δ*acsF*	Unmarked deletion mutant of *acsF* in Δ*bchE*	This study
Δ*bchE*Δ*acsF*::$acsF^{Rs}$	$acsF^{Rs}$ replacement of *acsF* in Δ*bchE*	This study
Δ*bchE*Δ*acsF*::*bciE-*$acsF^{Rs}$	*acsF* replaced with rsp_6110-$acsF^{Rs}$ in Δ*bchE*	This study
Δ*bchE*Δ*acsF*::*cycI*	*cycI* replacement of *acsF* in Δ*bchE*	This study
Δ*bchE*Δ*acsF*::*cycI-ycf54*	*cycI-ycf54* replacement of *acsF* in Δ*bchE*	This study
Synechocystis		
WT	sp. PCC6803	R. Sobotka†
$acsF^{Rg+}$	$acsF^{Rg}$ and Km^R replacement of *psbAII* in WT	This study
$acsF^{Rg+}$ Δ*cycI*	Cm^R replacement of *cycI* in $acsF^{Rg+}$	This study
$acsF^{Rg+}$ Δ*cycI*Δ*ycf54*	Zeo^R replacement of central portion of *ycf54* in $acsF^{Rg+}$Δ*cycI*	This study
Δ*ycf54*	Zeo^R replacement of central portion of *ycf54* in WT	(22)
Rba. sphaeroides		
WT	2.4.1	S. Kaplan‡
Δ*bchE*Δ*ccoP*	Unmarked deletion mutant of *bchE* and *ccoP* in WT	(15)
Δ*bchE*Δ*ccoP*Δ*acsF*	Unmarked deletion mutant of *acsF* in Δ*bchE*Δ*ccoP*	(15)
Δ*bchE*Δ*ccoP*Δ6110	Unmarked deletion mutant of rsp_6110 in Δ*bchE*Δ*ccoP*	This study
Plasmids		
pK18*mobsacB*	Allelic exchange vector, Km^R	J. Armitage§
pK18Δ$bchE^{Rg}$	Upstream-*NdeI*-downstream of $bchE^{Rg}$ cloned into *Bam*HI/*Hin*dIII sites of pK18*mobsacB*	This study
pK18Δ$acsF^{Rg}$	Upstream-*NdeI*-downstream of $acsF^{Rg}$ cloned into *Bam*HI/*Hin*dIII sites of pK18*mobsacB*	This study
pK18Δ6110	Upstream-downstream of rsp_6110 cloned into *XbaI*/*Hin*dIII sites of pK18*mobsacB*	This study
pK18[$acsF^{Rs}$]	$acsF^{Rs}$ cloned into the *NdeI* site of pK18Δ$acsF^{Rg}$	This study
pK18[6110-$acsF^{Rs}$]	rsp_6110-$acsF^{Rs}$ cloned into the *NdeI* site of pK18Δ$acsF^{Rg}$	This study
pK18[*cycI*]	*cycI* cloned into the *NdeI* site of pK18Δ$acsF^{Rg}$	This study
pK18[*cycI-ycf54*]	*cycI-ycf54* cloned into the *NdeI* site of pK18Δ$acsF^{Rg}$	This study
pPD-FLAG	Cloning site, Km^R, flanked by *psbAII* upstream and downstream regions, Amp^R	(21)
pPD[$acsF^{Rg}$]	$acsF^{Rg}$ cloned into *NdeI*/*BglII* sites of pPD-FLAG	This study
pBBRBB-$Ppuf_{843–1200}$	Expression vector carrying the 843–1,200 region of *puf* promoter of *Rba. sphaeroides*, Km^R	(27)
pBB[6110]	rsp_6110 cloned into the *BglII*/*NotI* sites of pBBRBB-$Ppuf_{843–1200}$	This study

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Table S3. Primers used in this study

Primer	Sequence (5′-3′)
6110UpF	GCTCTAGAGGAGCTGATCCCGCCCTTCC
6110UpR	GGAGAGCCCTCCGGCCGGCGCGTTCATGGGGGTTCCCTTCTCTTGG
6110DownF	CCAAGAGAAGGGAACCCCCATGAACGCGCCGGCCGGAGGGCTCTCC
6110DownR	GCAAGCTTCCCAGGTTCACCGCCACGCC
6110CheckF	GCCCCGGAGCGACAAGGAC
6110CheckR	GTATTTCTTGGCCTTGGTCAGG
6110F_NdeI	GAGTCTCATATGGGTCTGTTCACGAAACAAGCG
6110F_BglII	GGCAGATCTATGGGTCTGTTCACGAAACAAGCGGAA
6110R_NotI	TCTGCGGCCGCTCACAGCGTCACCTGCTCGGAGAA
0294F_NdeI	CCAGTACATATGTGAACGCGCCGGCCGGAGG
0294R_NdeI	CCAGTACATATGTCAATAGCTCGGCTCCAGTCGG
45840UpF	CTAGGTCAAGTAGGATCCTCATGCCGGCGGCGATCATG
45840UpR	CTAGGTCAAGTACATATGGGAAACGGCTCCTCGCGATTC
45840DownF	CTAGGTCAAGTACATATGCGACGGCTGGGTCACGATGC
45840DownR	CTAGGTCAAGTAAAGCTTTGCCGGTGTAGAAGTCGCACGC
45840CheckF	TAGCCGCCGACCATGCCGA
45840CheckR	GCGGTGCACCAGCACCGTGA
33550UpF	GAGTCTGGATCCCTGCATGAGCGACAACGCGTC
33550UpR	GAGTCTCATATGGAGGGTCTCCGTGGTGTGTCA
33550DownF	GAGTCTCATATGAAGCGAGGACAGGATGCTGAGC
33550DownR	GAGTCTAAGCTTGGAACTCCTCGCTCAGGTTGCG
33550CheckF	GAACGTTTGCCGGACACGGT
33550CheckR	ACGAGGTACTTCAGGTGCTCC
33550F_NdeI	GAGTCTCATATGCTCGCGACCCCGACGATCG
33550R_BamHI	GAGTCTGGATCCTCACCATGCCGGGGCCATG
1214UpF	GCCGATCCGGTTAACCTAGGCA
1214UpR	ATATCCAGTGATTTTTTTCTCCATAGAGTTGTTTAAAATAGTTTCC
1214UpCmF	GGAAACTATTTTAAACAACTCTATGGAGAAAAAAATCACTGGATAT
1214DownCmR	GGTGATCCAGCGGAAGACAACCTTACGCCCCGCCCTGC
1214DownF	GCAGGGCGGGGCGTAAGGTTGTCTTCCGCTGGATCACC
1214DownR	TGGAGTTGTTGGGAGAGTTCGGTC
1214F_NdeI	GGAATTCCATATGGTTAATACCCTCGAAAAGCCCG
1214R_NdeI	GGAATTCCATATGTTAGCGCACAGCTCCAGCCA
1214RBS1780F	GTTGGCTGGAGCTGTGCGCTAATATAGGAGCTTGGATTGTGGAAAGTTGGGCATTGACGA
1214RBS1780R	TCGTCAATGCCCAACTTTCCACAATCCAAGCTCCTATATTAGCGCACAGCTCCAGCCAAC
1780F	GTGGAAAGTTGGGCATTGACG
1780R	CTAATCCAGGGATGCAAGGGG
1780R_NdeI	GAGTCTCATATGCTAATCCAGGGATGCAAGGGG