Short communication

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More than meets the eye: regional specialisation and microbial cover of the blade of *Porphyra umbilicalis* (Bangiophyceae, Rhodophyta)

<https://doi.org/10.1515/bot-2018-0065>

Received 5 July, 2018; accepted 23 August, 2018; online first 12 September, 2018

Abstract: Completion of the *Porphyra umbilicalis* genome and ongoing research on this species by many investigators suggest the need for wider appreciation of regional specialisation of the *P. umbilicalis* blade. Here we use light and electron microscopy to describe four distinct regions of the blade: rhizoid cells with abundant floridean starch, vegetative cells, differentiating neutral sporangia, and mature neutral spores. The holdfast, densely covered by microorganisms, presents an intriguing biomechanical structure: thousands of very thin, long rhizoid tips course through the thick, secreted polysaccharide to the substratum. Wild blades in culture have more microorganisms than when collected, including filamentous cyanobacteria.

Keywords: algal SEM; floridean starch; holdfast; microbial architecture; rhizoid.

Recent sequencing of commercially important red algae (*Chondrus crispus* Stackhouse, Collén et al. 2013; *Gracilaria lemaneiformis* (Holmes) Ohmi, Lee et al. 2018; *Porphyra umbilicalis* Kützinger, Brawley et al. 2017; *Pyropia yezoensis* (Ueda) M.S.Hwang & H.G.Choi, Sasaki et al. 2013) has focused new attention on the value of these algae to understand many aspects of eukaryotic evolution. Here we demonstrate the underappreciated regional specialisation of the *Porphyra* blade, including attached microorganisms, that is relevant to future work with this model system (Blouin et al. 2011).

The gametophyte of *Porphyra sensu lato* (Sutherland et al. 2011) is often referred to as a blade with isodiametric cells, and it appears deceptively simple, despite the striking rhizoid cells (e.g. fig. 32 in Brodie and Irvine 2003; fig. 5 in Kikuchi et al. 2010; figs. 3–11, 3–12, 4–5 in Zhu et al. 2016). Polne-Fuller and Gibor (1984) recognised the importance of regional differentiation of the blade in their study of *Pyropia perforata* (J.Agardh) S.C.Lindstrom (as *Porphyra perforata*) because of the substantial variation in success of protoplast production from different regions of the blade, which they called “complex” (Polne-Fuller and Gibor 1984, p. 615). With publication of the nuclear genome of *Porphyra umbilicalis* Kützinger, renewed experimental studies need to subsample blades with an understanding of the gradient of differentiation across the blade from the holdfast to the outer margin where gametes (northeastern Atlantic) or neutral spores (NS, northwestern Atlantic, Blouin et al. 2007, Royer et al. 2018) are produced and released. Moreover, shotgun metagenomic sequencing and deep sequencing of hypervariable regions of the 16 S rDNA demonstrate the richness and functional diversity of bacterial species on many algae including *P. umbilicalis* (e.g. Miranda et al. 2013, Kim et al. 2016, Quigley et al. 2018; and references therein), but we lack a fundamental structural understanding of bacterial cover on the host, for which scanning electron microscopy (SEM) is an appropriate tool. Here we demonstrate the complexity of the blade of *P. umbilicalis* with the goal of advancing ongoing studies of these algae.

The *Porphyra umbilicalis* blade is attached to the substratum, usually rock, by a holdfast composed of thousands of green, pear-shaped rhizoid cells that have long, slender tips (Figure 1A and I). A small transition zone of nearly isodiametric cells borders the large vegetative area. Intercalary cell divisions and a graded change in colour of the plastid from green to red are typical of the central vegetative region (Figure 1). The region distal to the vegetative cells contains differentiating neutral sporangia (Figure 1D and E) and is where anticlinal and periclinal divisions begin to produce neutral spores (Figure 1B and C). Fully differentiated neutral sporangia containing

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Figure 1: Anatomy of a typical blade of *Porphyra umbilicalis* on the Maine coast.

Cartoons show the regions of the blade *en face* (A) and as a longitudinal cross-section from the margin to the holdfast (M mature spores, N developing neutral spores, V central vegetative area, R rhizoid cells), which is composed of thousands of rhizoid cells. The middle panel and right panels, confocal and brightfield images respectively, show representative areas of four distinct regions of the blade: mature spores – A, B; developing neutral spores – D, E; central vegetative area – F, G; and rhizoid cells – H, I. Note the change in spacing of cells and amount of intervening cell wall (confocal images generated by exciting photosynthetic pigments) and transition in colour from green to red (longitudinal cartoon, brightfield images). Scale bars = 20 μm.

packets of neutral spores are found from the blade margin to about 1 cm depth within the monostromatic blade.

With SEM, bacteria were observed in dense patches along some regions of the wild blade's margin, but not

others (Figures 2 and 3). The bulging, mature neutral sporangia gave contour to the smooth blade surface where it was not covered by bacteria (Figure 3). In cultured specimens (Figure 4), bacteria spread over more of the blade

Figures 2–4: Microbial colonisation of blade margins from a wild plant of *Porphyra umbilicalis* (Figures 2 and 3) and a cultured descendent (Figure 4) of a wild plant brought into laboratory culture and maintained without antibiotic treatment.

Bacteria occur in patches along portions (Figure 2, arrows) but not all (Figure 3, arrows mark neutral spores) of the same wild blade, with much greater density of filamentous bacteria in Figure 2. Cultured blades (Figure 4) have a dense cover of bacteria, including filamentous cyanobacteria (arrows); contemporaneous light microscopic observations found at least three distinct cyanobacteria on these cultured blades, including a probable *Calothrix*. Wild *P. umbilicalis* ($n=6$ plants) were collected along a 30-m transect (~ 5 m apart) in the high intertidal zone at Schoodic Point, Acadia National Park (44.33380000, -68.05805556 ; 30 December 2016, permit ACAD-2017-SCI-0006). For each plant, one piece of reproductive margin ($0.5\text{--}1\text{ cm}^2$) and the holdfast were removed with sterile techniques, transported on ice, fixed (4°C) in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0) containing 0.2 M sucrose, post-fixed in 1% OsO_4 in 0.07 M sodium cacodylate, dehydrated in an ethanol series, critical point dried, and sputter-coated with gold-palladium to give a final coating that was 27 nm thick; see Royer 2017 for complete details. All six plants were observed, and representative images are presented. The cultured specimens (Figure 4) were maintained through successive generations by standard techniques (Royer et al. 2018) after collection of the parent on 5 May 2015 at Lubec, Maine.

and included filamentous cyanobacteria (Figure 4; also, Royer 2017).

Regions near (Figure 5) and over (Figures 6–8) the holdfast were colonised by a greater diversity and more uniform cover of prokaryotic and eukaryotic organisms than the blade margin. The intimate association of different taxa of bacteria with the thallus surface is evident in exposures of peeling mucilage/biofilm and/or outer cell wall. Among the organisms forming a continuous layer around the base of holdfasts (Figure 6) were ones (Figures 7 and 8) that structurally resemble some Subsection II cyanobacteria (Castenholz 2015) that are classified

among the Pleurocapsales (Guiry and Guiry 2018). Some of these individuals appeared to be releasing cells that may be baeocytes (Figures 7 and 8). Overall, SEM demonstrates the considerable spatial diversity and architectural complexity of the blade microbiome. Rhizoid cells contain substantial floridean starch, and these cells are separated by large quantities of secreted polysaccharide in the holdfast (Figure 9). The slender tips of rhizoid cells make contact with the substratum, where a mucilage pad hypothetically provides strong anchorage for the blade (Figure 10). This mucilage pad and the cell walls of the rhizoid cells were metachromatic after toluidine blue O staining; such metachromasia often indicates the presence of sulfated polysaccharide (Brawley and Quatrano 1979).

Neutral spores are released from the margin of the blade along with large quantities of mucilage as neutral sporangia rupture (Figure 11). A large stellate chloroplast occupies most of the volume of the ejected neutral spore, and the cytoplasm is compact (Figure 12). Some floridean starch and vesicles that may contain mucilage are evident.

We show here that *Porphyra umbilicalis* has a remarkably polarised and complex structure, despite being a monostromatic blade with largely intercalary cell division. Moreover, epiphytic bacteria do not form a uniform biofilm

Figures 5–8: Microbial colonisation of holdfast regions of wild blades.

(5) A peeling area of mucilage and/or biofilm shows the intimate association of bacteria within mucilage on a flat surface adjacent to the holdfast, and most of the bacteria are rod-shaped or filamentous. Communities surrounding the holdfast disc contain dense, often encrusting, masses of epiphytic bacteria, but become patchier towards the centre of the blade (Figure 6). (7–8) At higher magnification of the area shown in Figure 6, microbial community diversity is apparent, and it includes bacteria that are structurally similar to some of the baeocyte-producing pleurocapsalean cyanobacteria. White arrow in Figure 7 shows an intact individual, and black arrows in Figures 7 and 8 indicate possible baeocyte/endospore release and mucilage trails from epiphytes on two different wild plants.

on wild blades, and bacterial patches have significant architectural substructure. Basal holdfast regions usually have thick coverings of microbial epiphytes compared to the rest of the blades, and we show that the neutral spores that recycle the blade (Blouin et al. 2007, Royer et al. 2018) emerge in thick mucilage as they are ejected from neutral sporangia at the blade margin. Hawkes (1980) demonstrated that mucilage was secreted from large and small fibrous vesicles in archeospores (monospores) of *Pyropia* (as *Porphyra*) *gardneri* (G.M.Smith & Hollenberg) S.C.Lindstrom.

Scanning electron microscopy is an important tool in studies of diatoms, coccolithophorid haptophytes, and dinoflagellates (Graham et al. 2016); however, relatively few studies have examined the macroalgal surface with SEM recently to describe the fine structure of epiphytic

bacteria. Sieburth and Tootle (1981) studied bacterial colonisation of *Fucus vesiculosus* Linnaeus, *Ascophyllum nodosum* (Linnaeus) Le Jolis and *Chondrus crispus* by filamentous, rod, and coccoid bacteria, and reported that cover was greatest during the colder months of the year. Specific bacteria, including some Actinobacteria, Bacterioidetes, and Proteobacteria (e.g. Matsuo et al. 2005, Ghaderiadekani et al. 2017, Weiss et al. 2017), are needed by young stages of macroalgae for normal development, and taxa from these phyla are common on wild and cultured blades of *Porphyra umbilicalis* (e.g. Miranda et al. 2013, Quigley et al. 2018). Epiphytic bacteria have the potential to change surface roughness and modify inorganic nutrient uptake, cause disease, and/or produce compounds that affect the blade nutritionally. For example, cyanobacteria may provide functional vitamin B₁₂ to the *Porphyra*

Figures 9 and 10: Holdfast structure.

(9) TEM of rhizoid cells in holdfast. The holdfast is a thick, flexible structure made of polysaccharide secreted by the pear-shaped rhizoid cells. Note the large amount of floridean starch (arrows) in the pear-shaped end of the rhizoid cell where the large plastid and its pyrenoid are evident. (10) Toluidine blue O-stained holdfast section. The polysaccharide that is secreted includes a thick, metachromatic pad at the base of the holdfast, and the cell walls of rhizoid cells are also metachromatic (arrows). Holdfasts from blades collected at Schoodic Point (ME) in November 2009 were fixed in 2% glutaraldehyde in seawater for 2 h, post-fixed in 4% OsO_4 , dehydrated in a standard series of ethanol and propylene oxide and embedded in Epon. Holdfasts from additional blades fixed as above were embedded in Spurr's resin (EM Sciences) and semithin sections stained with toluidine blue O (TBO) per Brawley and Quatrano (1979).

blade as a cofactor for methionine synthase; *P. umbilicalis* encodes both forms of methionine synthase [METH (B_{12} dependent), METE (B_{12} independent)] and the proteins needed to convert cyanobacterial pseudocobalamin to cobalamin (Helliwell et al. 2016, Brawley et al. 2017). Here we show that cyanobacterial filaments are part of the epiphytic microbiome on healthy *P. umbilicalis* brought into culture. Compared to cultured blades, microbial cover on wild plants was patchy, perhaps due to grazing (Royer et al. 2018), desiccation, and/or blades rubbing against other surfaces when plants are covered by seawater.

Although the large rhizoid cells at the base of *Porphyra sensu lato* blades are illustrated by several authors

Figure 11: Neutral spores during discharge through a thick covering of mucilage from the margin of a wild blade (see Figures 2–4 for techniques).

Figure 12: Neutral spore discharged from the neutral sporangium. Note residual mucilage around the bottom half of the neutral spore, the large stellate chloroplast (C), nucleus (N), mitochondria (M), some floridean starch (arrows), and vesicles (V) that may contain mucilage. (See Figures 2–4 for techniques). Scale bar = 1 µm.

with light microscopy (e.g. Brodie and Irvine 2003, Zhu et al. 2016), our ultrastructural studies show that these cells have large quantities of floridean starch, and that they form a large polysaccharide structure that anchors the blade to the substratum. Rhizoid cells appear to have more floridean starch than vegetative cells (fig S45a in Brawley et al. 2017) or neutral spores (Figure 12).

The thousands of thin, very long tips of rhizoid cells that run through the polysaccharide to the substratum are likely to contribute interesting mechanical properties to the holdfast, in addition to the primary role of the rhizoid cells in secretion of the extracellular matrix of the holdfast. Thin layers of non-geniculate coralline algae sometimes occur around holdfasts of wild blades based on our field observations, and our SEM observations showed diverse epiphytes uniformly coating the base of the holdfast. Physical disturbances such as ice shear and large storms that tear away the upper areas of blades require the ability of *P. umbilicalis* to regenerate from a basal section of the holdfast. These traits (e.g. large energy reserves, epiphyte-armoured bases) may be important to the ability to regenerate.

Molecular-based identifications of the macroalgal microbiome (e.g. Miranda et al. 2013, Quigley et al. 2018) and SEM analysis have provided novel information about the *Porphyra* blade and its microbial community that should inform studies of blade physiology going forward. For integration of structure and function, especially in algae where operational taxonomic units (OTUs) (or amplicon sequence variants, ASVs) are characterised, approaches such as fluorescence *in situ* hybridisation (FISH, e.g. Tujula et al. 2010) may help to understand effects of particular bacteria on host algae in the future.

Acknowledgements: This work was supported by awards from Maine Sea Grant (NOAA Contract NA14OAR4170072), the NSF (NSF RCN 0741907, NSF 1442231), and a University of Maine Graduate Student Research Fund award to CR to support her M.S. thesis research (Royer 2017). We thank Elisabeth Gantt (University of Maryland) for technical assistance with TEM. We are grateful to two anonymous reviewers and the Editor for their constructive suggestions that improved the manuscript.

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Bionotes

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