The ISME Journal (2018) 12, 556–567

www.nature.com/ismej

ORIGINAL ARTICLE

In situ metabolomic- and transcriptomic-profiling of the host-associated cyanobacteria *Prochloron* and *Acaryochloris marina*

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The tropical ascidian *Lissoclinum patella* hosts two enigmatic cyanobacteria: (1) the photoendosymbiont *Prochloron* spp., a producer of valuable bioactive compounds and (2) the chlorophyll-*d* containing *Acaryochloris* spp., residing in the near-infrared enriched underside of the animal. Despite numerous efforts, *Prochloron* remains uncultivable, restricting the investigation of its biochemical potential to cultivation-independent techniques. Likewise, in both cyanobacteria, universally important parameters on light-niche adaptation and *in situ* photosynthetic regulation are unknown. Here we used genome sequencing, transcriptomics and metabolomics to investigate the symbiotic linkage between host and photoendosymbiont and simultaneously probed the transcriptional response of *Acaryochloris in situ*. During high light, both cyanobacteria downregulate CO_2 fixing pathways, likely a result of O_2 photorespiration on the functioning of RuBisCO, and employ a variety of stress-quenching mechanisms, even under less stressful far-red light (*Acaryochloris*). Metabolomics reveals a distinct biochemical modulation between *Prochloron* and *L. patella*, including noon/midnight-dependent signatures of amino acids, nitrogenous waste products and primary photosynthates. Surprisingly, *Prochloron* constitutively expressed genes coding for patellamides, that is, cyclic peptides of great pharmaceutical value, with yet unknown ecological significance. Together these findings shed further light on far-red-driven photosynthesis in natural consortia, the interplay of *Prochloron* and its ascidian partner in a model chordate photosymbiosis and the uncultivability of *Prochloron*.

The ISME Journal (2018) **12,** 556–567; doi:10.1038/ismej.2017.192; published online 31 October 2017

Introduction

Colonial ascidians (family didemnidae) are the only chordates known to form an obligate photosymbiosis (Hirose *et al.*, 2009) with the chorophyll (Chl) *b*-containing cyanobacterium *Prochloron* spp. (Lewin and Withers, 1975). *Prochloron* is found at high cell densities in the cloacal cavity (=CC) of some didemnid species, or more rarely, as epibionts (Hirose *et al.*, 2009; Nielsen *et al.*, 2015). Certain aspects of this obligate symbiosis have been resolved, for example, it is known that *Prochloron* contributes to carbon fixation/nutrient translocation within its host (Pardy and Lewin, 1981; Kremer *et al.*, 1982; Griffiths and Thinh, 1983) and produces bioactive secondary metabolites such as patellamides (Schmidt *et al.*, 2005). Other physiological aspects, such as N_2 fixation (Kline and Lewin, 1999), reactive-oxygen species detoxification (Lesser and Stochaj, 1990), host interaction and the diel regulation of metabolic pathways have remained largely unstudied, mostly due to the inability to maintain cultures of *Prochloron*. Culture-independent methods have elucidated certain aspects of *Prochloron* biology, and the publication of a draft-genome (Donia *et al.*, 2011) allowed insights into the functional ecology of this symbiont. This first

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Received 8 July 2017; revised 25 September 2017; accepted 26 September 2017; published online 31 October 2017

genomic analysis (Donia *et al*., 2011) demonstrated that *Prochloron* carries all primary metabolic genes required for survival outside of its host, along with a large amount of paralogous high-light inducible genes (*Hli*). Additionally, *Prochloron* has genes coding for the nitrate reduction pathway but appears to lack the capability to fix dinitrogen (Donia *et al*., 2011), contrasting previous reports that demonstrated active N_2-fixation (Kline and Lewin, 1999).

Prochloron is the most abundant bacterium within the didemnid ascidian *Lissoclinum patella* (Behrendt *et al*., 2012) but other bacteria co-occur in the CC, the surface and the underside (US) of the animal (Behrendt *et al*., 2012). One of these bacteria is *Acaryochloris marina*, a Chl *d*-containing cyanobacterium residing below *Prochloron* on the US of the host (Kühl *et al*., 2005; Behrendt *et al*., 2012). In this particular microenvironment, *Acaryochloris* uses Chl *d* to sustain oxygenic photosynthesis under near-infrared radiation, that propagates through the overlying ascidian tissue and *Prochloron* cells which selectively filter out visible light (Kühl *et al*., 2005; Behrendt *et al*., 2012). *Acaryochloris* is a significant member of the microbial community found on the US of *L. patella*, accounting for up to 14% of all sequences (Behrendt *et al*., 2012). Because it contains Chl *d* and is easily cultivated, *Acaryochloris* has been studied extensively; yet little is known about its *in situ* ecology and functional niche adaptation mechanisms.

Most information about the diversity and physiology of *Prochloron* and *Acaryochloris* has been obtained through metagenomics and amplicon sequencing (Donia *et al*., 2011; López-Legentil *et al*., 2011; Behrendt *et al*., 2012), and a few studies on *in vitro* gene expression of *Acaryochloris* (Pfreundt *et al*., 2012; Yoneda *et al*., 2016). Here we used transcriptomics on *in vivo* microbial communities within the CC and the US of *L. patella* to obtain insights to the regulatory pathways employed by *Prochloron* and *Acaryochloris* during the noon and midnight. For the assessment of *in situ* transcripts, we used the published *A. marina* MBIC11017 genome (Swingley *et al*., 2008) and generated a new draft genome of *Prochloron* (=P5), using refined assembly methods (Albertsen *et al*., 2013). Moreover, metabolomics was used on the CC to assess the diel biochemical modulation between *Prochloron* and its host. The results are discussed against the background of the microenvironment of *Prochloron* and *Acaryochloris* (Behrendt *et al*., 2012; Kühl *et al*., 2012) and in the light of +40 years of unsuccessful attempts to cultivate *Prochloron*.

Materials and methods

Sampling

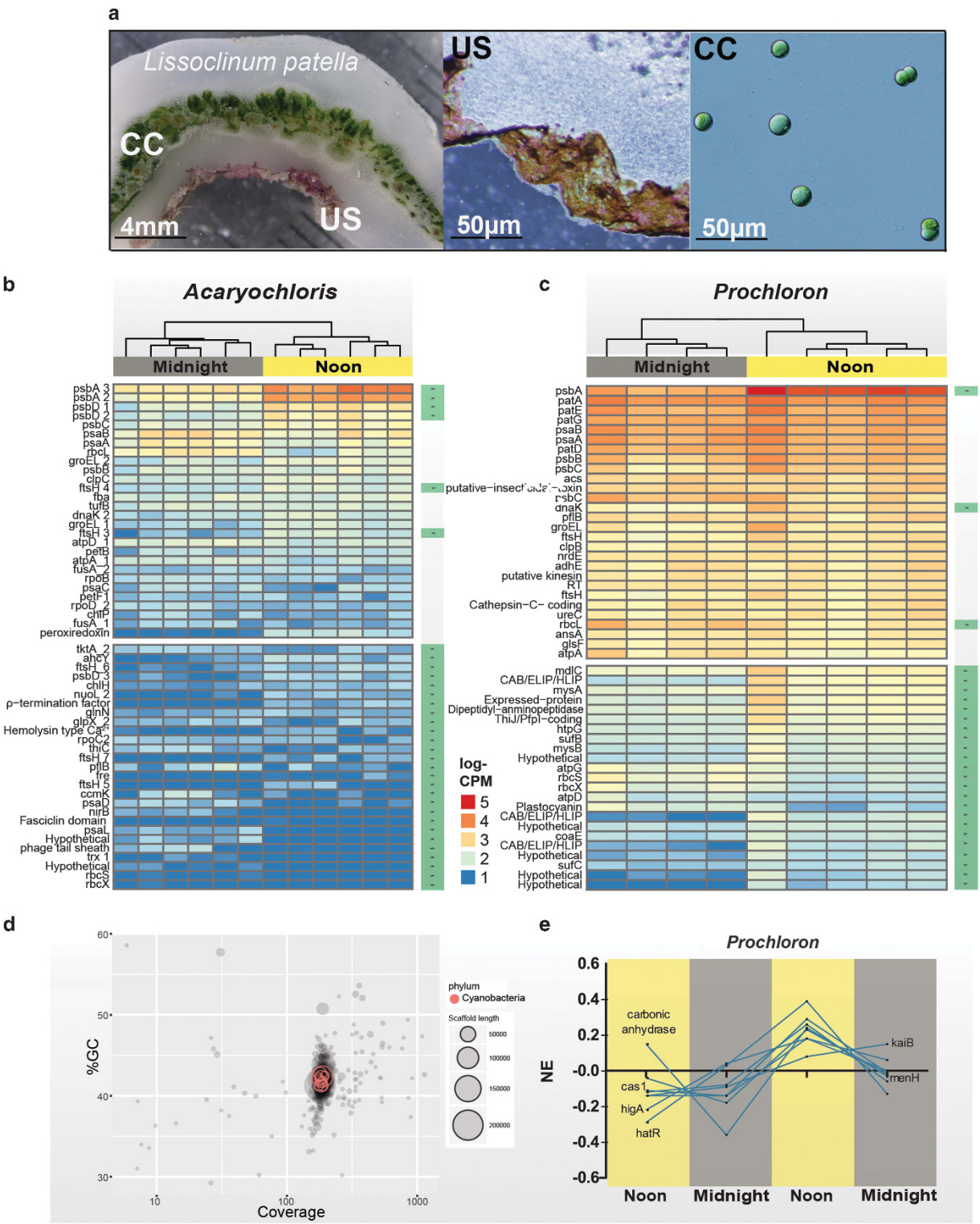
Intact specimens (5–15 cm^2) of 5–10 mm thick *L. patella* were collected on the outer reef flat off Heron Island (S23°26′055, E151°55′850) at ca. 30 cm water depth during low tide (~2.8 m tidal range). Collected specimens were kept in an outdoor aquarium (max. ~200 µmol photons m^{-2} s^{-1}) with a continuous supply of fresh seawater (26–28 °C). Sampling of *L. patella*-associated microbial biofilms for transcriptomics was performed during night-time (0000–0300 hours, hereafter referred to as 'midnight') or during midday (1200–1400 hours, hereafter referred to as 'noon') over 2 consecutive days (14–16.02.2011). During noon, photon irradiances at the sampling site routinely reached >1500 µmol photons m^2 s^{-1}. Sampling for metabolomic analysis was done for a single diel cycle during a different field campaign (07–18.02.2017) using *L. patella* specimens from the same sampling site and water depth. Sampling was done with a sharpened cork-borer (10 mm diameter), inserted into live *L. patella* specimen. The resulting core was subsampled into two microbial consortia: the CC, containing the internal symbiont *Prochloron* and the biofilm associated with the US of *L. patella* (Figure 1a and Behrendt *et al*., 2012). For transcriptomics, three subsamples were pooled and flash-frozen in liquid N_2. Three biological replicates were sampled for each time point and day. For genomic sequencing, *Prochloron* cells were collected via a Pasteur pipette inserted through the siphon into the CC of *L. patella*. The resulting cells were filtered through meshes (100 µm and 50 µm) and concentrated using centrifugation (2000 *g* for 2 min). The supernatant was removed and *Prochloron* cells were flash frozen in liquid N_2. For metabolomics, 10 biological replicates were sampled in a randomized fashion from four individual animals and the CC cores were snap frozen in liquid N_2 during the day and night. All samples were transported back to the laboratory on dry ice and stored at −80 °C upon arrival.

Prochloron genome sequencing

Frozen *Prochloron* cells were homogenized in liquid N_2 in a bleach-cleaned mortar. The resulting powder was processed using the standard FastDNA for soil kit (MP Biomedicals, Illkirch Cedex, France) with two additional bead-beating cycles. The resulting DNA was eluted in TAE buffer and quantified using a Qubit-fluorometer (Invitrogen, San diego, CA, USA), checked for integrity on a 0.8% agarose gel and stored at −20 °C. Sequencing was performed using Illumina technology (Illumina Inc., San diego, CA, USA) while assembly and binning procedures were done according to Albertsen *et al.* (2013). The resulting *Prochloron* genome consisted of 395 contigs with a combined genome size of 5 699 313 bp (=the P5 genome) and was submitted for Rapid Annotation using Subsystems Technology (RAST) (Aziz *et al*., 2008). The previously described patellamide gene cluster (Schmidt *et al*., 2005) was manually identified in the draft genome using BLAST.

Transcriptome analysis—experimental overview

Three reference genomes were used in the transcriptome analysis: (i) the *Prochloron* (P5) genome to which reads from the CC were mapped (Figure 1d); (ii) the *A. marina* genome MBIC11017 (Swingley *et al.*, 2008) to which reads from the US were mapped; and (iii) the *Ciona intestinalis* genome (Dehal *et al.*, 2002) to which all unmapped reads from (i) and (ii) were mapped (*Ciona intestinalis* is the most comprehensively annotated member of the ascidian family, phylogenetically belonging to a sister clade of didemnid-containing *Aplousobranchia* Tsagkogeorga *et al.*, 2009). For the metatranscriptomic analysis, all reads not aligning against (i), (ii) or (iii) were mapped against contigs ($n = 5769$) originating from a *de novo* assembly of the original



reads. For details on transcriptomic analysis, please refer to Supplementary Materials.

Metabolomics analysis

Metabolite extraction and quantitation followed established protocols (see Supplementary Materials) and was conducted by Metabolomics Australia, the University of Melbourne. Sugars, sugar alcohols and organic acids were quantified on dried aliquots using gas chromatography-mass spectrometry as described in Dias *et al.* (2015). Amino acids and biogenic amines were quantified using liquid chromatography-mass spectrometry as described by Boughton *et al.* (2011). Targeted metabolites were identified and quantified using corresponding calibration series of authentic standards using the Agilent MassHunter Quantitative and Quantitative Analysis software packages (B.07.00).

Results

The Prochloron (P5) draft genome and comparative genomics

Binning of metagenome reads, originating from extracted *Prochloron* cells (Figure 1a, CC), was done based on GC-content, taxonomic affiliation and read coverage and resulted in a total of 395 contigs hereafter referred to as P5 (Figure 1d). The complexity of the metagenome was low, resulting in good binning resolution and minimal contamination in the binned population genomes (Figure 2). A comparison of P5 with four published *Prochloron* draft-genome assemblies revealed G+C% contents ranging from 41.8% (P2) to 42% (P1). The quality of all five genomes was assessed using CheckM and demonstrated little contamination ($<1\%$) and high completeness ($>98\%$) in the P5 genome (Figure 2a). The average nucleotide identities based on BLAST (ANIb) in the *Prochloron* genomes were high and all tested pairs had ANIb values above the suggested species level cutoff ($>95\%$, Figure 2b).

Sequence overview

Despite an rRNA removal step, reads originating from the CC consisted of $\sim 40 \pm 12\%$ (mean ± s.d.; $n = 10$) rRNA (mostly eukaryotic 18S/28S, Supplementary Table S1) and $\sim 73 \pm 2\%$ in the US (mean ± s.d.; $n = 12$), again mostly 18S/28S, estimated using the SortMeRNA pipeline (Kopylova and Noe, 2012). A total of $\sim 8.4 \times 10^6$ ($n = 10$) of reads derived from the CC mapped against the P5 draft-genome (Supplementary Table S2). The mapping frequencies were low for *Acaryochloris* and a total of only $\sim 3.5 \times 10^5$ ($n = 12$) of the US reads mapped to this genome (Supplementary Table S2). Reads from the CC and US, not mapping against either of the two cyanobacterial genomes, were mapped against the *Ciona intestinalis* genome. Here a total of $\sim 2.2 \times 10^6$ ($n = 22$) of the reads mapped to the genome (Supplementary Table S2). Lastly, reads from the US and CC not mapping against any of the above reference genomes were mapped against *de novo* assembled transcriptome contigs, where a total of $\sim 2.1 \times 10^7$ (CC, $n = 10$) and $\sim 1.2 \times 10^7$ (US, $n = 12$) of reads mapped against 5.769 contigs above 1 kb.

Highly expressed genes in Acaryochloris and Prochloron

The 25 most transcribed genes for *Acaryochloris* and *Prochloron* were clustered according to their relative expression levels (Figures 1b and c, respectively). For P5, the majority of transcripts were at an abundance that allowed for a detailed transcriptomic investigation (Supplementary Figure S1A), whereas the low read-density for *Acaryochloris* demands careful interpretation of the differential expression patterns (Supplementary Figure S1B). Genes involved in the general maintenance of photosynthesis were constitutively expressed in both cyanobacteria. For *Acaryochloris*, this included genes coding for the photosystem (PS) I subunits (*psaA*, *psaB, psaC*), PSII CP47/43 (*psbB, psbC*), CO_2 fixation (*rbcL*) and stress mitigation *clpC*) (Lehel *et al.*, 1992). For *Prochloron*, photosynthesis-related genes were among the top 25 transcripts and encompassed genes coding for PSII CP47/43 (*psbB*, *psbC*), PSI subunits (*psaA*, *psaB*) and ATP synthase coding genes (*Acaryochloris*: *atpA*, *atpD*; *Prochloron*: *atpA*) (Alam *et al.*, 1986). Other highly expressed genes include those functionally related to nitrogen metabolism (*Prochloron*: *glsF, ureC*) and osmoregulation/

Figure 1 The *in situ* transcriptome of *Acaryochloris marina* MBIC11017 and *Prochloron* sp. associated with their ascidian host *Lissoclinum patella*. (**a**) Cross-section of the *L. patella* holobiont with the *Prochloron*-containing cloacal cavity (CC) and the underside (US) harboring the Chl *d*-containing cyanobacterium *Acaryochloris* sp. (**b** and **c**) Heatmaps displaying the 25 most expressed and differentially expressed genes ranked by the average expression during the noon (top-heatmap) for *Acaryochloris* (**b**) and *Prochloron* (**c**). The remaining statistically significant differentially expressed genes for *Acaryochloris* and *Prochloron* are found in the bottom heatmaps. Within heatmaps, the displayed values correspond to log10-transformed gene expression data normalized to counts per million (CPM) within each sample. Top dendrograms are based on Euclidean distances of values displayed and color-coded according to time of sampling (noon = yellow vs midnight = grey). Stars besides rows within the bottom heatmaps indicate genes (B = 30, C = 26) that were differentially expressed with statistical significance (adjusted *P*-values <0.05). (**d**) Percent GC vs sequence coverage of assembled scaffolds from extracted *Prochloron* cells. Taxonomic affiliation of the scaffolds (represented as circles) was based on the comparison with selected marker genes (where red indicates their affiliation to the phylum cyanobacteria). The size distribution of scaffolds is given by the relative size of radii. Note the absence of contamination, most likely attributable to the clean extraction of *Prochloron* cells from its host followed by a set of filtration steps. (**e**) K-medoid clustering of normalized gene expression (NE) over two diurnal cycles for *Prochloron* sp. The eight selected genes from the cluster were chosen based on their similarity in diurnal expression.

a

GENOME	COMPLETENESS	CONTAMINATION
P1	96.43	0.66
P2	98.03	2
P3	94.12	2.5
P4	98.18	0.51
P5	98.18	0.73

b

	P1	P2	P3	P4	P5
P1	*	97.28 [71.01]	97.16 [66.88]	98.91 [84.24]	97.82 [78.00]
P2	97.43 [74.71]	*	98.37 [77.17]	97.28 [71.83]	97.53 [73.98]
P3	97.67 [77.88]	98.79 [87.00]	*	97.59 [76.49]	97.71 [77.27]
P4	99.22 [90.69]	97.49 [74.42]	97.26 [70.63]	*	98.18 [81.56]
P5	98.17 [81.62]	97.77 [75.38]	97.38 [69.49]	98.17 [79.27]	*

Figure 2 Overall statistics and comparative genomics of the P5-*Prochloron* genome. (**a**) Genome quality parameters for four previously published *Prochloron* genomes (P1–P4) and the genome generated in this study (P5), all determined via the CheckM pipeline. The genomes were truncated to 1 kb scaffold sizes before analysis and the 'cyanobacterial marker lineage' used for quality assessment. Genome completeness and contamination was assessed by use of broad marker genes placed within a reference genome tree also taking into account co-location of genes. (**b**) Average nucleotide identity (ANIb) of all five sequenced *Prochloron* genomes (P1–P5) and their aligned nucleotide fraction (% nucleotides). All comparisons were done in JSpeciesWS and the P2–P4 genomes were truncated to contigs of >1 kb before analysis. Note the very high nucleotide conservation between all analyzed *Prochloron* genomes.

maintenance of photosynthesis (both *Prochloron* and *Acaryochloris*: *ftsH*) (Bailey *et al*., 2001; Stirnberg *et al*., 2007). For *Prochloron*, genes coding for cyclic peptides, patellamides, were transcribed at levels similar to regular photosynthesis housekeeping genes. Notably, all members of the patellamide gene cluster were transcribed, with *patA* and *patE* (the patellamide precursor molecule) (cf. Schmidt *et al*., 2005) reaching the highest expression levels.

Differentially expressed genes in Acaryochloris and Prochloron

Differential regulation of global gene expression was found in *Acaryochloris* ($n=30$; Figure 1b) and *Prochloron* ($n=26$; Figure 1c). Global gene expression patterns in both *Prochloron* and *Acaryochloris* were influenced by time of sampling, that is, noon vs midnight (PCoA plots in Supplementary Figures S2A and B, respectively). *Acaryochloris* displayed skewed expression patterns as determined via MA-plot characterization (Supplementary Figure S1B), demanding careful interpretation of the results. In *Acaryochloris*, differential regulation was found in 30 genes (Figure 1b), most of which were upregulated during noon and included photosynthesis-related genes, *psbA*, *psbD* and *psaD*, coding for the basic building blocks of PSII and PSI, and *chlH* a gene taking part in chlorophyll biosynthesis (Addlesee *et al*., 1996; Jensen *et al*., 1996). During midnight, several genes were upregulated: (i) *rbcX* and *rbcS* (coding for the assembly chaperone and small subunit of RuBisCO) (Saschenbrecker *et al*., 2007), (ii) *nirB* (involved in nitrite respiration) (Zumft, 1997), (iii) *psaL* (coding for the PSI reaction center subunit XI). During noon, significant upregulation of photosynthesis-related genes occurred in *Prochloron* (Figure 1c), where *psbA*, three members of the *CAB*/*ELIP*/*HLIP* superfamily (Kilian *et al*., 2008) and two genes coding for UV-protecting mycosporine molecules (*mysA*, *mysB*) (Gao and Garcia-pichel, 2011a,b) were upregulated. In *Prochloron*, significant upregulation during midnight occurred in three RuBisCO coding genes (*rbcL*, *rbcS, rbcX*) (Spreitzer and Salvucci, 2002; Emlyn-Jones *et al*., 2006) and a plastocyanin coding gene (*petE*) (Hervas *et al*., 1993). Diel expression patterns in *Prochloron* were further investigated via k-medoid clustering (Figure 1e). Genes involved in circadian rhythmicity (*kaiB*) (Johnson *et al*., 2011), CO_2 interconversion (carbonic anhydrase) (Badger and Price, 1994) and prokaryotic immune response (CRISPR *cas1*) (Makarova, 2011) were found to follow time-dependent expression patterns.

The metabolome of the L. patella cloacal cavity

Metabolomic investigation of the CC resulted in a total of 100 detected compounds, which were normalized to mg of fresh tissue weight and are reported in Supplementary Table S3. Distinctly regulated metabolite concentrations are evident by hierarchical clustering analysis (Figure 3a). Amino-acids were the most abundant metabolites with concentrations ranging from 71 ± 31 (mean ± s.d., Asp) to 575 ± 199 (Glu) pmol mg^{-1}. The microtubule-stabilizing Tau-protein reached a concentration of 2600 ± 520 pmol mg^{-1} and the neurotransmitter 4-amino butyric acid (GABA) a concentration of $\sim1600\pm560$ pmol mg^{-1}, while other vertebrate neurotransmitters were lower in concentration (that is, phenethylamine: 1.3 ± 0.3, octopamine: 0.38 ± 0.15, norepinephrine: 1.2 ± 0.9 pmol mg^{-1}). Saturated fatty acids occurred in low concentrations (octadecanoic acid: 2.9 ± 1.1, heptadecanoic acid: 0.1 ± 0.04 pmol mg^{-1}). Various sugars (that is, galactose, trehalose, arabinose, maltose, mannose, glucose, fucose) and their derivatives (that is, inositol, arabitol) had concentrations ranging from 0.39 ± 0.12 (galactose), 13 ± 13 (maltose) to 38 ± 15 (inositol) pmol mg^{-1}. Other notable metabolites include citrulline (3.1 ± 0.9 pmol mg^{-1}), an amino-acid and key intermediate in the urea cycle and L-alanyl-L-glutamine (AlnGln, 0.67 ± 0.14 pmol mg^{-1}) a soluble dipeptide of alanine and glutamine. Partial Least Square Discriminant Analysis (PLS-DA) clustered samples from noon/midnight according to the first (32.2%)

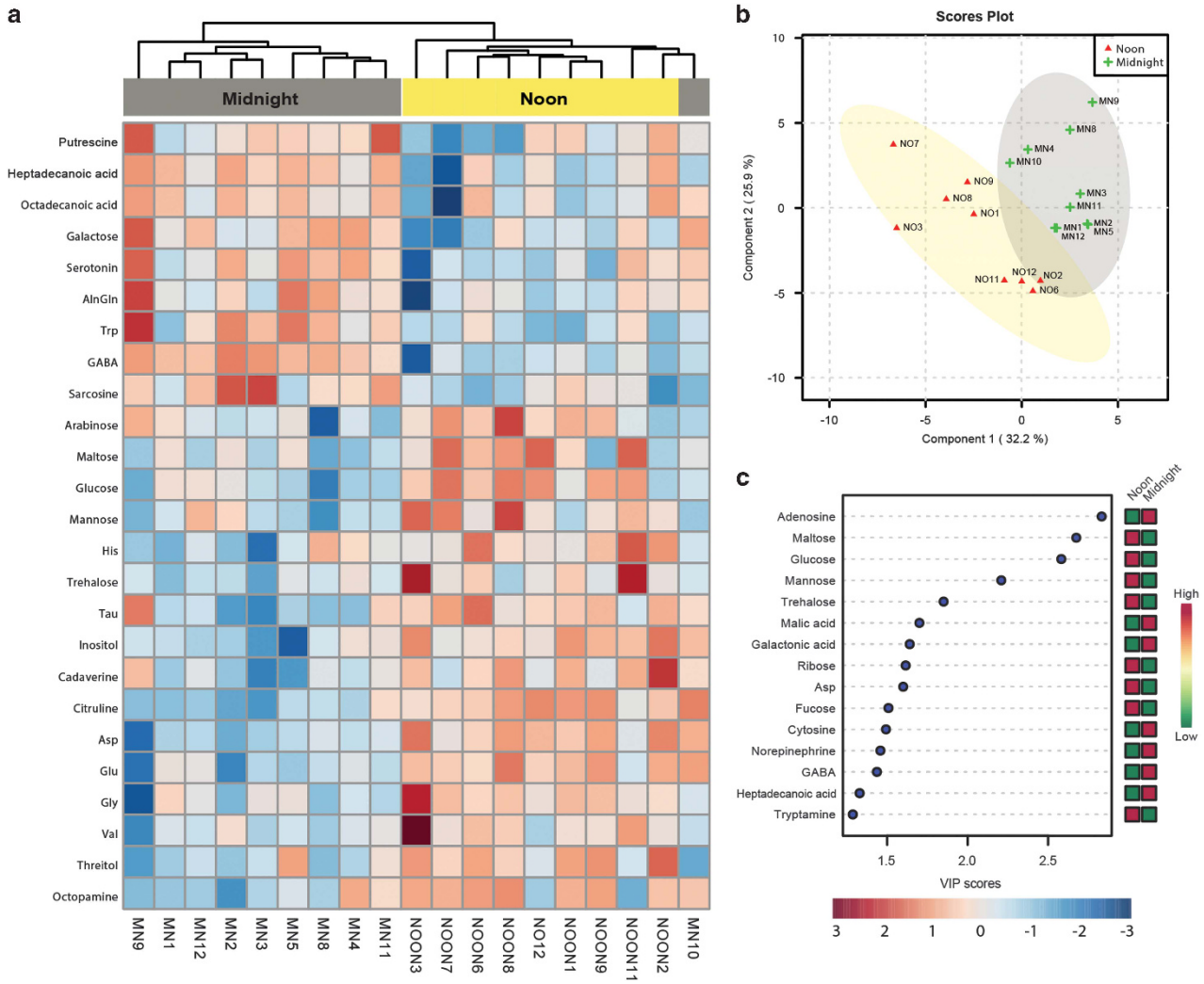


Figure 3 The *in situ* metabolome of the *Prochloron*-containing cloacal cavity of *Lissoclinum patella*. (**a**) Heatmap displaying the top 25 normalized and ranked metabolites resulting from GC-MS analysis of a total of 9 × CC samples taken during noon (yellow) and 10 × CC samples taken during midnight (gray). Ranking was performed via univariate *t*-testing and values within heatmaps correspond to the log2-transformed and sum-normalized values of pmol per mg^{-1} tissue fresh weight (scale in the bottom right). A single outlier was removed from the analysis due to missing metabolite information (=NOON10). Top dendograms are based on Euclidean clustering using Ward's method. (**b**) 2D score ordination plot of all 19 samples from the noon ('NO') or midnight ('MN') with displayed 95% confidence intervals (noon = yellow, midnight = gray). Data clustering was performed using Partial Least Square Discriminant Analysis (PLS-DA) after component optimization. (**c**) The most important metabolites ranked by their variable importance in projection (VIP) for PLS-DA. Higher VIP scores denote metabolites that are better at explaining the observed variance within PLS. The heatmap on the right indicates the detected concentration variations during noon or midnight. All data analysis and visualization was performed in MetaboAnalyst 3.0.

and second component (25.9%; Figure 3b). Pairwise statistical comparison of metabolite concentrations between midnight and noon ($P<0.05$, FDR = 0.1, fold-change >1.5) identified one upregulated metabolite during the night (GABA) and four upregulated metabolites during noon (glucose, Asp, mannose, maltose, trehalose) (Supplementary Table S4). Variables of importance projection (VIP) identified the metabolites that best explained the observed variance within PLS-DA, and included metabolites driving the separation at midnight (for example, adenosine, malic and galactonic acid and cytosine) and noon (for example, maltose, ribose, Asp and fucose) (Figure 3c).

Gene expression in L. patella

The *Ciona intestinalis* genome was used as a proxy for the investigation of gene expression in *L. patella*. Using a proxy genome is challenging, reflected in the overall low read mapping (see MA plot, Supplementary Figure S1C) and the absence of statistically differentially expressed genes. Given these obstacles, we found expressed genes that coded for translational products typically associated with general housekeeping; actin isoforms, histones (H3, H2B), tubulins and actin filament assembly (talin2). More specific for ascidians, expression of genes involved in the formation of metalloproteins, vanabins was observed in the CC (vanadium binding

protein 1, Supplementary Figure S3) (Ueki *et al*., 2015).

The metatranscriptome of the cloacal cavity and underside of L. patella

To investigate the transcription of other *L. patella*-associated organisms all remaining reads (=not mapping to any reference genome) were mapped onto contig-assemblies generated via *de novo* assembly. Quality control via MA-plot characterization revealed disproportionate expression patterns (Supplementary Figures S1D and E), possibly affecting subsequent normalization and testing for differential expression. Owing to this bias, we refrain from inferring statistical significance and will only provide expression values. Reads re-mapped from both the US (Supplementary Figure S4A) and CC (Supplementary Figure S4B) displayed high expression of genes involved in sulfate metabolism (*cysD*) (Malo and Loughlin, 1990), L-lysine transport (*lysE*) (Vrljic *et al*., 1996) and the entry of bacterial pathogens into eukaryotic cells (*Invasin*) (Palumbo and Wang, 2006). Within the US, expression of genes related to the production of bioactive compounds (*TOMM C/D*)(Melby *et al*., 2011) and heat-shock proteins/chaperones (*groEL, dnaK, clpB*) (Eriksson and Clarke, 1996; Török *et al*., 1997; Calloni *et al*., 2012) was observed.

Discussion

We present the first *in situ* metabolomic- and transcriptomic investigation of *Prochloron* and *Acaryochloris* in association with *L. patella*.

Photosynthesis: light utilization

Accurate timing of gene expression is crucial for cyanobacteria, enabling appropriate responses to anticipated daily fluctuations and allowing for, for example, temporally decoupled N_2 fixation and modulated metabolic responses to changes in extracellular pH, temperature and O_2 (Steunou *et al*., 2008; Jensen *et al*., 2011). Circadian rhythmicity is entrained by a light-induced phosphorylation cycle between kaiA/B/C proteins, a mechanism leading to sustained and anticipatory gene-expression in cyanobacteria (Liu *et al*., 1995; Johnson *et al*., 2011). In *Prochloron*, the gene coding for one of the clock proteins (*kaiB*) is expressed in a cyclic fashion, suggesting light as a primary stimulus for the activation of oxygenic photosynthesis during the noon and respiration during the night (Kim *et al*., 2012). As expected for such phototrophs, both *Prochloron* and *Acaryochloris* express the D1, D2 and CP43/CP47 coding genes during noon. To sustain photosynthesis under high-light regimes (=noon), appropriate stress quenching and repair mechanisms are required. For chloroplasts, the repair mechanisms of the reaction center proteins D1 and D2 are well understood and characterized by a high turnover rate of these proteins during high irradiance (Melis, 1999). Metalloproteases such as FtsH can expedite the rate limiting step of D1 protein degradation (Bailey *et al*., 2001) and high expression of the *ftsH* gene in *Acaryochloris* and *Prochloron* supports FtsH-facilitated D1-protein degradation during noon in order to maintain maximum photosynthetic quantum yield. In *Prochloron,* photoprotection is likely provided by translational products of *mysA*/*mysB* (producing UV-screening mycosporine amino-acids) and CAB/ELIP/HLIP genes, known to maintain photosynthesis under high-light conditions (HLIP), protect against oxidative damage (ELIP), and extend light harvesting capabilities by binding to Chl *a*/*b* (CAB) (Kilian *et al*., 2008). Earlier metagenomic analysis of *Prochloron* revealed such genes (Donia *et al*., 2011) and our expression data substantiate their use as photoprotectants. *Acaryochloris* resides in a near-infrared radiation-enriched microniche below *Prochloron*, relatively shielded from shorter, more damaging wavelengths (Kühl *et al*., 2005). Yet, the expression of peroxiredoxin-like genes in *Acaryochloris* (involved in oxidative stress mitigation) advocates the occurrence of light generated reactive-oxygen species (Dietz, 2011). A study on reactive oxygen species production under near-infrared radiation demonstrated that *Acaryochloris* does encounter light-stress, albeit at a lower level than under white light (Behrendt *et al*., 2013). Although light stress must be reduced in *Acaryochloris*, because of its cryptic location, the expression of *clpC*, *groEL* and *dnaK* genes (all expressed during noon and involved in protein stabilization under stress/heat-shock conditions) (Török *et al*., 1997; Charpentier *et al*., 2000; Horváth *et al*., 2008) still suggests a requirement for reactive oxygen species quenching.

Photosynthesis: CO_2 fixation

Photosynthesis drives carbon fixation, a process linked to RuBisCO, the carboxylase enzyme which facilitates chemical incorporation of CO_2 into organic molecules (Spreitzer and Salvucci, 2002). Early studies on ascidians used $^{14}CO_2$ labeling to demonstrate that glycolic acid is produced under light and accounts for up to 7% of isotopes fixed by *Prochloron* (Fisher and Trench, 1980). Illumination of isolated *Prochloron* cells triggered the accumulation of amino-acids (Glu, Gly, Ala and Asp) (Kremer *et al*., 1982). Our sampling procedure cannot distinguish between host- and *Prochloron*-derived metabolites, yet, amino acids and primary photosynthates (that is, maltose, glucose, fructose, mannose and sucrose) were elevated during noon, and so was the expression of an ATP-synthase-coding gene in *Prochloron*. This suggests the active production (and possible translocation) of *Prochloron*-derived photosynthates in the CC. In *Prochloron* and *Acaryochloris,* RuBisCO-associated genes (*rbcL, rbcS,*

rbcX) and *ccmK* (a gene coding for the basic building block in carboxysome formation, Kerfeld *et al*., 2005) were elevated during the night. While this was unexpected—given the high demand for CO_2 in photosynthetic carbon fixation during the day—it has been previously reported for other cyanobacteria (Huang and McCluskey, 2002). This trend could relate to photorespiration, that is, the displacement of CO_2 in RuBisCO by O_2, which induces its oxygenase function and production of phosphoglycolate (2PG), acting as a sensor molecule for the regulation of carbon concentration mechanisms (Haimovich-Dayan *et al*., 2015). At a photon irradiance of 250 μmol photons m^{-2} s^{-1}, the CC and US of *L. patella* reach hyperoxic O_2 and very high pH levels (Kühl *et al*., 2012), a microenvironment where O_2 could outcompete CO_2 in RuBisCO. Within the metabolomics data set, we find no direct evidence of 2PG accumulation, yet in other cyanobacteria 2PG can re-enter the Calvin-Benson-Bassham cycle through a metabolic conversion via several intermediary products and pathways (Eisenhut, 2006; Eisenhut *et al*., 2008). One of these intermediary species is glycine, the transamination product of glyoxylate (Maurino and Peterhansel, 2010), which was found to occur predominantly during noon in the metabolome. The utilization of the C2 pathway in *Prochloron* is supported by the transcription (data not shown) of most components of the glycine cleavage system (the H, T and P protein coding genes are present, L appears missing), suggesting that 2PG accumulates and is biochemically converted to glycine during high-light. Yet, we cannot exclude that glycine is associated with host-specific behavior, for example, ascidian locomotion via glycine-specific receptors (Nishino *et al*., 2010) or the biosynthesis of UV-protective mycosporine-glycine (Dunlap and Yamamoto, 1995; Maruyama *et al*., 2003). However, previous microenvironmental measurements, current expression data on RuBisCO genes and metabolomic signatures give strong indications for high photorespiration during noon. We hypothesize that more efficient CO_2 fixation occurs pre- and post-noon under less hyperoxic conditions.

Nitrogen metabolism

The uptake and utilization of nitrogen in *Prochloron* was suggested to be driven by nitrogen fixation (Paerl, 1984; Kline and Lewin, 1999). Contrary to these findings, no essential dinitrogen fixation (*nif*) genes were found within the P1–P4- (Donia *et al*., 2011) or the P5-*Prochloron* genome. In P5, the occurrence of a full ferredoxin-dependent glutamate synthase pathway (Fd-GOGAT) and its partial expression (*glsF*) suggests that *Prochloron* instead utilizes host-excretion products as its primary nitrogen source, a mechanism proposed earlier (Parry, 1985; Donia *et al*., 2011). In addition to transcriptional evidence for ammonium utilization, we find that the CC contains chemical signatures of citrulline, a chemical intermediate in the urea cycle. Citruline can react with aspartate and via additional steps form urea (Herman and Shambaugh, 1977); a compound which ascidians excrete (Markus and Lambert, 1983). Urea can be decomposed, via the urease enzyme, into ammonia and carbonic acid (Solomon *et al*., 2010) and be used as a nitrogen source. In *Prochloron*, urea decomposition is corroborated by the expression of the urease subunit-α coding gene (*ureC*), additional β/γ subunits (data not shown) and earlier suggestions on the genetic potential of *Prochloron* to utilize urea (Donia *et al*., 2011). These results substantiate that *Prochloron* relies on host-associated nitrogen recycling and is not capable of fixing dinitrogen.

For *Acaryochloris*, N_2 fixation has only been described in strain HICR111A (Pfreundt *et al*., 2012) and the remaining *Acaryochloris* genomes, MBIC11017 (Swingley *et al*., 2008) and CCMEE5415 (Miller *et al*., 2005) contain no N_2 fixation genes. In this study most reads mapped to strain MBIC11017, suggesting that the *Acaryochloris* ecotypes found on *L. patella* are most closely related to this non-N_2 fixing strain (or, alternatively, that the genome coverage is better). The absence of N_2 fixation is (non-statistically) supported by metatranscriptomic analysis; only two solitary *nif*-related genes (=*nifU*) were detected within 9.744 coding sequences, and both demonstrated only weak expression. In an *in vitro* nitrogen starvation experiment *Acaryochloris* CCMEE5410 upregulated *glnN*, a gene coding for a type III glutamine synthetase (Yoneda *et al*., 2016) which provides an advantage during periods of nitrogen starvation (Sauer *et al*., 2000). We find *glnN* expressed in *Acaryochloris* during noon, suggesting a low availability of nitrogen and a fine-tuned adaptation to conserve limited nitrogen resources during times of oxygenic photosynthesis.

Why does Prochloron resist cultivation?

The presence of all primary metabolic genes and little genome modifications (P1–P5 are >95% identical across a >8000 km geographic transect), suggested that *Prochloron* sp. is associating with its host in a facultative manner (Donia *et al*., 2011). This hypothesis is corroborated by the finding of surface-associated *Prochloron* cells (for example, Nielsen *et al*., 2015). However, *Prochloron* was successfully cultivated in only one (unreplicated) occasion (Patterson *et al*., 1982); here the addition of tryptophan or serine + indole initiated growth, particularly at low pH (~5.5), a proton concentration almost two orders of magnitude lower than measured in the CC of intact *L. patella* specimens (Kühl *et al*., 2012). The authors attributed the observed growth to a biochemical deficit in the first steps of tryptophan biosynthesis (the shikimate pathway). An inspection of the P5 genome revealed all genes involved in the shikimate pathway and *Prochloron* appears fully equipped to biosynthesize tryptophan, confirming earlier reports

(Donia *et al*., 2011). Within the metabolome, amino-acids (including Trp and Ser) are abundant and the expression of amino-acid transporters such as TRAP (in *Prochloron*, Mulligan *et al*., 2011) underscores that translocation could occur across the membrane of *Prochloron* and its host. In our experience, adding Trp/Ser/Ind and lowering the pH does not sustain cell divisions in *Prochloron* (Behrendt *et al*., unpubl), questioning the amino acid auxotrophy of this cyanobacterium.

Other clues to the uncultivability of *Prochloron* might lie hidden in its microenvironment. Most ascidians, including *L. patella*, accumulate trace metals at high concentrations, often 2–4 orders of magnitude higher than in surrounding waters (Krupp *et al*., 2012). Specifically for vanadium, concentration mechanisms involve the chemical reduction by microbial communities and transport within blood plasma using vanabins (Ueki *et al*., 2015), specialized carrier proteins for which we find expression signatures in *L. patella*. Whether *Prochloron* benefits, suffers or is involved in facilitating such metal concentration is unknown, but we can reason that unspecific uptake would cause an influx of metals at high concentrations, instigating the disruption of regular physiological functions (Baptista and Vasconcelos, 2006). To this end, *Prochloron* appears well equipped to maintain metal homeostasis, and the P5 genome contains signatures of various metal influx/efflux transporters most of which are transcribed (data not shown), for example, for Cu^{2+} (P-type ATPases), Zn^{2+} (ZnuABC, Patzer and Hantke, 1998), Mn^{2+} (MgtE, Hattori *et al*., 2009) and Fe^{3+} (*futA1*/*futB*/*futC,* Morrissey and Bowler, 2012). Besides these metal influx and efflux mechanisms, *Prochloron* produces a whole array of cyanobactins, prominently patellamides, microcin-like cyclic peptides (Long *et al*., 2005; Schmidt *et al*., 2005). *Prochloron* expresses the patellamide gene cluster with levels of *patE* (the precursor molecule) on-par with levels for *psbA*, the major D1 protein for PSII. This suggests an important role of patellamides for the metabolism and fitness of *Prochloron*. We speculate that patellamides might have the potential to scavenge metal cations, a theory put forward earlier (Bertram and Pattenden, 2007; Hirose *et al*., 2009), but never confirmed experimentally. Patellamide-like peptides comfortably bind Cu^{2+} and Zn^{2+} (Comba and Eisenschmidt, 2017), and do so even within *Prochloron* cells (Comba *et al*., 2017), yet show no affinity for binding to Mg^{2+} and Ca^{2+} (Bertram and Pattenden, 2007). Intriguingly, Cu (II)-complexed patellamide pseudo-peptides exhibit a wide spectrum of pH-dependent hydrolytic activities, *in vitro* these copper-bound analogs function as carbonic anhydrase or phosphatases at pH 7–8 (Comba *et al*., 2013, 2012), as glycosidases at pH ~10, and as lactamases at pH ~11.5 (Comba *et al*., 2016), evocative of a 'chemical Swiss-army knife'. We speculate that Patellamides might thus 'be imbued with biological activity' upon metal complexation (Krupp *et al*., 2012), opening a functional parameter space that adjusts with the above indicated influx/efflux mechanisms. Given the strong diel pH fluctuations in *L. patella* (Kühl *et al*., 2012), we hypothesize that this spectrum of catalytic patellamide functions could be accessed by *Prochloron* and/or *L. patella* and might present an essential component of their host–microbe interaction.

We anticipate that future experiments with metabolite-augmented media will enable the establishment of stable *Prochloron* cultures. Further, we foresee that such an approach will highlight the value of metabolomics-assisted cultivation efforts and facilitate the artificial reconstruction of other symbiotic relationships *in vitro*.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

This study was supported by a *Sapere Aude* starting postdoc grant (LB, DFF-1323-00747), a *Sapere-Aude* advanced grant (MK, DFF-1323-00065B), a research project grant from the Danish Council for Independent Research (MA, PH-N, DFF-4005-00369) and a research grant from VILLUM FONDEN (MA, 15510). We thank the staff at Heron Island Research Station for assistance (conducted under permit no. G12/35118.1 and G16/38423.1). Metabolite analysis was conducted at Metabolomics Australia (The University of Melbourne, Australia), a NCRIS initiative under Bioplatforms Australia Pty Ltd.

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