

SHORT COMMUNICATION

Endolithic chlorophyll *d*-containing phototrophs

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Cyanobacteria in the genus *Acaryochloris* are the only known oxyphototrophs that have exchanged chlorophyll *a* (Chl *a*) with Chl *d* as their primary photopigment, facilitating oxygenic photosynthesis with near infrared (NIR) light. Yet their ecology and natural habitats are largely unknown. We used hyperspectral and variable chlorophyll fluorescence imaging, scanning electron microscopy, photopigment analysis and DNA sequencing to show that *Acaryochloris*-like cyanobacteria thrive underneath crustose coralline algae in a widespread endolithic habitat on coral reefs. This finding suggests an important role of Chl *d*-containing cyanobacteria in a range of hitherto unexplored endolithic habitats, where NIR light-driven oxygenic photosynthesis may be significant.

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Chlorophyll *d* (Chl *d*) is only found in the cyanobacterium *Acaryochloris marina*, where it has replaced Chl *a* as the dominant pigment in the antennae and in the reaction centers extending the range of photosynthetic active radiation into the near infrared (NIR) light region (700–740 nm; Miyashita *et al.*, 1996; Kühl *et al.*, 2007). Only four *A. marina* strains have so far been isolated (Table 1), and they have been subject to detailed biochemical and genomic analysis (for example, Miller *et al.*, 2005; Swingley *et al.*, 2008). Although there is increasing evidence that Chl *d* is more widely distributed in both aquatic and terrestrial systems (Kashiyama *et al.*, 2008; Table 1), the ecology of Chl *d*-containing cyanobacteria is still largely unknown and in only one case, has a detailed description of its habitat been considered with actual *in situ* measurements of photosynthetic activity (Kühl *et al.*, 2005). Among cyanobacteria, *A. marina* has an unusually large genome with a significant proportion of its DNA localized to 9 plasmids (Swingley *et al.*, 2008), and it was speculated that such high capacity for genetic mobility and genome expansion could facilitate its adaptation to a variety of ecological niches. In this study, we show that Chl *d*-containing cyanobacteria can colonize widespread

endolithic habitats on coral reefs, where they occupy a distinct optically defined niche deprived of visible light.

We combined hyperspectral and variable chlorophyll fluorescence imaging (Kühl and Polerecky, 2008) with pigment extraction (Mohr *et al.*, 2010), scanning electron microscopy and DNA pyrosequencing (see Materials and Methods in Supplementary Online Material) to investigate the occurrence of Chl *d*-containing phototrophs on patches of dead coral branches from the reef flat and shallow outer reef crest off Heron Island, Australia. Such branches were almost completely covered by a diversity of crustose coralline algae (predominantly *Porolithon* spp. and *Lithophyllum* spp.; Ringeltaube and Harvey, 2000) and other epiphytic macroalgae, sponges and didemnid ascidians. Careful mechanical removal of the coralline algal layer revealed a thin yellow–green, biofilm closely associated with the carbonate skeleton immediately below (Figures 1a, 2a and Supplementary Figure S1). Hyperspectral imaging in combination with variable chlorophyll fluorescence imaging and scanning electron microscopy confirmed that this biofilm contained photosynthetically active Chl *d* in patches of *A. marina*-like cells (1–2 µm) intermixed with other endolithic filamentous cyanobacteria and the green alga *Ostreobium* sp. (Figures 1b–d and Figures 2b–f).

We did not attempt a quantification of light attenuation in the crustose coralline layer, but earlier studies of the light microenvironment in coral skeleton (Magnusson *et al.*, 2007) showed a

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very strong light attenuation of visible light (down to 0.1–1% of the incident irradiance) in the uppermost millimetres, whereas NIR light penetrated significantly deeper reaching 10–100 times the level of visible wavelengths in the skeleton matrix. However, coral skeleton density varies with species affecting light penetration (Enriquez *et al.*, 2005; Magnusson *et al.*, 2007).

Vertical slices through the encrusted coralline layer further revealed that Chl *d* is found in a thin layer in between the coralline alga and deeper parts of the coral skeleton (Supplementary Figures S1A, B), which was harboring other phototrophic endoliths, such as the green algae *Ostreobium* sp. These siphonaeal green algae can thrive on NIR (Halldal, 1968) due to antenna chlorophylls bound to the Lhca1-type protein (Koehne *et al.*, 1999) that absorb light beyond the typical absorption maximum of Chl *d* (Kühl *et al.*, 2007). We found shoulders between 710 and 725 nm in the reflec-

tance spectra indicative of such far-red antenna pigments (Figures 1a, 2b, Supplementary Figure S1C). Pigment extraction analysis of crustose coralline samples with ~1–2 mm of attached skeleton showed a distinct spectral signal of Chl *d*, albeit on a large background of coralline algal photopigments (Supplementary Figure S2). Pyrosequencing analysis of DNA extracted from similar samples revealed an endolithic microbial community with cyanobacteria as the dominant oxyphototrophs, of which ~5% could be assigned to *A. marina* (Supplementary Figure S3); *A. marina* accounted for ~1.8% of the total microbial community. In this first attempt of quantifying the presence of *A. marina* in a natural habitat, a total of five operational taxonomic units (OTUs) showed significant homology to known *Acaryochloris* species.

Crustose coralline algae are ubiquitous on coral reefs and are essential for stabilizing corals against mechanical erosion by sealing the porous carbonate

Table 1 Habitats for Chlorophyll *d*-containing and/or *Acaryochloris marina*-like phototrophs

Habitat	Evidence	Geographic location
<i>Marine</i>		
Coral reefs and mediterranean:		
Epizotic biofilms on didemnid ascidians	<i>A. marina</i> isolate (MBIC11017); absorbance and fluorescence spectra; pigment extraction; cell morphology.	Palau (Miyashita <i>et al.</i> , 1996) and Australia (Kühl <i>et al.</i> , 2005)
Epizotic biofilms on colonial ascidian	Fluorescence microscopy, pigment extraction, cell morphology.	Mediterranean sea (Martínez-García <i>et al.</i> , 2010)
Epilithic biofilms on dead coral	<i>A. marina</i> isolate (HICR111A); absorbance and fluorescence spectra; pigment extraction; cell morphology; genetic analysis.	Australia (Mohr <i>et al.</i> , 2010)
Red algae		
Epiphytic patches on thallus	<i>A. marina</i> isolate (AWAJI-1); absorbance and fluorescence spectra; pigment extraction; 16S rRNA gene sequence.	Japan (Murakami <i>et al.</i> , 2004; Ohkubo <i>et al.</i> , 2006)
Endolithic biofilm under crustose coralline algae	Absorbance and fluorescence spectra; pigment extraction; 16S rRNA gene sequence; SEM, cell morphology	Australia (this study)
Brown and green algae	16S rRNA gene sequence	Japan (Ohkubo <i>et al.</i> , 2006)
Sediments		
Temperate coastal basins	Pigment extraction	Japan (Kashiyama <i>et al.</i> , 2008)
Subarctic and arctic basins	Pigment extraction	Bering Sea and Arctic Sea (Kashiyama <i>et al.</i> , 2008)
Stromatolites	16S rRNA gene sequence	Australia (Goh <i>et al.</i> , 2009)
<i>Saline and freshwater</i>		
Sediments/biofilms		
Hypertrophic lake	<i>A. marina</i> isolate (CCMEE 5410); absorbance and fluorescence spectra; pigment extraction; morphology; 16S rRNA gene sequence	USA (Miller <i>et al.</i> , 2005)
Temperate freshwater lake	Pigment extraction	Japan (Kashiyama <i>et al.</i> , 2008)
Saline lakes	Pigment extraction	Antarctica (Kashiyama <i>et al.</i> , 2008)
High altitude lakes	16S rRNA gene sequence	Bolivia (Fleming and Prufert-Bebout, 2010)
<i>Terrestrial</i>		
Epilithic biofilms	16S rRNA gene sequence	Mexico (McNamara <i>et al.</i> , 2006)
Epi- and endolithic biofilms	TEM; SEM; 16S rRNA gene sequence; cell morphology	Antarctica (de los Rios <i>et al.</i> , 2007)

Abbreviations: rRNA, ribosomal RNA; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

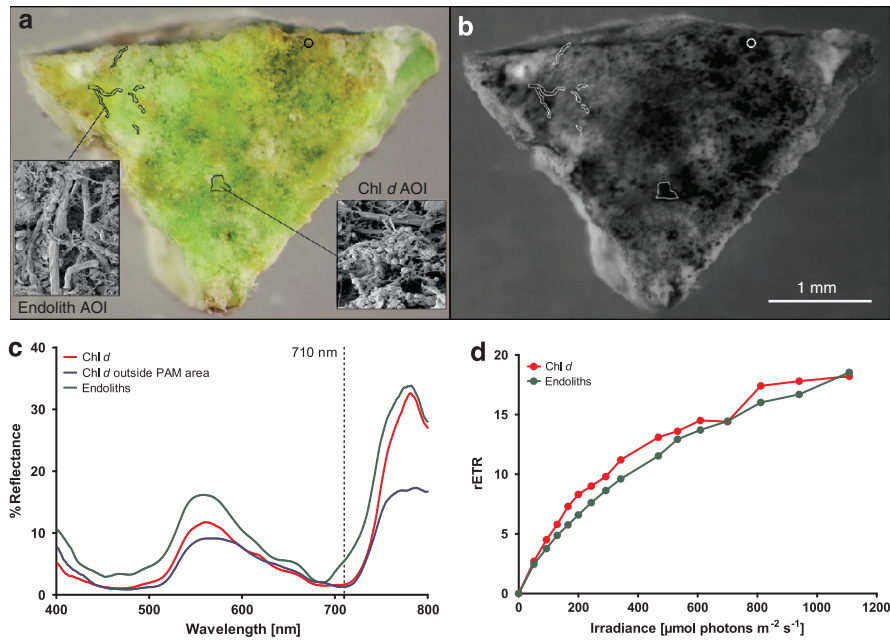
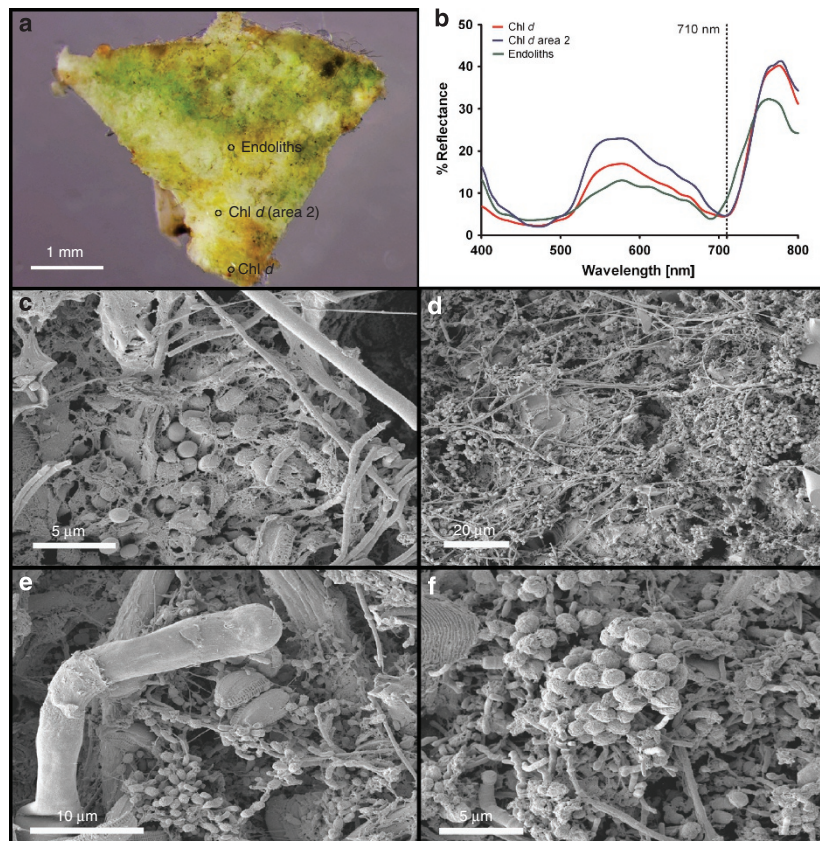


Figure 1 Endolithic habitat of Chl *d*-containing cyanobacteria. (a) Typical Chl *d*-containing yellow-greenish biofilm found directly under encrusting coralline algae. Labels indicate two different areas of interest (AOI) where pulse-amplitude modulated (PAM) variable chlorophyll fluorescence imaging was carried out: (1) Endolith AOI is dominated by filamentous cyanobacteria and green algae, and (2) Chl *d* AOI is dominated by *Acaryochloris*-like Chl *d*-containing cyanobacteria. Another circular AOI indicates a Chl *d*-containing area where no PAM analysis was carried out. Inserts show scanning electron microscopy pictures for the respective AOI; note the presence of round 1–2 μm large bacteria, similar in size and shape to *Acaryochloris marina*. (b) Hyperspectral reflectance image at 710 nm, that is, the peak absorbance of Chl *d* *in situ*. Dark areas indicate strong Chl *d* absorption. (c) Reflectance spectra of the two AOI showing distinct spectral signatures of Chl *d* around 710 nm. (d) Photosynthetic activity versus irradiance measured as the PSII related relative electron transport rate (rETR) in the two AOI.



skeleton matrix and consolidating the reef framework. Inside shallow water patches of coralline encrusted corals NIR light prevails (Kühl *et al.*, 2007) and our data indicate that *A. marina*-like Chl *d*-containing cyanobacteria are much more common on coral reefs than previously thought. Interestingly, a new *A. marina*-like isolate was reported from similar habitats (Mohr *et al.*, 2010). We have recently started a survey of Chl *d* underneath crustose coralline algae from different climatic zones, and first preliminary results obtained with pigment extraction and hyperspectral imaging (data not shown) confirmed the presence of Chl *d* underneath coralline specimens encrusting sandstone rock platforms around Sydney in more temperate coastal waters. Our results can also explain several sequence-based findings of *A. marina* and/or Chl *d* in other endolithic habitats (Table 1) and thus provide strong evidence for a more widespread occurrence of *A. marina*-like phototrophs, supporting the hypothesis that these unique oxyphototrophs exhibit a large capacity for niche adaptation (Swingley *et al.*, 2008). Recently, a new chlorophyll, that is, Chl *f*, has been identified in cyanobacteria from stromatolites (Chen *et al.*, 2010), and this pigment shows an even more red-shifted absorption than Chl *d*. It is not known whether this new pigment is indeed involved in active photosynthesis, but Chl *f*-containing phototrophs may colonize similar habitats where Chl *d*-containing cyanobacteria thrive. Thus Chl *d*-containing cyanobacteria and other phototrophs with far-red absorbing chlorophylls may have an important yet unexplored role as NIR-driven oxygenic primary producers in both terrestrial and aquatic ecosystems.

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Figure 2 Scanning electron microscopy and hyperspectral measurements performed on a dead coral sample with the crustose coralline algal layer removed. (a) Biofilm found under encrusting coralline algae. (b) Reflectance spectra extracted from hyperspectral imaging of three AOI in (a) showing Chl *d* absorption around 710 nm. (c–f) Scanning electron microscopy pictures portraying biofilms growing below encrusting coralline algae. Note frequent occurrence of 1–2- μ m large *A. marina* like cells (c–f), along with filamentous cyanobacteria (c, d) and *Ostreobium*-like filamentous green algae (e).

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Supplementary Information accompanies the paper on The ISME Journal website (<http://www.nature.com/ismej>)