

GENETICS

Complete enzyme set for chlorophyll biosynthesis in *Escherichia coli*

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Chlorophylls are essential cofactors for photosynthesis, which sustains global food chains and oxygen production. Billions of tons of chlorophylls are synthesized annually, yet full understanding of chlorophyll biosynthesis has been hindered by the lack of characterization of the Mg–protoporphyrin IX monomethyl ester oxidative cyclase step, which confers the distinctive green color of these pigments. We demonstrate cyclase activity using heterologously expressed enzyme. Next, we assemble a genetic module that encodes the complete chlorophyll biosynthetic pathway and show that it functions in *Escherichia coli*. Expression of 12 genes converts endogenous protoporphyrin IX into chlorophyll a, turning *E. coli* cells green. Our results delineate a minimum set of enzymes required to make chlorophyll and establish a platform for engineering photosynthesis in a heterotrophic model organism.

INTRODUCTION

Chlorophylls (Chls) underpin photosynthesis, which generates the oxygen that supports all complex life on Earth and the reducing potential to fix carbon dioxide as carbohydrates, the ultimate source of all the organic compounds required for life on Earth. The annual production of Chls, on land and in the oceans, is on the scale of billions of tons, yet the enzyme components of the biosynthesis pathway have neither been fully determined nor assembled to define the minimal set of genes required to make Chl. The Chl biosynthetic pathway (Fig. 1A) is a branch of tetrapyrrole biosynthesis, and it begins with protoporphyrin IX (PPIX), which is also the precursor for heme biosynthesis. Most life forms, including those able to photosynthesize, make PPIX and convert it to heme by inserting Fe²⁺ into the porphyrin macrocycle. Heme is a cofactor for respiratory proteins, and it is also converted into bilins, linear tetrapyrroles that are used as light-harvesting pigments in cyanobacteria.

Chl biosynthesis is initiated when the magnesium chelatase enzyme complex (ChlIDH, Gun4) inserts Mg²⁺ into PPIX, so the Mg²⁺/Fe²⁺ Chl/heme branchpoint in photosynthetic bacteria, algae, and plants requires fine control to ensure that the correct amounts of hemes, bilins, and Chls are produced (1). Following the production of Mg–PPIX (MgP), six more enzymatic steps culminate in the production of Chl a, the ubiquitous pigment of oxygenic photosynthesis (see Fig. 1A). The MgP methyltransferase (ChlM) produces the substrate for the MgP monomethyl ester (MgPME) cyclase (AcsF), which produces 3,8-divinyl protochlorophyllide a (DV PChlide a); this intermediate has acquired a fifth ring that imparts the green color characteristic of Chls. Protochlorophyllide oxidoreductase (POR) produces 3,8-divinyl chlorophyllide a (DV Chlide a), which is reduced by divinyl reductase (DVR) and then esterified with geranylgeranyl pyrophosphate (GGPP) by Chl synthase (ChlG) to produce GG–Chl a. Finally, the reduction of the GG “tail,” catalyzed by GG reductase (ChlP), completes the pathway, and Chl a is produced. At this point, the pigment is hydrophobic and enters

the membrane-bound assembly pathway for photosynthetic complexes, where it engages with the Sec/YidC-assembly machinery that coordinates the cotranslational insertion of nascent photosystem polypeptides with pigment production (2). Many steps of the Chl biosynthesis pathway have been studied individually in mutant strains of phototrophs, and some of the early steps have been characterized to define kinetic parameters (3), but it is not known whether these enzymes are sufficient to assemble the pathway and convert PPIX to Chl a. Apart from the global significance of Chl biosynthesis, the bottom-up construction of this pathway represents the first step in reprogramming a heterotrophic bacterium for growth under a variety of predetermined conditions of light intensity and wavelength. This metabolic engineering, which would also require genes encoding photosynthetic apoproteins, not only addresses important biological problems such as the concept of the minimal amount of genetic information required for photosynthetic life but also lays the groundwork for engineering cell factories with light-powered metabolism.

RESULTS AND DISCUSSION

In vivo cyclase activity of heterologously expressed AcsF

From the outset, the major obstacle for constructing a complete Chl biosynthetic pathway was the cyclase step, the activity of which has never been demonstrated using a recombinant enzyme. There are two mechanistically unrelated cyclases: an oxygen-sensitive, radical-SAM enzyme with a [4Fe-4S] cluster encoded by the *chlI* gene in most anoxygenic phototrophic bacteria (4) and an oxygen-dependent di-iron enzyme in some purple proteobacteria, as well as cyanobacteria, algae, and higher plants (5). We previously delineated three distinct classes of the oxygen-dependent cyclase in terms of subunit composition (6); one class comprises only one known subunit, AcsF, and the other two cyclases require an additional subunit, either BciE or Ycf54, for activity. Studies on barley mutants (7) indicated that additional subunits were required to form an active cyclase complex, and substantial effort had been made to identify a missing component. To verify that AcsF is the sole cyclase component, we transferred the *chlI* gene (8) from one purple bacterial phototroph, *Rhodospirillum rubrum* (9), to another that lacks the oxygen-dependent cyclase, *Escherichia coli* (10). The *chlI* gene encoding the oxygen-sensitive cyclase was then deleted from the recipient (fig. S1A), as was the *chlP* gene encoding a subunit of the

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oxygen scavenging O_2 oxidase in *R. rubrum* (fig. S1B). This latter manipulation should increase the intracellular oxygen concentration and allow the foreign AcsF cyclase to function. Bacteriochlorophyll biosynthesis and assembly of photosynthetic complexes were abolished in the Δhcr mutant, which, when harboring the pBB[*hcr*] plasmid-containing *R. rubrum*, regained nearly wild-type levels of pigmentation (Fig. 2, A and B).

To demonstrate AcsF activity in a nonphotosynthetic bacterium, we conducted an in vivo cyclase assay with an *R. rubrum* strain expressing the

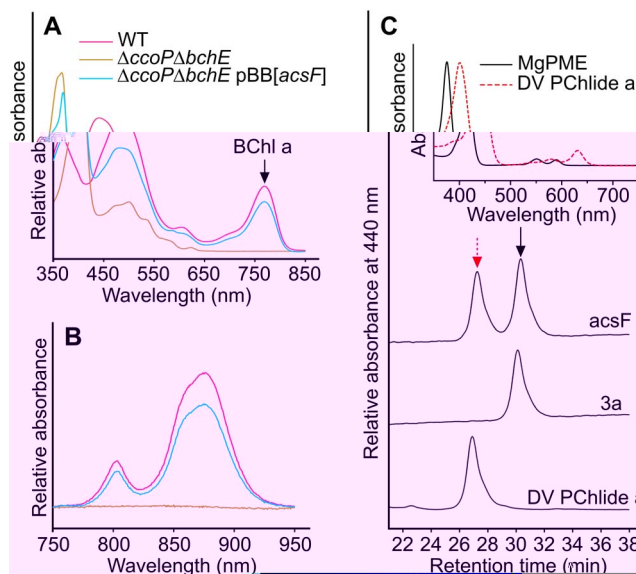


Fig. 2. In vivo cyclase activity of heterologously expressed AcsF. (A and B) The cyclase activity is linked to the (A) production of bacteriochlorophyll a (BChl a) and (B) assembly of photosynthetic complexes in *R. rubrum*. (C) HPLC analysis of pigment methanol extracts from in vivo cyclase assays conducted with *E. coli* strains harboring either the pET3a (3a) or pET3a- F(acsF) plasmid. *E. coli* cultures were grown to an optical density at 600 nm (OD_{600}) of 0.4 to 0.6 and then induced with isopropyl- β -D-thiogalactopyranoside (IPTG) and incubated with MgPME.

Assembly of the Chl biosynthesis pathway in *E. coli*

After demonstrating the in vivo cyclase activity of the *E. coli* AcsF, we tested the known components of Chl biosynthesis by engineering the whole pathway (Fig. 1A) in the nonpigmented enteric bacterium *E. coli*. Genes encoding enzymes that mediate the six enzymatic steps of the Chl biosynthesis pathway were assembled into one operon (Fig. 1B), starting from the conversion of PPIX to MgP. We used the genes for Chl biosynthesis from the cyanobacterium *Spirulina* sp. PCC 6803 (hereafter referred to as *S. sp.*), except for the cyclase-encoding gene *acsF*, which was from *R. rubrum*. The *gcpA* gene, encoding the GGPP synthase, was also included to modify the nonmevalonate pathway in *E. coli* to produce GGPP, a substrate for Chl synthase. To ensure sufficient isopentenyl PP for GGPP production, we overexpressed the native *ispA* gene, encoding one of the rate-limiting steps of the nonmevalonate pathway (1). A sequence encoding the *BoWSCP* (var. *BoWSCP*) water-soluble Chl protein (BoWSCP) (10) was included, in an attempt to sequester the hydrophobic and phototoxic Chl molecules produced by this engineered pathway. All genes used are listed in table S1. The progressively larger constructs depicted in Fig. 1B represent an increasingly complete pathway and contain genes from *S. sp.*, then to *R. rubrum*, to *E. coli*, and finally to *S. sp.*; they were named IM, IA, ID, and IG, respectively. In addition, the *acsF* cyclase-encoding genes *acsF* and *acsF* were cloned into the IM plasmid, resulting in the IM-4 construct (fig. S3).

The *E. coli* C43(DE3) strain (11), a strain effective for expression of toxic proteins, was used as the host. Single transformations of *E. coli* C43 (DE3) yielded the IM, IA, ID, IG, and IM-4 strains; two and three sequential transformations were conducted to obtain the DE/IG and DE/BoP/IG strains, respectively. The resulting strains were assayed for their capacities to synthesize Chl intermediates at various stages and

the final product, Chl a (see Materials and Methods). Pigments were extracted from the harvested *E. coli* cells using methanol and analyzed by HPLC with elution profiles monitored by absorbance at 416, 440, and 665 nm (Fig. 3, B to E; see figs. S3 to S5 for additional controls).

Control strain 3a, containing the empty pET3a vector, did not accumulate any Chl intermediates (Fig. 3). MgPME was produced in the IM strain (Fig. 3B), and the IA strain accumulated DV PChlide a (Fig. 3C), as did the IM-4 strain containing the *acsF* genes (12-1) from the cyanobacterium *S. sp.* (fig. S3). On the basis of retention times and positions of Soret absorption bands, the pigments in the ID strain were identified as monovinyl (MV) PChlide a and MV Chlide a (Fig. 3D). The trace level of MV Chlide a in the dark sample was likely produced by unavoidable light exposure during experimental procedures. The increased level of MV Chlide a and substantial decrease in MV PChlide a upon light treatment demonstrate that the ID strain harbors an active light-dependent POR enzyme and show that we have built a biosynthetic pathway that converts PPIX to MV Chlide a. Further biosynthetic steps (Fig. 3E) do not alter the absorption properties of the pigment, so HPLC was used to assess the completion of the pathway, which relies on the availability of GGPP to esterify MV Chlide a. The IG strain accumulated a new pigment (retention time, 46.9 min; Fig. 3E), which is much more hydrophobic than MV Chlide a (retention time, 20.2 min) and likely to be Chl a esterified with a farnesyl moiety; the immediate precursor of GGPP, farnesyl PP, is produced in *E. coli*, and Chl synthase has been reported to be able to use farnesyl PP as a substrate (1). The combination of the DE and IG plasmids enables *E. coli* to produce GG-Chl a. Three successive reductions of the GG tail, catalyzed by the GG reductase ChlP, complete the Chl pathway. The noticeable green color of the DE/BoP/IG strain and identification of Chl a by HPLC and liquid chromatography-mass spectrometry (LC-MS; Fig. 3E and fig. S6) demonstrate the successful assembly of the Chl biosynthetic pathway in *E. coli*. We estimate that the productivity of Chl a in the DE/BoP/IG strain under the tested conditions was ~1000 molecules per cell, equivalent to a concentration of ~1 μ M in *E. coli* cells.

Finally, the pigmentation of the engineered *E. coli* cells was imaged at the single-cell level by structured illumination microscopy (SIM) (1) and fluorescence spectroscopy. Figure 4A demonstrates that newly synthesized Chl a molecules are exclusively localized in the native cytoplasmic membrane [with an in vivo emission maximum at 676 nm (Fig. 4H)] rather than in the cytosolic recombinant BoWSCP (see fig. S7 for immunodetection of the protein), a natural scavenger of Chls (1). In plants, cyanobacteria, and algae, the destination of Chls is a series of transmembrane multisubunit complexes, the assembly pathways of which are under active investigation in many laboratories (1-21). BoWSCP was included here because it represents a relatively simple, cytosolic target for binding newly synthesized Chls. Previously, we have shown that the phototrophic bacterium *R. rubrum*, which normally makes bacteriochlorophyll, can be engineered to synthesize Chl a, which was subsequently assembled in vivo into the BoWSCP (10). Although this protein is synthesized by *E. coli* (fig. S7), evidence for Chl binding was inconclusive because of the low levels of BoWSCP synthesized. The factors that determine in vivo assembly of Chl into water-soluble and membrane-bound complexes in *E. coli* require further study.

Concluding remarks

This research completes our understanding of how the tetrapyrrole pigments of life, namely, siroheme (22), hemes (23, 24), bilins (2, 2), vitamin B₁₂ (2), coenzyme F₄₃₀ (2, 2), and now Chls, are synthesized from a common template. By identifying the elusive oxidative ring

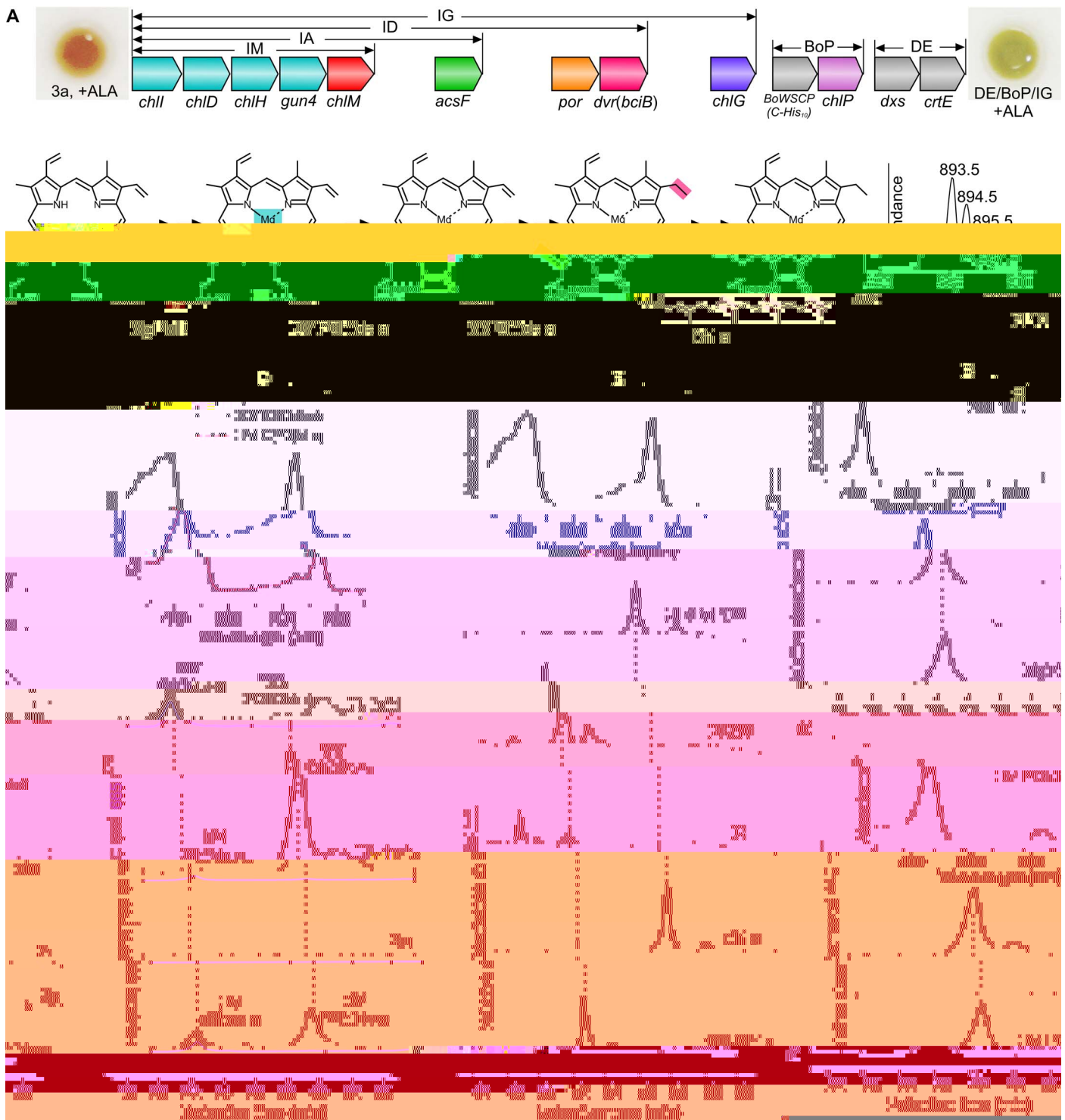


Fig. 3. Pigment accumulation in *E. coli* strains expressing Chl biosynthesis genes. (A) Photographs of cell pellets of the *E. coli* control strain accumulating PPIX (3a) and the Chl a–producing strain (DE/BoP/IG) grown with supplementation of δ -aminolevulinic acid (ALA). Pigment production in described *E. coli* strains was analyzed by HPLC. *m/z*, mass/charge ratio. (B) Accumulation of MgPME in the IM strain monitored by absorbance at 416 nm. (C) Accumulation of DV PChlide a in the IA strain monitored by absorbance at 440 nm. (D) Light-dependent production of MV Chlide a in the ID strain monitored by absorbance at 440 nm (shown in black) and 665 nm (shown in blue). POR was activated by treatment with $5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ light at the latter stage of the incubation. The small MV Chlide a peak detected in the dark sample is due to unavoidable light exposure during experimental procedures. DV and MV pigments are differentiated by positions of Soret bands in the absorption spectra. (E) Accumulation of GG–Chl a in the DE/IG strain and of Chl a in the DE/BoP/IG strain monitored by absorbance at 665 nm. The production of authentic Chl a was further confirmed by LC–MS with the acquired mass spectrum shown in the inset (see fig. S6 for details). The pigment accumulated in the IG strain was not assigned because of lack of an appropriate pigment standard.

The allelic exchange suicide vector pK18mobsacB (34) was used to construct marker-free in-frame deletion mutant of *chlA*. For construction of the pK18Δ plasmid, the upstream and downstream regions (approximately 500 base pairs) of the *chlA* gene were

cyclase step that confers the green color to Chl, we have been able to assemble a cyclase gene, together with 11 others, to form a Chl biosynthetic pathway in *S. obliquus*. The PPIX substrate for this new pathway is furnished by the host's tetrapyrrole metabolism, and the widespread availability of PPIX in most forms of life could provide the basis for installing Chl biosynthesis in a variety of heterotrophic organisms. This work forms the basis for performing in vitro cyclase assays with defined components and will enable molecular biologists to reprogram the cellular energetics of various platform organisms to include solar energy capture, commencing a new era of light-powered synthetic biology.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in table S2. Primers used for plasmid construction and screening mutants are listed in table S3. *S. obliquus* strains were grown in LB medium at 37°C. If required, antibiotics were added at 30 μg ml⁻¹ for kanamycin, 100 μg ml⁻¹ for ampicillin, and 34 μg ml⁻¹ for chloramphenicol. *S. obliquus* strains were grown in polypeptone-yeast extract-sodium succinate (PYS) medium (30) at 30°C. *S. obliquus* strains were grown in blue-green 11 (BG-11) medium (31) buffered with 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 8.3, adjusted by KOH) at 30°C with continuous illumination. Glucose was added at 5 mM if required. The *S. obliquus* strain (SB1003) (32), resistant to rifampicin, was obtained from C. Bauer (Indiana University) and grown in mineral-peptone-yeast extract (MPYE) medium (33) at 30°C. If required, antibiotics were added at 30 μg ml⁻¹ for kanamycin and 20 μg ml⁻¹ for rifampicin.

Construction and phenotypic analysis of *Rba. capsulatus* mutants

The deletion plasmids pK18Δ and pK18Δ were transformed into the *S17-1* strain (3), and transformants were selected on LB agar with kanamycin (30 μg ml⁻¹). A single colony from the plate was inoculated to 5 ml of LB medium with kanamycin (30 μg ml⁻¹) and grown at 37°C for 24 hours. *S17-1* cells harvested from 3 ml of the culture were resuspended in 50 μl of LB medium and mixed with *Rba. capsulatus* cells, which were harvested from 30 ml of liquid culture and resuspended in 100 μl of LB medium. The mating mixture was spotted onto solid LB medium and incubated at 30°C overnight. The mixture was streaked out onto MPYE agar supplemented with kanamycin (30 μg ml⁻¹) and rifampicin (20 μg ml⁻¹). The obtained kanamycin-resistant transconjugants were subcultured three times in nonselective MPYE medium to allow a second homologous recombination. Then, the culture was diluted and spread onto MPYE agar with 10% (w/v) sucrose. Colonies were then replica-plated onto MPYE plates containing 10% (w/v) sucrose, containing and lacking kanamycin (30 μg ml⁻¹). The desired mutants were screened from the kanamycin-sensitive and sucrose-resistant colonies by PCR with the geneScreenF/geneScreenR primers. The expression plasmid pBB[*chlA*] was conjugated into *Rba. capsulatus* via *S17-1* using the same method as described above. Transconjugants harboring the plasmid were selected on MPYE agar with kanamycin (30 μg ml⁻¹) and rifampicin (20 μg ml⁻¹).

For phenotypic analysis, *Rba. capsulatus* strains were grown in 10 ml of MPYE medium in 50-ml Falcon tubes with shaking at 230 rpm. Cells were harvested and resuspended in 1 ml of 60% (w/v) sucrose before recording absorption spectra of whole cells. Pigments were extracted from cells standardized by OD₆₈₀ with methanol. The absorption spectra of whole cells and pigment extracts were recorded on a Cary 60 UV-Vis spectrophotometer, whereas those for whole cells were normalized and corrected for light scattering, as described previously (3).

In vivo enzymatic assay of cyclase in *E. coli*

E. coli C43(DE3) was transformed with either the pET3a or the pET3a-*chlA* plasmid and selected on LB agar with ampicillin (100 μg ml⁻¹). For in vivo assays, a single colony was used to inoculate 10 ml of LB medium with ampicillin (100 μg ml⁻¹) and grown overnight at 37°C, with shaking at 220 rpm. The next day, 30 to 50 μl of the resulting culture were used to inoculate 10 ml of LB medium with ampicillin (100 μg ml⁻¹) and grown as above until the OD₆₀₀ reached 0.4 to 0.6. IPTG was added at 0.5 mM to induce gene expression. Meanwhile, purified MgPME dissolved in methanol was directly added to cultures. The cultures were incubated for a further 24 hours at 30°C, with shaking at 175 rpm, before pigments were extracted from the cells.

Synthetic production of Chl a in *E. coli*

E. coli C43(DE3) was transformed with the pET3a-based plasmids containing Chl biosynthesis genes and selected on LB agar with ampicillin (100 μg ml⁻¹). For the DE/IG strain, two sequential transformations were conducted, and the second transformation was selected on LB agar with ampicillin (100 μg ml⁻¹) and kanamycin (30 μg ml⁻¹). For the DE/BoP/IG strain, three sequential transformations were conducted, and the third transformation was selected on LB agar with ampicillin (100 μg ml⁻¹), kanamycin (30 μg ml⁻¹), and chloramphenicol (34 μg ml⁻¹). A single colony was used to inoculate liquid medium and cultured as above, except that, at the point of induction, ALA and Mg²⁺ (equimolar mixture of MgCl₂ and MgSO₄) were also added at 10 mM.

Cultures requiring light activation were illuminated with 5 μmol photons m⁻² s⁻¹ light for the final 4 hours.

Pigment extraction

S17-1 cells were harvested from liquid cultures and washed in 25 mM Hepes buffer (pH 7.4). Pigments were extracted with an excess of methanol by vigorous shaking using a Mini-Beadbeater (BioSpec), incubated on ice for 10 min, and centrifuged at 16,000 g for 5 min at 4°C. The resulting supernatant containing extracted pigments was transferred to a new tube and analyzed immediately or vacuum-dried at 30°C using a Concentrator plus (Eppendorf) and stored at -20°C for future analysis. GG-Chl a was extracted from a *S17-1* Δ mutant (10) using the same method as described above. MgPME was extracted from a *S17-1* Δ mutant (), which excreted the pigment into the growth medium as granules. Cells were harvested from liquid culture and washed in ultrapure water, and the pellet was suspended in methanol with gentle shaking to facilitate dissolution of the pigment into the solvent. MgPME solution was collected from the colored supernatant after centrifugation at 5000 g at 4°C for 10 min.

High-performance liquid chromatography

A methanolic pigment solution, either freshly extracted or reconstituted from dried samples, was analyzed on an Agilent 1200 HPLC system equipped with a diode array detector and a fluorescence detector. A published method (3) with some modifications was used for separation of Chl a and its precursors. The pigment solution was loaded onto a Sigma-Aldrich Discovery C18 reversed-phase column (particle size, 5 μm; 250 mm × 4.6 mm). Solvent A was methanol/500 mM ammonium acetate (30/70, v/v). Solvent B was methanol. Pigment species were eluted at 40°C at a flow rate of 1 ml min⁻¹ with a linear gradient of 65 to 75% solvent B over 35 min, followed by column wash with 100% solvent B for 15 min. The eluates were monitored by absorbance at 416, 440, and 665 nm.

Liquid chromatography–mass spectrometry

An Agilent 1200 HPLC system coupled to a 6410 QQQ mass spectrometer was used for confirmation of production of authentic Chl a. Data were collected and analyzed with MassHunter Software. Pigments were initially separated by reversed-phase HPLC using an identical program to that described above. The elution of Chl a from the column was monitored by checking absorbance at 665 nm. The assignment of *chlA* to eluates was performed using in-line electrospray ionization in positive mode monitoring *chlA* between 800 and 1000.

Structured illumination microscopy

S17-1 cells were pelleted and washed with phosphate-buffered saline and resuspended to an appropriate density for microscopy. Cell suspensions were incubated on an agarose pad for 30 min and mounted in VECTASHIELD Antifade Medium (Vector Laboratories). Samples were imaged on a DeltaVision OMX V4 microscope with the Blaze 3D structured illumination module equipped with a 60× oil objective [numerical aperture (NA), 1.42]. Illumination was performed with a 642-nm laser with the emitted light collected through a 683/40 band-pass filter. For each SIM image, nine axial planes were captured with a spacing of 125 nm, and the data were reconstructed with the SoftWoRx 6.0 software package (GE Healthcare).

Fluorescence spectroscopy

Resuspended frozen *S17-1* cells (20 μl) were spotted onto LB agar and left until the liquid was absorbed into the agar. The cell spot was cut out

of the agar and flipped over onto a glass-bottomed petri dish (WillCo Wells, GWSt-3522). The petri dish was then mounted in the microscope sample holder for imaging. Fluorescence imaging and emission spectral measurements were performed on a home-built imager equipped with a spectrometer (Acton SP2558, Princeton Instruments) and an electron-multiplying charge-coupled device camera (ProEM 512, Princeton Instruments). Dual-sample illumination was provided by a 470-nm light-emitting diode light source (M470L2, ThorLabs) to provide Köhler illumination for wide-field imaging and a 485-nm pulsed laser (LDH-D-C-485, PicoQuant) for a critical illumination when recording the emission spectra of individual cells. The laser beam was focused on the sample surface to a diffraction-limited spot using a 100× objective (PlanFluorite; NA, 1.4; oil immersion, Olympus), illuminating only a portion of the individual cell. Fluorescence signal was filtered through a 495-nm dichroic mirror (FF495-Di03, Semrock) and a 594-nm long-pass filter (BLP01-594R-25, Semrock). The emission spectra were recorded using a diffraction grating of 150 g mm⁻¹ with an 800-nm blaze, resulting in a wavelength range of 114 nm. The central wavelength was always selected to match the peak emission wavelength of the sample.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/4/1/eaag1407/DC1>

- fig. S1. Deletions of the *E* and *P* genes in *R. rubrum*.
- fig. S2. Diagram of the link-and-lock method for plasmid construction.
- fig. S3. The production of DV PChlide a in the IA and IM-*Δ*54 strains.
- fig. S4. The light-dependent production of MV Chlide a in the ID strain.
- fig. S5. The production of GG-Chl a in the DE/IG strain and of Chl a in the DE/BoP/IG strain.
- fig. S6. Verification of the production of Chl a in *E. coli* by LC-MS.
- fig. S7. Western blot analysis of the BoWSCP-His₁₀ expression in the DE/BoP/IG strain.
- table S1. List of genes used to assemble the Chl biosynthesis pathway in *E. coli*.
- table S2. Strains and plasmids described in this study.
- table S3. Oligonucleotide primers used in this study.

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