ORIGINAL ARTICLE

Cell wall proteomic analysis of the cnidarian photosymbionts *Breviolum minutum* and *Cladocopium goreaui*

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Abstract

The algal cell wall is an important cellular component that functions in defense, nutrient utilization, signaling, adhesion, and cell–cell recognition—processes important in the cnidarian–dinoflagellate symbiosis. The cell wall of symbiodiniacean dinoflagellates is not well characterized. Here, we present a method to isolate cell walls of Symbiodiniaceae and prepare cell-wall-enriched samples for proteomic analysis. Label-free liquid chromatography–electrospray ionization tandem mass spectrometry was used to explore the surface proteome of two Symbiodiniaceae species from the Great Barrier Reef: *Breviolum minutum* and *Cladocopium goreaui*. Transporters, hydrolases, translocases, and proteins involved in cell-adhesion and protein–protein interactions were identified, but the majority of cell wall proteins had no homologues in public databases. We propose roles for some of these proteins in the cnidarian–dinoflagellate symbiosis. This work provides the first proteomics investigation of cell wall proteins in the Symbiodiniaceae and represents a basis for future explorations of the roles of cell wall proteins in Symbiodiniaceae and other dinoflagellates.

KEYWORDS
cell surface, Great Barrier Reef, mass spectrometry, proteins, Symbiodiniaceae, symbiosis

DINOFLAGELLATES are a large and diverse group of unicellular protists with a great variety of forms. Dinoflagellates mostly inhabit marine environments, including benthic and sea-ice systems, but can also be found in freshwater habitats (Taylor, 1974). They are important planktonic primary producers and prominent components of aquatic food webs (Hackett et al., 2004). Many dinoflagellates are photosynthetic, including members of the family Symbiodiniaceae, many of which are endosymbionts of cnidarians (LaJeunesse et al., 2018). The symbiosis between Symbiodiniaceae and stony corals underpins tropical coral reef ecosystems, because it is the main primary producer and builds the three-dimensional reef framework (Davy et al., 2012).

Symbiodiniaceae deliver photosynthate to their hosts, typically meeting most of the host's energetic requirements for growth, reproduction, and survival. In return, the host provides the symbionts with shelter and inorganic nutrients from its metabolism. The algal symbionts reside within the endodermal cells that line the gastrovascular cavity of the host wherein they are bounded by a specialized set of membranes, the symbiosome membrane complex, which seems to prevent the digestion of the dinoflagellate cell by lysosomal fusion (Davy et al., 2012).

The cell surface of dinoflagellates consists of a robust and intricate multilayered mesh of cellulose, polysaccharides (Morrill & Loeblich, 1981), and protein (Wang et al., 2011). In Symbiodiniaceae, a relatively thick internal cell wall is positioned between thinner membranous layers. Although the exact structure of the cell wall remains unknown, there is evidence of the presence of a large amount of cellulose and the existence of a variable number of amphiesmal plates (Markell et al., 1992; Morrill & Loeblich, 1981; Wakefield et al., 2000). A few studies have localized glycoconjugates to the Symbiodiniaceae cell surface and have investigated their role in the onset of symbiosis with cnidarians. Particular protein moieties on the surface of the alga seem to participate in the onset of symbiosis with cnidarians, and chemical alteration of cell wall glycoproteins negatively affects the colonization of the host by the symbiont (Bay et al., 2011; Lin et al., 2000; Logan et al., 2010; Markell et al., 1992; Tortorelli et al., 2021; Wood-Charlson et al., 2006). This field is still in its infancy, and although some proteomic studies have been performed on the host (Oakley et al., 2016, 2017; Sproles et al., 2019; Weston et al., 2015), a thorough inventory of Symbiodiniaceae cell wall proteins (CWPs) has never been done.

In this study, we present a new method for the isolation of the Symbiodiniaceae cell wall and preparation of cell-wall-enriched samples. We used label-free liquid chromatography–electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) proteomics to characterize CWPs of two species of Symbiodiniaceae: *Breviolum minutum* and *Cladocopium goreaui*. Members of the genus *Breviolum* are relatively small (6–10 µm in diameter), are abundant in the Atlantic Ocean, and rare throughout the Indo-Pacific, where they associate with selected cnidarian hosts (LaJeunesse et al., 2018). Algae of the genus *Cladocopium* have larger cells (9–11 µm) and are extremely diverse in terms of ecological distribution, geographical prevalence, and physiological attributes (LaJeunesse et al., 2018).

The goal of this work was to provide an overview of the proteins populating the Symbiodiniaceae cell wall, to address the variability of the wall peptides between the two species, and to offer a foundation for future investigations of the functions of CWPs in Symbiodiniaceae and other dinoflagellates.

MATERIALS AND METHODS

Symbiodiniaceae cultures

Two Symbiodiniaceae species were used in this study: *B. minutum* (Parkinson and LaJeunesse; MMSF 01, ITS2 type B1) and *C. goreaui* (LaJeunesse and Jeong; SCF 055-01.10, ITS2 type C1). *B. minutum* was originally isolated from the anemone *Exaiptasia diaphana* (Tortorelli et al., 2020), whereas *C. goreaui* was obtained from the scleractinian coral *Acropora tenuis*. All organisms were sourced from the Great Barrier Reef. Cultures were maintained in 1× IMK⁻ culture medium (1% w/v, prepared in 0.2-µm filtered, reconstituted seawater prepared with Red Sea Salt (Red Sea) dissolved in deionized water; 34 ppt; Daigo's IMK, Novachem) in 1-liter Schott bottles, with 0.2-µm membrane vented caps in a growth chamber (740FHC LED, HiPoint) under constant temperature (27℃), 12 h:12 h light: dark photoperiod cycle, and 60 µmol photons/m²/s.

Cell wall isolation and protein extraction

Breviolum minutum and *C. goreaui* were sampled in the middle of the culture exponential growth phase. Six replicates of 5×10^6 cells/ml were grown for each algal species, three of which were used for cell wall protein extraction and three for protein extraction of the whole algal cells. Proteins were extracted from whole cells for comparison with the cell wall fractions and enrichment analysis of proteins exclusive to the cell wall.

Algal cell wall isolation was adapted from Feiz et al. (2006). Cell aliquots were washed twice in ice-cold MilliQ water to remove salts and resuspended in 500 µl of ice-cold acetate buffer (5 mM, pH 4.6, 0.4 M sucrose, 20 µl protease inhibitor cocktail [Sigma-Aldrich]). To lyse the cells, 300 mg of sterile glass beads (Sigma-Aldrich G8772) was added and the samples bead-beaten for 20 min at 30 Hz (Qiagen Tissue-Lyser II). Cell walls were concentrated (along with the glass beads) by centrifugation at 16,000 *g* at 4℃ for 15 min, and the pellet was then washed off the beads with ice-cold acetate buffer and transferred to a new tube. Soluble cytoplasmic fluid and organelles were separated from the cell wall fraction by centrifuging the homogenate at 2500 *g* at 4℃ for 30 min, and cell walls were further purified by two consecutive washes in ice-cold acetate buffer at 0.6 and 1 M sucrose and two more washes in MilliQ water. The cell wall isolation procedure is summarized in Figure 1A. To ensure that the isolation of algal cell walls was successful, the cellulosic Symbiodiniaceae cell wall was stained with calcofluor white (Levin et al., 2017; Markell et al., 1992), and the fractions were visualized with a Nikon A1R confocal laser scanning microscope (Nikon) with a 409-nm laser to detect stained cellulose.

Whole algal cell samples were washed twice in ice-cold MilliQ water, resuspended in 500 µl of 5% sodium deoxycholate (SDC; Sigma-Aldrich) detergent with 20 µl protease inhibitor cocktail (Sigma-Aldrich) and cells were ruptured

(A)

1. Symbiodiniaceae cells

Centrifugation:
MilliQ
16,000 ×*g*, 5 min

→ Supernatant discarded

WASH

2. Symbiodiniaceae cells

Bead beater 30 Hz for 20 min :
acetate buffer (5mM, pH 4.6, 0.4 M sucrose) + PI + glass beads

LYSIS

3. Homogenate

Centrifugation:
16,000 ×*g*, 4°C, 15 min

→ Supernatant discarded

WASH

4. Pellet + beads

Beads wash w/
acetate buffer (5mM, pH 4.6, 0.4 M sucrose)

→ Beads discarded

5. Pellet

Centrifugation:
acetate buffer (5mM, pH 4.6, 0.4 M sucrose)
2500 ×*g*, 4°C, 30 min

→ Supernatant discarded

6. Cell wall pellet

Centrifugation:
acetate buffer (5mM, pH 4.6, 0.6 M sucrose)
2500 ×*g*, 4°C, 30 min

→ Supernatant discarded

PURIFICATION

7. Cell wall pellet

Centrifugation:
acetate buffer (5mM, pH 4.6, 1 M sucrose)
2500 ×*g*, 4°C, 30 min

→ Supernatant discarded

8. Cell wall pellet

Centrifugation:
MilliQ
2500 ×*g*, 4°C, 30 min

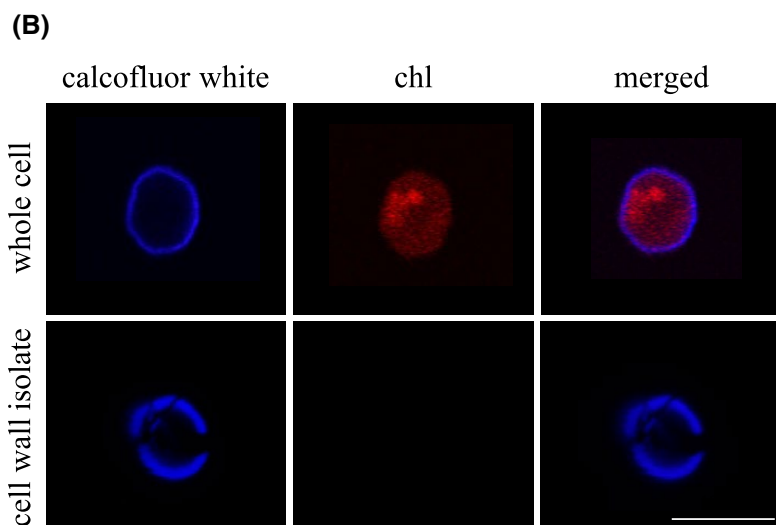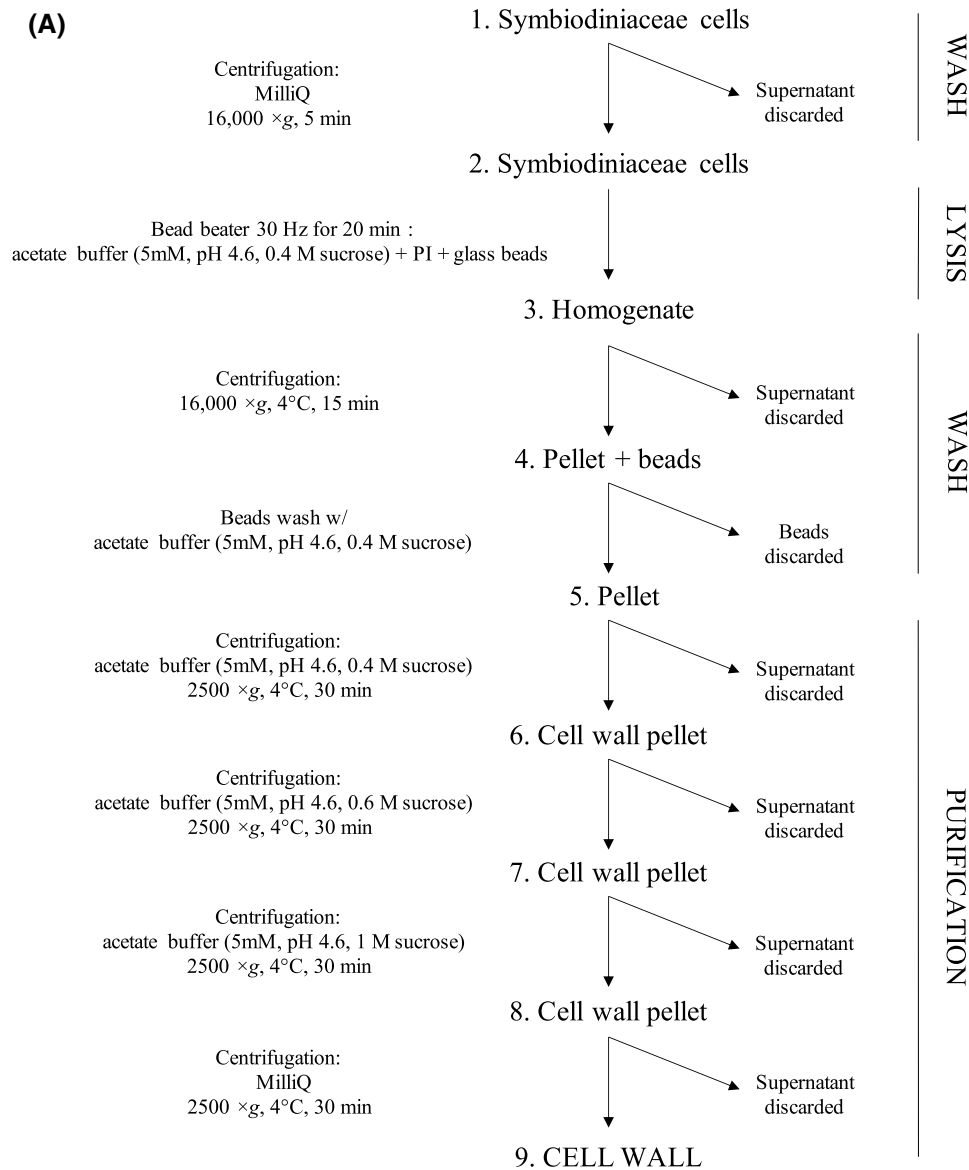→ Supernatant discarded

9. CELL WALL

(B)

FIGURE 1 Symbiodiniaceae cell wall isolation. (A) Schematic representation of the cell wall isolation procedure. (B) Confocal microscopy of Symbiodiniaceae whole cell and of cell wall isolate. The whole cell refers to step 1 and the cell wall isolate refers to step 9 of the cell wall isolation procedure. Calcofluor white is in blue; Symbiodiniaceae chlorophyll autofluorescence (chl) is in red. Scale 10 μm

as described above. The homogenate was then decanted into a new tube, to which B-mercaptoethanol (BME, 1% total volume; Sigma-Aldrich) was added.

The cell wall pellet was suspended in 100 μl 5% SDC 1% BME, and both cell wall and whole cell samples were incubated at 85℃ for 20 min to solubilize proteins. Whole cell protein samples were concentrated with Amicon 0.5 ml 3-kDa molecular weight cut-off filters (Sigma-Aldrich). Both concentrated samples were diluted to 1% SDC with 50 mM Tris pH 8.5, incubated in 10 mM tris(2-carboxyethyl)phosphine (TCEP; Sigma-Aldrich) reducing agent at 95℃ for 10 min to denature disulfide bonds and alkylated with 55 mM iodoacetamide at 37℃ for 45 min. Trypsin (Sigma-Aldrich) digestion was carried out at 37℃ and terminated after 18 h by addition of formic acid (Sigma-Aldrich) to 1% final concentration. Debris and precipitated SDC were pelleted at 16,000 g for 10 min, and peptides transferred to a new tube for LC-ESI-MS/MS analysis.

Liquid chromatography–mass spectrometry

Samples were analyzed by nano-electrospray ionization–liquid chromatography coupled to tandem mass spectrometry (LC-ESI-MS/MS). An Ultimate 3000 RSLC nano-LC system (Thermo Fisher Scientific) was equipped with an Acclaim Pepmap nano-trap column (C18, 100 Å, 75 μm × 2 cm, Thermo Fisher Scientific) and an Acclaim Pepmap RSLC analytical column (C18, 100 Å, 75 μm × 50 cm, Thermo Fisher Scientific) maintained at a temperature of 50℃. Peptides were loaded onto the trap column at an isocratic flow of 5 μl/min of 3% acetonitrile (ACN)/0.05% trifluoroacetic acid (TFA) for 6 min before the trap column was switched in-line with the analytical column. The eluents used were water with 0.1% v/v formic acid (FA) and 5% v/v dimethyl sulfoxide (DMSO) for solvent A and ACN with 0.1% v/v FA and 5% DMSO for solvent B. A 300 nl/min gradient was run from 3% B to 25% B in 34 min, 25% B to 45% B in 8 min, and 45% B to 80% B in 2 min, followed by holding at 80% B for the final 3 min before dropping to 3% B in 1 min and equilibration for 11 min at 3% B prior to the next analysis. Peptides were ionized with a spray voltage of 1.9 kV and analyzed by a Q Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific). The full MS scans were acquired in the Orbitrap at m/z 375–1400, a resolving power of 70,000, an AGC target value of $3.0 × 10^6$ and a maximum injection time of 50 ms. The top 15 most abundant ions in the MS1 spectrum were subjected to higher energy collisional dissociation (HCD) and analyzed in the ion trap at a resolving power of 17,500, auto gain control (AGC) target value of $5 × 10^4$, isolation window of m/z 1.2, and normalized collision energy (NCE) of 30%. Dynamic exclusion of 30 s was enabled.

Protein identification

The LC-ESI-MS/MS spectra were searched against a sequence database constructed from *B. minutum* and *C. goreaui* transcriptomes (Ros, 2020) using MaxQuant (v1.6.12.0; Cox & Mann, 2008). Transcriptomes of *B. minutum* and *C. goreaui* (isolate C124) cultures were generated by RNA extraction using a RNeasy Plant Mini Kit (Qiagen) and Illumina TruSeq library preparation kit, then sequenced using an Illumina HiSeq2500 (Ros, 2020; NCBI SRA: BioProject #PRJNA723630). The transcripts were then assembled in Trinity (v.2.8.4, Haas et al., 2013) using default settings and translated. This resulted in 74,281 *B. minutum* and 87,371 *C. goreaui* amino acid sequences, which formed the database for protein identification. Identified protein sequences were functionally annotated by searching against the publicly available *Symbiodinium microadriaticum* genome (UniProt taxon identifier 2951; Aranda et al., 2016) using diamond-BLAST (v0.9.24; Buchfink et al., 2015). The *B. minutum*/*C. goreaui* sequences were annotated with the best *S. microadriaticum* match for each (maximum expected value 0.001). GO term enrichment was performed by using aGOtool (Schölz et al., 2015), and each GOterm assessed manually to identify potential CWPs.

In MaxQuant, trypsin was designated as the digest enzyme with a maximum of two missed cleavages. The first and main search peptide tolerances were 20 and 4.5 ppm, respectively, and the ion trap MS2 search used a mass tolerance of 0.5 Da. Protein and peptide false discovery rates were both set to 1% with a minimum of two peptides for identification. Methionine oxidation and n-terminus acetylation were allowed as variable modifications and cysteine carbamidomethylation was allowed as a fixed modification. Label-free quantification was enabled with a minimum of two unique peptides. Contaminant proteins and known false hits were filtered out using Perseus (v1.6.10.45) and the data log$_2$-transformed. The matrix was exported to classify proteins that were found either in the whole cell, just in the cell wall, or both in the whole cell and cell wall fractions. We discarded the whole cell sequences and created a list of sequences identified exclusively in the cell wall and the ones common to both the whole cell and cell wall fractions.

TABLE 1 List of cell wall proteins identified in the Symbiodiniaceae species *Breviolum minutum* and *Cladocopium goreaui*

GOterm	Protein ID	Protein name	Gene name	Functional category	Peptide counts	B. minutum	C. goreaui
A0A1Q9E3Y3	TRINITY_DN81331_c0_g1_i1.p1	Choline transporter-like protein 5-A	slc44a5a AK812_SmicGene15055	Transporters	4		x
A0A1Q9DXM6	TRINITY_DN12400_c0_g1_i1.p1	Divalent metal cation transporter MntH	mntH AK812_SmicGene17473	Transporters	4;3;3;1		x
A0A1Q9CX31	TRINITY_DN81400_c0_g1_i1.p1	GTP-binding protein yptV4	YPTV4 AK812_SmicGene31283	Transporters	3		x
A0A1Q9EVT1	TRINITY_DN8565_c0_g2_i3.p1	High-affinity nitrate transporter 2.5	NRT2.5 AK812_SmicGene4635 AK812_SmicGene4659	Transporters	2;2;2;2;2;2;2;1;1	x	
A0A1Q9F6C9	TRINITY_DN75323_c0_g1_i1.p1	Protein ZINC INDUCED FACILITATOR-LIKE 1	ZIFL1 AK812_SmicGene469	Transporters	17		x
A0A1Q9D599	TRINITY_DN589_c0_g1_i1.p1	Putative ABC transporter ATP-binding protein	AK812_SmicGene28158	Transporters	8;3	x	
A0A1Q9EZY9	TRINITY_DN1165_c0_g1_i4.p1	Putative E3 ubiquitin-protein ligase HERC1	HERC1 AK812_SmicGene3039	Transporters	3;3;3;3;2	x	
A0A1Q9E7B9	TRINITY_DN4547_c0_g4_i1.p1	Putative transporter YrhG	yrhG AK812_SmicGene13733	Transporters	2	x	x
A0A1Q9E7B9	TRINITY_DN2129_c0_g1_i3.p1	Putative transporter YrhG	yrhG AK812_SmicGene13733	Transporters	2;2;2;2;2		x
A0A1Q9CTV0	TRINITY_DN331_c0_g1_i9.p1	Sodium-dependent phosphate transport protein 2A	SLC34A1 AK812_SmicGene32588	Transporters	2;2;2;2;1;1;1;1	x	
A0A1Q9D6M3	TRINITY_DN22423_c0_g2_i1.p1	Solute carrier family 12 member 7	SLC12A7 AK812_SmicGene27515	Transporters	2		x
A0A1Q9CNB5	TRINITY_DN66597_c0_g1_i1.p1	Synaptic vesicle 2-related protein	SVOP AK812_SmicGene34731	Transporters	2	x	
A0A1Q9EBY9	TRINITY_DN61236_c0_g1_i1.p1	Vignain	CYSEP AK812_SmicGene1951	Transporters	4;1		x
A0A1Q9E5T8	TRINITY_DN68576_c0_g1_i1.p1	(3S)-malyl-CoA thioesterase	mcl2 AK812_SmicGene14316	Hydrolases	15		x
A0A1Q9D1Y5	TRINITY_DN1384_c0_g1_i12.p1	AAA domain-containing protein	AK812_SmicGene29413	Hydrolases	2;2;2;2;2;1;1	x	
A0A1Q9F599	TRINITY_DN2468_c0_g1_i1.p1	Metalloendopeptidase (EC 3.4.24.-)	AK812_SmicGene955	Hydrolases	4		x
A0A1Q9CVW0	TRINITY_DN12416_c0_g1_i1.p1	Putative isochorismatase family protein YddQ	yddQ AK812_SmicGene31815	Hydrolases	3		x
A0A1Q9CTN2	TRINITY_DN6337_c0_g1_i1.p1	Acyl_transf_3 domain-containing protein	AK812_SmicGene32637	Translocases	4	x	
A0A1Q9C4Z0	TRINITY_DN8204_c0_g1_i1.p1	Canalicular multispecific organic anion transporter 2 (Fragment)	Abcc3 AK812_SmicGene41887	Translocases	2;1	x	
A0A1Q9BYL0	TRINITY_DN294_c0_g1_i6.p1	dTDP-4-amino-4,6-dideoxygalactose transaminase	rffA AK812_SmicGene44383	Translocases	12;12;3;3;3;3;3;1;1	x	
A0A1Q9EF77	TRINITY_DN3047_c0_g1_i4.p1	H(+)-exporting diphosphatase (EC 7.1.3.1)	AK812_SmicGene10685	Translocases	6;1	x	
A0A1Q9EF77	TRINITY_DN1694_c0_g1_i1.p1	H(+)-exporting diphosphatase (EC 7.1.3.1)	AK812_SmicGene10684;	Translocases	7		x

(Continues)

TABLE 1 (Continued)

GOterm	Protein ID	Protein name	Gene name	Functional category	Peptide counts	B. minutum	C. goreaui
A0A1Q9EUX6	TRINITY_DN1401_c0_g1_i3.p1	Hematopoietic prostaglandin D synthase	HPGDS AK812_SmicGene4996	Translocases	5;5		x
A0A1Q9CDQ7	TRINITY_DN3840_c0_g1_i1.p1	Proton-translocating NAD(P)(+) transhydrogenase (EC 7.1.1.1)	NNT AK812_SmicGene38460;	Translocases	6;6	x	
A0A1Q9CDQ7	TRINITY_DN1231_c0_g1_i1.p1	Proton-translocating NAD(P)(+) transhydrogenase (EC 7.1.1.1)	NNT AK812_SmicGene38460;	Translocases	13		x
A0A1Q9EXD2	TRINITY_DN11568_c0_g1_i2.p1	Proton-translocating NAD(P)(+) transhydrogenase (EC 7.1.1.1)	pntA AK812_SmicGene3948	Translocases	13;13;13		x
A0A1Q9EGY9	TRINITY_DN55363_c0_g1_i1.p1	Protein translocase subunit SecA	secA AK812_SmicGene9992	Translocases	5;1		x
A0A1Q9FIJ3	TRINITY_DN14529_c0_g1_i1.p1	Putative serine/threonine-protein kinase-like protein CCR3	CCR3 AK812_SmicGene2484	Translocases	2	x	
A0A1Q9CEJ3	TRINITY_DN47369_c0_g1_i1.p1	Serine/threonine-protein kinase Nek5	NEK5 AK812_SmicGene38102	Translocases	14		x
A0A1Q9EGG1	TRINITY_DN2237_c0_g1_i2.p1	14 kDa zinc-binding protein	ZBP14 AK812_SmicGene10174	Other	5;5		x
A0A1Q9D706	TRINITY_DN775_c0_g1_i1.p1	ANK_REP_REGION domain-containing protein	AK812_SmicGene27441	Other	8		x
A0A1Q9DP71	TRINITY_DN19289_c0_g1_i3.p1	ANK_REP_REGION domain-containing protein	AK812_SmicGene20729	Other	4;4;3;2;2		x
A0A1Q9F7U4	TRINITY_DN2962_c0_g1_i1.p1	Ankyrin repeat domain-containing protein 50	ANKRD50 AK812_SmicGene83	Other	3;3;3;2;1;1;1		x
A0A1Q9CVK2	TRINITY_DN47063_c0_g1_i1.p1	Ankyrin-1	ANK1 AK812_SmicGene31912	Other	10	x	
A0A1Q9CVK2	TRINITY_DN49615_c0_g1_i1.p1	Ankyrin-1	ANK1 AK812_SmicGene31912	Other	10		x
A0A1Q9CRV7	TRINITY_DN9643_c0_g1_i2.p1	Long-chain fatty acid--CoA ligase 4	ACSL4 AK812_SmicGene33346	Other	2;2;2;2;2;2;2		x
A0A1Q9EJE9	TRINITY_DN55895_c0_g1_i1.p1	Long-chain fatty acid--CoA ligase ACSBG2	ACSBG2 AK812_SmicGene9054	Other	4	x	
A0A1Q9D7L1	TRINITY_DN1621_c0_g1_i1.p1	Oxygen-independent coproporphyrinogen-III oxidase-like protein	AK812_SmicGene27203	Other	2	x	
A0A1Q9DK01	TRINITY_DN757_c0_g4_i1.p1	Potassium voltage-gated channel subfamily H member 5	Kcnh5 AK812_SmicGene22354	Other	2		x
A0A1Q9CCS4	TRINITY_DN55369_c0_g1_i1.p1	Reticulocyte-binding protein 2-like a	AK812_SmicGene38827	Other	3	x	
A0A1Q9ERN9	TRINITY_DN16676_c0_g1_i1.p1	Reticulocyte-binding protein 2-like a	AK812_SmicGene6218	Other	3		x
A0A1Q9D5J5	TRINITY_DN67878_c0_g1_i1.p1	Transmembrane protein 87A	tmem87a AK812_SmicGene27971	Other	2		x

Note: GOterm, protein ID, protein name, gene name, functional category, and number of peptides identified are reported for each protein. Uncharacterized proteins have been omitted and are included in Table S1.

RESULTS AND DISCUSSION

In this study, we prepared CWPs of two Symbiodiniaceae species (*B. minutum* and *C. goreaui*) by adapting a method developed in the model plant *Arabidopsis* (Feiz et al., 2006). Complete separation of cell wall from internal membranes and organelles of the algal cell was difficult to achieve. Our cell-wall-enriched fraction likely includes algal membranes, particularly the plasma membrane and the alveolar sac membranes (also known as amphiesmal vesicles). A series of centrifugations, aimed to separate cell walls from membranes and organelles of the Symbiodiniaceae cell, was performed (Figure 1A). The efficacy of the cell wall isolation was then confirmed microscopically (Figure 1B and Figure S1). Intact Symbiodiniaceae cells showed algal chlorophyll autofluorescence (in red) surrounded by a continuous layer of cellulose (stained in blue by calcofluor white). In comparison, the cell wall isolates appeared as calcofluor white-stained cellulosic fractions, and the chlorophyll autofluorescence was not detected, suggesting that the algal cell lost pigments and inner cell structures during the cell wall isolation procedure. This permitted the proteomic analysis of Symbiodiniaceae cell-wall-enriched samples.

We identified a total of 786 proteins for *B. minutum* and 1487 proteins for *C. goreaui*. Filtering of these proteins according to their potential relevance to the cell wall resulted in 70 high confidence CWPs, of which 25 were identified for *B. minutum* and 54 for *C. goreaui* (Table 1 and Table S1). It is likely that a few scarce or labile proteins were lost during preparation of the samples. The identified CWPs clustered accordingly to the Symbiodiniaceae species. Surprisingly, only a few proteins were shared between cultured *B. minutum* and *C. goreaui* (Figure 2), suggesting that the phylogenetic differences between the two Symbiodiniaceae may be reflected by their surface proteome. The proteins were annotated as integral components of the membrane and grouped into five main functional categories: transporters (12), hydrolases (4), translocases (9), uncharacterized proteins (32), and other functions (12; Figure 3).
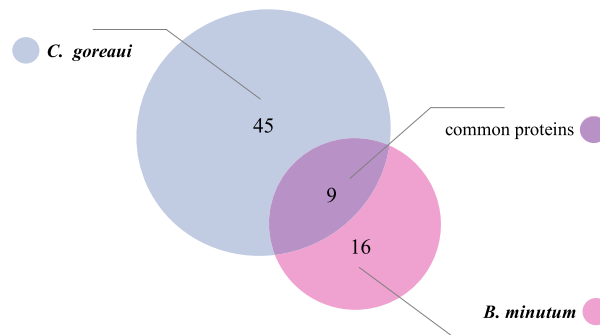

FIGURE 2 Venn diagram of cell wall proteins identified in *Breviolum minutum* (purple) and *Cladocopium goreaui* (blue). *B. minutum* = 16 proteins; *C. goreaui* = 45 proteins; *B. minutum* and *C. goreaui* = 9 proteins
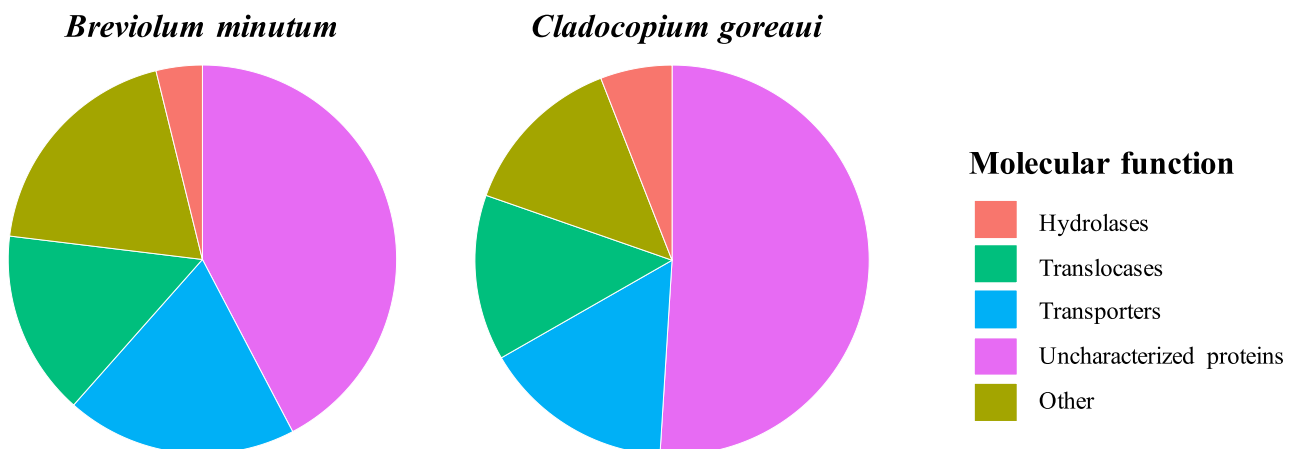

FIGURE 3 Relative proportions of proteins identified on the cell wall of *Breviolum minutum* and *Cladocopium goreaui*. The proteins are grouped in five main functional categories: hydrolases, translocases, transporters, uncharacterized proteins, and other (oxidases, protein–protein interactions, cell adhesion)

Transporters

Membrane transport proteins regulate the selective passage of molecules across membranes, and so their presence in the algal cell wall is unsurprising (Table 1, Figure 3, Table S1).

We identified zinc (Zn) and nitrate (NO_3^-) transporters as potential CWPs. Both Zn and NO_3^- are used by the dinoflagellate cell in several biological functions, ranging from cell growth to metabolism (Karim et al., 2011) and must be acquired from the surrounding environment. The presence of Zn induced facilitator-like-1a and high affinity NO_3^- transporters has been previously shown in plants (Lezhneva et al., 2014; Ricachenevsky et al., 2011), so their presence is not surprising in the cell wall of Symbiodiniaceae. The high affinity NO_3^- transporter 2.5, in particular, is well studied in higher plants, where it is localized to the plasma membrane and involved in NO_3^- acquisition and re-mobilization (Lezhneva et al., 2014). Nitrate—together with ammonium (NH_4^+)—are two common forms of available nitrogen (N) found in natural ecosystems and are key nutrients for cellular growth and development. In the cnidarian–dinoflagellate symbiosis, there is a highly efficient system of N cycling and assimilation (Pernice et al., 2012). Indeed, Symbiodiniaceae are specialized in assimilating dissolved inorganic nitrogen (DIN) from the environment and, although both host and symbiont have the enzymes to incorporate DIN, it is the symbiont that accounts for the majority of NO_3^- and NH_4^+ acquired from the surrounding water (Pernice et al., 2012). N transporters are, therefore, important players in the regulation of the mutualism between cnidarians and their symbiotic dinoflagellates.

A choline-like transporter (CTL) involved in the movement of choline for phospholipid synthesis (Michel et al., 2006) was detected among the possible CWPs of *C. goreaui*. CTLs are enriched in the Symbiodiniaceae genome compared to other eukaryotes (Aranda et al., 2016), and genes for this class of transporters were found to be differentially expressed in the algae when in culture compared to *in hospite* (Maor-Landaw et al., 2020). When symbiotic with cnidarians, the dinoflagellate produces lipids that are transferred to the host (Peng et al., 2011). CTL proteins may, thus, have a role in regulating lipid biosynthesis and metabolism of the cnidarian–dinoflagellate mutualism (Maor-Landaw et al., 2020).

ABC transporters belonging to the ATP-binding cassette (ABC) superfamily are characterized by two regions: a highly conserved ABC and a transmembrane domain. These transporters hydrolyze ATP to promote the import/export of various substrates, including small ions, metabolic products, lipids, and sterols (Higgins, 2001; Wang et al., 2011). Here, a putative ABC transporter was found in the CWPs of *B. minutum*. This transporter has previously been identified in the cnidarian–Symbiodiniaceae symbiosis in both the symbiont (Aranda et al., 2016) and the host (Meyer & Weis, 2012), and on the symbiosome membrane (Peng et al., 2010). The cell walls of the green alga *Haematococcus pluvialis* (Wang et al., 2004) and the dinoflagellate *Alexandrium catenella* (Wang et al., 2011) also possess several ABC transporters, suggesting a role for these proteins in nutrient transport. Although these transporters remain uncharacterized in the cnidarian–dinoflagellate symbiosis, it is possible that they are involved in inter-partner nutrient flux.

Hydrolases

Hydrolases catalyze hydrolysis reactions, the cleavage of a covalent bond by the addition of a water molecule. This class of enzymes is commonly found on the walls of bacteria (Gumucio & Ostrow, 1991) and higher plants (Minic, 2008). In this study, we identified four hydrolases: (3S)-malyl-CoA thioesterase, AAA domain-containing protein, metalloendopeptidase, and putative isochorismatase family protein YddQ (Table 1, Figure 3, Table S1).

Breviolum minutum CWPs showed an AAA domain-containing protein belonging to a large family of proteins that are characterized by a conserved 230 amino acid residues. The protein identified here contains an AAA ATPase domain in residues 87–180, which has 71% similarity to the canonical conserved AAA domain. AAA domain protein activity combines the chemical energy provided by the hydrolysis of ATP and alterations in their structural conformation to induce conformational changes in a wide range of macromolecules (Erzberger & Berger, 2006). They are involved in many cellular processes and their functions range from membrane fusion to signal transduction (Hanson & Whiteheart, 2005).

Cladocopium goreaui CWPs contained a metalloendopeptidase, a protein belonging to a diverse group of enzymes that catalyzes the hydrolysis of internal, α-peptide bonds of a polypeptide chain. Previously found in Symbiodiniaceae (Baumgarten, 2013), the integral membrane metalloendopeptidases play a role in physiological and pathological processes and are involved in cell adhesion (Bond & Beynon, 1995; Sauer et al., 2004).

Translocases

Translocases are proteins that use enzyme activity to move molecules across membranes. Nine translocases were found in the cell wall fractions of *B. minutum* and *C. goreaui* (Table 1, Figure 3, Table S1).

Two translocases were common to the cell wall of both Symbiodiniaceae species: H(+)-exporting diphosphatase and proton-translocating NAD(P)(+) transhydrogenase. The first enzyme uses the energy from diphosphate hydrolysis to move protons across the membrane, while the second one catalyzes the transfer of hydride equivalents from NADH to NADP+, hence regenerating NADPH in the cell (Sauer et al., 2004).

We identified the hematopoietic prostaglandin D synthase (HPGDS) among the CWPs of *C. goreaui*. HPGDS is a key enzyme in the synthesis of prostaglandins (PGs; Kanaoka & Urade, 2003), as the name suggests. PGs are derivatives of polyunsaturated fatty acids that commonly act as mediators in a range of physiological and pathological processes (Di Dato et al., 2020). First described in higher vertebrates, these molecules have also been discovered in marine invertebrates (Cnidaria, Mollusca and Crustacea), macroalgae, and microalgae (Di Costanzo et al., 2019). Animal-like PGs in unicellular photosynthetic eukaryotes have been proposed to play a role in mediating intracellular and extracellular (cell–cell) signaling (Di Dato et al., 2017; Rosset et al., 2020). Although the function of these proteins has not yet being described in Symbiodiniaceae, they might play a similar role in dinoflagellates.

A subunit of the Sec pathway, the SecA protein, was also found. The SecA translocase protein is a cell membrane-associated subunit of the Sec pathway and has been described in diatoms (Chan et al., 2011). It has the functional properties of an ATPase and translocates macromolecules involved in the biogenesis of cell walls and signaling (Vrontou & Economou, 2004).

Other

Proteins with several other functions (oxidase, protein–protein interaction, and cell adhesion) were also identified on the cell wall of Symbiodiniaceae and are considered here (Table 1, Figure 3, Table S1).

The ANK repeat (ANKr) is a conserved domain of approximately 33 amino acids, characteristic of the components of the Ankyrin family, one of the most common protein families across all kingdoms of life (Brüwer et al., 2017). We found proteins containing this domain in both *B. minutum* and *C. goreaui* CWPs. In general, ANKr functions as a protein–protein interaction domain and mediates cross-talk between host and symbiont in various endosymbioses (Liu et al., 2018; Thomas et al., 2010). For instance, four ANKr proteins were discovered in γ-proteobacteria when symbiotic with sponges and proposed to prevent phagocytosis, thus allowing the symbiont to escape digestion by the sponge host (Nguyen et al., 2014). In some cases, this protein motif is part of the signaling network of viral infections and works against host innate immunity by preventing apoptosis of infected cells (Al-Khodor et al., 2010; Brüwer et al., 2017). In the Symbiodiniaceae genome, ANKrs represent the second largest gene family (after the EF-hand family; Shinzato et al., 2014). Although the function of ANKr is not well described in the Symbiodiniaceae, these polypeptides may play a role in suppressing phagocytosis, as they appear to do in the bacterial-sponge symbiosis.

One CWP that caught our attention is a putative homologue of the malaria parasite reticulocyte-binding-like (RBL) protein 2a. RBL proteins are sialic acid receptors crucial to host cell recognition and invasion by *Plasmodium*, the malaria-causing pathogen (Rayner et al., 2001). Dinoflagellates and *Plasmodium* are closely related, both being members of the Infra-Kingdom Alveolata, and it is widely held that Apicomplexa, the group of parasites to which *Plasmodium* belongs, arose from photosynthetic, endosymbiotic mutualists akin to modern day Symbiodiniaceae (Berney & Pawlowski, 2006). Thus, at face value, RBL homologues in the cell walls of Symbiodiniaceae might point to an ancient, conserved mechanism of host cell recognition/invasion. However, the sequence similarity between A0A1Q9CCS4, A0A1Q9ERN9 in the *S. microadriaticum* genome (Aranda et al., 2016) and RBL homologues of *Plasmodium* is confined to low complexity repeats. Whether or not CWPs A0A1Q9CCS4 and A0A1Q9ERN9 have a role in dinoflagellate–cnidarian symbiosis remains to be determined.

CONCLUSIONS

This study explored the cell wall protein composition of two cultured Symbiodiniaceae species: *B. minutum* and *C. goreaui*. We developed a method to isolate Symbiodiniaceae cell walls and performed a proteomic analysis of cell-wall-enriched samples. Transporters, translocases, hydrolases, and polypeptides involved in protein–protein interactions and cell signaling were identified in this analysis of the algal cell wall proteome. The majority of proteins identified have yet to be functionally characterized in Symbiodiniaceae, so this study flags numerous interesting candidates for further investigation to better understand this iconic symbiosis. We found little overlap between the cell wall proteomes of *B. minutum* and *C. goreaui*. The proteins identified here refer to cultured algae. Various traits of Symbiodiniaceae biology are likely to change between the two lifestyles of the organism (free-living and *in hospite*) as a result of symbiosis (Maruyama & Weis, 2020). Furthermore, previous studies showed that the Symbiodiniaceae cell wall adjusts its thickness when switching from cultured to being *in hospite* (Palincsar et al., 1988; Wakefield et al.,

2000). Thus, it is feasible that the suite of proteins populating the cell wall might change as the algae switch between their two lifestyles. Our development of protocols to isolate and characterize CWPs in free-living algae paves the way for future work to compare the cell wall proteome of free-living vs. symbiotic Symbiodiniaceae. This would provide precious insights into the molecules that are critical to the mutualistic state and the effect that symbiosis has on the mutualistic partners.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.