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Chlorophyll *f* and chlorophyll *d* are produced in the cyanobacterium *Chlorogloeopsis fritschii* when cultured under natural light and near-infrared radiation

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ABSTRACT

We report production of chlorophyll *f* and chlorophyll *d* in the cyanobacterium *Chlorogloeopsis fritschii* cultured under near-infrared and natural light conditions. *C. fritschii* produced chlorophyll *f* and chlorophyll *d* when cultured under natural light to a high culture density in a 20 L bubble column photobioreactor. In the laboratory, the ratio of chlorophyll *f* to chlorophyll *a* changed from 1:15 under near-infrared, to an undetectable level of chlorophyll *f* under artificial white light. The results provide support that chlorophylls *f* and *d* are both red-light inducible chlorophylls in *C. fritschii*.

1. Introduction

Chlorophyll *f*, the longest wavelength absorbing chlorophyll of oxygenic photosynthesis, was first discovered in samples taken from a stromatolite and enriched under near-infra red (near-IR) light [1]. A cyanobacterium was subsequently isolated and found to produce chlorophyll *f* when cultured under near-IR, but not white light [2,3]. A chlorophyll *f-producing* cyanobacterium has also been found in a Japanese Lake [4]. In organic solvent, the absorption spectrum of chlorophyll *f* shows a relatively large Q_Y transition at 706 nm, and a Soret band at 406 nm [1,5]. Stromatolite environments like that of the chlorophyll *f* producing filamentous cyanobacteria, *Halomicronema hongdechloris*, can be comparatively enriched in near-IR compared to UV/vis wavelengths [6], analogous to habitats of the chlorophyll *d*-producing cyanobacterium *Acaryochloris marina* [7–9]. Chorophyll *d* was first discovered in 1943 [10] but was thought to be an artefact of extraction for many years [11]. Chlorophyll *d* has been mistakenly associated with red algae in the past [10,12], whereas it is now known to be produced by epiphytic *Acaryochloris* sp. growing on red algae [13]. Chlorophyll *d* is the major chorophyll in *A. marina* [14,15]. Like chlorophyll *f*, chlorophyll *d* also has a red-shifted Q_Y transition (696 nm) compared to chlorophyll *a* [5,16], enabling light harvesting in the near-IR region [17,18].

Chlorogloeopsis fritschii is a subsection V cyanobacterium [19] which has a diverse morphology and diversity of function [20–22]. With a tolerance to a variety of growth conditions [22], and being amenable to large-scale culture, *C. fritschii* has potential for biotechnological applications [23,24]. We report production of chlorophyll *f* and chlorophyll *d* in the cyanobacterium *C. fritschii* cultured under near-IR and natural light conditions. We discuss these findings in context of the organism's natural habitat, morphotype and genome sequence.

Abbreviations: LC/MS^n, liquid chromatography/multistage mass spectrometry; PDA, photodiode array detector; HPLC, high performance liquid chromatography; APCI, atmospheric pressure chemical ionisation; CAO, chlorophyllide *a* oxygenase; HMM, Hidden Markov Model; t_R, retention time; *m/z*, mass to charge ratio

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2. Materials and methods

2.1. Culture conditions

2.1.1. Large scale

C. fritschii was inoculated into BG11 media and grown on a large scale (50 L) in purpose built Perspex bubble columns, illuminated either by natural light (average light intensity 100 μmol m^{-2} s^{-1} (PAR), or by artificial white light (90 μmol m^{-2} s^{-1}; Grolux tubes). The bubble columns were inoculated with 500 mL culture of approximately 0.9 g L^{-1} density. The columns illuminated by natural light were set up on the roof of Plymouth Marine Laboratory, and the temperature of these bubble columns was maintained in the range 25–35 °C by use of a heat exchanger. The columns illuminated by artificial white light (Grolux tubes) were maintained at 35 °C in a temperature controlled room.

2.1.2. Small scale

Small scale cultures (100 mL) were grown in Pyrex culture dishes. *C. fritschii* (PCC6912) was inoculated into BG11 media at a concentration of 1 g L^{-1}, and grown under artificial white light at 60 μmol m^{-2} s^{-1} (Grolux fluorescent tubes) or near-IR light (720 nm) at 10 μmol m^{-2} s^{-1} provided by an LED light source (20 nm half width; 20° viewing angle; Epitex, Japan) in a light box that excluded external sources of light. The cultures were grown at 28 °C in continuous light for 7 days, after which time the cells were harvested by centrifugation and stored at −80 °C until analysis.

2.2. Pigment extraction

A known mass of frozen cell paste was transferred to an extraction tube containing 1 mL HPLC grade acetone and 1 mm glass beads. The sample was then lysed in a tissue lyser (Qiagen) at 30 Hz for 15 min. The sample was centrifuged (5 min at 20,000*g*, Microcentrifuge 5415, Eppendorf), and the supernatant removed, and transferred immediately to −20 °C. For exhaustive extraction, the pellet, which still appeared green, was subjected to 5 freeze-thaw cycles using dry ice. 1 mL of acetone was then added to the pellet and the tissue lyser program was repeated. The extraction process was repeated three times, after which the pellet appeared blue. Immediately after extraction, the samples were analysed by liquid chromatography/multistage mass spectrometry LC/MS^n.

2.3. LC/MS^n

The pigment extract was analysed using a high performance liquid chromatography (HPLC) method described previously (Method C in [25]). Pigment extracts (90 μL) were mixed with water (10 μL) in the autosampler and injected (100 μL) onto the HPLC column (2 Waters Spherisorb ODS2 cartridges coupled together, each 150 × 4.6 mm, particle size 3 μm, protected with a precolumn containing the same phase). Elution was carried out using a mobile phase comprising methanol, acetonitrile., ammonium acetate (0.01 M) and ethyl acetate (Method C in [25]) at a flow rate of 0.7 mL min^{-1}. HPLC was performed using an Agilent 1200 system comprising a degasser (G1379B), binary pump (G1312A), thermostated autosampler (G1367B and G1330B), thermostated column compartment (G1316A) and photodiode array (PDA) detector (G1315A). The PDA detector was set to monitor wavelengths at 406, 696 and 706 nm, in addition to the wavelengths routinely used in our laboratory for carotenoid and chlorophyll detection (440 and 660 nm). The HPLC was coupled to an Agilent 6330 ion trap mass spectrometer via an atmospheric pressure chemical ionisation (APCI) source. Ionisation conditions were as follows: ion polarity positive; drying temperature 350°C, vapouriser temperature 450°C; nebulizer pressure 60 psi; drying gas flow rate 5 L min^{-1}, high voltage capillary −4500 V. Mass spectra were collected over the range 400–1100 *m/z*. Post column addition of formic acid was used to improve mass spectrometric detection of the chlorophylls [26]. Quantification of long wavelength chlorophylls was calculated based on their HPLC peak areas, HPLC flow rate, path length of the flow cell and published extinction coefficients [27]. The extinction coefficients in 100% methanol were used [27], as this most closely matched the HPLC mobile phase at the elution point of chlorophylls *f* and *d* [25].

2.4. Phylogeny of genes putatively involved in the synthesis of chlorophyll *f* in *C. fritschii*

Bioinformatic analyses of the phylogeny of genes involved in the putative biosynthesis of chlorophyll *f* in *C. fritschii* are fully described in the Supplementary information (all analyses are available on GitHub at https://github.com/btemperton/airs). Briefly, chlorophyllide *a* oxygenase (CAOs) and CAO-like genes in *Prochlorococcus* and closely related taxa were aligned with MUSCLE (v. 3.8.31) and used to create a Hidden Markov Model (HMM). The CAO HMM was used to query the predicted proteins of *C. fritschii* to identify putative CAOs and CAO-like proteins, with a cutoff *e*-value of 1×10^{-5}, identifying 8 putative proteins. One of these proteins (WP_016873721) was excluded as it was much shorter than other candidates (115 amino acids vs. 4–500 amino acids). Sequences used to construct the HMM and putative targets in *C. fritschii* were used in an iterative process of identifying closely-related sequences with best-BLASTP hit analysis against the NCBI nr database, alignment, automatic gap removal with GBlocks (V. 0.91) and tree generation using FastTree (v. 2.1.7) [28]. Trees were left unrooted due to the lack of a suitable outgroup of known function in chlorophyll *f* or chlorophyll *d* biosynthesis.

3. Results

A pigment extract from *C. fritschii* cells grown to high cell density in a bubble column photobioreactor (50 L) under natural light was analysed using an HPLC method described previously (Method C in [25]). The resulting chromatogram (Fig. 1) showed four peaks with absorption at 706 nm. The peak eluting at t_R 42 min (peak 4; Fig. 1), gave retention time, UV/vis spectrum (Fig. 2A) and mass spectra characteristic of chlorophyll *a*. Although chlorophyll *a* has negligible absorbance at 706 nm, its high relative concentration in the extract (see absorbance scale in Fig. 2A) gave rise to absorbance at 706 nm on a scale comparable with peaks 1–3 (ie. <6 mAU). The predominant component absorbing at 706 nm and eluting at t_R 35 min (peak 2; Fig. 1), gave a UV/vis spectrum (Fig. 2B) characteristic of chlorophyll *f* [1,5,29]. Under LC/MS^n conditions using post-column addition of formic acid to aid ionisation of chlorophylls [26], peak 2 gave rise to ions in full MS at *m/z* 907 and *m/z* 885, corresponding to $[M+H]^+$ and $[M+H-Mg]^+$ of chlorophyll *f* [1,5,30]. Isolation and fragmentation of the ion at *m/z* 907 in the ion trap resulted in MS^2 spectra typical of a formyl substituted chlorophyll macrocycle [31], consistent with chlorophyll *f* [5], namely an ion at *m/z* 879 corresponding to loss of 28 daltons from $[M+H]^+$, and ions at *m/z* 629 and 569, corresponding to $[M+H-phytyl]^+$ and $[M+H-phytyl-CO_2Me]^+$, respectively (Fig. 3A). Isolation and fragmentation of the ion at *m/z* 885 in the ion trap resulted in MS^2 spectra dominated by ions at *m/z* 607 and 547 (Fig. 3B) corresponding to $[M+H-Mg-phytyl]^+$ and $[M+H-Mg-phytyl-CO_2Me]^+$, respectively [5]. Notably, the full mass spectra were dominated by ions of mass to charge ratio (*m/z*) corresponding to

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