INTRODUCTION

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 (Morrell & 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 algae 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 goreau. 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. goreau (LaJeunesse and Jeong; SCF 055-01.10, ITS2 type Cl). B. minutum was originally isolated from the anemone Exaiptasia diaphana (Tortorelli et al., 2020), whereas C. goreau 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 Exaiptasia diaphana (Tortorelli et al., 2020), whereas the anemone

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 μg 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°C 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°C 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 AIR confocal laser scanning microscope (Nikon) with a 409-nm laser to detect stained cellulose.
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.
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 as described above. The homogenate was then decanted into a new tube, to which B-mercaptopethanol (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°C 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°C for 10 min to denature disulfide bonds and alkylated with 55 mM iodoacetamide at 37°C for 45 min. Trypsin (Sigma-Aldrich) digestion was carried out at 37°C 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°C. 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 × 106 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 × 104, 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 log2-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.
| 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 | Transports | 4 | x |
|        | A0A1Q9DXM6 | TRINITY_DN12400_c0_g1_i1.p1 | Divalent metal cation transporter MntH | mtH AK812_SmicGene17473 | Transports | 4;3;3;1 | x |
|        | A0A1Q9CX31 | TRINITY_DN81400_c0_g1_i1.p1 | GTP-binding protein yptV4 | YPTV4 AK812_SmicGene31283 | Transports | 3 | x |
|        | A0A1Q9ETI1 | TRINITY_DN8565_c0_g2_i3.p1 | High-affinity nitrate transporter 2.5 | NRT2.5 AK812_SmicGene4635 AK812_SmicGene4659 | Transports | 2;2;2;2;2;2;2;1;1 | x |
|        | A0A1Q9FC9 | TRINITY_DN75323_c0_g1_i1.p1 | Protein ZINC INDUCED FACILITATOR-LIKE 1 | ZIFL1 AK812_SmicGene469 | Transports | 17 | x |
|        | A0A1Q9D599 | TRINITY_DN589_c0_g1_i1.p1 | Putative ABC transporter ATP-binding protein | AK812_SmicGene28158 | Transports | 8;3 | x |
|        | A0A1Q9EZY9 | TRINITY_DN1165_c0_g1_i4.p1 | Putative E3 ubiquitin-protein ligase HERC1 | HERC1 AK812_SmicGene3039 | Transports | 3;3;3;3;2 | x |
|        | A0A1Q9EB9 | TRINITY_DN4547_c0_g4_i1.p1 | Putative transporter YrhG | yrhG AK812_SmicGene13733 | Transports | 2 | x |
|        | A0A1Q9EB9 | TRINITY_DN2129_c0_g1_i3.p1 | Putative transporter YrhG | yrhG AK812_SmicGene13733 | Transports | 2;2;2;2;2;2 | x |
|        | A0A1Q9CTV0 | TRINITY_DN331_c0_g1_i9.p1 | Sodium-dependent phosphate transport protein 2A | SLC34A1 AK812_SmicGene32588 | Transports | 2;2;2;1;1;1;1 | x |
|        | A0A1Q9C5M3 | TRINITY_DN22423_c0_g2_i1.p1 | Solute carrier family 12 member 7 | SLC12A7 AK812_SmicGene27515 | Transports | 2 | x |
|        | A0A1Q9CNB5 | TRINITY_DN66597_c0_g1_i1.p1 | Synaptic vesicle 2-related protein | SVOP AK812_SmicGene34731 | Transports | 2 | x |
|        | A0A1Q9EB9 | TRINITY_DN61236_c0_g1_i1.p1 | Vignain | Vignain AK812_SmicGene1951 | Transports | 4;1 | x |
|        | A0A1Q9EST8 | TRINITY_DN68576_c0_g1_i1.p1 | (3S)-malyl-CoA thioesterase | md2 AK812_SmicGene4316 | Hydrolases | 15 | x |
|        | A0A1Q9D1Y5 | TRINITY_DN1384_c0_g1_i2.p1 | AAA domain-containing protein | AK812_SmicGene29413 | Hydrolases | 2;2;2;2;1;1 | x |
|        | A0A1Q9F9R9 | 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 | rfaA 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 |
| GOterm          | Protein ID                | Protein name                                                                 | Gene name                  | Functional category | Peptide counts | B. minutum | C. goreaui |
|-----------------|---------------------------|------------------------------------------------------------------------------|----------------------------|---------------------|----------------|------------|------------|
|                 | TRINITY_DN1401_c0_g1_i3.p1| Hematopoietic prostaglandin D synthase                                      | HPGDS AK812_SmicGene4996  | Translocases        | 5;5            |           | x          |
|                 | TRINITY_DN3840_c0_g1_i1.p1| Proton-translocating NAD(P)(+) transhydrogenase (EC 7.1.1.1)                | NNT AK812_SmicGene38460;  | Translocases        | 6;6            |           | x          |
|                 | TRINITY_DN1231_c0_g1_i1.p1| Proton-translocating NAD(P)(+) transhydrogenase (EC 7.1.1.1)                | NNT AK812_SmicGene38460;  | Translocases        | 13             |           | x          |
|                 | TRINITY_DN11568_c0_g1_i2.p1| Proton-translocating NAD(P)(+) transhydrogenase (EC 7.1.1.1)                | pntA AK812_SmicGene3948    | Translocases        | 13;13;13      |           | x          |
|                 | TRINITY_DN53363_c0_g1_i1.p1| Protein translocase subunit SecA                                            | secA AK812_SmicGene9992    | Translocases        | 5;1            |           | x          |
|                 | TRINITY_DN14529_c0_g1_i1.pl| Putative serine/threonine-protein kinase-like protein CCR3                  | CCR3 AK812_SmicGene2484    | Translocases        | 2              |           | x          |
|                 | TRINITY_DN47369_c0_g1_i1.p1| Serine/threonine-protein kinase Nek5                                        | NEK5 AK812_SmicGene38102   | Translocases        | 14             |           | x          |
|                 | TRINITY_DN2237_c0_g1_i2.p1| 14 kDa zinc-binding protein                                                  | ZBP14 AK812_SmicGenel0174   | Other               | 5;5            |           | x          |
|                 | TRINITY_DN775_c0_g1_i1.p1  | ANK_REP_REGION domain-containing protein                                    | AK812_SmicGene27441        | Other               | 8              |           | x          |
|                 | TRINITY_DN19289_c0_g1_i3.p1| ANK_REP_REGION domain-containing protein                                    | AK812_SmicGene20729        | Other               | 4;4;3;3;2;2    |           | x          |
|                 | TRINITY_DN2962_c0_g1_i1.p1  | Ankyrin repeat domain-containing protein 50                                 | ANKRD50 AK812_SmicGene83    | Other               | 3;3;2;1;1;1    |           | x          |
|                 | TRINITY_DN47063_c0_g1_i1.p1| Ankyrin-1                                                                    | ANK1 AK812_SmicGene31912   | Other               | 10             |           | x          |
|                 | TRINITY_DN49615_c0_g1_i1.p1| Ankyrin-1                                                                    | ANK1 AK812_SmicGene31912   | Other               | 10             |           | x          |
|                 | 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          |
|                 | TRINITY_DN55895_c0_g1_i1.p1| Long-chain fatty acid--CoA ligase ACSBG2                                     | ACSBG2 AK812_SmicGene9054  | Other               | 4              |           | x          |
|                 | TRINITY_DN1621_c0_g1_i1.p1  | Oxygen-independent coproporphyrinogen-III oxidase-like protein               | AK812_SmicGene27203        | Other               | 2              |           | x          |
|                 | TRINITY_DN757_c0_g4_i1.p1  | Potassium voltage-gated channel subfamily H member 5                         | Kcnh5 AK812_SmicGene22354  | Other               | 2              |           | x          |
|                 | TRINITY_DN55569_c0_g1_i1.p1| Reticulocyte-binding protein 2-like a                                       | AK812_SmicGene38827        | Other               | 3              |           | x          |
|                 | TRINITY_DN16676_c0_g1_i1.p1| Reticulocyte-binding protein 2-like a                                       | AK812_SmicGene6218         | Other               | 3              |           | x          |
|                 | 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. goreau) 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. goreau. 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. goreau (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. goreau (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 goreau (blue). B. minutum = 16 proteins; C. goreau = 45 proteins; B. minutum and C. goreau = 9 proteins

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

An 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-associate 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).

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 A0AIQ9CCS4, A0AIQ9ERN9 in the S. microadriaticum genome (Aranda et al., 2016) and RBL homologues of Plasmodium is confined to low complexity repeats. Whether or not CWPs A0AIQ9CCS4 and A0AIQ9ERN9 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.

ACKNOWLEDGMENTS
We thank Shuai Nie from the Melbourne Mass Spectrometry and Proteomics Facility (MMSPF) at Bio21 at the University of Melbourne for assistance with the preparation of samples for proteomics analysis. We also thank David Suggett, Emma Camp, and Tim Kahilke for the Brevelium minutum and Cladocopium goreaui transcriptome sequences. G.T. acknowledges receipt of the University of Melbourne International Research Scholarship and Fee Remission Scholarship, the EMBL Australia travel grant fund and the Science Abroad Travelling Award at the University of Melbourne that supported this work. MJHvO acknowledges Australian Research Council Laureate Fellowship FL180100036. GMcF and MJHvO acknowledge Australian Research Council Discovery Grant DP160101539. GMcF acknowledges Australian Research Council Discovery Grant DP210100639. Financial support was provided by the Marsden Fund of the Royal Society Te Apārangi to S.K.D. and C.A.O.

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How to cite this article: Tortorelli, G., Oakley, C.A., Davy, S.K., van Oppen, M.J.H. & McFadden, G.I. (2021) Cell wall proteomic analysis of the cnidarian photosymbionts Breviolum minutum and Cladocopium goreau. Journal of Eukaryotic Microbiology, 00, e12870. https://doi.org/10.1111/jeu.12870
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Title:
Cell wall proteomic analysis of the cnidarian photosymbionts Breviolum minutum and Cladocopium goreaui

Date:
2022-01

Citation:
Tortorelli, G., Oakley, C. A., Davy, S. K., van Oppen, M. J. H. & McFadden, G. (2022). Cell wall proteomic analysis of the cnidarian photosymbionts Breviolum minutum and Cladocopium goreaui. JOURNAL OF EUKARYOTIC MICROBIOLOGY, 69 (1), https://doi.org/10.1111/jeu.12870.

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