Absence of the \( cbb_3 \) Terminal Oxidase Reveals an Active Oxygen-Dependent Cyclase Involved in Bacteriochlorophyll Biosynthesis in \textit{Rhodobacter sphaeroides}

Guangyu E. Chen, Daniel P. Canniffe,* Elizabeth C. Martin, C. Neil Hunter
Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, United Kingdom

ABSTRACT
The characteristic green color associated with chlorophyll pigments results from the formation of an isocyclic fifth ring on the tetrapyrrrole macrocycle during the biosynthesis of these important molecules. This reaction is catalyzed by two unrelated cyclase enzymes employing different chemistries. Oxygenic phototrophs such as plants and cyanobacteria utilize an oxygen-dependent enzyme, the major component of which is a diiron protein named AcsF, while BchE, an oxygen-sensitive [4Fe-4S] cluster protein, dominates in phototrophs inhabiting anoxic environments, such as the purple phototrophic bacterium \textit{Rhodobacter sphaeroides}. We identify a potential \( acsf \) in this organism and assay for activity of the encoded protein in a strain lacking \( bchE \) under various aeration regimes. Initially, cells lacking \( bchE \) did not demonstrate AcsF activity under any condition tested. However, on removal of a gene encoding a subunit of the \( cbb_3 \)-type respiratory terminal oxidase, cells cultured under regimes ranging from oxic to micro-oxic exhibited cyclase activity, confirming the activity of the oxygen-dependent enzyme in this model organism. Potential reasons for the utilization of an oxygen-dependent enzyme in anoxygenic phototrophs are discussed.

IMPORTANT
The formation of the E ring of bacteriochlorophyll pigments is the least well characterized step in their biosynthesis, remaining enigmatic for over 60 years. Two unrelated enzymes catalyze this cyclization step; \( O_2 \)-dependent and \( O_2 \)-independent forms dominate in oxygenic and anoxygenic phototrophs, respectively. We uncover the activity of an \( O_2 \)-dependent enzyme in the oxygen-purple phototrophic bacterium \textit{Rhodobacter sphaeroides}, initially by inactivation of the high-affinity terminal respiratory oxidase, cytochrome \( cbb_3 \). We propose that the \( O_2 \)-dependent form allows for the biosynthesis of a low level of bacteriochlorophyll under oxic conditions, so that a rapid initiation of photosynthetic processes is possible for this bacterium upon a reduction of oxygen tension.

The \((\text{bacterio})\text{chlorophylls} ([\text{B}]\text{Chls})\) are ubiquitous pigments employed by chlorophototrophic organisms for both light harvesting and photochemistry; thus, the elucidation of their biosynthetic pathways is of great importance. The least well characterized step in the common pathway for all of the \((\text{B})\text{Chls}\) is the formation of the isocyclic E ring, occurring via the oxidation and cyclization of the C-13 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 employing different chemistries: an oxygen-sensitive protein containing [4Fe-4S] and cobalamin prosthetic groups (1), which derives oxygen from water (2), and a diiron enzyme that requires molecular oxygen (3). Although an \textit{in vitro} assay has not yet been described, the MgPME (oxygen-independent) cyclase enzyme is believed to be encoded by a single gene, \( bchE \) (4–6), which is essential for \( BChl \) biosynthesis in bacterial phototrophs inhabiting anoxic environments. The MgPME (oxygen-dependent) cyclase (EC 1.14.13.81) catalyzes this reaction in plants and cyanobacteria (7, 8) and has been demonstrated to require both soluble and membrane-bound components (9, 10). Interestingly, the first subunit assigned to the oxygen-dependent reaction was identified in the anoxygenic purple phototrophic bacterium \textit{Rubrivivax gelatinosus} and was named AcsF (gerbic cyclization system Fe-containing subunit) (11); while the wild-type (WT) strain was able to synthesize \( BChl \) under oxic conditions, a mutant in \( acsf \) accumulated MgPME. It was subsequently discovered that \textit{Rbv. gelatinosus} contained both forms of the cyclase, conferring the ability to synthesize \( BChl \) under different oxygen concentrations (12). Orthologs of \( acsf \) have since been studied in higher plants (13, 14) and cyanobacteria (15) as well as the green nonsulfur bacterium \textit{Chloroflexus aurantiacus} (16). Recently, the distribution of \( acsf \) and \( bchE \) in the genomes of phototrophic proteobacteria has been investigated in detail (17); \( acsf \) is present in all of the aerobic anoxygenic phototrophs but is absent in the purple sulfur bacteria, while the majority of purple nonsulfur bacteria were found to contain both \( acsf \) and \( bchE \).

Unlike the situation described for \textit{Rbv. gelatinosus}, it has been well documented that \( BChl \) biosynthesis, and thus photosynthetic
membrane assembly, is repressed by the presence of oxygen in *Rhodobacter* spp. of purple phototrophic bacteria (18). Under oxic conditions, it is possible to reduce cellular BChl contents to less than 1% of those of photosynthetic cells. As *Rhodobacter sphaeroides* transitions from oxic to micro-oxic conditions, this repression is lifted and the cell develops a system of pigmented membranes that house the photosynthetic apparatus (19–21). It was demonstrated that disruption of the *Rba. sphaeroides ccoNOQP* operon, previously shown to encode the *cbb*3-type terminal oxidase in *Rhodobacter capsulatus* (22), resulted in the development of this membrane system in the presence of O2 (23). Samuel Kaplan’s laboratory studied various elements involved in the regulation of the maturation of this membrane in *Rba. sphaeroides*, showing that the rate of electron flow through the *cbb* oxidase and the redox state of the quinone pool in the photosynthetic membrane generate signals that regulate photosynthesis gene expression in this organism (24–28). An inhibitory signal generated by the *cbb*3 oxidase is transduced to the PrrBA two-component activation system, which controls the expression of most of the photosynthesis genes in response to O2, while the AppA/PpsR antirepressor/repressor system, modulated by TspO, monitors the redox state of the quinone pool. These systems, along with the assembly factors of the light-harvesting complexes (29), control the ultimate cellular levels and composition of the photosynthetic membrane.

Aside from various antirepressor/repressor systems, the assembly of photosynthetic membranes will also be influenced by the characteristics of the biosynthetic enzymes involved, in terms of their tolerance to oxygen and/or their ability to use it as a substrate. A transition from oxic conditions to oxygen-limited growth initiates a developmental process that culminates in the assembly of the photosynthesis apparatus, and the early stages have to tolerate, and even use, the available oxygen. Thus, the presence of an oxygen-dependent cyclase could be beneficial, even though later stages of assembly rely on the oxygen-sensitive BchE cyclase. Thus, it is important to find out if there is an oxygen-dependent cyclase in *Rba. sphaeroides*. In this study, we identify an ortholog of *acsF* in *Rba. sphaeroides*, rsp_0294 (Fig. 2), which resides in the photosynthesis gene cluster (29). In order to test for activity of RSP_0294 as an oxygen-dependent cyclase component, we constructed a mutant lacking *bchE* in which we were initially unable to detect BChl a. Removal of the *cbb*3 oxidase in this background resulted in the accumulation of this pigment, confirming that rsp_0294 encodes an AcsF component of the cyclase enzyme. Subsequently, we discovered that in the presence or absence of this cytochrome, the Δ*bchE* mutant accumulates Zn-BChl a, potentially due to replacement of the central magnesium ion, as the pigment is not sequestered by the light-harvesting polypeptide apparatus. The reasons for the employment of an oxygen-dependent cyclase by an anoxygenic phototroph are discussed.

**MATERIALS AND METHODS**

**Growth conditions.** *Rba. sphaeroides* strains were grown in a rotary shaker at 30°C in liquid M22+ medium (30) supplemented with 0.1% Casamino Acids. Differential aeration of cultures between oxic and micro-oxic was achieved by filling 250-ml Erlenmeyer flasks with 20 ml, 40 ml, 80 ml, and 160 ml of medium, with agitation at 150 rpm. *Escherichia coli* strains JM109 (31) and S17-1 (32) transformed with pK18mobsacB plasmids were grown in a rotary shaker at 37°C in LB me...

**FIG 1** Isocyclic ring formation catalyzed by AcsF (solid arrow) and BchE (dashed arrow). IUPAC numbering of the relevant macrocycle carbons is indicated, and the catalyzed formation of ring E is highlighted. The oxygen sources for AcsF and BchE are molecular oxygen and water, respectively. X denotes the as-yet-unassigned subunit required for the oxidative reaction.

**FIG 2** Amino acid sequence alignments of known AcsF proteins with RSP_0294. Sequences are those from *Rbv. gelatinosus* (AcsF), *Synechocystis* sp. PCC 6803 (CycI), *Chlamydomonas reinhardtii* (CRD1), and *Arabidopsis thaliana* (CHL27), aligned with RSP_0294 (0294). Conserved, highly similar, and similar residues are highlighted in black, dark gray, and light gray, respectively.
dium supplemented with 30 µg · ml⁻¹ kanamycin. All strains and plasmids used in this study are listed in Table 1.

Construction of mutants of *Rba. sphaeroides*. *Rba. sphaeroides* genes were deleted using the allelic exchange vector pK18mobacB (33). Sequences up- and downstream of target genes were amplified with the relevant UpF and UpR primers and DownF and DownR primers, respectively. Sequences of all of the primers used in the present study can be found in Table S1 in the supplemental material. The up- and downstream PCR products were fused by overlap extension PCR, digested with the relevant restriction enzymes, and ligated into cut pK18mobacB. Sequenced clones were conjugated into *Rba. sphaeroides* from *E. coli* S17-1, and transconjugants in which the clone had integrated into the genome by homologous recombination were selected on M22+ medium supplemented with kanamycin. Transconjugants that had undergone a second recombination event were then selected on M22+ supplemented with 10% (wt/vol) sucrose, lacking kanamycin. Sucrose-resistant kanamycin-sensitive colonies had excised the allelic exchange vector through the second recombination event (34). The deletion of the desired gene was confirmed by colony PCR using relevant CheckF and CheckR primers.

Whole-cell absorption spectroscopy. Cell pellets were resuspended in 60% sucrose to reduce light scattering, and absorption between 350 to 850 nm was recorded on a Cary 60 UV-Vis spectrophotometer.

Extraction of pigments. Pigments were extracted twice from cell pellets after washing in 20 mM HEPES (pH 7.2) by adding an excess of 0.2% (vol/vol) ammonia in methanol, bead beating for 30 s, and incubating on ice for 20 min (35). The extracts were then dried in a vacuum concentrator at 30°C. The dried pigments were reconstituted in 0.2% (vol/vol) ammonia in methanol-acetone, bead beating for 30 s, and incubating on ice for 20 min (35). The extracts were then dried in a vacuum concentrator at 30°C and reconstituted in a small volume of the same solvent. The extracts were clarified by centrifugation (15,000 g for 5 min at 4°C), and the supernatants were immediately analyzed on an Agilent 1200 high-performance liquid chromatography (HPLC) system.

Analysis of pigments by HPLC. BChl a species were separated on a Fortis UniverSil C18 reverse-phase column (5-µm particle size, 150 mm by 4.6 mm) using a method modified from that of van Heukelem et al. (37). Solvents A and B were 80:20 (vol/vol) methanol–500 mM ammonium acetate and 80:20 (vol/vol) methanol-acetone, respectively. Pigments were eluted at 1 ml/min at 40°C on a linear gradient of 92 to 93% solvent B over 10 min, increasing to 100% to wash the column. Elution of BChl a species was monitored by checking absorbance at 770 nm.

**RESULTS**

Deletion of *bchE* does not reveal a functional AcsF in *Rba. sphaeroides*. *Rba. gelatinosus* is able to synthesize BChl a under conditions ranging from oxic to anoxic by employing O₂-dependent and O₂-independent cyclase enzymes, respectively (11, 12). In order to determine whether a similar situation exists in *Rba. sphaeroides*, in which BChE dominates but a putative acsF gene (rps_0294) exists, a strain lacking *bchE* was constructed (see Fig. S1 in the supplemental material). The ability of the Δ*bchE* mutant to produce BChl a via AcsF under a range of oxygen tensions (see Materials and Methods) was tested by HPLC analysis of pigments extracted from cultures standardized by cell number (Fig. 3). BChl a production by the Δ*bchE* strain was not detected under any of the conditions tested (Fig. 3, profiles A to D), while the WT accumulates BChl a as expected (Fig. 3, profile E). These data suggest that a functional AcsF is not found in *Rba. sphaeroides*.

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|---------------------|
| **E. coli**       |             |                     |
| JM109             | Cloning strain for pK18mobacB constructs | Promega |
| S17-1             | Conjugation strain for pK18mobacB constructs | 32 |
| **Rba. sphaeroides** |             |                     |
| 2.4.1             | WT strain | S. Kaplan⁶ |
| Δ*bchE* mutant    | Unmarked deletion mutant of *bchE* in WT | This study |
| Δ*ccoP* mutant    | Unmarked deletion mutant of *ccoP* in WT | This study |
| Δ*bchE ΔccoP* mutant | Unmarked deletion mutant of *ccoP* in Δ*bchE* strain | This study |
| Δ*bchE ΔccoP Δrps_0294* mutant | Unmarked deletion mutant of rps_0294 in Δ*bchE ΔccoP* strain | This study |
| Plasmid pK18mobacB | Allelic exchange vector; Km⁷ | J. Armitage⁶ |

⁶ Department of Microbiology and Molecular Genetics, The University of Texas Medical School, Houston, TX.
⁷ Department of Biochemistry, University of Oxford, Oxford, United Kingdom.
Effect of the deletion of $ccoP$ in *Rba. sphaeroides*. The sub-units of the $cbb_3$-type terminal respiratory oxidase are encoded by the genes found in the $ccoNOQP$ operon, and disruption of this stretch of open reading frames (ORFs) results in the assembly of the photosynthetic architecture under oxic conditions (22, 23). The $ccoP$ gene, which encodes a membrane-bound diheme $c$-type cytochrome subunit, was deleted in the WT in order to replicate these conditions (Fig. 4A). When grown under oxic conditions achieved via high aeration, the $\Delta ccoP$ strain was visibly pigmented compared to the WT (Fig. 4B). Whole-cell absorption spectra of these strains standardized by cell number demonstrated that photosynthetic apparatus assembly was derepressed upon deletion of $ccoP$ (Fig. 4C). Pigments extracted from these standardized samples also show that removal of $ccoP$ results in greatly increased accumulation of BChl $a$ in this strain relative to the WT (Fig. 4D). This background was considered to be ideal for testing the activity of RSP_0294.

RSP_0294 activity can be detected in a strain lacking $ccoP$. In order to determine if the derepression of the BChl biosynthesis pathway in the $\Delta ccoP$ strain could reveal if RSP_0294 is an active AcsF component of the oxygen-dependent cyclase, this mutation was combined with $\Delta bchE$. The resulting $\Delta bchE \Delta ccoP$ strain was grown under the range of oxygen tensions described earlier for the $\Delta bchE$ strain, and the pigments extracted from these strains were analyzed by HPLC. Peaks corresponding to BChl $a$ were present in the traces from all of the samples (Fig. 5, profiles A to D), although the BChl $a$ extracted from the $\Delta bchE \Delta ccoP$ strain grown at the highest oxygen tensions approached the detection limit of the instrument (Fig. 5, profile D). To confirm that the cyclization reaction in this strain was dependent on the presence of RSP_0294, this ORF was deleted in the $\Delta bchE \Delta ccoP$ strain (see Fig. S2 in the supplemental material). The resulting strain, $\Delta bchE \Delta ccoP$

![FIG 3](image-url) HPLC elution profiles of pigments extracted from the $\Delta bchE$ mutant grown under differing aeration conditions. Pigments were extracted from the $\Delta bchE$ strain grown at increasing aeration (A to D) as described in Materials and Methods. Pure BChl $a$ (E) was used as a standard. Retention times and absorption spectra of peaks are used to identify BChl $a$ (inset).

![FIG 4](image-url) Construction and phenotype of the $\Delta ccoP$ strain. (A) Diagram depicting the deletion of $ccoP$ and confirmation by colony PCR (inset). (B) Illustration of differential pigmentation of WT and $\Delta ccoP$ cultures grown at high aeration. (C and D) Whole-cell absorption spectra (C) and absorption spectra of pigments (D) extracted from WT and $\Delta ccoP$ strains, standardized by cell number.
as an internal reference (38). The primer efficiency was deduced, encoding the rpoZ qRT-PCR was performed in triplicate. The housekeeping gene conditions for BChl tal RNA was isolated from cultures grown to provide the optimum /H9004 ccoP component of the enzyme. We therefore propose that rsp_0294 be detected BChl a (see peak at 9.3 min in Fig. 3 and 5), properties indicative of a bacteriochlorin pigment carrying a hydrophobic alcohol moiety, the addition of which is the last step in mature photopigment production. It has been demonstrated that an Rba. sphaeroides Tn5 mutant in a gene encoding a subunit of magnesium chelatase (bchD), the enzyme catalyzing the first committed step in BChl biosynthesis, is able to assemble photosynthetic apparatus containing Zn-BChl a, able to utilize both oxygen-dependent and oxygen-independent forms of the cyclase enzyme for the biosynthesis of BChl a. The activity of the oxygen-dependent form was revealed after the expression of genes involved in photosynthetic processes was derepressed under high aeration by the inac-
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FIG 6 HPLC elution profiles of pigments extracted in order to assign the peak at 9.3 min. (A) Pigments extracted from ΔbchE ΔccoP cells cultured in 40 ml of medium in a 250-ml Erlenmeyer flask. (B and C) Mg-BChl a extracted from WT (B) and Zn-BChl a (C), prepared as described in the text. Retention times and Soret/Qₐ maxima of peaks are used to identify BChl a species (inset).

activation of the cbb₃ terminal respiratory oxidase, when we were able to detect Mg-BChl a in a strain lacking the dominant, oxygen-sensitive enzyme. This derepression led to a >2-fold increase in the level of acsF transcript. The inactivation of the cbb₃ oxidase was initially the difference between undetectable and apparent Mg-BChl a accumulation, although the increase in acsF expression was relatively modest. Therefore, it may be that the large increase in the amount of Mg-BChl a in particular, but also Zn-BChl a, formed in the ΔbchE ΔccoP mutant was due not only to this increased expression of acsF but also to the increase in available substrate for this enzyme; in WT cells, O₂ is both the terminal electron acceptor for the cbb₃ oxidase, which it binds with high affinity, and a substrate for the oxidative cyclase; thus, the competition for O₂ in a strain lacking ccoP is greatly reduced.

In addition to cbb₃, Rba. sphaeroides also employs a lower-affinity aa₃-type terminal respiratory oxidase (26). The genes encoding the subunits of this cytochrome could also have been viable targets for disruption in order to increase the cellular level of O₂. However, proteomic work published by our laboratory has indicated that the majority of the enzymes involved in tetrapyrrole biosynthesis are found concentrated in the precursor of the mature, photosynthetic apparatus-containing intracytoplasmic membrane (43). The precursor membrane, known as UPB (upper pigmented band, migrating slower than intracytoplasmic membranes in rate zone sedimentation), was also found to contain the majority of the cbb₃ oxidase, unlike other proteins involved in photosynthetic energy transduction, which were all more abundant in the intracytoplasmic membrane. These data suggested proximity between the cbb₃ oxidase and enzymes involved in BCHl biosynthesis; thus, we chose this cytochrome as our target for disruption.

Deletion of ccoP led to the detectable accumulation of Mg-BChl a in the ΔbchE mutant, although further analysis revealed the presence of Zn-BChl a in the ΔbchE strain in both the presence and absence of ccoP. The occurrence of BCHls containing zinc rather than magnesium has been previously documented. It has been discovered that when cultured heterotrophically to late stationary phase in acidic medium, the unicellular alga Chlorella kessleri accumulates Zn-Chl a (44). Additionally, the acidophilic aerobic anoxygenic phototroph Acidiphilium rubrum assembles functional light-harvesting apparatus solely with Zn-BChl a (45).

It was subsequently shown that the magnesium chelatase enzyme of this organism catalyzed insertion of Mg₂⁺ into the pigment macrocycle (46), and thus it was proposed that the insertion of Zn follows dechelation of Mg at a later stage in the biosynthesis of the photopigment. Recently, the first phototroph identified from the phylum Acidobacteria, Chloracidobacterium thermophilum, was discovered to contain both Mg- and Zn-BChls a in its homodimeric type I photosynthetic reaction center, although the exact role of each pigment is not currently known (47). It was hypothesized that, in the absence of an active magnesium chelatase enzyme, the accumulation of Zn-BChl a in the bchD mutant of Rba. sphaeroides was due to insertion of Zn²⁺ into the macrocycle of protoporphyrin IX, catalyzed by ferrochelatase (42). The in vivo role of this enzyme is the insertion of Fe²⁺ into protoporphyrin IX during the biosynthesis of hemes, but it has been shown to chelate Zn²⁺ in vitro (48, 49). However, the strains described in this study contain a functional magnesium chelatase enzyme, and accumulation of Mg-BChl a in the ΔbchE ΔccoP strain suggests that, as in the cases of Chlorella kessleri and Acidiphilium rubrum, zinc insertion may occur after dechelation of magnesium. We propose that the high O₂ tension in the ΔbchE strains, containing or lacking ccoP, coupled with the low level of Mg- or Zn-chelated BChl a formed via the AcSF route, prevents assembly of the photosynthetic apparatus. It may be that the unbound Mg-BChl a is susceptible to dechelation, either by a spontaneous reaction or catalyzed by an as-yet-unidentified dechelatase enzyme, while bound Mg-BChl a in the photosynthetic apparatus may be effectively shielded from this process.

The presence of Zn-BChl a in the ΔbchE mutant indicates that AcSF-catalyzed formation of the Mg-chelated pigment occurs under oxic conditions, but replacement of the central metal of the unbound Mg-BChl occurs with high efficiency. Although the overall contribution to BChl a biosynthesis is small, the role of AcSF in Rba. sphaeroides may be to ensure a modest level of mature pigment in cells switching from aerobic respiration to phototrophy. BCHl biosynthesis is likely initiated at the indented regions of the cytoplasmic membrane identified as sites for preferential synthesis of BChl and photosystem apoproteins (21, 50–52). AcSF might ease the transition from oxic growth, by providing BCHl for the earliest stage of photosystem assembly, which mainly involves
the synthesis of the reaction center-light harvesting 1-PufX complex (53, 54).

We have demonstrated that *Rba. sphaeroides*, like *Rbv. gelatinosus*, is able to use both oxygen-dependent and oxygen-independent cyclases for BCHl biosynthesis. Many other purple phototrophs contain genes assigned to both enzymes, and it may be that the ability to utilize both forms of the enzyme for pigment production, or to switch between them according to the balance of oxic versus anoxic conditions, is conserved in these strains. Outside the purple bacteria, organisms containing orthologs of both *bchE* and *acsF* have been reported. The green filamentous anoxygenic phototroph *Chloroflexus aurantiacus* primarily relies on BchE for the production of BCHls, yet AcsF rather than BchE can side the purple bacteria, organisms containing orthologs of both enzymes (139). Expression of *acsF* does not change with O$_2$ tension (16). These observations led the authors to hypothesize alternative functions for AcsF in *C. aurantiacus*: the diiron protein may have evolved to play a role in electron transfer or iron transport under anoxic conditions. Conversely, the cyano bacterium *Synechocystis* sp. strain PCC 6803 relies on different AcsF proteins for Chl a biosynthesis under oxic and micro-oxic conditions but contains three orthologs of *bchE*, none of which appears to play a role in pigment production under any oxygen tension (15). Recently, cyanobacterial *bchE* orthologs from two strains of *Cyanothecae* were shown to be able to restore Bchl a biosynthesis in a *bchE* mutant of *R. capsulatus*, demonstrating activity of oxygen-independent ChlE proteins from oxygenic phototrophs for the first time (55). Boldareva-Nuijanizna *et al.* propose that *acsF* was adopted by the *bchE*-containing proto bacterium via horizontal gene transfer from cyanobacteria, in which this gene evolved (17). They suggest that acquisition after the Great Oxygenation Event in the early Proterozoic era, when the surface of the oceans became mildly oxic while deep waters remained anoxic, allowed the early purple phototrophs to adapt to these new conditions in water supporting both oxygenic and anoxygenic photosynthesis (56).

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