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# Proteomics of a toxic dinoflagellate *Alexandrium catenella* DH01: Detection and identification of cell surface proteins using fluorescent labeling

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*Alexandrium catenella* DH01 is a toxic dinoflagellate species that is able to not only produce paralytic shellfish toxins, but also cause harmful algal blooms along the coast of China. In this study, we presented a new protocol for specific labeling and detection of the cell surface proteins (CSPs) of *A. catenella* DH01 cells using CyDye difference gel electrophoresis (DIGE) fluor minimal dyes. CSPs were identified using two-dimensional gel electrophoresis (2-DE) and MALDI TOF-TOF mass spectrometry (MS). The results showed that the fluorescent cyanine dye Cy3 could specifically label the CSPs of *A. catenella* DH01, with minimal labeling of intracellular proteins. Among three protein extraction methods evaluated, the Trizol method was the most efficient to extract CSPs with respect to protein spot number and resolution. Forty-one CSPs were separated and identified from *A. catenella* DH01 by 2-DE, in which 14 were identified in the protein database using MALDI TOF-TOF MS analysis. This work represents the first attempt to investigate the CSPs of *A. catenella* using the CyDye DIGE fluor dyeing method that provides a potentially important tool for future comprehensive characterization of CSPs and elucidation of the physiological functions of CSPs in dinoflagellates.

***Alexandrium catenella*, cell surface protein, cyanine dye, protein extraction, two-dimensional gel electrophoresis, MALDI TOF-TOF**

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*Alexandrium* is a dinoflagellate genus that is widely spread throughout many regions of the world [1,2]. Many species within this genus are able to produce paralytic shellfish toxins (PSTs), a family of potent neurotoxins, which specifically bind to sodium channels in neural cells and result in paralytic shellfish poisoning [3]. In the past few decades, harmful algal blooms (HABs) formed by *Alexandrium* have been significantly increasing in frequency, intensity and distribution, resulting in serious economic and public health related problems, which has attracted significant attention to this genus from various aspects [4].

A free-living *Alexandrium* cell is covered by theca or amphiesma consisting of a continuous outermost membrane, an outer plate membrane and a single-membrane bound thecal vesicle [5]. Inside this vesicle, a number of cellulosic thecal plates are subtended by a pellicular layer. Thecal plates usually consist primarily of cellulose and polysaccharides, with a small amount of protein [6,7]. Previous studies of *Alexandrium* cell walls have been mainly based on electron microscopic and cytochemical observations [8], and molecular information on cell wall biogenesis and dynamics is lacking. For example, no cell surface proteins (CSPs) have been identified from *Alexandrium*, and this lack of characterization has impeded our understanding of *Alexandrium*.

A number of proteins and enzymes reside on the cell surface and outer membrane of phytoplankton, such as high-affinity binding proteins [9–11], transporters [12–16], stress proteins [17,18], signaling proteins [19] and ectoenzymes [20–27]. These proteins play important roles in

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nutrient utilization, defense, signaling, cell adhesion, and cell recognition. The outmost membrane of the *Alexandrium* thecal wall is a subcellular component of substantial interest with regard to the various aspects of cell surface associated ecophysiology. However, there are few experimental data available concerning the outer membrane of *Alexandrium*, compared with those of other organisms, because of incomplete genome sequences of this genus. Thus far, only limited CSPs and enzymes have been identified and characterized at biochemical and functional levels, and the mechanisms of their functions and localization have not been elucidated [28–32]. Some studies indicate that CSPs and their activities are induced or increased by factors that limit the growth of these eukaryotic phytoplankton because of potentially enhanced cell scavenging of nutrients [28–30]. However, dinoflagellate CSPs may also have other important properties. For example, in *Dictyostelium discoideum*, CSPs have been reported to be involved in signaling pathways [19]. Evidently, CSPs present an important site of interaction between algal cells and their environment. Therefore, a better understanding of the structure and composition of the dinoflagellate cell surface may contribute to revealing important physiological activities on the cell surface and the mechanism that causes blooming of dinoflagellate species.

The study of CSPs has relied on various methods to isolate and identify CSPs from the cell surface or outmost membrane of dinoflagellates. A current strategy is labeling the CSPs of intact cells using vectorial labeling [31] or biotinylation [32] reagents prior to extraction. The major problem with these pre-labeling systems is the loss of protein solubility due to multiple additions of large hydrophobic groups to the proteins. Moreover, these methods can only address one or a few proteins at one time. Another strategy is isolation of CSPs by sequential extraction from whole cells. However, this approach not only extracts CSPs, but also other cell wall-associated proteins and results in potential cross contamination with cytosolic proteins [33]. Recent modifications of traditional DIGE procedures have enabled an elegant examination of CSPs by protein labeling prior to lysis [34,35]. This method has been used to label human cell lines *in vitro* and *in vivo* [34], bacterial CSPs [36] and the monocyte plasma membrane [37], and has been shown to be a powerful tool to study CSPs.

*A. catenella* DH01, a major HAB species that is widely spread in the coastal waters of China, not only produces PSTs, but also has formed extensive blooms in the East China Sea in recent years [38]. Thus, *A. catenella* DH01 has become an economic and public health concern, and losses in mariculture and the threat to human life due to PST exposure are increasingly documented. In this study, we developed a simple and rapid method to label *A. catenella* DH01 cells with the fluorescent cyanine dye Cy3, compared three extraction methods for CSPs and identified CSPs using MALDI-TOF-TOF mass spectrometry (MS) and database searching. The goals of this study were to establish an efficient and reliable detection and extraction method for CSPs from dinoflagellates, construct a two-dimensional CSP reference map of *A. catenella* and characterize putative proteins to provide a foundation for future investigation of the function and expression of CSPs in *A. catenella*.

## 1 Materials and methods

### 1.1 Organism and culture conditions

*A. catenella* DH01 was provided by the Culture Collection Center of Marine Bacteria and Algae of the State Key Laboratory of Marine Environmental Science, Xiamen University, China. A unialgal isolate was routinely maintained in K medium [39] at 20°C under a 14 h:10 h light:dark photoperiod at a light intensity of approximately 100 μmol photons $m^{-2}$ $s^{-1}$ provided by fluorescent lamps. Cells for surface labeling and CSP analysis were grown in 5 L flasks containing 4 L K medium under the culture conditions described above. K medium did not contain protein.

### 1.2 Cell surface labeling

The CSPs of *A. catenella* DH01 cells were labeled using the Ettan DIGE dyeing protocol with a minor modification. Algal cells ($1\times10^7$) were collected by centrifugation at 800 ×*g* for 5 min at 20°C. The supernatant was removed, and the cell pellet was resuspended in 1 mL phosphate-buffered saline (PBS; 0.02 mol $L^{-1}$ phosphate and 0.15 mol $L^{-1}$ NaCl, pH 7.5) and 1 mL ice-cold Hank's Balanced Salt Solution (HBSS; pH 8.5) successively, followed by centrifugation at 800×*g* at 4°C for 2 min. The supernatant was removed, and the cell pellet was resuspended in 200 μL ice-cold labeling buffer (HBSS, pH 8.5, and 1 mol $L^{-1}$ urea). Intact cells were labeled with 600 pmol Cy3 for 20 min on ice in the dark. The reaction was stopped by adding 20 μL 10 mmol $L^{-1}$ lysine and incubating for 10 min. Labeled cells were washed twice with 500 μL HBSS (pH 7.4), followed by centrifugation at 800×*g* at 4°C for 2 min. The fluorescence of intact Cy3-labeled algal cells was observed under a fluorescence microscope (Leica DM 4500 B, Germany) using Leica FW4000 software.

### 1.3 Protein extraction

Three methods of protein preparation were used to extract the Cy3-labeled CSPs of *A. catenella* DH01 [40–42].

(i) The urea/amidosulfobetaine-14 (ASB-14) extraction method. Pre-chilled urea/ASB-14 extraction buffer (0.5 mL) containing 7 mol $L^{-1}$ urea, 2 mol $L^{-1}$ thiourea, 1% ASB-14 (w/v), 1% DTT (w/v) and 2% carrier ampholytes was added to the cell pellet. Then, the cell pellet was sonicated on ice. Cell debris was removed by centrifugation at 20000×*g* for 30 min at 4°C, and then 0.5 mL pre-chilled 20% trichloracetic acid (TCA)/acetone (w/v) was added to

the supernatant followed by incubation for 30 min at –20°C to precipitate proteins. The solution was then centrifuged at 20000×*g* for 30 min at 4°C, and the supernatant was removed. The cell pellet was washed three times with ice-cold acetone containing 20 mmol $L^{-1}$ DTT by centrifugation at 20000×*g* for 30 min at 4°C each time. Residual acetone was removed in a speed Vac, and proteins were dissolved in 50 μL rehydration buffer containing 7 mol $L^{-1}$ urea, 2 mol $L^{-1}$ thiourea, 1% ASB-14 (w/v), 2 mmol $L^{-1}$ tributylphosphine (TBP) (w/v), 0.5% immobilized pH gradient (IPG) buffer and a trace amount of bromophenol blue.

(ii) The $CaCl_2$-LiCl sequential extraction method. The cell pellet was extracted twice with 2 mL $CaCl_2$ solution (5 mmol $L^{-1}$ acetate buffer, pH 4.6, and 0.2 mol $L^{-1}$ $CaCl_2$), followed by two extractions with 2 mL LiCl solution (5 mmol $L^{-1}$ acetate buffer, pH 4.6, and 2 mol $L^{-1}$ LiCl) [33]. Protein precipitation was performed as described above. Proteins were dissolved in 50 μL rehydration buffer containing 7 mol $L^{-1}$ urea, 2 mol $L^{-1}$ thiourea, 4% CHAPS, 65 mmol $L^{-1}$ DTT (w/v) and 0.5% IPG buffer.

(iii) The Trizol extraction method. Trizol reagent (1 mL) was added to the cell pellet, followed by sonication on ice. Then, 200 μL chloroform was added to the cell lysate before shaking vigorously for 15 s. The mixture was allowed to stand for 5 min at room temperature before centrifugation at 12000×*g* for 15 min at 4°C. The top pale-yellow to colorless layer was removed, 300 μL ethanol was added to resuspend the reddish bottom layer, and the mixture was centrifuged at 2000×*g* for 5 min at 4°C. The supernatant was transferred to a new tube and 2 mL isopropanol was added. Then, the mixture was stored at –20°C for at least 1 h to precipitate proteins. The mixture was then centrifuged at 14000×*g* for 30 min at 4°C, and the recovered pellet was briefly washed with 95% ethanol and then air dried. Fifty microliters of rehydration buffer (7 mol $L^{-1}$ urea, 2 mol $L^{-1}$ thiourea, 4% CHAPS, 65 mmol $L^{-1}$ DTT (w/v) and 0.5% IPG buffer) was added to dissolve proteins.

For the three extractions described above, protein quantification was performed using a 2D Quant kit (GE Healthcare, Piscataway, NJ).

### 1.4 Two-dimensional gel electrophoresis (2-DE) analysis

Rehydration, isoelectric focusing (IEF) and equilibration were performed as described elsewhere [40]. Briefly, protein (400 μg) obtained from dye-labeled *A. catenella* DH01 cells was subjected to IEF using an IPGphor III system (Amersham Biosciences, Xiamen, China) with 24 cm IPG strips (Immobiline Drystrip™, pH 3–10 and 4–7; Amersham Biosciences) and then resolved on a 12.5% slab gel with sodium dodecylsulfate polyacrylamide gel electrophoresis. The gel was overlaid with 0.5% agarose (dissolved in running buffer containing bromophenol blue) and 2-DE was run using an Ettan DALTsix Vertical System (GE Healthcare, USA) at 1 W/gel for 30 min, and then at 15 W/gel until the dye front reached the bottom of the gel.

### 1.5 Protein visualization

After 2-DE, gels were scanned for Cy3-labeled proteins using a Typhoon™ 9400 Imager (GE Healthcare) at a resolution of 100 pixels and an excitation wavelength of 532 nm. Total proteins were visualized by staining gels with SYPRO Ruby (Molecular Probes™, Invitrogen, USA), following the standard protocol, and then scanned. After scanning, proteins were visualized using silver staining according to the method by Wang et al. [41].

### 1.6 MS analysis

CSP spots were manually excised from silver stained 2-DE gels, and then gel pieces were destained for 5 min with 200 μL destaining solution (15 mmol $L^{-1}$ $FeK_3(CN)_6$ and 50 mmol $L^{-1}$ $Na_2S_2O_3$). After removing the destaining solution, gel pieces were washed three times with 800 μL water and vortexed for 10 min each time at 50°C. Acetonitrile (200 μL) was added to the mixture, followed by incubation at room temperature with occasional vortexing until gel pieces became white and shrunken, and then the acetonitrile was removed. Trypsin buffer (typically, 5 ng $\mu L^{-1}$ Promega Sequencing Grade Modified Trypsin in 10 mmol $L^{-1}$ $NH_4HCO_3$) was added, depending on the quantities of protein loaded for 2-DE, to cover the dry gel pieces that were then placed in an ice bucket or fridge. After saturation for 30 min and when the gel pieces were completely rehydrated, the trypsin was removed. Next, 10–15 μL 10 mmol $L^{-1}$ ammonium bicarbonate buffer was added to cover the gel pieces for enzymatic cleavage. Tubes containing the gel pieces were then placed into an air circulation incubator at 37°C for 4–16 h.

After gel digestion, 1.4 μL peptide solution was mixed with 0.4 μL matrix (4-hydroxy-α-cyanocinnamic acid) in 30% acetonitrile (CAN) and 0.1% trifluoroacetic acid (TFA) before spotting onto the target plate. MALDI-TOF and tandem TOF/TOF MS were then carried out using an AB SCIEX MALDI TOF-TOF™ 5800 Analyzer (AB SCIEX, Shanghai, China). Peptide mass maps were acquired in positive reflection mode, averaging 1000 laser shots per MALDI-TOF spectrum and 2000 shots per TOF/TOF spectrum (the resolution was 20000). Calibration mixtures (Applied Biosystems) were used to calibrate the spectrum to a mass tolerance within 0.1 Da. Parent mass peaks with a mass range of 800–4000 Da and a minimum signal to noise ratio of 50 were chosen for tandem TOF/TOF analysis.

### 1.7 Database search

The contaminant *m/z* peaks originating from human keratin, trypsin autodigestion or matrix were included in the exclusion list used to generate the peptide mass list used for

database searching. Database searching used internal MASCOT (Version 2.2, Matrix Science, London, UK) software to match MS and MS/MS data against NCBInr databases (updated December, 2010, containing 4607655 entries) without taxonomic restriction. Searches were conducted using the following setting: one missed cleavage, $P < 0.05$ significance threshold, 50 ppm peptide mass tolerance, 0.25 Da fragment mass tolerance, 50 ppm peptide mass tolerance, 0.1 Da MS/MS ion tolerance, carbamidomethylation of cysteine as a fixed modification, and methionine oxidation as a variable modification. Once the confident identifications were removed after searching against the NCBInr database, the rest was searched against the dinoflagellate expressed sequence tag (EST) database (downloaded from NCBI, updated December, 2010, containing 171550 entries). Protein identification, performed using MASCOT software, was considered to be correct at a > 95% confidence interval for the protein score, and hits were considered to be significant when the total ion confidence interval (C.I., %) was ⩾ 95 and the *E*-value was below $e^{-20}$ or less for EST search results.

## 2 Results and discussion

### 2.1 Surface labeling of *A. catenella* DH01 cells

The principle of CyDye surface labeling is based on the chemistry of minimal labeling CyDyes to covalently bind the ε-amine of lysine with their *N*-hydroxysulfosuccinimide ester group via an amide linkage. Impermeability of the amphiesma of *A. catenella* to fluorescent dyes is a crucial requirement for selective labeling of CSPs. Thus, in this study, both amphiesma permeability and active uptake of CyDye fluorophores by cells were conducted in suspension. About $1\times10^7$ viable *A. catenella* cells were labeled with Cy3. After labeling, excess dye was removed and cells were observed under fluorescence microscopy. Cy3-labeled cells were red with a ring-shaped membranous staining pattern (Figure 1). Such fluorescent images of Cy3-labeled cells indicated that these cells remained intact and their CSPs were labeled with Cy3.

Several cell surface labeling methods, such as isotope vectorial labeling [31] and biotinylation labeling [32], have been developed to investigate the CSPs of dinoflagellates. However, these methods lead to loss of CSP solubility due to the complex procedure involved in labeling intact cells. Moreover, these methods can only address one or a few proteins at one time. Fluorescent labeling of CSPs with Ettan DIGE dyes is a newly developed method and has been widely applied to study the CSPs of various human cell lines [34], bacteria [36] and the monocyte plasma membrane [37]. The results demonstrate that CyDyes can specifically bind to CSPs, with minimal labeling of cytosolic proteins because CyDyes cannot penetrate the outmost membrane of living cells [43–47]. Moreover, labeling is performed under mild conditions (HBSS, pH 8.5) that are less likely to alter the structure of the algal cell surface [36]. Importantly, cyanine dyes are small and do not alter protein migration in gels [48]. The present study used Cy3 to label the CSPs of *A. catenella* DH01 by a similar method used for labeling human cell lines [34]. This method was simple compared with other methods to detect algal CSPs (e.g. biotinylation), and did not require specific treatment or designated facilities to perform, as is the case using radioactive materials to label proteins.

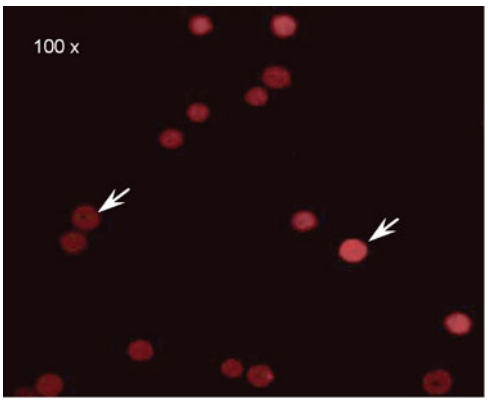


**Figure 1** Surface labeling of living cells. Cy3-labeled algal cells were examined using a fluorescence microscope with the excitation wavelength of Cy3. Images show how Cy3 selectively labels the CSPs of live cells, a ring-shaped staining pattern (marked by arrows) and that the staining procedure does not compromise the integrity of the cell wall.

### 2.2 CSP extraction

The 2-DE profiles of *A. catenella* DH01 CSPs prepared using three extraction methods are shown in Figure 2. Among them, the Trizol method obtained the optimal result with regard to protein spot number and resolution, and 41 CSP spots were identified by 2-DE (Figure 2(c)). The majority of proteins were separated in the molecular mass range of 20–98 kD and had an isoelectric point range of 4.5–7.0. The urea/ASB-14 extraction with TCA/acetone precipitation method (Figure 2(a)) also yielded 34 CSPs spots, while the $CaCl_2$/LiCl extraction method yielded just 12 CSPs (Figure 2(b)).

The 2-DE patterns, stained using SYPRO Ruby (Figure 3(b)) and silver staining (Figure 3(c)), were also determined in this study. Cy3-labeled proteins (Figure 3(a) and (b)) could be accurately aligned and silver stained spots corresponding to SYPRO Ruby and Cy3-labeled CSPs were easily discerned. The results suggested that we selectively targeted proteins by fluorescent labeling of algal cells to identify CSPs using MALDI TOF/TOF MS analysis.

### 2.3 Protein identification

Thirty-eight protein spots were visualized using fluorescence scanning and silver staining, which were excised

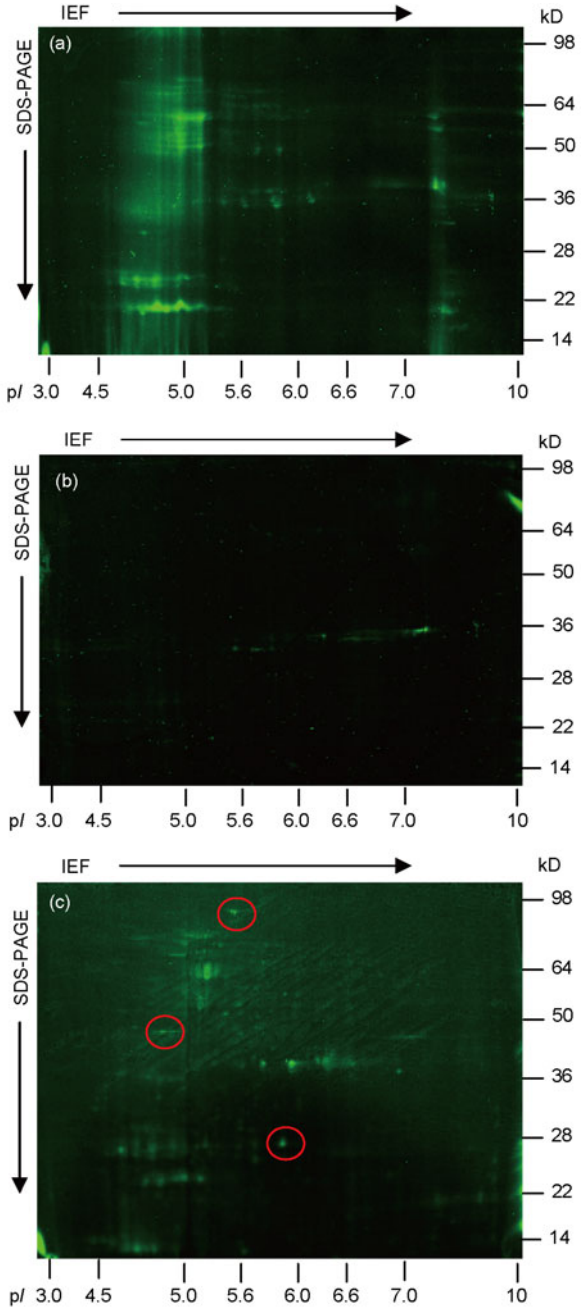


**Figure 2** 2-DE protein profiles of CSPs extracted using three methods (pH 3–10). (a) Urea/ASB-14 extraction; (b) $CaCl_2$/LiCl sequential extraction; (c) Trizol extraction. Protein spots in the circular regions in (c) show unique proteins on the 2-DE gel obtained using Trizol extraction, compared with that of the other two methods.

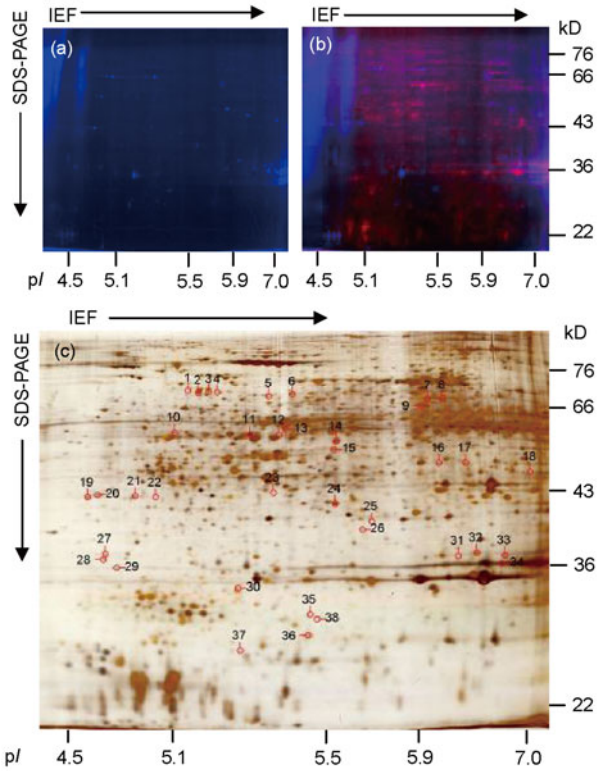


**Figure 3** Fluorescence and silver stained 2-DE pattern of proteins extracted from the Cy3-labeled cells of *A. catenella* using the Trizol method. Proteins were resolved on non-linear, pH 4–7 IPG strips in the first dimension followed by separation on precast polyacrylamide gels (12.5%) in the second dimension. (a) A typical 2-D pattern of the CSPs of Cy3-labeled *Alexandrium* cells; (b) two-color image of 2-D-separated CSPs (blue) and total proteins (red) labeled with Cy3 and SYPRO Ruby, respectively; (c) the corresponding silver stained image. Spots marked with a number were subjected to MALDI TOF/TOF analysis.

from silver stained gels (Figure 3(c)) and trypsinized prior to MALDI TOF/TOF MS analysis. The nine confident identifications of Cy3-labeled CSPs searched against the NCBInr database are shown in Table 1, and the five EST search results are listed in Table 2. The remaining 24 protein spots could not be positively identified as protein orthologs in the protein database and were assigned as unknown or novel proteins.

MS analysis of the CSPs of *A. catenella* DH01 led to identification of two proteins with chaperone functions, namely heat shock protein (HSP) 70C (spot 2) and HSP70 (spot 3). Cell surface localization of chaperones has been reported in several studies [49–51]. HSP70, previously characterized as a chaperone, regulates apoptosis in response to heat shock and oxidative stress by preventing the release of cathepsins and cytochrome *c* [52]. This protein prevents lysosome-mediated sudden death by binding to lysosomal cathepsins B, D, L and H and providing structural stability to lysosomal membranes [37,53]. It is unclear how the localization of these HSPs is targeted to the cell surface, but it is unlikely to be the classic secretory pathway (endoplasmic reticulum (ER) to Golgi to plasma membrane). A previous study demonstrats that thioredoxin H-type 5 (spot 28) is secreted in a non-classic manner that is independent of the ER-Golgi pathway [51].

Spot 5 was identified as a homologue of the cell division protein FtsH that has an integral cytoplasmic membrane protein spanning the membrane twice and a large cytoplasmic carboxyterminal with a putative ATP-binding domain [54]. FtsH participates in the assembly of proteins into and through the membrane and is required for cells to ensure

**Table 1** Confident identifications of Cy3-labeled CSPs searched against the NCBInr database[a)]

| Spot No. | Protein name | Species | Accession No. | Theoretical Mr (Da) | Theoretical p*I* | Peptide count | Protein score | Protein score C.I. (%) | Total ion score | Total ion C.I. (%) | Sequence coverage (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | heat shock protein 70C | *Chlamydomonas reinhardtii* | 159472671 | 65434.8 | 5.6 | 10 | 94 | 99.52 | 65 | 98.665 | 23 |
| 3 | Hsp70 | *Crypthecodinium cohnii* | 20143982 | 70865.7 | 5.09 | 11 | 92 | 99.256 | 44 | 0 | 21 |
| 5 | cell division protein FtsH | *Sphingobacterium spiritivorum ATCC 33300* | 227539016 | 76531 | 6.18 | 8 | 87 | 97.421 | 68 | 99.299 | 11 |
| 6 | luciferin-binding protein | *Alexandrium catenella* | 166030338 | 75057.6 | 5.59 | 22 | 124 | 100 | – | – | 38 |
| 20 | chloroplast sedoheptulose-1,7-bisphosphatase | *Lingulodinium polyedrum* | 99903657 | 45360.9 | 5.18 | 7 | 170 | 100 | 144 | 100 | 20 |
| 21 | chloroplast sedoheptulose-1,7-bisphosphatase | *Lingulodinium polyedrum* | 99903657 | 45360.9 | 5.18 | 8 | 141 | 100 | 94 | 99.998 | 21 |
| 28 | thioredoxin H-type 5 | *Arabidopsis lyrata subsp. lyrata* | 297852252 | 13371.8 | 5.37 | 11 | 94 | 99.485 | – | – | 85 |
| 32 | alpha S1 casein | *Bos taurus* | 159793187 | 13896.3 | 5.43 | 5 | 97 | 99.759 | 66 | 98.855 | 41 |
| 38 | hypothetical protein RHA1_ro11142 | *Rhodococcus jostii RHA1* | 111026969 | 19384 | 9.82 | 11 | 88 | 98.257 | – | – | 80 |

a) The peptide sequences assigned and the mass/charge of Table S1.

**Table 2** Confident identifications of Cy3-labeled CSPs against the EST of dinoflagellates

| Spot No. | Protein name [species] | Accession No. | *E*-value | Peptide count | Total ion score | Total ion C.I. (%) | Query coverage (%) | Peptide sequences | Calculate mass | Observed mass |
|---|---|---|---|---|---|---|---|---|---|---|
| 7 | conserved hypothetical protein [*Perkinsus marinus* ATCC 50983] | XP_002788434.1 | $1.00\times10^{-98}$ | 3 | 271 | 100 | 79 | FGAHSTIIATGGFGR-<br>GADWLGDQDAIQHMCR-<br>LAPEVILELESFGLPFSR | 1491.7704<br>1872.8116<br>2017.1005 | 1491.7673<br>1872.8103<br>2017.1012 |
| 8 | conserved hypothetical protein [*Perkinsus marinus* ATCC 50983] | XP_002765468.1 | $7.00\times10^{-111}$ | 3 | 192 | 100 | 89 | LGANSLLDLVVFGR-<br>DHIYLHLDHLPPETLAER-<br>QAADTTAELVKPNSPPVQLP<br>ANAGEATIAR | 1473.8424<br>2169.1086<br>3030.5854 | 1473.8285<br>2169.1106<br>3030.5862 |
| 19 | chloroplast sedoheptulose-1,7-bisphosphatase [*Lingulodinium polyedrum*] | ABF68590.1 | $3.00\times10^{-103}$ | 2 | 98 | 100 | 83 | IFAPANMR-<br>LAFEAAPFGR | 919.4818<br>1078.568 | 919.4791<br>1078.5706 |
| 30 | triose-phosphate isomerase [*Karlodinium micrum*] | ABV22237.1 | $2.00\times10^{-45}$ | 3 | 170 | 100 | 71 | CVFAIGEK-<br>ENVSEAVAEGIR-<br>VVIAYEPVWAIGTGVTAT<br>PEQAQETHAEIR | 923.4655<br>1273.6383<br>3236.6587 | 923.4512<br>1273.6434<br>3236.6631 |
| 37 | predicted protein [*Phaeodactylum tricornutum* CCAP 1055/1] | XP_002184440.1 | $4.00\times10^{-31}$ | 2 | 61 | 99.973 | 63 | EALYYVSR-<br>YMPEDVVQAAR | 1000.5098<br>1294.6096 | 1000.5095<br>1294.597 |

efficient stop-transfer of some transmembrane proteins [55]. Moreover, FtsH is significantly homologous to members of an ATPase family found in eukaryotic cells [56].

It should be noted that several intracellular proteins were identified in this study, such as luciferin-binding protein (spot 6) and chloroplast sedoheptulose-1,7-bisphosphatases (spots 19, 20 and 21). Presently, we have no explanation for these results. One possibility is that CSPs were contaminated by intracellular proteins during labeling, but no other intracellular high abundance proteins were found in this study. Moreover, all proteins identified in this study were low abundance proteins in silver stained gels. Therefore, we postulate that these proteins might be transmembrane proteins or channel proteins associated with the outermost membrane. It is known that the chloroplast of *A. catenella* has a radiating structure from the central pyrenoid complex to the cell wall, which contacts the plasma membrane [58]. In addition, cells organized as chains are interconnected by a cytoplasmic connection that passes through the so-called attachment pore of the pore plate and a pore in the posterior sulcal plate [57,58]. This connection between cells is prone to breakage by mechanical disturbance, and proteins located on or associated with the plasma membrane are released from the attachment pore and labeled with CyDye. Potential direct physical connections may occur between the plasma membrane and the cell wall and/or interactions at the plasma-cell wall interface [59]. In addition to the above proteins, a small number of proteins were identified as hypothetical proteins or functionally unknown proteins. This result was not surprising because the dinoflagellate genome has not been completely sequenced and few studies have been conducted on the CSPs of dinoflagellates.

In conclusion, this study developed a rapid and simple method for specific labeling and detection of the CSPs of *A. catenella* cells using CyDye DIGE fluor minimal dyes, which provides a potential tool for further proteomic studies of CSPs from *Alexandrium* and other dinoflagellate species. The fluorescent labeling used in this study does not interfere with subsequent identification of proteins excised from gels by MS because most peptides do not contain the label. Because cyanine dyes are compatible with 2-DE and MS, they can be used in proteomic approaches to identify dinoflagellate CSPs. More insights can be expected into the rapid analysis of many CSPs, as well as the characterization of the proteomic changes occurring at the cell surface in response to environmental stress, which will ease the identification of new surface-exposed targets that may improve our understanding of the relationship between cells and environmental variations.

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## Supporting Information

**Table S1** The mass/charge and peptide sequences of identified proteins

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