Absence of the *cbb*$_3$ terminal oxidase reveals an active oxygen-dependent cyclase involved in bacteriochlorophyll biosynthesis in *Rhodobacter sphaeroides*

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Running head: An O_2-dependent cyclase in *Rhodobacter sphaeroides* (50 characters)

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Abbreviations

BChl, bacteriochlorophyll; Chl, chlorophyll; MgPME, magnesium protoporphyrin IX monomethyl ester; Pchlide, 8-vinyl protochlorophyllide

ABSTRACT

The characteristic green color associated with chlorophyll pigments results from the formation of an isocyclic fifth ring on the tetrapyrrole macrocyle 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 *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 microoxic 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.

IMPORTANCE

The formation of the E ring of (bacterio)chlorophyll 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 anoxygenic purple phototrophic bacterium *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.

INTRODUCTION

The (bacterio)chlorophylls ((B)Chls) are ubiquitous pigments employed by chlorophototrophic organism 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 (B)Chls is the formation of the isocyclic E ring, occurring via the 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 employing different chemistries; an oxygen-sensitive protein containing [4Fe-4S] and cobalamin prosthetic groups [1] which derives oxygen from water [2], and an oxidative diiron enzyme which requires molecular oxygen [3]. Although an *in vitro* assay has not yet been described, the magnesium protoporphyrin IX monomethyl ester (oxygen-independent) enzyme is believed to be encoded by a single gene, *bchE* [4-6], that is essential for BChl biosynthesis in bacterial phototrophs inhabiting anoxic environments. The magnesium protoporphyrin IX monomethyl ester (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 purple phototrophic bacterium *Rubrivivax* (*Rbv*.) *gelatinosus* and was named AcsF (aerobic cyclization system Fe-containing subunit) [11]; while the WT strain was able to synthesize BChl under oxic conditions, a mutant in *acsF* accumulated MgPME. It was subsequently discovered that *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 *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 non-sulfur bacteria were found to contain both *acsF* and *bchE*.

Unlike the situation described for *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* (*Rba.*) *sphaeroides* transitions from oxic to microoxic conditions, this repression is lifted and the cell develops a system of pigmented membranes which 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 *Rba. capsulatus* [22], resulted in the development of this membrane system in the presence of O_2 [23]. Samuel Kaplan's laboratory studied various elements involved in the regulation the maturation of this membrane in *Rba. sphaeroides*, showing that the rate of electron flow through the cbb_3 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 O_2, 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, Δ*bchE* 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. 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 the dark 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 microoxic 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.

E. coli strains JM109 [31] and S17-1 [32] transformed with pK18*mobsacB* plasmids were grown in a rotary shaker at 37°C in LB medium supplemented with 30 $\mu g \cdot ml^{-1}$ 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 pK18*mobsacB* [33]. Sequences up- and down-stream of target genes were

amplified with the relevant UpF and UpR, and DownF and DownR primers, respectively. Sequences of all of the primers used in the present study can be found in **Table S1**. The up- and down-stream PCR products were fused by overlap extension PCR, digested with the relevant restriction enzymes and ligated into cut pK18*mobsacB*. 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% (w/v) 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 350-850 nm were 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% (v/v) 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 and reconstituted in a small volume of the same solvent. The extracts were clarified by centrifugation (15000 *g* for 5 min at 4°C) and the supernatants were immediately analyzed on an Agilent 1200 HPLC system.

Preparation of Zn-BChl *a*. Zn-BChl *a* was prepared from Mg-BChl *a* extracted from WT *Rba. sphaeroides* using a method modified from one previously described [36]. Briefly, one volume of extracted Mg-BChl *a* in methanol was mixed with an excess of anhydrous zinc acetate, 50 mM sodium ascorbate and 6 volumes of glacial acetic acid. The mixture was incubated in boiling water for 2 hours in an uncapped 1.5 ml Eppendorf tube. The remaining supernatant was transferred to a new tube, clarified by centrifugation (15000 *g* for 5 min at 4°C) and then dried in a vacuum

concentrator at 30°C. The dried pigments were reconstituted in 0.2% (v/v) ammonia in methanol and clarified by centrifugation as above before analysis by HPLC.

Analysis of pigments by HPLC. BChl *a* species were separated on a Fortis UniverSil C18 reverse-phase column [5 µm particle size, 150 mm × 4.6 mm] using a method modified from that of van Heukelem *et al*. [37]. Solvents A and B were 80:20 (v/v) methanol/500 mM ammonium acetate and 80:20 (v/v) methanol/acetone respectively. Pigments were eluted at 1 ml/min at 40°C on a linear gradient of 92–93% 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.

RNA isolation and quantitative Real Time-PCR (qRT-PCR). *Rba. sphaeroides* cultures were grown in 40 ml of medium in 250 ml Erlenmeyer flasks, shaken at 150 rpm, which represents intermediate oxygenation conditions suitable for BChl *a* production. Total RNA was isolated at mid-exponential growth phase using the RNeasy Protect Bacteria Mini Kit (Qiagen). The cell disruption was performed by treatment with 10 mg/ml lysozyme for 30 min at room temperature with constant shaking. The isolated RNA was treated with the TURBO DNA-*free*™ Kit (Ambion) to eliminate genomic DNA contamination. One microgram of RNA was used for reverse transcription using the SensiFAST™ cDNA Synthesis Kit (Bioline) according to the manufacturer's instructions. No-RT controls were included for RNA samples by omitting the reverse transcriptase in the reaction.

Gene expression levels were analyzed by qRT-PCR using the SensiFAST™ SYBR Lo-ROX Kit (Bioline) with a Stratagene Mx3005P system (Agilent). Primers RT0294F/RT0294R were used to detect RSP_0294 transcripts and RTrpoZF/RTrpoZR were used to detect *rpoZ* transcripts which served as an internal reference [38]. The qRT-PCR reactions were set up in a 20 µl volume containing 10 µl of 2x SensiFAST™ SYBR Lo-ROX mix, 0.4 µM primers and 6.25 ng cDNA template. The primer efficiency was determined using ten-fold serial dilutions of genomic DNA from *Rba. sphaeroides*. No-RT controls and no-template controls were also included. The qRT-PCR conditions

were as follows: 3 min at 95 °C, 40 cycles of 5 s at 95 °C and 30 s at 60 °C, and followed by melting curve analysis. The relative expression ratios were calculated and statistically analyzed using the REST© software [39] based on the Pfaffl method [40].

RESULTS

Deletion of *bchE* does not reveal a functional AcsF in *Rba. sphaeroides*. *Rbv. gelatinosus* is able to synthesize BChl *a* under conditions ranging from oxic to anoxic by employing O_2-dependent and O_2-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 (rsp_0294) exists, a strain lacking *bchE* was constructed (**Fig. S1**). The ability of Δ*bchE* to produce BChl *a* via AcsF under a range of oxygen tensions (see Materials & Methods) was tested by HPLC analysis of pigments extracted from cultures standardized by cell number (**Fig. 3**). BChl *a* production by Δ*bchE* was not detected under any of the conditions tested (**Fig. 3A-D**), while the WT accumulates BChl *a* as expected (**Fig. 3E**). These data suggest that a functional AcsF is not found in *Rba. sphaeroides*.

Effect of the deletion of *ccoP* in *Rba. sphaeroides*. The subunits of the cbb_3-type terminal respiratory oxidase are encoded by the genes found in the *ccoNOQP* operon, and disruption of this stretch of 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 Δ*ccoP* strain was visibly pigmented when 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 determined in a strain lacking *ccoP*. In order to determine if the derepression of the BChl biosynthesis pathway in the Δ*ccoP* strain could reveal if RSP_0294 is an active AcsF component of the oxygen-dependent cyclase, this mutation was combined with Δ*bchE*. The resulting Δ*bchE*Δ*ccoP* strain was grown under the range of oxygen tensions described earlier for Δ*bchE*, 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. 5A-D**), although the BChl *a* extracted from Δ*bchE*Δ*ccoP* grown at the highest oxygen tensions approached the detection limit of the instrument (**Fig. 5D**). To confirm that the cyclization reaction in this strain was dependent on the presence of RSP_0294, this ORF was deleted in Δ*bchE*Δ*ccoP* (**Fig. S2**). The resulting strain, Δ*bchE*Δ*ccoP*Δrsp_0294, was again cultured under the previously described oxygen tensions. Extracts from each culture contained no detectable BChl *a* (**Fig. 5E**). These data confirm that oxygen-dependent cyclase activity in *Rba. sphaeroides* is reliant on the presence of RSP_0294, and that this protein is the active AcsF component of the enzyme. We therefore propose that rsp_0294 be reassigned as *acsF*.

Deletion of *ccoP* slightly increases the expression level of *acsF*. The expression levels of *acsF* in WT, Δ*bchE* and Δ*bchE*Δ*ccoP* strains of *Rba. sphaeroides* were analyzed by qRT-PCR. Total RNA was isolated from cultures grown to provide the optimum conditions for BChl *a* production based on the HPLC results. Each qRT-PCR reaction was performed in triplicate. The housekeeping gene *rpoZ* encoding the ω-subunit of RNA polymerase was included as an internal reference [38]. The primer efficiency was deduced from a standard curve generated by using genomic DNA as a PCR template in a series of ten-fold dilutions. These efficiencies were 99.09% for *acsF* and 97.35% for *rpoZ*. The primer specificity and the absence of primer dimers were confirmed by melting curve analysis. By giving the WT a value of 1, the relative expression ratio of *acsF* was calculated using the threshold cycle deviation between a mutant strain and WT, with primer efficiency correction and normalisation to the internal reference gene *rpoZ*. The Pair Wise Fixed Reallocation Randomisation

Test© was performed to test whether there was a significant difference between the described mutants and WT. As shown in **Table 2**, the expression level of *acsF* in Δ*bchE* was not significantly different to WT (P value=0.1). However, *acsF* expression was increased by a factor of 2.293 in Δ*bchE*Δ*ccoP*, significant (P value < 0.05) when compared to WT. Thus, deletion of *ccoP* results in increased expression of *acsF*; this result agrees with data indicating that the cbb_3-type cytochrome *c* oxidase, encoded by the *ccoNOQP* operon, can generate an inhibitory signal to repress photosynthesis gene expression in *Rba. sphaeroides* [23].

Zn-BChl *a* accumulates in Δ*bchE* mutants of *Rba. sphaeroides.* In both the presence and absence of *ccoP*, the Δ*bchE* mutant accumulates a pigment that can be detected by absorbance at 770 nm and has a retention time longer than that of BChl *a* (see peak at 9.3 min in **Figs. 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 a *Rba. sphaeroides* Tn*5* mutant in a gene encoding a subunit of magnesium chelatase (*bchD*), the enzyme catalyzing the first committed step in (B)Chl biosynthesis, is able to assemble photosynthetic apparatus containing Zn-BChl *a* [41,42], indicating that the BChl biosynthetic enzymes demonstrate plasticity with regard to the divalent metal within the pigment macrocycle. To determine whether the pigment accumulated in the Δ*bchE* strains was Zn-BChl *a*, this pigment was prepared from Mg-containing BChl *a* extracted from WT *Rba. sphaeroides* via an acid reflux method described previously by Hartwich *et al*. [36] (see Materials & Methods). The retention time and absorption spectrum of the prepared Zn-BChl *a* were identical to those of the 9.3 min peak in the pigments extracted from highly aerated Δ*bchE*Δ*ccoP* (**Fig. 6**). The longer retention time, as well as blue-shifts in both the Soret and Q_y absorption bands of Zn-BChl *a*, when compared to Mg-BChl *a*, are in agreement with published data [42].

DISCUSSION

Here we demonstrate that the model purple phototrophic bacterium *Rba. sphaeroides* is 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 inactivation of the *cbb*$_3$ 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 more than two-fold increase in the level of *acsF* transcript. The inactivation of the *cbb*$_3$ 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 Δ*bchE*Δ*ccoP* was due not only to this increased expression of *acsF*, but also the increase in available substrate for this enzyme; in WT cells O_2 is both the terminal electron acceptor for the *cbb*$_3$ oxidase, which it binds with high affinity, and a substrate for the oxidative cyclase, thus the competition for O_2 in a strain lacking *ccoP* is greatly reduced.

In addition to *cbb*$_3$, *Rba. sphaeroides* also employs a lower affinity *aa*$_3$-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 O_2. 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 *cbb*$_3$ oxidase, unlike other proteins involved in photosynthetic energy transduction which were all more abundant in the intracytoplasmic membrane. These data suggested proximity between the *cbb*$_3$ 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 Δ*bchE*, although further analysis revealed the presence of Zn-BChl *a* in Δ*bchE* in both the presence and absence of *ccoP.* The occurrence of (B)Chls containing zinc rather than magnesium have 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 Mg^{2+} into the pigment macrocycle [46], thus it was proposed that the insertion of Zn follows de-chelation 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 Zn^{2+} into the macrocycle of protoporphyrin IX, catalyzed by ferrochelatase [42]. The *in vivo* role of this enzyme is the insertion of Fe^{2+} into protoporphyrin IX during the biosynthesis of hemes, but it has been shown to chelate Zn^{2+} *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 Δ*bchE*Δ*ccoP* strain suggests that, as in the cases of *Chlorella kessleri* and *Acidiphilium rubrum*, zinc insertion may occur after de-chelation of magnesium. We propose that the high O_2 tension in the Δ*bchE* 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 de-chelation, 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 Δ*bchE* 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 centre-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 vs anoxic conditions, is conserved in these strains. Outside of 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 been detected in the specialized chlorosome antenna under anoxic conditions, and unlike *bchE*, 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 cyanobacterium *Synechocystis* sp. PCC 6803 relies on different AcsF proteins for Chl *a* biosynthesis under oxic and microoxic conditions, but contains three orthologs of *bchE*, none of which appears to play a role in pigment production under any oxygen tensions [15]. Recently, cyanobacterial *bchE* orthologs from two strains of *Cyanothece* were shown to be able to restore BChl *a* biosynthesis in a *bchE* mutant of *Rba. capsulatus*, demonstrating activity of

oxygen-independent ChlE proteins from oxygenic phototrophs for the first time [55]. Boldareva-Nuianzina *et al*. propose that *acsF* was adopted by the *bchE*-containing proteobacteria 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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FIGURES, TABLES & LEGENDS

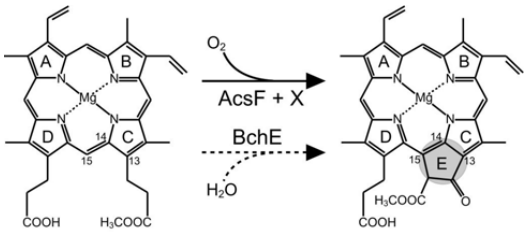

Fig. 1. Isocyclic ring formation catalyzed by AcsF (solid arrow) and BchE (dashed arrow). IUPAC numbering of the relevant macrocycle carbons are indicated and the catalyzed formation of the ring E is highlighted. The oxygen sources for AcsF and BchE are molecular oxygen and water, respectively. X denotes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

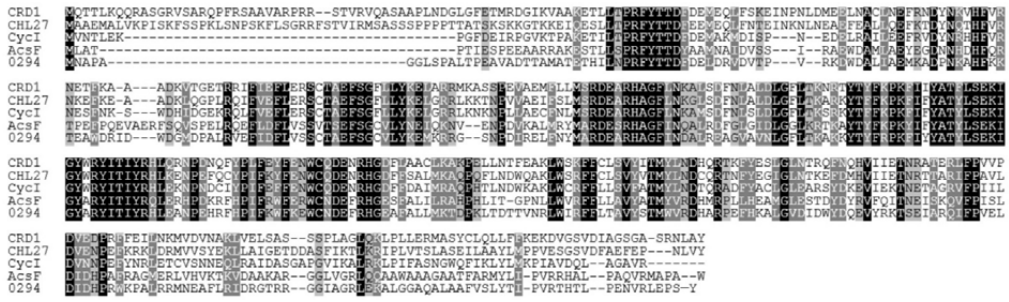

Fig. 2. Amino acid sequence alignments of known AcsF proteins with RSP_0294

Sequences are those from *Chlamydomonas reinhardtii* (CRD1), *Arabidopsis thaliana* (CHL27), *Synechocystis* sp. PCC 6803 (CycI) and *Rbv. gelatinosus* (AcsF), aligned with Rsp_0294 (0294). Conserved, highly similar and similar residues are highlighted in black, dark grey and light grey, respectively.

503

Strain/Plasmid	Genotype/characteristics	Source/Reference
E. coli		
JM109	Cloning strain for pK18*mobsacB* constructs	Promega
S17-1	Conjugation strain for pK18*mobsacB* constructs	[32]
Rba. sphaeroides		
WT	2.4.1	S. Kaplan*
Δ*bchE*	Unmarked deletion mutant of *bchE* in WT	This study
Δ*ccoP*	Unmarked deletion mutant of *ccoP* in WT	This study
Δ*bchE*Δ*ccoP*	Unmarked deletion mutant of *ccoP* in Δ*bchE*	This study
Δ*bchE*Δ*ccoP*Δrsp_0294	Unmarked deletion mutant of rsp_0294 in Δ*bchE*Δ*ccoP*	This study
Plasmid		
pK18*mobsacB*	Allelic exchange vector, Km^R	J. Armitage †

504 **Table 1. List of strains and plasmids described in this study**

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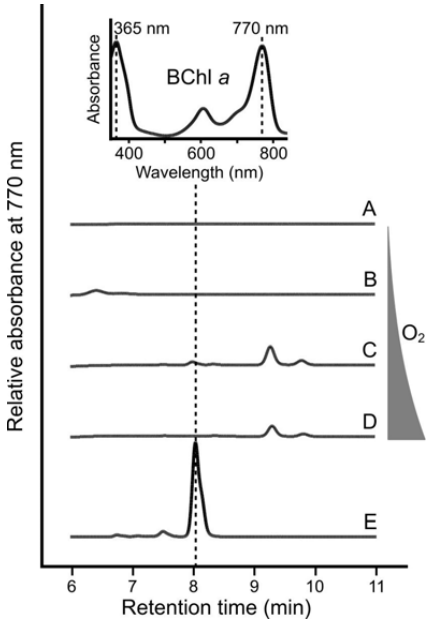
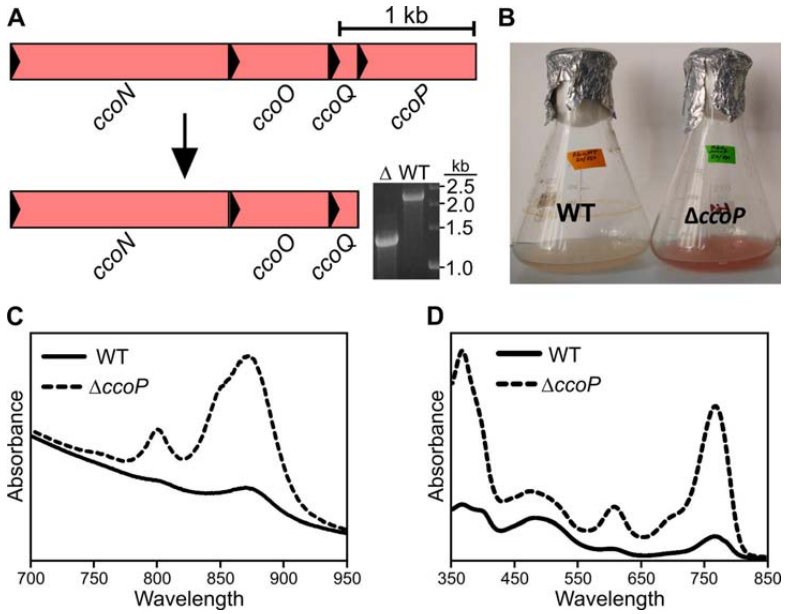


Fig. 3. HPLC elution profiles of pigments extracted from Δ*bchE* grown under differing aeration

Pigments were extracted from Δ*bchE* grown at increasing aeration (A-D) as described in the Materials and Methods section. Pure BChl *a* (E) was used as a standard. Retention times and absorption spectra of peaks are used to identify BChl *a* (inset).

514



515

516 **Fig. 4. Construction and phenotype of Δ*ccoP***

517 (A) Diagram depicting the deletion of *ccoP* and confirmation by colony PCR (inset). (B) Illustration

518 of differential pigmentation of WT and Δ*ccoP* cultures grown at high aeration. (C) Whole-cell

519 absorption spectra, and (D) absorption spectra of pigments extracted from WT and Δ*ccoP* strains,

520 standardized by cell number.

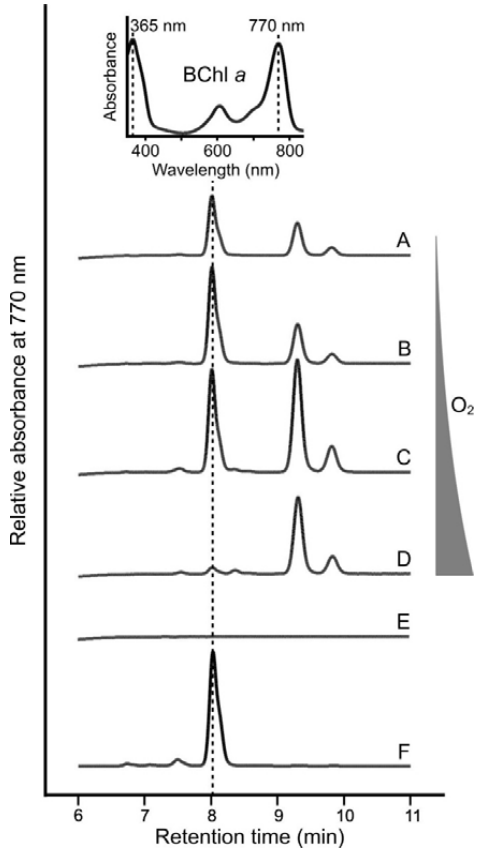


Fig. 5. HPLC elution profiles of pigments extracted from strains lacking *ccoP* grown under differing aeration

Pigments were extracted from Δ*bchE*Δ*ccoP* grown at increasing aeration (A-D) as described in the Materials and Methods section. Trace (E) represents a typical elution profile from extracts of Δ*bchE*Δ*ccoP*Δrsp_0294 cultured under all tested conditions. Pure BChl *a* (F) was used as a standard. Retention times and absorption spectra of peaks are used to identify BChl *a* (inset).

529

Strain	Expression level	95% confidence interval	P(H1)[a]
WT	1	N/A	N/A
Δ*bchE*	1.202	1.004 - 1.470	0.1
Δ*bchE*Δ*ccoP*	2.293	1.839 - 3.247	0.017

530 **Table 2. Expression levels of *acsF* in described strains determined by qRT-PCR**

531 [a] P(H1) represents the probability of the alternative hypothesis that the difference between a

532 mutant and WT is due only to chance. N/A, not applicable.

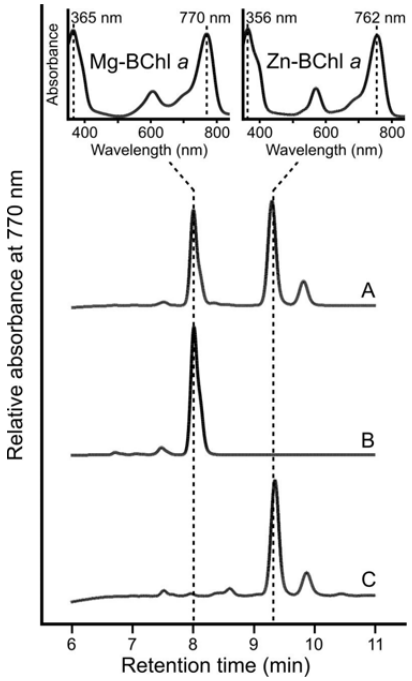


Fig. 6. HPLC elution profiles of pigments extracted in order to assign the peak at 9.3 min

Pigments extracted from Δ*bchE*Δ*ccoP* (A), cultured in 40 ml of medium in a 250 ml Erlenmeyer flask. (B) Mg-BChl *a* extracted from WT, and (C) Zn-BChl *a*, prepared as described in the text. Retention times and Soret/Q_y maxima of peaks are used to identify BChl *a* species (inset).